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OMISSION

In Vol. 36, No. 1, the title page was omitted, for which the C.U.P. apologise. Enclosed with this part, as a loose insert, is the missing title page.

The major fatty acids in whole milk fat and in a fraction obtained by crystallization from acetone

By SONJA MATTSSON, P. SWARTLING* and R. NILSSON†
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(*Received* 28 *September* 1968)

Summary. Summer and winter milk-fat samples from 14 dairies in Sweden were fractionated by crystallization from acetone solution (1:8) at 15 °C. The composition of the major fatty acids of the parent milk fat and of the acetone insoluble fraction were examined by GLC, and the gross triglyceride pattern by TLC on plates of silicic acid treated with silver nitrate.

The fatty acid composition of the milk fat was similar to that of milk fat from other countries and varied according to season and also, to a smaller extent, from region to region. Four fractions, representing 33–45, 41–34, 18–14 and 7–6 % of the fat and which contained progressively smaller proportions of saturated acids, were obtained by TLC.

The acetone insoluble glyceride (AIG) fraction was characterized by a smaller content of short-chain fatty acids and unsaturated fatty acids, and a larger content of saturated long-chain fatty acids, than the parent milk fat. AIGs from summer milk fat contained a larger proportion of C_{18} acids and a smaller proportion of C_6–C_{16} acids than AIGs from winter milk fat.

Four fractions representing 62–70, 15–8, 16–15 and 7 % of the AIG fraction were obtained by TLC. The distribution of the triglycerides in the AIG fraction differed from that in the parent milk fat, mostly in the relative amounts of glycerides in the 2 most saturated TLC fractions. The seasonal variation was largely confined to these 2 fractions.

The composition of milk fat and of fractions of milk fat obtained in various ways has been widely studied in the last 20 years (Antila, 1966; Chen & deMan, 1966; Jensen, Quinn, Carpenter & Sampugna, 1967; Jensen & Sampugna, 1966; Mattsson, 1962*b*; Svensen & Ystgaard, 1966). In the course of a series of investigations on the composition of milk lipids carried out at Alnarp (Kishonti & Sjöström, 1965; Mattsson, 1949, 1962*a*, 1966; Mattsson & Swartling, 1958, 1963; Mattsson, Thomé & Swartling, 1951*a*, *b*; Nilsson, 1965), Nilsson (1966) determined the content in milk fat of a high-melting triglyceride fraction from numerous milk fat samples collected during all seasons of the year in various parts of Sweden. The high-melting triglyceride fraction was obtained by fractional crystallization from an acetone solution. The purpose of the present investigation was to elucidate the fatty acid pattern of

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Swedish milk fat and to further characterize the fraction obtained with Nilsson's technique. Summer and winter butter from several dairies in various parts of Sweden was used.

MATERIAL AND METHODS

Milk-fat samples

Butter samples were collected in August and in December from 14 dairies in various parts of the country (Nilsson, 1966). Milk fat was obtained from the fresh butters by melting at 50 °C and removal of the serum by decantation and filtration.

Refractive number

The refractive number of the milk fat was determined at 40 °C with a Zeiss butter refractometer.

Acetone insoluble triglyceride (AIG) fraction

The AIG fraction was prepared from the milk fat as described by Nilsson (1966). Filtered milk fat (5 g) was dissolved in 40 ml acetone, A.R., and kept at 15 °C for 3 h. The precipitate was collected on a glass filter and washed with a cold mixture of ethanol and ethyl ether (2:3, v/v). The precipitate was dried under vacuum at room temperature and weighed.

Methyl esters

Methyl esters were prepared from the various milk fats and AIG fractions by the method of Smith (1961).

Gas chromatography

A Perkin-Elmer vapour fractometer, model 116E, equipped with a flame ionization detector, was used with 2-m columns of ethylene glycol succinate (Perkin-Elmer 2S 42.14) at 195 °C and with helium as carrier gas. The units were calibrated with standard fatty acid methyl esters (obtained from Hormel Foundation).

Butyric acid

The values found for butyric acid by the gas chromatographic method were evidently too small. The butyric acid in the milk fats and in the AIG fractions was therefore determined by column chromatography on silicic acid by the method of Kishonti & Sjöström (1965).

Thin-layer chromatography (TLC)

TLC of the milk fat samples and the AIG fractions was carried out on 20 × 20 cm glass plates coated with a 0·30 mm layer of silicic acid impregnated with silver nitrate as described by Barret, Dallas & Padley (1963). The plates were activated by heating for 1 h at 130 °C.

Samples (20 mg) of milk fat or AIG fraction were dissolved in 400 μl chloroform and applied as a band to the plate, and developed with 0·5 % acetonitrile in chloroform. The plates were sprayed with a 0·1 % ethanolic solution of 2,7-dichlorofluorescein and viewed under UV light.

The bands were scraped off the plate into a small column (1·1 cm diam.) loaded with 1 g silicic acid in chloroform. The glycerides were eluted with 25 ml chloroform.

The amount of triglycerides in the chloroform eluates was determined by the acyl ester method of Antonis, Platt & Thorp (1965) as modified by Mattsson (1966).

RESULTS AND DISCUSSION

Fatty acids of milk fat

The average fatty-acid composition in mole per cent of the 14 milk-fat samples in August and in December are given in Table 1. The variation between samples from different regions is apparent from the standard deviations included in the table. The relative amounts of the individual fatty acids in Swedish milk fat were found to be about the same as in milk fat from other countries. The major components were the C_4, C_{14}, C_{16} and C_{18} acids.

Table 1. *Average major fatty acid composition (mole %) of fat and acetone insoluble glycerides in milk fat samples collected from 14 dairies in August and in December*

	Mole % fatty acids in			
	Milk fat		AIG	
	August	December	August	December
C_4	9·5 ± 0·4	9·4 ± 0·4	1·1 ± 0·3	1·8 ± 0·4
C_6	3·3 ± 0·5	3·7 ± 0·3	0·3 ± 0·1	0·6 ± 0·1
C_8	1·9 ± 0·3	2·2 ± 0·2	0·4 ± 0·1	0·6 ± 0·1
C_{10}	3·7 ± 0·5	4·4 ± 0·4	1·5 ± 0·2	2·4 ± 0·3
	0·4 ± 0·1	0·5 ± 0·1	0·1 ± 0·0	0·2 ± 0·1
C_{12}	3·8 ± 0·5	4·7 ± 0·5	3·0 ± 0·3	4·5 ± 0·5
	0·7 ± 0·2	0·7 ± 0·1	0·5 ± 0·1	0·5 ± 0·2
C_{14}	10·9 ± 0·5	12·4 ± 0·5	13·3 ± 0·8	15·8 ± 0·6
	4·0 ± 0·3	4·0 ± 0·4	3·9 ± 0·3	3·8 ± 0·4
C_{16}	22·7 ± 1·3	28·1 ± 1·9	35·4 ± 1·7	41·4 ± 2·2
	4·8 ± 0·5	4·8 ± 0·2	5·2 ± 0·4	4·9 ± 0·6
C_{18}	9·2 ± 0·7	6·8 ± 1·0	23·0 ± 1·4	15·0 ± 2·4
$C_{18}^{1=}$	21·5 ± 1·3	16·0 ± 1·9	10·8 ± 1·1	7·3 ± 0·9
$C_{18}^{2=}$	1·5 ± 0·2	1·3 ± 0·2	0·5 ± 0·1	0·3 ± 0·1
C_{20}	0·2 ± 0·1	0·2 ± 0·1	0·5 ± 0·1	0·5 ± 0·1
$C_{18}^{3=}$	2·1 ± 0·4	1·1 ± 0·1	0·6 ± 0·1	0·4 ± 0·2

Summer milk fat contained more C_{18} acids and less C_6–C_{16} acids than did winter milk fat. As in Kishonti & Sjöström's (1965) investigation the seasonal variation in the butyric acid content was only slight.

The samples collected during August and December were from cows grazing pasture or fed winter diets, respectively. The samples were rather uniform, as is shown by the standard deviations, but within these periods differed significantly from one part of the country to another in the ratio of saturated to unsaturated acids. This regional variation in composition is smaller than the seasonal variation. Owing to climatic differences the time of change from summer to winter systems of feeding varies between the different regions of the country, which causes a very strong regional variation in the composition of the milk fat during the period of change-over

as has long been observed in studies of the refractive number of Swedish milk fat (for references see Mattsson, 1962). In the present investigation, the correlation coefficient: Σ unsaturated C_{18} acids – refractive number was +0·96.

Fatty acids of the AIG fraction

The average fatty acid composition of the insoluble triglyceride fraction obtained at 15 °C from an acetone solution of the milk fat samples is shown in Table 1.

The AIG fraction was characterized by a smaller content of short-chain fatty acids and of unsaturated fatty acids, and a larger content of saturated long-chain fatty acids than the parent milk fat.

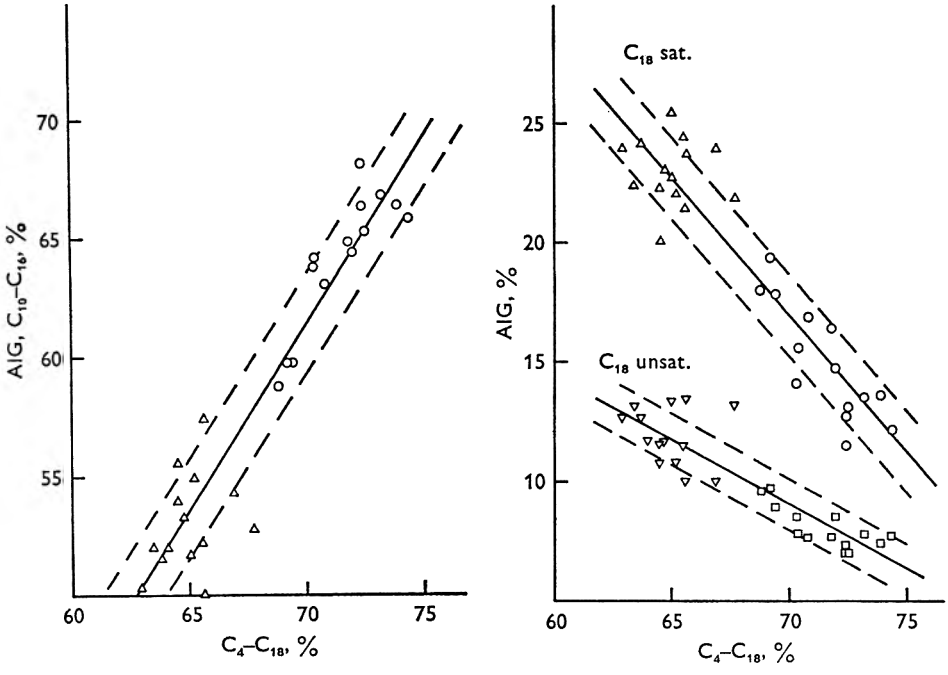

Fig. 1. Content of C_{10}–C_{16} acids of the AIG in relation to the content of the major saturated fatty acids in the parent milk fat. $r = +0{\cdot}95$; $y = 1{\cdot}56x - 48{\cdot}1$. △, August; ○, December.

Fig. 2. Content of stearic acid and of unsaturated C_{18} acids of the AIG in relation to the content of the major saturated fatty acids in the parent milk fat. C_{18} sat: $r = -0{\cdot}93$; $y = -1{\cdot}15x + 97{\cdot}6$; C_{18} unsat: $r = -0{\cdot}89$; $y = -0{\cdot}54x + 47{\cdot}0$. △, ▽, August; ○, □, December.

The AIG fractions obtained from the various milk fat samples differed in size (Table 2) and in fatty acid composition both between sampling areas and between seasons. The seasonal variation corresponded largely to that of the parent milk fat, i.e. AIGs from summer milk fat contained a larger proportion of C_{18} acids and a smaller proportion of C_6–C_{16} acids than the AIGs from winter milk fat.

The relationship between the composition of the AIG fraction and the parent milk fat is demonstrated in Figs 1 and 2.

The AIG fraction of milk fat obviously consists of a mixture of triglycerides, which are different in different milk-fat samples. Common to these glycerides is their insolubility in acetone rather than detailed similarities in their fatty acid composition.

In Nilsson's (1966) investigation, winter milk fat from 4 dairies in north Sweden, Örnsköldsvik, Östersund, Lycksele and Skellefteå, was found to contain relatively large amounts of AIGs. The AIG fractions of the milk fat from these dairies were now found to contain less C_{18} acids and more C_{10}–C_{16} acids than the AIG fraction of milk fat from other dairies, but the differences were only small.

TLC glyceride fractions

TLC of the AIGs and of the parent milk-fat samples revealed 4 fractions. The average yield of each fraction is shown in Table 2, where the fractions are numbered 1–4 in order of increasing R_F value. The band with the highest R_F value has been shown (Barret, Dallas & Padley, 1963; Blank & Privett, 1964; Blank, Verdino & Privett, 1965; Lichtfield, Farquhar & Reiser, 1964) to consist of the saturated triglycerides.

Table 2. *Proportionate yield of the 4 fractions obtained by TLC on silver nitrate–silicic acid (moles %)*

TLC fraction	Milk fat		AIG	
	August	December	August	December
1	7±2	6±1	7±2	7±1
2	18±3	14±1	16±2	15±1
3	41±3	34±2	15±3	8±1
4	33±2	45±3	62±3	70±3

On TLC of milk fat, 70–80 % of the fat appeared in the 2 most saturated fractions (3 and 4). Compared with the fat of summer milk the winter fat contained a somewhat smaller amount of glycerides in fractions 1 and 2, a smaller amount of glycerides in fraction 3 and a larger amount of glycerides in fraction 4.

The relative proportions of fat in fraction 4 varied closely with the saturated fatty acid content of the parent milk fat, the correlation coefficient being +0·94 (Fig. 3).

The amount of fat in fraction 4, 31–50 %, agrees with the figure, 38 %, given by Chen & deMan (1966) for the content of saturated triglycerides in a sample of Canadian milk fat. Using the mercuric acetate separation method Kerkhoven & deMan (1966) investigated 19 Canadian milk fat samples collected during all seasons of the year. They found the saturated triglyceride content to vary between 33 and 44 %.

From the known fatty acid composition and from the determinations of the content of saturated glycerides of 3 milk-fat samples Kerkhoven & deMan (1966) calculated the concentrations of the other possible glyceride types GS_2U, GSU_2, GU_3, containing, respectively 1, 2 and 3 unsaturated fatty acids. The levels found were approximately 40, 20 and 3 %, respectively. Similar results, obtained partly by calculation, were reported by Hilditch & Williams (1964) and by Bhalerao, Johnson & Kummerow (1959). A comparison between these values and the results given in Table 2 supports but does not prove the calculation method used by the aforementioned authors, because the fatty acid composition of the 4 TLC fractions obtained on silver nitrate–silicic acid has not yet been determined.

Calculation according to Hammond & Jones (1960), assuming the TLC fraction 4 to

be fully saturated and using the values given in Table 1 for the fatty acid composition of the fat, gave substantially higher values for glycerides of type GUS_2 and correspondingly lower values for glycerides GU_2S and GU_3 than those found in TLC fractions 3, 2 and 1, respectively.

The distribution of the triglycerides of the acetone insoluble fraction differed from that of the parent milk fat mostly in the relative amounts of glycerides in fractions 3

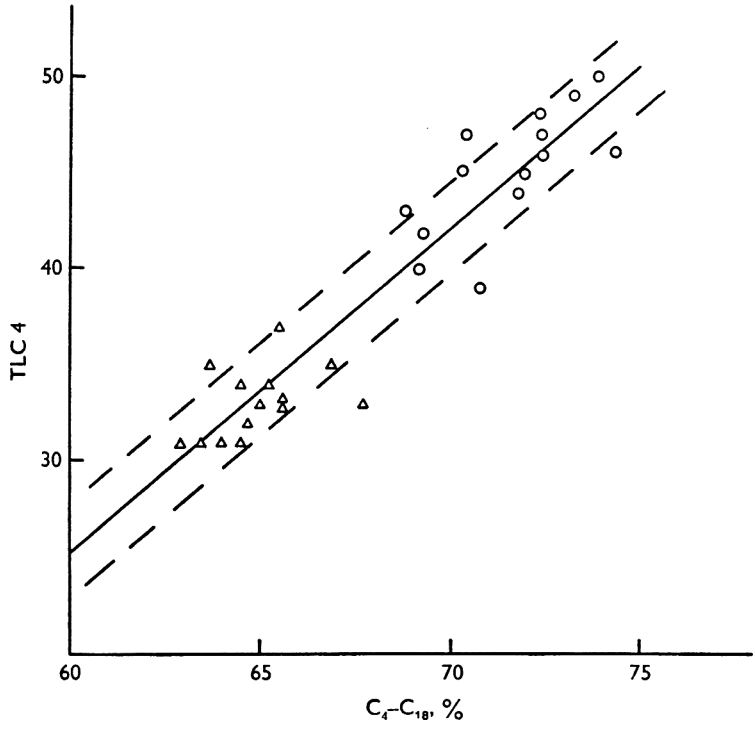

Fig. 3. Amount of fat in TLC fraction 4 in relation to the content of the major saturated fatty acids in the parent milk fat. $r = +0{\cdot}94$; $y = -75{\cdot}4 + 1{\cdot}68x$. △, August; ○, December.

Table 3. *Refractive number and content of acetone insoluble glycerides, saturated C_{10}–C_{18} acids and TLC fraction 4 of milk fat samples collected in August and December*

	Refractive number		AIG, %		C_{10}–C_{18}, mole %		TLC 4, mole %	
	August	December	August	December	August	December	August	December
Vallberga	43·6	41·6	6·2	9·0	50·1	56·5	37	44
Vetlanda	43·7	41·8	6·4	8·6	50·0	54·2	31	42
Ljungby	43·6	41·8	6·2	9·8	50·2	55·7	33	47
Visby	43·7	41·4	6·6	8·8	50·7	57·3	33	46
Borgholm	44·1	41·1	7·2	10·2	48·3	58·3	31	50
Norrköping	43·9	41·9	7·2	8·4	49·3	52·9	35	43
Örebro	43·6	42·3	6·6	8·4	49·6	54·1	32	40
Grådö	43·7	41·6	6·6	8·6	49·6	55·9	33	39
Östersund	43·6	41·3	8·0	11·6	51·2	56·8	34	47
Örnsköldsvik	43·5	41·3	8·8	12·6	51·1	58·8	31	46
Ljusdal	43·2	41·4	6·4	9·2	51·8	56·3	35	45
Lycksele	43·3	41·4	8·2	11·6	51·3	57·9	33	48
Skellefteå	43·9	41·4	7·4	11·0	50·8	58·1	34	48
Hedenäset	43·4	41·8	7·0	9·8	49·5	55·4	31	45

and 4. The seasonal variation, which was evident also for the AIGs, was practically confined to these 2 fractions. The high value found for the content of the most saturated glycerides, 62–70 %, agrees well with that given by Chen & deMan (1966), who estimated the content of fully saturated triglycerides as 67 % in a fraction of milk fat obtained by crystallization from an acetone solution (slightly more diluted than ours) at 15 °C.

Though samples from the dairies varied somewhat in the distribution of the glycerides among the 4 TLC fractions neither the milk fat nor the AIGs from the 4 above-mentioned dairies in north Sweden showed any particular distinguishing characteristics.

Observations on the content of AIG

The AIG content of the various milk fat samples is given in Table 3 together with the refractive number of the milk fat, its content of some saturated fatty acids and the percentage of the milk fat found in TLC fraction 4. As expected (Nilsson, 1966), the AIG content in the milk fat varied roughly inversely with the refractive number of the milk fat. Consequently, the AIG content varied directly with the content of saturated C_{10}–C_{18} acids in the parent milk fat. The correlation coefficients were $-0{\cdot}83$ and $+0{\cdot}88$, respectively.

The AIG content was also closely related to the most saturated TLC fraction of the milk fat, the correlation coefficient being $+0{\cdot}83$.

The December values for the AIG content of the milk fat from the 4 dairies in north Sweden seem to be anomalously high. For an explanation further investigation of the glyceride structure is required.

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Size distribution of fat globules in cow's milk during milking, measured with a Coulter counter

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Summary. A Coulter counter was used to measure the diameters and numbers of fat globules in serial samples taken during a single milking of each of 8 cows. Milk fat percentages of samples were estimated by the Babcock method. The average globule diameter, globule number and fat percentage all increased during milking. Regression analyses indicated that fat percentage was a stronger function of fat globule diameter than of globule number. The results are discussed with relation to the rise in fat percentage that occurs during milking.

It was van Leeuwenhoeck in 1674 who first discovered the globular nature of milk fat and since then there have been many studies of the size distributions and concentrations of globules in cow's milk. There have also been many studies describing the influence of breed, stage of lactation, nutrition and disease on these variables. On the other hand, there are relatively few reports on changes in the distribution of globule sizes during a single milking. A knowledge of the latter is considered important (Whittlestone, 1953) in relation to the rise in milk-fat percentage that occurs during milking.

Collier (1891), using Babcock's (1885) classical technique for measuring globule size, was the first to report an increase in the proportion of large globules in successive samples taken during a milking. This observation was confirmed by Woods (1905) and by Bitting (1905). The next major study in this field was carried out by Johansson (1952) who used the euscope method described by Campbell (1932) to study the distribution of fat globule size throughout a milking. He found little difference between the average globule diameter of the last 200 ml milking sample (3·59 μm) and that obtained after one injection of oxytocin (3·57 μm); the average diameter in fore-milk samples was only 2·60 μm.

Whittlestone (1952) modified a camera lucida technique for measuring fat globule diameter and studied the size distribution of fat globules during a single milking of the sow (1952), cow (1954) buffalo (1958) and human (Whittlestone & Perrin, 1954). Apart from small differences between the earliest fore-milk and the next sample, no changes in globule size distribution were found. The first reported use of the Coulter

counter for measuring milk fat globules was by Whittlestone (1962). The results obtained in the latter study of cow's milk confirm his earlier findings. The actual range of sizes measured in his study was not known, as a calibrating emulsion was not available.

During an experiment using Whittlestone's camera lucida technique, one of us (E.A.K.) observed a definite increase in average globule diameter in samples obtained successively during the milking of a single quarter in the cow. It was considered therefore that a further examination of the distribution of milk fat globules during milking was required and the present paper describes the results of such an investigation using a Coulter counter. The use of this instrument enabled a very large number of globules to be objectively counted and measured.

METHODS

Milk samples (approx. 100 ml) were collected serially by means of a teat siphon from the right front quarters of 8 cows. The cows were of mixed breed and at different stages of lactation, each producing at least 800 ml milk from the test quarter.

The other 3 quarters of each cow were milked by machine in the usual way. When milk flow ceased, 5 i.u. of oxytocin (Syntocinon, Sandoz Aust. Pty. Ltd.) was administered via a polythene cannula inserted into the jugular vein and 100 ml serial sample of residual milk obtained.

Samples of fore-milk, mid-milking, strippings and residual milk were selected and their milk-fat concentrations and globule-size distributions determined. The milk-fat concentration was estimated by the Babcock method (Davis, 1959).

Estimates of the size distributions of fat globules were then made using a model A Coulter counter having a 50-μl manometer volume and fitted with a tube having a 30-μm diam. orifice. The operation of the machine is well documented (Coulter, 1956; Mattern, Brackett & Olson, 1957; Ullrich, 1960) and will not be described here. The instrument was calibrated according to the manufacturer's recommendations using monosized polystyrene latex beads 3·49 μm diam. (Particle Information Service, 600 South Springer Road, Los Altos, California, U.S.A.).

The electrolyte used was 2% potassium chloride in distilled water containing potassium and sodium phosphate buffers of pH 6·9. Formaldehyde (0·1%) was added to the electrolyte to prevent bacterial contamination and the solution filtered through a millipore filter (0·45 μm porosity) to remove particulate matter. Immediately before use the electrolyte was again millipore filtered to remove any phosphate deposits.

After collection, 1 ml of each milk sample was immediately diluted to 100 ml with prepared electrolyte in a volumetric flask. This was further diluted to 1:10000 in a similar manner. These dilutions were mixed at 10-min intervals to prevent clumping of fat globules. Microscopic examination of these samples made at varying intervals after dilution confirmed that this treatment prevented clumping. During the counting procedure the sample was intermittently mixed with a stirring device fitted to the Coulter counter.

Counts on the diluted (1:10000) samples for 6 cows were performed at 15 threshold settings covering a diameter range of 0·8–9·5 μm. With the technique used, 0·8 μm

was the lowest measure of diameter obtainable. In an attempt to obtain more detailed information on samples from the other 2 cows the diameter range was extended to 12·75 μm.

RESULTS

The percentage of total globules at each Coulter counter threshold level was calculated for every sample. The results for the first 6 cows were averaged for each stage of milking and are illustrated as histograms (Fig. 1). These histograms represent the percentage of the fat globules at each threshold level; the latter being expressed in terms of actual globule diameter.

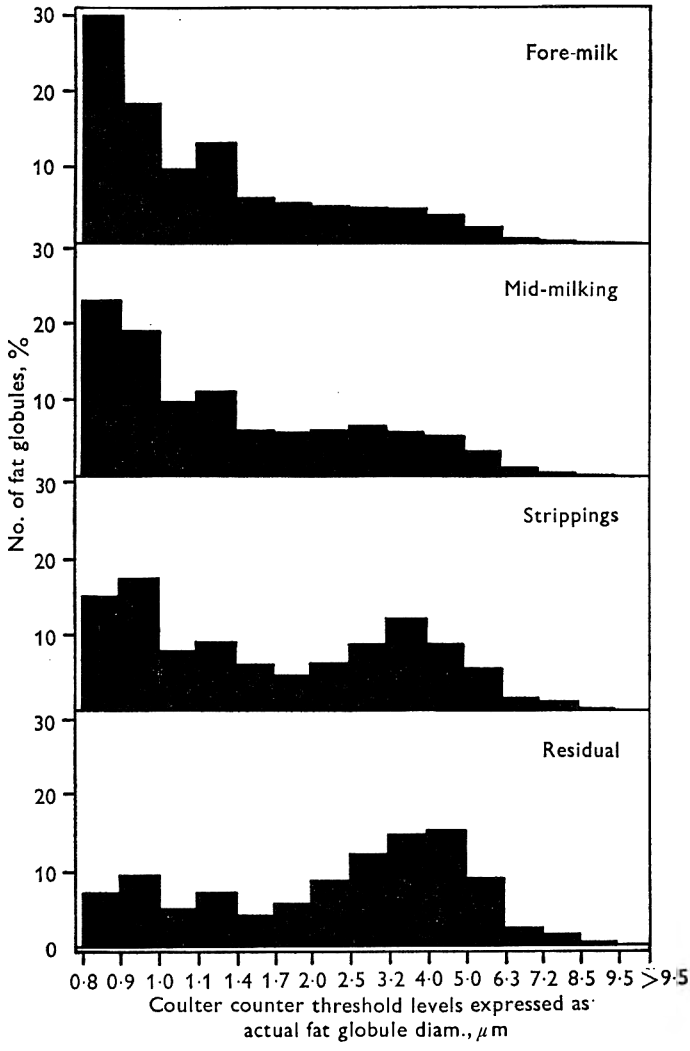

Fig. 1. The percentage of fat globules at each Coulter counter threshold level for each of the 4 stages of milking. (Average for 6 cows.)

The average globule diameter of every sample was calculated and an analysis of variance carried out (Table 1). The average globule diameters and least significant differences (LSD) for the 4 stages of milking (samples) are presented in Table 2. It

may be seen that the average globule diameters of residual milk samples were greater than those for fore-milk, milking and strippings ($P < 0{\cdot}001$ or $0{\cdot}01$). The average diameter of globules in strippings was also greater than in fore-milk and milking samples ($P < 0{\cdot}001$). However, there was no significant difference in diameters between fore-milk and milking samples. Although there was a significant difference in globule diameter between cows ($P < 0{\cdot}05$—Table 1), the variation due to cow differences was only 15·18% of the total variation compared with 69·89% for differences between samples.

Table 1. *Summary of analyses of variance of results of average globule diameter, number of globules/ml > 0·8 μm diam. and milk-fat percentage in samples taken throughout 1 milking of 8 cows*

Source of variation	Degrees of freedom	Mean squares: Globule diam., μm	Globules/ml	Milk fat, %
Cows	7	2·60*	10·89	11·72*
Samples	3	11·95***	40·49**	266·52***
Cows × samples	21	2·55	5·52	3·42
Total	31	17·10	10·11	30·76

* $P < 0{\cdot}05$. ** $P < 0{\cdot}01$. *** $P < 0{\cdot}001$.

Table 2. *Mean values with least significant difference (LSD) for globule diameter, globules/ml > 0·8 μm diam. and milk-fat percentage of samples taken throughout 1 milking of 8 cows*

Variable	Mean: Globule diam., μm	Globules/ml	Milk fat, %
Fore-milk	2·34	$4{\cdot}53 \times 10^9$	1·75
Milking	2·60	$7{\cdot}71 \times 10^9$	3·58
Strippings	3·28	$8{\cdot}17 \times 10^9$	7·01
Residual	3·91	$9{\cdot}93 \times 10^9$	14·80
LSD			
$P < 0{\cdot}001$	0·68	$4{\cdot}48 \times 10^9$	3·53
$P < 0{\cdot}01$	0·49	$3{\cdot}32 \times 10^9$	2·62
$P < 0{\cdot}05$	0·36	$2{\cdot}44 \times 10^9$	1·92

The summary of results of the analyses of variance for number of globules/ml greater than 0·8 μm in diam., and the milk-fat percentage during the 4 stages of milking, are also shown in Table 1. The means and LSD values for these variables are given in Table 2. The number of globules/ml in the fore-milk samples was significantly less than the number in the residual ($P < 0{\cdot}001$), strippings ($P < 0{\cdot}01$) and milking ($P < 0{\cdot}05$) samples. No significant differences were found between milking, strippings and residual milk samples.

Highly significant differences ($P < 0{\cdot}001$ or $0{\cdot}01$) were found in the milk-fat percentages between all milk samples except fore-milk and milking in which there was no difference. The variation in fat percentage due to significant differences between cows ($P < 0{\cdot}05$—Table 1) was only 8·61% of the total variation compared with 83·9% due to stage of milking.

The data were subjected to a regression analysis (Table 3) and the regression equation calculated:

$$Y = -14{\cdot}65 + 5{\cdot}90X_1 + 0{\cdot}47X_2,$$

where Y = milk-fat percentage, X_1 = average globule diam., X_2 = average globule number.

A multiple correlation coefficient of 0·92 was also calculated. Partial regression coefficients for average globule diam. (+0·79) and average globule number (+0·27) indicated that average globule diameter had a greater effect than average globule number on fat percentage.

Table 3. *Summary of multiple regression analysis of the effect of average globule diameter* (X_1) *and average globule number* (X_2) *on milk-fat percentage* (Y) *of samples taken throughout the milking of 8 cows*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Variance ratio
Regression	2	806·51	403·25	79·60***
Deviations	29	146·91	5·07	—
Total	31	953·42	—	—

*** $P < 0{\cdot}001$.

DISCUSSION

Results obtained by this method show a clear-cut increase in average globule diameter and globule number throughout a milking. Only globules greater than 0·8 μm diam. were measured, but it is well known that small globules (< 1·0 μm diam.) contribute only a small percentage to the total fat in a milk sample (Webb & Johnson, 1965; Cornell & Pallansch, 1966). The multiple correlation coefficient of 0·92 in this experiment would indicate that a maximum of 8 % of the total variation could be attributed to the omission of these small globules. Moreover, as these small globules predominate in fore-milk (Johansson, 1952; Whittlestone, 1954) any differences in globule diameter between samples would probably be increased by their inclusion. Certain technical difficulties associated with some milks of very high fat percentage were encountered which necessitated extrapolation of results to the 0·8 μm level. This gave results for globule number of the order of those obtained by Cornell & Pallansch (1966).

The presence of large numbers of somatic cells in milk samples could affect results for globule diameters greater than 5·25 μm (Phipps & Newbould, 1966). However, results of the California Mastitis Test (Schalm & Noorlander, 1957) on the quarter milk samples indicated that less than 500 000 cells/ml were present. As these samples were diluted 1 : 10 000 for counting it is unlikely that somatic cells would significantly contribute to the results.

The size distribution patterns (Fig. 1) were similar to those in the earlier study using the camera lucida techniques (E. A. Kernohan, unpublished) except that in the present study the distribution of globules in the strippings was bimodal (Fig. 1). Irrespective of this possible bimodal effect, the trend of increasing average globule diameter in successive samples during a milking is apparent from the histograms.

The existence of bimodal peaks in fat globule distributions has been previously indicated with respect to stage of lactation effects (van Dam & Sirks, 1922). However, we have generally assumed that the diameter of fat globules in samples taken throughout a milking follows a normal distribution pattern and on this basis have used the Coulter counter and standard statistical analyses.

The results obtained in this study agree with those of earlier investigators (Collier, 1891; Woods, 1905; Bitting, 1905; Campbell, 1932; Johansson, 1952), but are contrary to those of Whittlestone (1953, 1954, 1962). This would appear not to support the currently held theory explaining the rise in fat percentage that occurs during milking (Whittlestone, 1953) since one of the premises of the theory is that fat globule diameter does not significantly change throughout milking. His theory assumes that fat globules cluster within the alveolus and these clusters are partly filtered out by the ducts, so that the last milk to leave the alveolus is very rich in fat. Originally Whittlestone called this a modified filtration theory and stated it did not exclude the possibility of some filtering of larger globules. It is suggested that this filtering of large globules *per se* may contribute more to the rise of fat percentage during milking than has previously been thought.

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The action of rennet on whole milk

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Summary. The release, by the action of rennet, of peptides which are soluble in trichloroacetic acid (TCA) has been studied using milk samples from individual cows. This release showed an initial marked increase with time of rennet action. After the milk clotted there was no further increase for at least 30 min. There was considerable variation in the total amount of N and of sialic acid released from milk samples taken from individual animals at different times during the lactation.

For a given milk sample treated with rennet, the amount of sialic acid in the filtrate obtained after precipitation with 2 % TCA was similar to that obtained with 10 % TCA, whereas the amount of nitrogen was much greater with the 2 % TCA. It appeared that the peptides containing sialic acid were released at a slower rate.

On average, about half the total sialic acid in the casein was recovered in the TCA filtrate after rennet action, which suggests that either a proportion of the κ-casein was not accessible to enzyme action or the technique used did not permit full recovery of the sialic acid-containing peptides.

The clotting of milk takes place in at least 2 phases (Berridge, 1954). During the primary phase, peptides are released from κ-casein by the action of the enzyme rennin. This loss of peptides destroys the micelle stabilizing property of the κ-casein so that, in the presence of Ca^{2+} ions, the casein micelles aggregate to form a clot (Waugh & Von Hippel, 1956).

The primary stage has been examined by Nitschmann & Bohren (1955) who determined the increase in N soluble in 2 and 12 % TCA at intervals after the beginning of the enzyme action on casein or on whole milk. Alais (1963) also carried out extensive investigations on the release of N soluble in 12 % TCA after the action of rennin on whole milk, casein and κ-casein. He considered that the release of N represents the primary phase of rennin action.

In view of the importance of κ-casein in the coagulation of milk, there is considerable interest in the structure of the protein, and it has been established that κ-casein is heterogeneous. MacKinlay & Wake (1965) have shown that this heterogeneity is due partly to the fact that different amounts of carbohydrate are attached to a common amino acid skeleton. Virtually all the sialic acid present in κ-casein is attached to that part of the molecule which is released by the action of rennin (Gibbons & Cheeseman, 1962).

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In the present investigation the action of rennet on the whole milk of individual cows has been studied to obtain further information on the primary phase of the enzymic action and on the sialic acid and N content of the peptides released.

EXPERIMENTAL

Milk samples were obtained from individual cows of the Institute herd. For certain animals samples were obtained on several occasions during the lactation. Bacteriological tests showed that all the animals were free from bacterial infections of the udder at the time of the experiment.

A preparation of a solution of rennet (Chr. Hansen, Reading) was diluted to give a clotting time of 5–15 min in the following procedure. To 40 ml portions of whole milk in 200 ml flasks were added 10 ml rennet solution. The flasks were put to incubate at 37 °C and at intervals up to 1 h pairs of flasks were taken and their contents diluted with TCA to contain 2 and 10% TCA. The digests were filtered through no. 40 Whatman filter paper and the concentrations of N and of sialic acid in the filtrates determined.

The TCA also precipitated the milk proteins, and in order to determine the distribution of N and sialic acid between the whole milk and the non-casein fraction prepared according to the method of Aschaffenburg & Drewry (1959), this latter also was analysed for N and sialic acid.

N was determined by the Kjeldahl method. Sialic acid was determined by the method of Warren (1959). Before hydrolysis, the preparations were dialysed for 2 days at 4 °C against distilled water to remove lactose and TCA. Portions (1 ml) were then diluted with 0·2 ml of 0·1 N-H_2SO_4 and heated for 1 h at 80 °C.

RESULTS

Rate of release of peptides by the action of rennet

In all the experiments there was a rapid rise in the peptides and glycopeptides soluble in 10% TCA, determined as N and sialic acid, during the first few minutes after the addition of rennet. Then the rate of increase fell until shortly after clotting was first observed, when the concentration of peptides remained constant for at least 30 min. This finding is illustrated in Fig. 1.

Effect of TCA concentration on the solubility of peptides released by the action of rennet

Two of the experiments were repeated using 2% TCA to stop the reaction and precipitate the proteins. Figure 2 shows that the maximum increase in sialic acid in the 2% TCA filtrate was almost the same as that obtained with the 10% TCA filtrate. There was, however, a markedly greater increase in the N soluble in 2% TCA as compared with that soluble in 10% TCA.

Distribution of N and of sialic acid in the milk

In milk samples taken during the mid-lactation period there was considerable variation in the amount of N released by the action of rennet and soluble in 10%

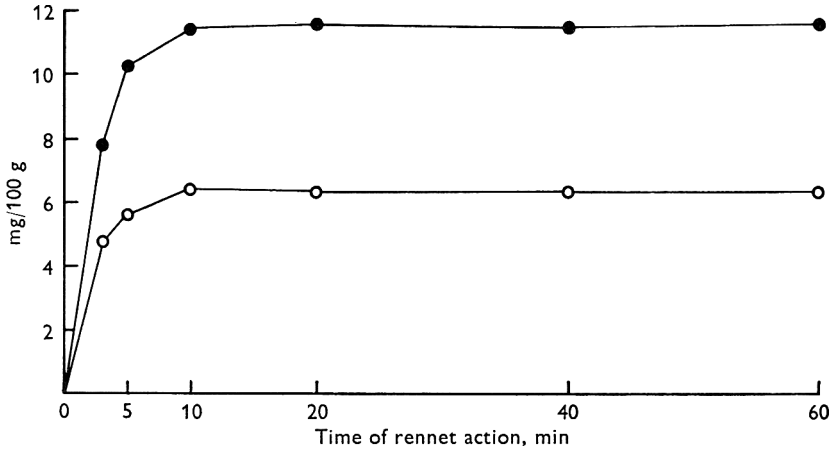

Fig. 1. The increase in the concentrations of N and of sialic acid, soluble in 10 % TCA, after the action of rennet. ●, N; ○, sialic acid.

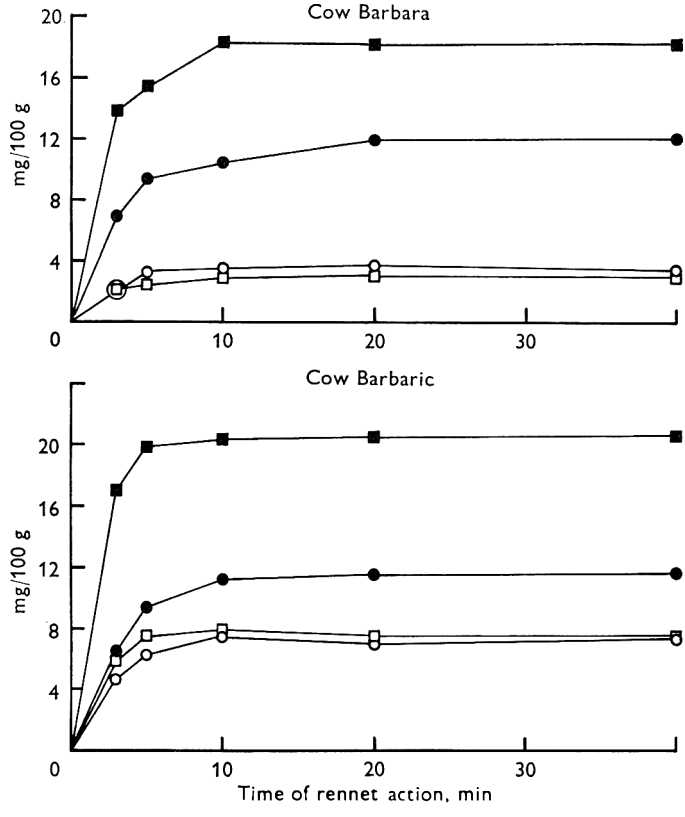

Fig. 2. The increase in the concentrations of N and of sialic acid, soluble in 10 % TCA and in 2 % TCA, after the action of rennet. ■, N, soluble in 2 % TCA; ●, N, soluble in 10 % TCA; □, sialic acid, soluble in 2 % TCA; ○, sialic acid, soluble in 10 % TCA.

TCA. In milk from cow 160, the N released varied from 4·8 to 11·6 mg/100 g milk. Values higher than those obtained during the mid-lactation period were found for milk samples from cows 204 and 160 taken just after parturition. An exceptionally high value of 21 mg N/100 g was observed for 1 sample from cow 204, and this did not appear to be related to any marked change in any of the other components measured in the experiment.

Table 1. *The distribution of N in the milk fractions examined*

(N, mg/100 g)

Cow	Days after parturition	Total	Non-casein N	N soluble in 10 % TCA	Casein N	N released by the action of rennin and soluble in 10 % TCA	% Casein N released by the action of rennin and soluble in 10 % TCA
323	155	464	103	29·7	361	11·5	3·2
	176	445	101	33·9	344	9·3	2·7
	186	444	104	31·8	340	10·0	2·9
324	164	425	103	28·7	322	8·6	2·7
	169	445	102	32·1	343	8·2	2·4
	175	450	108	39·2	342	8·3	2·4
	185	420	106	33·8	314	7·5	2·4
Georgina 14	155	—	—	24·4	—	8·2	—
Barbara	162	—	—	25·3	—	12·0	—
	176	—	—	28·6	—	12·0	—
Barbaric	133	—	—	15·4	—	11·6	—
204	2	642	172	37·3	470	13·9	3·0
	14	498	128	31·5	370	10·4	2·8
	79	559	134	27·5	425	21·9	5·2
	89	565	138	33·9	427	6·9	1·6
	97	528	141	36·4	387	7·9	2·0
	117	571	148	44·4	423	10·6	2·5
	131	541	138	44·0	403	8·4	2·1
160	3	659	211	38·8	448	14·8	3·3
	20	494	122	33·7	372	10·5	2·8
	94	566	136	40·5	430	4·8	1·1
	106	608	143	42·5	465	7·7	1·7
	122	561	147	44·0	414	11·6	2·8
	136	508	129	43·2	379	10·1	2·7
250	13	514	124	41·8	390	13·0	3·3
	129	510	127	42·3	383	11·0	2·9
	142	555	136	49·0	419	11·9	2·8
	156	521	119	39·0	402	9·9	2·5
161	26	455	118	35·4	337	9·1	2·7
197	45	496	118	36·0	378	10·3	2·7
	73	534	130	33·0	404	11·2	2·8

With the exception of the sample from cow 324, from which 2·4 % of the casein N was released in 3 of the 4 experiments, there was no consistent pattern in the proportion of the casein N released.

The sialic acid content of whole milk varied within individual cows. The values obtained for the 2 samples taken shortly after parturition from cows 204 and 160 were the highest recorded, and were about twice the average value obtained for later samples from the same animals.

DISCUSSION

In the present experiments, N and sialic acid soluble in 10 % TCA increased with time after the action of rennet on whole milk and finally reached a value which remained constant. Our results are similar to those obtained by Kim, Arima & Yasui (1967) who studied the effect of rennet on skim-milk, casein and κ-rich casein. The

Table 2. *The distribution of sialic acid in the milk fractions examined*

(Sialic acid, mg/100 g)

Cow	Days after parturition	Total sialic acid	Non-casein sialic acid	Sialic acid soluble in 10 % TCA	Casein sialic acid	Sialic acid released by the action of rennin and soluble in 10 % TCA	% Casein sialic acid released by the action of rennin and soluble in 10 % TCA
323	155	12·7	—	0·5	—	6·4	—
	176	13·9	2·6	0·5	11·4	5·7	50
	186	11·4	2·5	0·6	8·9	4·7	53
324	164	10·0	—	0·0	—	4·5	—
	169	10·9	2·4	0·3	7·5	4·3	57
	175	11·8	2·4	0·6	9·4	4·7	50
	185	9·1	2·5	0·5	6·5	3·2	49
Georgina 14	155	—	—	0·7	4·5	—	—
Barbara	162	—	—	0·4	—	4·8	—
	176	9·3	—	0·7	—	3·5	—
Barbaric	133	8·8	—	0·4	—	7·1	—
204	2	20·1	4·4	0·6	15·7	8·0	51
	14	9·9	2·1	0·3	7·8	4·6	59
	79	14·9	2·6	1·2	12·3	5·4	44
	89	11·0	2·6	1·1	8·3	3·2	39
	97	11·6	2·4	0·6	9·2	4·8	52
	117	12·8	2·6	1·2	10·2	5·0	49
	131	12·4	1·5	1·0	10·9	5·8	53
160	3	19·3	4·2	1·0	15·1	6·9	46
	20	7·2	2·0	0·4	5·3	2·5	47
	94	9·4	2·6	0·8	7·7	2·5	32
	102	8·8	1·9	0·7	6·9	3·0	43
	122	9·9	2·9	0·9	7·0	3·5	50
	136	10·6	1·2	0·5	9·4	4·2	45
250	13	13·3	2·0	0·4	11·3	5·1	45
	129	10·2	2·1	0·2	8·1	4·1	51
	142	13·2	3·2	0·8	10·0	5·3	53
	156	12·5	1·2	0·8	11·3	5·8	51
161	26	9·9	2·4	0·5	7·5	3·7	49
197	45	11·3	2·3	0·5	9·0	3·7	41
	73	9·6	2·5	0·6	7·1	3·4	48

results for N were also similar to those obtained using the purified enzyme rennin on skim-milk (Alais, 1963; Foltmann, 1959); on casein (Alais, 1963; Nitschmann & Bohren, 1955) and on κ-casein (Alais, 1963; Beeby, 1963).

The action of rennet in milk coagulation consists of a primary phase, in which peptides are split from κ-casein, and also a general proteolysis of milk proteins. The primary phase proceeds at a much faster rate than the general proteolysis (Alais,

Mocquot, Nitschmann & Zahler, 1953). It follows that the primary phase would give rise to a rapid increase in the peptides released, while there would be a more gradual increase with the general proteolysis (Nitschmann & Keller, 1955; Cerbulis, Custer & Zittle, 1959). In our experiments, the values for N remained constant for an appreciable period after the initial rise, which shows that general proteolysis was not being detected. Alternatively, under the conditions of the experiments, the rate of general proteolysis was so slow as to be negligible. The pepsin, which was probably present in the rennet preparation used, would also contribute to the primary phase (Cerbulis *et al.* 1959), but the peptides released would be the same as those released by rennin

Table 3. *The rate of release, by the action of rennet, of peptides from whole milk*

Time of rennet action, min	Increase in peptides containing sialic acid, mg N/100 g milk*	Increase in peptides which lack sialic acid, mg N/100 g milk†
	Cow Barbara	
3	6·9	6·8
5	9·3	6·1
10	10·4	7·8
20	11·9	6·2
40	12·0	6·2
	Cow Barbaric	
3	6·5	10·5
5	9·4	10·4
10	11·2	9·1
20	11·5	9·0
40	11·5	9·0

* Values represent the increase in the concentration of N soluble in the 10 % TCA after the action of rennet.

† Values were obtained by subtracting the increase in the concentration of N soluble in 10 % TCA from the corresponding increase in the concentration of N soluble in 2 % TCA after the action of rennet.

(Haberman, Mattenheimer, Sky-Peck & Singhara, 1961). In view of these considerations, and of the fact that similar results were obtained by the action of rennin on κ-casein, casein and skim-milk, it is probable that the peptides and glycopeptides detected in 10 % TCA in the present experiments were the same as those released from isolated κ-casein by rennin.

The experiments of MacKinlay & Wake (1965) have shown that the heterogeneity of κ-casein is due partly to differences in the amount of carbohydrate attached to the κ-casein molecule. It has been demonstrated that most of the carbohydrate present in the κ-casein is attached to the peptides released by rennin action (Gibbons & Cheeseman, 1962). MacKinlay & Wake (1965) concluded that the solubility of these peptides in TCA is a function of the carbohydrate content, so that the carbohydrate-rich peptides are soluble in both 2 and 12 % TCA, while those which contain no carbohydrate are soluble in 2 % TCA but not in 12 % TCA. Our results are wholly in agreement with these conclusions. Thus, the increase in N in the 10 % TCA would be due to the release of peptides which contained sialic acid (Alais, Blondell-Quéroix & Jollès, 1964). The peptides which contained sialic acid would also be soluble in 2 % TCA, as is shown by the similar increase in sialic acid in both 10 and 2 % TCA

after rennet action (Fig. 2). The greater increase in the N after rennet action in 2% TCA as compared with 10% TCA would therefore be due to the solubility of other peptides not containing sialic acid.

Our results show that the amount of N and of sialic acid released from casein varied considerably throughout lactation for an individual animal. There did not appear to be any consistent pattern for this variation except that in the 2 samples obtained within 3 days of parturition the values for the sialic acid released were markedly higher than in the samples taken later in lactation. The concentration of sialic acid in casein varied between 0·20 and 0·60%—values which approximate to those obtained by Marier, Tessier & Rose (1963). Our values may be slightly underestimated since Cheeseman (1966) has shown that some of the casein sialic acid is not precipitated at pH 4·6. Since the proportion of casein sialic acid released by rennet was found by us to be about 50%, the variations in the amount of sialic acid released would be largely a reflexion of variations in the casein sialic acid. Malpress & Hytten (1964) studied the action of rennin on human casein and obtained similar values for the proportion of casein sialic acid released by the enzyme in samples taken in mid-lactation. Experiments performed by Kim *et al.* (1967) showed that only 37% of the sialic acid was released from bovine casein by the action of rennet.

It has been shown that the sialic acid attached to κ-casein is not essential for rennin action (Gibbons & Cheeseman, 1962) or for stabilization of casein micelles (MacKinlay & Wake, 1965). However, the possibility that sialic acid may affect the rate of rennin action does not appear to have been examined. Table 3 shows that there was no increase in the peptides, which lacked sialic acid, after 3 min of rennet action, but that the peptides which did contain sialic acid continued to be released for 10 min. This evidence suggests that the peptides which did not contain sialic acid were released at a faster rate than those which did. It follows therefore that the sialic acid or other carbohydrates normally associated with sialic acid in casein may be responsible for retarding the rate of the rennet action. If this is so, then the wide variation in casein sialic acid within animals and between animals may contribute to differences in clotting times of different milks. This conclusion is in line with the findings of Verpoorte, Green & Kay (1965), who showed that removal of sialic acid causes a marked increase in the rate of the proteolytic action of trypsin on fetuin.

If the increase in sialic acid and N following the action of rennet is entirely due to sialic acid-containing peptides, it should be possible to estimate the number of residues of sialic acid/molecule in the peptides released from κ-casein. Since all sialic acid of the κ-casein is present in the peptide released by rennin (Gibbons & Cheeseman, 1962; Beeby, 1963) this value will be equivalent to the number of molecules of sialic acid/molecule of κ-casein. Taking 7500 for the molecular weight of the peptide (Nitschmann, Wissman & Henzi, 1957), values varying between 1 and 3 are obtained for the molecular ratio of sialic acid/peptide. Deviations from whole numbers may be due to a combination of experimental error and of the assumptions involved in the calculations. Alternatively, the deviations may indicate that the peptides released are a mixture containing 1, 2, 3 or more molecules of sialic acid in varying proportions, as observed for bovine transferrin (Chen & Sutton, 1967).

It is noteworthy that the lowest values obtained were close to unity since it would

be expected that at least 1 residue of sialic acid would be required for the peptide to be soluble in 10% TCA.

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The influence of the milk ejection reflex on the flow rate during the milking of ewes

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Summary. In order to obtain more information on the importance of the milk ejection reflex in the lactation of normal ewes, experiments were conducted to determine whether the neuroendocrine reflex is able to modify the parameters which characterize the flow rate of the milk under normal milking conditions.

Ewes were allowed to suckle their lambs freely during the 3 days following parturition. They were then milked with a machine and the individual milk production was measured volumetrically at each milking. Different fractions obtained during milking ('machine milk', 'machine strippings' and 'hand strippings'), and the milk flow, were measured using a sensitive recording system. In further experiments, machine milking was performed after intravenous administration of oxytocin, during general anaesthesia, and after unilateral or bilateral denervation of the mammary gland.

The ewes fell into 2 categories: those giving most of their milk in 1 rapid emission, and those giving it in 2 quite separate emissions. The results indicated that the second emission found in some of the ewes was the milk emptied from the acini by the neuroendocrine ejection reflex. Disappearance of the second emission resulted from the administration of oxytocin before milking—which induces passage of the milk from the acini into the mammary cistern—and also from general anaesthesia or denervation of the mammary gland.

The role of the neuroendocrine ejection reflex during the milking of the ewe and the goat is still incompletely understood (Denamur, 1965). The reflex disappears after section of the mammary nerves (Denamur & Martinet, 1954, 1959*a*, *c*, 1960, 1961), of the spinal cord (Tverskoĭ, 1958; Denamur & Martinet, 1959*b*, *c*), of the pituitary stalk (Tverskoĭ, 1960), and following spinal medullectomy (Denamur & Martinet, 1959*b*), ectopic graft of the mammary gland (Linzell, 1963), and during general anaesthesia (Yokoyama & Ôta, 1965); but, nevertheless, under all these conditions there persists an abundant secretion of milk. The existence of the milk ejection reflex can easily be demonstrated, however, in both species (see Denamur, 1965), but the importance of its participation in the lactation of normal animals remains to be ascertained.

In order to obtain more information on this last point, we have conducted experiments on the ewe to determine whether the neuroendocrine reflex can modify the parameters which determine, under normal milking conditions, the flow rate of the

milk from the mammary gland. There have been few studies on the measurement of the milk flow during the milking of ewes. Ricordeau, Martinet & Denamur (1963) observed an increased flow 30–45 sec after the application of the teat cups. It was assumed without proof that this flow of milk (which, depending on the stage of lactation, represented 10–30% of the total amount of milk obtained) corresponded to the neuroendocrine ejection reflex, triggered by application of the cups of the milking machine. Labussière & Martinet (1964) and Labussière (1966), using a more sensitive recording system than the previous authors, noticed that ewes milked without preliminary massage of the mammary gland could be divided into 2 categories, those which gave most of their milk in 1 rapid emission and those which gave it in 2 quite separate emissions. The following experiments performed on ewes lead us to presume that it is during the second milk emission that the milk is emptied from the acini by a neuroendocrine reflex. Oxytocin administered before milking induced passage of the milk from the acini into the mammary cistern, and general anaesthesia during milking, or chronic denervation of the mammary gland, led to the disappearance of the second emission.

MATERIAL AND METHODS

Care of the flock and milking technique

The ewes suckled their lambs freely for 3 days after parturition. On the fourth day they were separated from their offspring and were then milked twice daily at 06.00 and 16·15 h, on a 32-place 'carrousel' with an Alfa–Laval machine, the essential characteristics of which were as follows: vacuum 33 cmHg; pulsation speed 180/min; vacuum massage ratio 1/1. Teat cups of average weight 240 g were applied without previous massage of the udder. They were removed approximately 3 min later, machine stripping being carried out before removal (the length of machine milking is effectively less than 2 min in 86% of ewes—Ricordeau *et al.* 1963). Finally, a more complete milk extraction was assured by hand stripping.

The individual milk yield was measured volumetrically at each milking.

Measurement of the milk flow

The equipment used to measure the milk flow was as described by Labussière & Martinet (1964). The flow from right and left mammary glands was measured separately and simultaneously. The different fractions obtained during the milking were defined as follows:

Machine milk (ml). The volume of milk obtained by machine alone. This portion included, depending on the animal, either 1 fraction (V 1) or 2 fractions (V 1 + V 2) (see Fig. 1). The fraction V 1 always refers to the volume collected immediately after application of the cups, without taking into consideration the filling time of the tubes (approximately 8 sec). We have assumed that this fraction corresponds to the cisternal milk (V 1). Machine milking was stopped when the milk flow dropped below 5 ml/4 sec.

Machine stripping (ml). The volume of milk obtained by machine aided by manual massage, subsequent to the main flow of machine milk.

Hand stripping (ml). The volume of milk obtained by hand milking after removal of the teat cups.

Other definitions are summarized in Fig. 1.

Milking after the intravenous administration of oxytocin

Four Prealpine ewes were selected showing 2 peaks in the milk flow curve. They were given on alternate days, 1 min before application of the teat cups, at the morning milking, 1 i.u. oxytocin (Syntocinon, Sandoz). Each animal received 5 in-

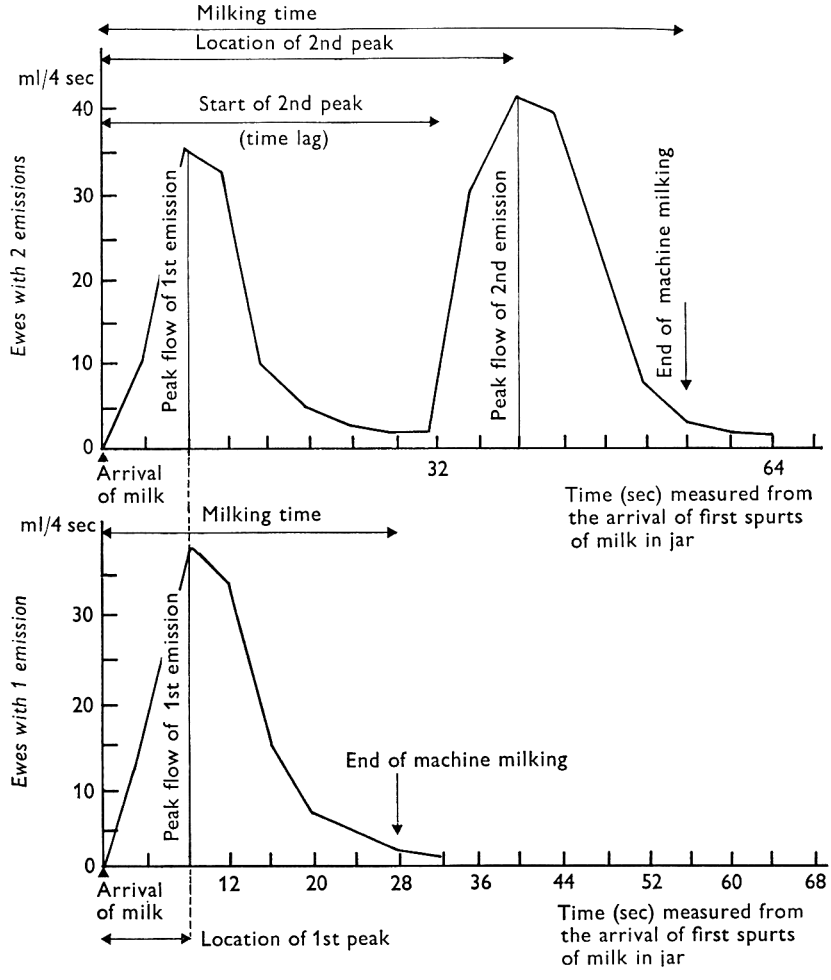

Fig. 1. Milk flow curves of animals exhibiting 1 or 2 peaks.

jections in all, between the 110th and the 128th day of lactation. For the intravenous injections a polyethylene catheter (Clay Adams Inc., type PE 200; internal diam. 1·40 mm, external diam. 1·90 mm) was placed permanently in the jugular vein. To keep the catheter patent it was filled 2 or 3 times a day with heparin solution (0·05 %).

The milk flow of the 4 animals was recorded twice daily, morning and evening, between the 110th and the 128th day of lactation.

Milking during general anaesthesia

The experiment conducted on 22 Prealpine ewes included 2 successive periods during which the pattern of milk release was recorded in all the animals.

The first period, from the 30th to the 34th day of lactation, allowed a classification of the ewes into animals exhibiting either 1 or 2 peaks in the milk flow curve (17 with 2 peaks, 5 with 1 peak), and an evaluation of the respective importance of the milk evacuation parameters.

In the second period, from the 35th to the 55th day of lactation, on every fourth day before morning milking the animals were anaesthetized by intravenous injection of 20 ml of a mixture containing 5 ml sodium pentobarbitone (Nembutal; Abbot Labs. Ltd) and 45 ml 2% sodium thiopentone (Pentothal; Abbot Labs. Ltd).

Ewes normally exhibiting 2 peaks in the milk flow curves were anaesthetized in the milking room immediately before the experimental milking. Those with 1 peak were placed the night before in an unfamiliar pen where they were anaesthetized the next morning in the same manner as the other ewes.

Immediately after the induction of anaesthesia the ewes were placed in an apparatus which maintained them in a normal position and permitted free access to their udders. After machine milking and machine and hand stripping, the milker injected 2 i.u. of oxytocin into the jugular vein, thereby obtaining a second hand stripping.

Milking after unilateral or bilateral denervation of the mammary gland

The surgical techniques used for this experiment have been described by Denamur & Martinet (1959*c*).

Mammary denervation during pregnancy

The surgical process involved the excision and then the reattachment of the mammary gland. Bilateral denervations were performed in 2 stages, on 7 Prealpine ewes (in second, third and fourth lactations) which had shown 2 peaks in their flow curve during the milkings in the preceding year.

The first operation was on the left half shortly after the 100th day of pregnancy, and the second on the right half, 2 weeks later and about 3 weeks before parturition. Four ewes underwent only a unilateral denervation.

Mammary denervation during lactation

This was carried out by severing the first 4 pairs of lumbar nerves, the perineal nerve, and the lumbar sympathetic system.

Twenty-three Prealpine ewes (second, third, fourth lactations) were operated on bilaterally. The first operation took place on the right side immediately after the morning milking of the 35th day of lactation. The denervation of the left mammary gland was performed 2 weeks later. Seven Prealpine × East Friesian ewes were unilaterally denervated.

Experimental checks

(1) *Absence of mammary sensitivity.* Pain-producing stimuli were applied on the surface and in depth to the udders of all the animals, using a needle. If the stimuli

were perceived, the denervation was considered incomplete. Examinations, repeated several times during the milking, eliminated animals that revealed even a very localized sensitivity to pain.

(2) *The milk flow.* The kinetics of the milk flow were measured morning and evening during 5 consecutive days, at 3 periods during lactation: from the 30th to the 34th day; from the 44th to the 48th day, and from the 58th to the 62nd day.

For the ewes whose mammary gland was denervated during lactation, the 3 check periods furnished characteristic milking parameters for the period before denervation then after unilateral and, finally, after bilateral denervation.

(3) *Fat content of the milk.* Eight days after the first denervation performed during lactation (35th day), the successive milk fractions obtained during milking, as well as the residual milk obtained by injection of 2 i.u. oxytocin into the jugular vein, were collected separately from each half of the mammary gland by suitable manipulation of the taps.

The fat content of each fraction was determined by the Gerber method.

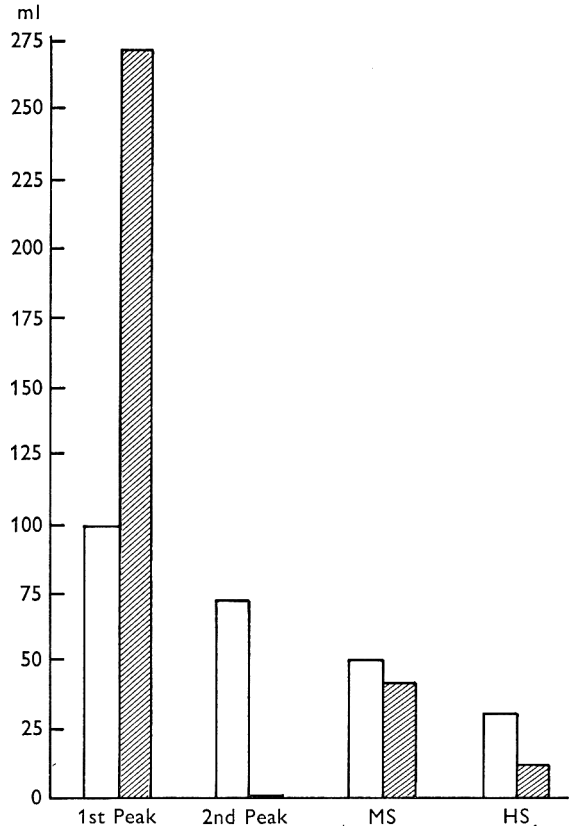

Fig. 2. Modification of the milk distribution (percentage) following an intravenous injection of 1 i.u. of oxytocin preceding the application of the teat cups. □, Normal distribution; ▨, distribution following oxytocin injection. MS = machine stripping; HS = hand stripping.

RESULTS

Effect of oxytocin administered before milking

The effects of the intravenous injection of oxytocin shortly before placing of the teat cups, are shown in Fig. 2. In all cases, the second peak (V2) disappeared in favour of the first, which was noticeably increased, whereas the hand-stripping fraction was slightly reduced.

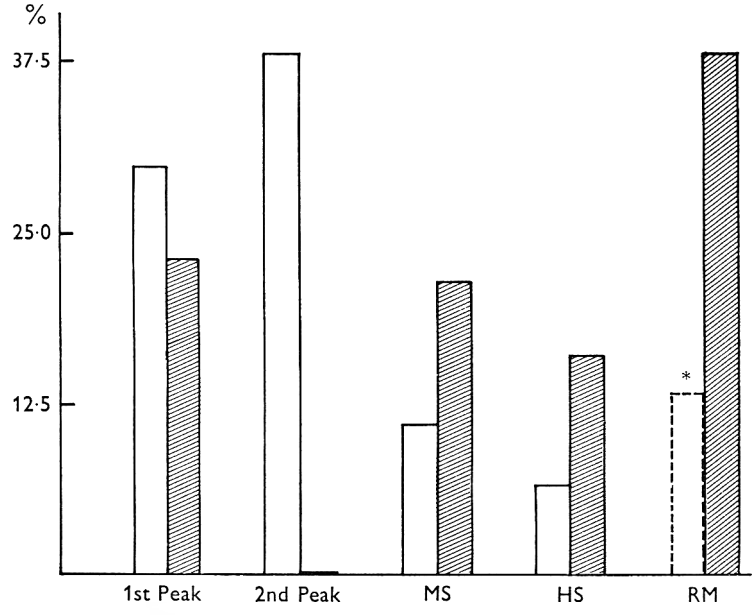

Fig. 3. Milking during general anaesthesia in ewes showing 2 peaks. Effects of milk distribution: □, without anaesthesia; ▨, with anaesthesia. MS = machine stripping; HS = hand stripping; RM = residual milk. * Estimated RM.

Effect of general anaesthesia

General anaesthesia administered on 75 occasions to the 17 animals with 2 peaks caused a disappearance of the second peak in all animals and an appreciable milk retention in the alveolar regions of the udder. The volumetric increase of the machine stripping, of the hand stripping, and of the residual milk which thus resulted is shown in Fig. 3.

On the other hand, general anaesthesia administered on 27 occasions to 5 animals exhibiting 1 peak did not affect the distribution of the milk during the milking. The cisternal milk (V1) represented 56·02 % of the total amount of milk in normal ewes and 55 % in the anaesthetized ewes.

Effect of bilateral denervation of the mammary gland

The qualitative results of the different denervation procedures are summarized in Table 1.

Thus, the second emission was absent in all the ewes with insensitive mammary glands, whereas it was present in ewes where partial sensitivity had persisted. It is

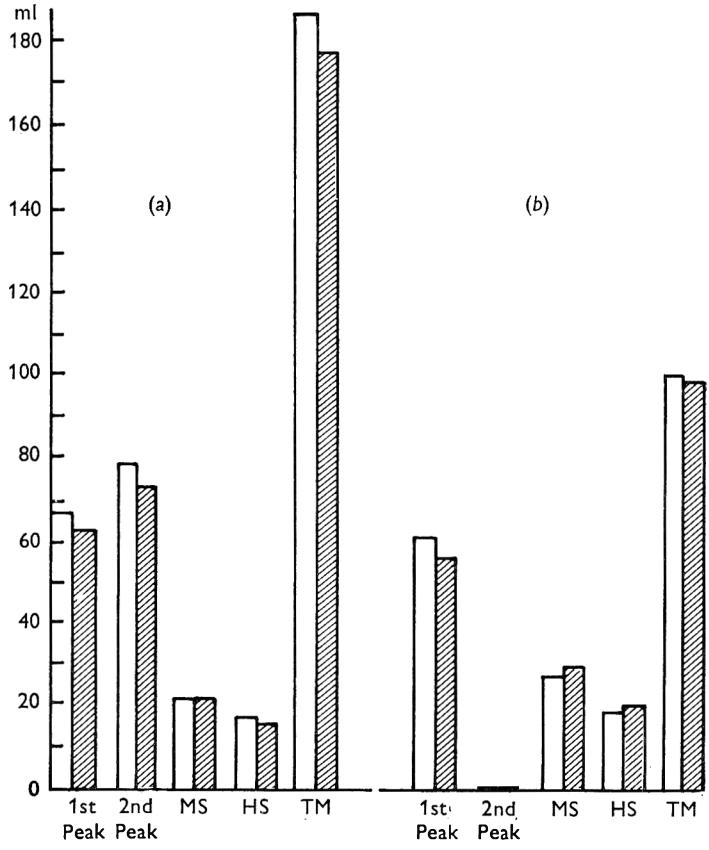

Fig. 4. Effects of bilateral denervation of the mammary gland on the milk distribution. (*a*) Check test (normal ewes) 30th, 34th days of lactation. (*b*) Test after bilateral denervation (58th, 62nd days). □, Left half; ▨, right half. MS = machine stripping; HS = hand stripping; TM = total milk.

Table 1. *Influence of bilateral denervation of the mammary gland on the milk flow*

Denervation	No. of animals operated on	Mammary sensitivity test	No. of animals still presenting a second peak
Bilateral (during gestation)	7	Insensitive in all	0
Bilateral (during lactation)	23	6 remained sensitive 17 were insensitive	6 0

Table 2. *Influence of unilateral denervation of the mammary gland on the milk flow*

Denervation	No. of animals operated on	Sensitivity test	No. of animals still presenting a second peak
Unilateral (during gestation)	4	Insensitive on the operated side	4
Unilateral (during lactation)	7	Insensitive on the operated side	7

possible that in these latter animals, the fifth pair of lumbar nerves may have contributed to the innervation of the mammary gland (Linzell, 1959).

The quantitative results of bilateral denervation during the lactation period are presented in Fig. 4. It may be noted that the disappearance of the second peak resulted in a simultaneous increase in the machine- and hand-stripping fractions.

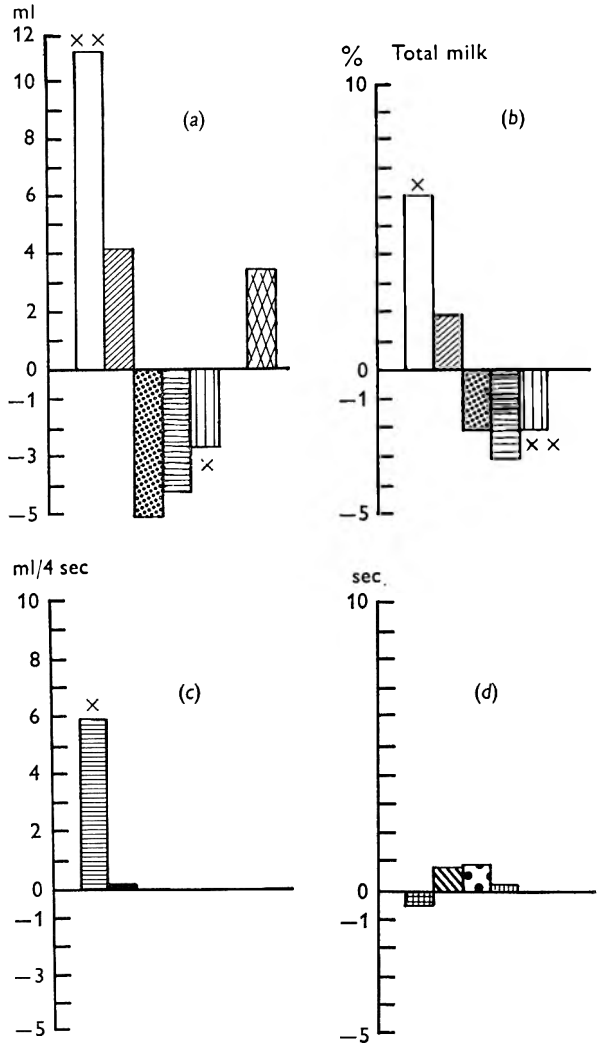

Fig. 5. Effects of unilateral denervation of the mammary gland on: (*a*) actual volume (in ml) of the different samples and (*b*) relative volume (in % of total milk) of the different samples. ▢, First peak; ▨, second peak; ⊞, machine strippings; ⊟, hand strippings; ▥, residual milk; ▩, total milk. (*c*) The peak flow: ▤, first peak; ■, second peak. (*d*) The sequence of principal events occurring during the milking period (time in sec). ⊞, Location of first peak; ▧, time lag; ⊡, location of second peak; ▥, milking time. The results are expressed in relation to those for the normal half of the udder which are considered as a control check. ×, Significant difference in relation to the intact side; × ×, highly significant difference in relation to the intact side.

Effect of unilateral denervation

In the case of unilateral denervation the second peak persisted in the insensitive half of the mammary gland when the machine cups were applied simultaneously on both teats (see Table 2).

Nevertheless, unilateral denervation resulted in a slightly different distribution of the milk fractions recorded during the milking of the denervated half of the udder.

The absolute or relative volume of the first peak fraction (V1) was always increased, especially in the morning when the result was highly significant. This increase in the cisternal milk of the denervated mammary gland without significant modification of the level of secretion was accompanied by a decrease in the amount of residual milk, both absolute and relative (see Fig. 5(*a*) (*b*)). This last observation

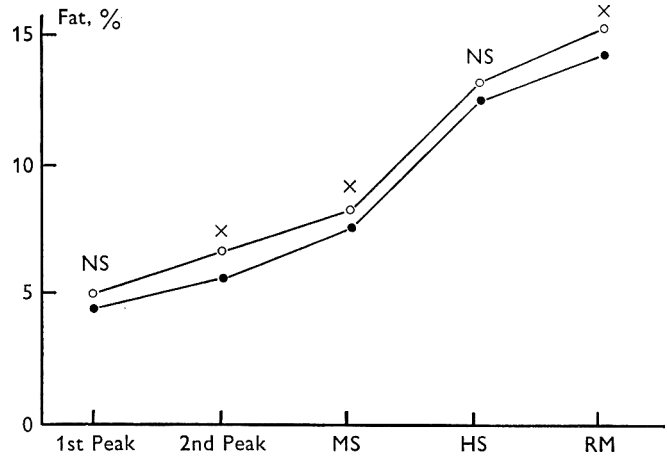

Fig. 6. The effect of unilateral denervation of the mammary gland on the fat content of the different fractions collected during milking. ○—○, denervated half; ●—●. normal half. NS, No significant difference; ×, significant difference. MS = machine stripping; HS = hand stripping; RM = residual milk.

indicates that the transfer of milk from the alveolar regions of the udder towards the cistern, between milkings, is facilitated by denervation. Furthermore, mammary denervation never decreased the fat content, but rather enriched all the fractions analysed (Fig. 6).

DISCUSSION

The experiments described in this study confirm several conclusions already reached concerning ewes. First, total denervation of the mammary gland permits an abundant lactation in ewes as Denamur & Martinet (1954, 1959*c*, 1960) have previously shown. Thus, nervous impulses are not essential to secretion, nor to the emptying of the udder. Secondly, the secretion of fat is not appreciably modified by denervation. There was even a tendency toward an increase in the fat content of the milk secreted by the denervated mammary gland (see unilateral denervation experiment). These findings agree with those obtained for the goat by Denamur & Martinet (1959*a*, *c*), Linzell (1963), Ward & Huskisson (1966) and Tverskoĭ (1962, 1966*a*–*c*). They are at variance with those of Zaks (1962), who presupposes liposecretory reflexes in the goat.

The sensitivity of the denervated mammary gland to oxytocin was little changed, or even slightly increased (see experiment with unilateral denervation). The volume of the second peak fraction, and the time lag between the application of teat cups and its appearance, were not significantly changed. These results confirm the observations of Martinet & Denamur (1960) obtained after the injection of oxytocin into ewes with denervated mammary glands. Finally, the increase in fraction V 1 observed in the denervated side probably indicates a facilitated milk flow from alveoli to larger ducts and cistern between milkings, perhaps due to the modification of the tonus of the supporting tissues.

Our experiments also contribute original information concerning the origins of the different milk fractions obtained during machine milking. A second peak in the flow curve necessitates first the anatomical and functional integrity of the nervous system, since the denervation of the mammary gland as well as a general anaesthesia caused its disappearance, and secondly, the participation of a humoral link since the second peak persisted in the denervated side when the normal laterally opposite side was milked. The humoral link is probably represented by oxytocin, for if this hormone was administered before milking, it caused the second peak fraction to shift to the cisternal fraction V 1.

These findings generally confirm the hypothesis set forth by Ricordeau *et al.* (1963). The second milk peak seems to correspond to the expulsion of the milk contained in the acini through the effect of oxytocin, which constitutes the hormonal link of the milk-ejection reflex (for a discussion on the respective roles of oxytocin and vasopressin see Denamur, 1965). The reflex was triggered in our experimental conditions by the application of the teat cups. However, mammary massage before milking seemed also to represent an effective stimulus, since it eliminated the second peak fraction which combines with the cisternal fraction (Martinet, Labussière & Richard, 1963).

Animals with only one milk flow peak (V 1) probably do not have a reflex reaction to the application of the cups. Nevertheless, we cannot ignore the 2 following hypotheses:

First, in such animals, the ejection reflex could be conditioned by the environment of the milking parlour before the application of the teat cups thus inducing the passage of the alveolar milk into the mammary cistern. This hypothesis can be rejected, however, since general anaesthesia, induced before milking, did not modify the distribution of milk between the different fractions (see p. 196) nor did it diminish the volume of cisternal milk (V 1). In addition, there was no significant difference in the volume of cisternal milk between animals exhibiting 1 or 2 flow peaks (Labussière, 1966).

Secondly, it is possible that ewes showing 1 flow peak differ from those showing 2 peaks, not in the absence of the milk ejection reflex, but rather in a reduced sensitivity of their myoepithelial cells to oxytocin. Indeed, Martinet & Denamur (1960) have shown that the sensitivity threshold of the myoepithelial cells is quite high in the ewe.

The hypothesis that the myoepithelial cells of ewes with 1 peak have a different sensitivity to oxytocin from those with 2 peaks has, however, not been confirmed in recent *in vivo* and *in vitro* studies on mammary strips (Labussière & Martinet, unpublished).

In conclusion, under usual machine milking conditions and with no mammary massage before application of the teat cups, a certain proportion of ewes, which varies according to the length of maternal suckling, do not show a second peak in the flow curve and therefore exhibit no milk ejection reflex. This agrees with the findings of previous studies which showed that the reflex is not essential to the course of lactation in the ewe. When the reflex does occur, the milk obtained by its intervention represents 10–35 % of the total milk. Moreover, Labussière (1966) showed that ewes exhibiting 2 peaks had a total milk production 25–30 % higher than those having only 1 peak in the flow. The latter had a higher proportion of machine strippings, of hand strippings and of residual milk.

Our results confirm a positive effect of the milk-ejection reflex on facility of milking and on lactation level in the ewe. They also confirm the galactopoietic effects of repeated injections of oxytocin in this species (Denamur & Martinet, 1961; Martinet & Denamur, unpublished; Morag & Fox, 1966).

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The flavour of milk protein

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Summary. The flavour stability of casein and co-precipitated milk proteins has been examined organoleptically. Differences in flavour stability were correlated with processing conditions and the early stages of the non-enzymic browning reaction were implicated in the off-flavour development. The 'gluey' flavour present in stored casein could be removed and the flavour stability improved by treatment with activated carbon or Sephadex. However, a better solution to the problem was to avoid the development of off-flavour by careful choice of conditions of preparation, drying and storage to minimize heat damage and browning. Conditions were established under which milk protein could be expected to have flavour stability during at least 6 months storage at ambient conditions.

Casein is one of the most nutritionally complete proteins as well as being one of the cheapest animal proteins. It has a wide potential use in foodstuffs owing to this high nutritional value and also to its emulsifying and stabilizing properties. Casein is used, for instance, in hamburger and sausage meat, breakfast cereals, dessert mixes and toppings, coffee whiteners, concentrated baby and invalid foods, and in low calorie diets. Unfortunately, such uses are restricted at present by an unpleasant gluey or 'stale' off-flavour and the major consumption of casein is not in the food industry but in paper coating, in the preparation of adhesives, and in the production of artificial fibres.

Cayen & Baker (1963) showed that a fairly tasteless sodium caseinate could be prepared from a well-washed freshly precipitated wet curd by freeze-drying or tunnel-drying. In industrial practice, fresh casein is usually bland in flavour but acquires the gluey off-flavour on storage. Several patented processes claim improved flavour characteristics, including the preparation of calcium caseinate following a hot-water wash stage (Srinivasan, 1961), partial enzymic degradation (Wingerd, 1962) or oxidation by iron and ascorbic acid (Wingerd, Bauer & Damisch, 1962). These processes are effective to some degree on the laboratory scale but none has been accepted commercially—presumably because they are time-consuming or expensive or both.

Although there are few references in the literature to gluey off-flavour in casein, several authors have recognized a similar flavour—'stale', 'gluey' or 'burnt feathers'—in dried skim-milk powder (cf. Henry, Kon, Lea & White, 1948) and in dried whole-milk powder (Supplee, 1923; Tillmans & Strohecker, 1924; Coulter, Jenness & Geddes, 1951). This off-flavour was believed to be associated with protein breakdown, especially when it was associated with storage at high moisture levels. Coulter *et al.*

(1951) concluded that the typical off-flavour of dried whole-milk powder was a composite of 2 distinct flavours one of which arose from oxidation of lipids and the other from the non-enzymic browning reaction; in high moisture conditions a burnt feather (gluey) flavour predominated. Radema (1954) found that tallowy flavours in stored whole-milk powder were associated with low moisture and high peroxide values whereas gluey flavours were correlated with high moisture, low peroxide value and high ferricyanide reducing power.

Buchanan, Snow & Hayes (1965) showed that casein and whey proteins could be co-precipitated by heat in the presence of added calcium ions, and Muller, Hayes & Snow (1967) prepared co-precipitates of varying calcium content (high 2·5–3·0 % Ca, medium about 1·5 % Ca, low 0·5–0·8 % Ca). Early consumer trials using high calcium co-precipitate suggested that it may have better flavour stability than casein. Laboratory ageing trials on casein and the 3 types of co-precipitate, all obtained from different factories, were inconclusive and differences could be explained most readily by variation in lactose content or heat damage by varying factory procedures.

For the present study, casein and co-precipitates were prepared on a pilot-scale under as nearly identical conditions as possible, and their flavour stability examined using accelerated ageing trials and evaluated as a function of lactose content, calcium content and acidity (all of which may influence the browning reaction) and of colour development and ferricyanide reducing value, which are measures of the extent of the browning reaction (Part I). A further examination was then made of the effect of processing conditions on the reducing value and flavour of co-precipitated milk proteins (Part II). Finally, methods of removing gluey off-flavour from casein and for preparing casein of stable flavour were evaluated.

PART I. COMPARISON OF THE FLAVOUR STABILITY OF CASEIN AND CO-PRECIPITATED MILK PROTEIN

Experimental

Casein and co-precipitate. These were prepared using the plant and methods described by Muller *et al.* (1967) and dried in a fluidized bed using hot air at an inlet temperature of 50 or 70 °C.

Lactose. This was determined by the method of Lawrence (1968).

Calcium content. This was determined by the method of Sawyer & Hayes (1961).

Colour differences. These were determined by measuring colour intensity before and after heating for 5 h at 102 °C. An 'Agtron' model 'F22 Green' (Agtron CB, Monterey, California, U.S.A.) was used to compare reflectance of the sample in the 546 nm region with that of black and white standards and express it on a 0–100 scale.

Ferricyanide reducing values (FRV). The test protein (0·5 g) was made up to 30 ml with distilled water, 3 ml 5 % (v/w) acetic acid added and the procedure of Choi, Koncus, Cherrey & Remaley (1953) used to determine the FRV expressed in mg potassium ferricyanide/g protein.

Accelerated storage test. The sample was heated in an open container in an air oven at 80 °C saturated with water vapour, stored for 24 h in a closed container under refrigeration, then evaluated organoleptically at room temperature.

Organoleptic evaluation. One per cent solutions at pH 7 were prepared by dissolving the samples in water by addition of NaOH, and adding about 1 % sodium tripolyphosphate in the case of the calcium co-precipitates. A laboratory panel of 8–10 judges scored the flavour of solutions using a scale 0–3 (0, clean flavour; 1, just detectable gluey flavour; 2, distinct gluey flavour; 3, strong gluey flavour). Two labelled controls (clean and gluey) were available and were also presented unlabelled with the samples. The results were evaluated by calculating the least significant difference at the 5 % level. Samples were rated gluey if their score exceeded the score of the unlabelled control by this difference.

Results

Samples of the 4 types of milk protein (casein, high calcium co-precipitate, medium calcium co-precipitate and low calcium co-precipitate) were prepared from the same milk with 2 levels of washing and dried with hot air at 2 inlet temperatures. The flavour stability of each sample was examined using the accelerated storage test, and assessed in relation to the differing experimental variables and analytical data. The results are shown in Table 1.

Table 1. *Analytical data and flavour stability in milk protein*

Sample no.	Type	No. of washes	Drying temp., °C	Ca^{2+} content, mg/g	Lactose content, %	Colour difference, 5 h at 102 °C	Flavour after heating at 80° C for 0 h	20 h	40 h	FRV, mg/g
362 E	Casein	3	50	0·62	0·22	4·1	N	N	N	0·51
F	Casein	3	70	0·59	0·14	4·1	N	N	N	0·54
G	Casein	5	50	0·24	0·06	2·1	N	N	N	0·47
H	Casein	5	70	0·30	0·07	1·4	N	N	N	0·50
361 A	Hi Ca	3	50	28·5	0·85	11·3	N	G	G	0·71
B	Hi Ca	3	70	29·0	0·61	9·7	N	G	G	0·71
C	Hi Ca	5	50	26·6	0·07	4·4	N	N	N	0·33
D	Hi Ca	5	70	26·5	0·04	3·1	N	N	N	0·30
362 A	Med Ca	3	50	15·6	0·85	12·9	N	G	G	1·14
B	Med Ca	3	70	15·6	0·85	13·7	N	G	G	0·95
C	Med Ca	5	50	13·0	0·27	7·3	N	N	G	0·89
D	Med Ca	5	70	13·1	0·16	6·4	N	N	G	0·89
361 E	Lo Ca	3	50	3·8	0·59	13·1	N	G	G	1·19
F	Lo Ca	3	70	4·6	0·62	12·4	N	G	G	1·25
G	Lo Ca	5	50	2·9	0·22	7·5	N	G	G	0·99
H	Lo Ca	5	70	2·6	0·16	7·5	N	N	G	0·96

N = not gluey; G = gluey.

Effect of drying temperature. Comparison of pairs of samples dried at different temperatures showed no difference in flavour. However, it must be remembered that these drying conditions (fluidized bed with inlet air at 50 or 70 °C) were not as severe as those encountered in normal commercial practice.

Effect of Ca content. Simon, Wagner, Silveira & Hendel (1955) reported that calcium at a level of 8·4 mg/g inhibits the browning of dried potato flakes. There was little evidence of any effect of Ca content on flavour stability (Table 1), but some slight suggestion (cf. 361 A *vs.* 362 B; 361 B *vs.* 361 E or 361 F) that, at similar lactose levels, a higher calcium level allowed less colour development on heating. Several further samples

were prepared by addition of aqueous $CaCl_2$ and redrying but none showed significant variation in flavour stability.

Effect of acidity. The pH value of a 10 % aqueous slurry of each of the 4 samples of each type of dried protein was constant. The values were: casein, 4·15; high calcium co-precipitate (Hi Ca), 6·60; medium calcium co-precipitate (Med Ca), 5·90; low calcium co-precipitate (Lo Ca), 5·20, but there was no obvious correlation between acidity and flavour stability.

Table 2. *Lactose level and flavour stability in milk protein*

Sample no.	Type	Lactose, %	Colour difference, 5 h at 102 °C.	Flavour after heating at 80 °C for: 0	20 h	40 h	FRV, mg/g
362 H 1	Casein	0·084	4·2	N	N	N	0·41
2	Casein	0·161	5·5	N	N	N	0·36
4	Casein	0·314	9·0	N	N	G	0·39
10	Casein	0·880	17·4	N	N	G	0·41
361 D 1	Hi Ca	0·110	3·6	N	N	N	0·33
2	Hi Ca	0·204	5·9	N	N	N	0·33
4	Hi Ca	0·423	8·9	N	N	G	0·36
10	Hi Ca	0·850	15·9	N	N	G	0·36

Effect of lactose. Table 1 shows that the best flavour stability of the milk protein was associated with the lower lactose levels. There were apparent differences between the different types of milk protein—thus casein with 0·22 % lactose was stable, whereas Med Ca and Lo Ca with 0·22 % lactose became gluey—but in all instances the samples of lower lactose content were more stable than those of higher. To investigate this relation further, varying amounts of an aqueous solution of lactose were added to casein (362H) and Hi Ca (361D) and the slurry redried. The flavour stability and analysis of these samples is shown in Table 2. Once again, lower lactose levels correlate with better flavour stability and, both for casein and Hi Ca, lactose levels below about 0·3 % gave high flavour stability.

Correlation of colour and flavour stability. The relation between flavour stability and lactose level suggests implication of the browning reaction. If this is so, there may be a relation between colour development (which must be a measure of the amount of browning) and flavour development. It was decided to measure colour differences before and after heating for 5 h at 102 °C as this is the test used in commercial practice but, in fact, the colour difference was of the same order as that produced by 40 h at 80 °C. Tables 1 and 2 show that the correlation between colour difference and flavour stability was slightly better than that between lactose and flavour stability. It is apparent that the best flavour stability was obtained when the colour difference was below 6 units. The anomaly noted above for casein and Med Ca or Lo Ca of 0·22 % lactose can be explained as the latter 2 samples had a higher colour difference, indicating that they browned more on heating.

Correlation of ferricyanide reducing value (FRV) and flavour stability. It is apparent from the results so far reported that the browning reaction plays a significant part in the formation of gluey flavour, and some measure of its extent was needed. Although the exact mechanism of the reaction is not known, FRV has been accepted

as an index of the formation and degradation of a protein-sugar complex in milk powder (Lea, 1947). Richards (1963) suggested that the reducing substances are the L-amino-1-deoxy-2-ketones formed as intermediates in the browning reaction. Table 1 shows that gluey off-flavour development was associated with high FRV and indicates that a value below 0·60 mg/g is necessary for good flavour stability. The high FRV for Med Ca or Lo Ca at low lactose level is indicative of incipient browning, presumably initiated during their preparation. The values of samples listed in Table 2 did not change with added lactose and were close to the values for untreated samples 361D and 362H.

Discussion

Development of the accelerated storage test. In initial attempts to develop an accelerated storage test, heating or irradiation with UV light were used. Gluey flavours were induced by both treatments but a more typical gluey flavour was produced by heating; UV irradiation produced a flavour described as gluey but also as burnt, unpleasant, or dirty. In prolonged heat treatment the protein tended to become insoluble but this insolubility could be reduced by saturating the oven with water vapour. There was little difference in flavour between samples treated in open or closed containers or under wet or dry conditions provided the initial moisture content was not above 10 %. If the moisture content was above 10 % unpleasant, dirty, burnt flavours and excessive browning were produced on heating in a closed container.

Cayen & Baker (1963) presented 10 % solutions of sodium caseinate at 40–50 °C to their panel of 3 judges. In the present investigation, a panel of 8–10 judges with experience of organoleptic testing was used. They found 10 % too strong and detected no significant differences between 1 % solutions presented at 20, 30 or 40 °C. Thus, 1 % solutions were presented at room temperature.

Limited experience suggests that samples which remained clean in flavour after 40 h accelerated storage would remain of good flavour for at least 6 months under ambient conditions. This corresponds to an increase in reaction rate of about 3-fold for a rise in temperature of 10 °C which is in good agreement with the figure of 2–5 found by Henry *et al.* (1948) for stored whole-milk powder.

Mechanism of off-flavour development. The best milk protein flavour stability was associated with a low lactose level, low colour development on heating and low production of ferricyanide reducing substances. These facts all support the theory that gluey off-flavour is developed during the early stages of the non-enzymic browning reaction between the milk sugar and the milk protein. Removal of most of the lactose —one of the reactants—reduces the extent of the browning reaction but it is not always sufficient to prevent it altogether. Samples of Med Ca and Lo Ca of low lactose content exhibited higher colour development and higher FRV owing to initiation of browning during their preparation, which included 30 min preheating at 90 °C, a treatment known to increase FRV and initiate the protein–lactose reaction (Choi *et al.* 1953; Kumetat & Beeby, 1957).

PART II. THE EFFECT OF PROCESSING CONDITIONS ON THE FERRICYANIDE REDUCING VALUE AND ON THE FLAVOUR OF CO-PRECIPITATED MILK PROTEINS

Processing may involve severe heating which could promote the early stages of the lactose–protein interactions. Part II describes an investigation of the effect of varying the initial preheating time, and 3 different methods of drying co-precipitates that had been washed for 3 different periods of time and therefore contained different levels of lactose.

Experimental

Co-precipitated milk proteins were prepared with the plant and by the general method of Muller *et al.* (1967). Lo Ca was prepared from milk heated for 10 or 30 min at 90 °C and Hi Ca was prepared from milk pre-heated for 1 or 30 min at 90 °C. In each case, samples were taken after 1, 3 and 5 washes and freeze-dried or dried in a fluidized bed with inlet air at 70 °C and cooled immediately or kept at 50 °C for 2 h.

Results

The analytical data obtained for the 4 samples of co-precipitate are compared with their flavour stability in Table 3.

Table 3. *Analytical data and flavour stability of milk protein*

Method of drying	Lactose, %	Colour difference	FRV, mg/g	Flavour	Lactose, %	Colour difference, 5 h at 102 °C	FRV, mg/g	Flavour
	Low calcium, 10-min preheat				Low calcium, 30-min preheat			
F	1·89	23·1	1·27	G	1·59	12·7	1·62	G
C	1·96	20·9	1·36	G	1·63	20·3	1·89	G
H	1·59	26·1	1·86	G	1·46	24·7	1·86	G
F	0·404	7·0	0·95	sl	0·336	4·9	1·40	sl
C	0·406	8·4	1·18	G	0·300	11·1	1·48	G
H	0·373	10·2	1·01	G	0·234	8·3	1·63	G
F	0·042	2·2	0·78	N	0·333	4·5	1·29	sl
C	0·043	3·8	1·09	N	0·075	8·4	1·63	G
H	0·062	3·3	1·09	sl	0·048	5·1	1·72	G
	High calcium, 1-min preheat				High calcium, 30-min preheat			
F	2·15	12·3	0·83	G	2·41	32·9	1·80	G
C	2·05	24·4	1·27	G	1·96	22·2	1·78	G
H	1·71	17·3	2·96	G	2·02	30·0	3·00+	G
F	0·124	4·2	0·53	sl	0·058	4·3	1·09	G
C	0·121	2·4	0·80	N	0·048	4·6	1·86	G
H	0·102	2·6	0·65	N	0·040	8·9	1·86	G
F	0·230	4·0	0·67	sl	0·051	4·0	1·12	N
C	0·052	2·7	0·59	N	0·046	4·9	1·72	G
H	0·038	2·7	0·47	N	0·045	5·5	2·07	N

F = freeze-dried; C = fluidized-dried and cooled; H = fluidized-dried and stored hot; G = gluey; sl = slightly gluey; N = not gluey.

Effect of drying conditions. There was no clear correlation between drying conditions and lactose level, colour difference or flavour stability. However, in most series, FRV increased with increasing severity of drying conditions.

Effect of lactose level. There was a general trend towards lower colour difference,

lower FRV and better flavour stability as the lactose level was decreased. Samples with less than 0·3 % lactose did not develop gluey flavour after 40 h accelerated storage at 80 °C unless they had been subjected to heat damage in the preheating.

Effect of preheating conditions. Although it is difficult to obtain a clear picture, there was a general trend to higher colour differences with longer preheating times. FRVs are a good indication of damage in preheating and in all cases the FRV increased with preheating time. When samples which should differ only in preheating treatment are compared, the best flavour stability was always associated with the shortest preheating time. Excellent examples of the effect of the incipient browning caused by preheating are the Hi Ca, 30 min preheat samples of lactose content 0·058, 0·048 and 0·040 %. Although these have satisfactorily low lactose level, their colour difference, FRV and rate of gluey off-flavour development were all high when compared with similar Hi Ca samples that had been preheated for only 1 min, even with considerably higher lactose levels.

Discussion

Owing to the complex nature of the flavour and the difficulties associated with the human element in organoleptic evaluation, no single chemical or physical test can be expected to correlate accurately with flavour development. Nevertheless, the FRV provides an excellent index of the amount of heat damage to the protein caused by processing conditions. A ferricyanide reducing value less than 0·60 mg/g protein combined with a colour difference of less than 6 units and a lactose content below 0·3 % ensure a protein of good flavour stability. The results of this study confirm the hypothesis advanced in Part I that the development of gluey off-flavour is associated with the early stages of the non-enzymic browning reaction: samples of the best flavour were obtained when processing conditions were chosen to minimize browning.

PART III. REMOVAL OF GLUEY OFF-FLAVOUR FROM CASEIN AND PREPARATION OF CASEIN OF STABLE FLAVOUR

As commercially produced, casein is not a pure protein but contains minor quantities of other compounds including up to 2 % lactose and up to 2 % fat. Cayen & Baker (1963) showed that casein of good flavour could be produced from a well-washed curd and it is possible that the minor constituents play a major role in the formation of gluey off-flavour. The practical problems caused by gluey flavour may be overcome in 2 ways—either by treatment designed to remove the off-flavour already present in stored casein or by preparation of a flavour-stable casein of acceptable shelf-life. Although the latter solution would obviously be the more efficient, many products would allow a treatment stage to be incorporated in their formulation—for example, if the raw material, casein, is converted to sodium caseinate before mixing with other ingredients.

Part III describes a study of flavour stability of caseins prepared with low fat and with low lactose content and records the effect of some selected treatments on the flavour and flavour stability of aged casein.

Experimental

Organoleptic evaluation. Accelerated storage trials were performed and evaluated as described in Part I. To compare the effect of treatments on flavour, judges were presented with a geometric concentration series (e.g. 0·1, 0·32, 1·0 %, etc.) and the threshold concentration estimated as that concentration at which more than half the panel could perceive the gluey off-flavour.

Preparation of casein of low lactose content. Casein was precipitated in the normal fashion and given 3 additional washes before freeze-drying. A control 'lactose' casein was prepared without these additional washes.

Preparation of casein of low fat content. Four litres of skim-milk were centrifuged at 10000*g* for 15 min at 30 °C followed by 15 min at 5 °C and the upper layer of solid fat removed. To the milk at 35 °C was added HCl to pH 4·5. The precipitate was filtered and washed with 3 separate portions of 1 l distilled water acidified to about pH 4·5. The damp precipitate was soaked for 1 h in 500 ml freshly distilled ethanol, filtered and dried under vacuum, then washed with 3 separate portions of freshly distilled diethyl ether, filtered and again dried under vacuum. The casein was dissolved in water by addition of NaOH to pH 7, reprecipitated, washed 3 times and freeze-dried. A control sample of 'fat' casein was prepared in a similar manner but omitting only the initial centrifugation and solvent washing stages.

Treatment of gluey casein. The following treatments were investigated:

(i) *Vacuum distillation.* The casein was placed in a round-bottom flask connected to a vacuum line through a trap immersed in liquid nitrogen. The flask was heated in a water bath at 50–70 °C and evacuated at 5×10^{-5} Torr, for 4 h.

(ii) *Steam distillation.* In initial experiments, a 3 % solution of sodium caseinate was distilled almost to dryness in a rotary evaporator at 15 Torr and 25 °C. The distillate was condensed at 0 °C and reconstituted with fresh freeze-dried sodium caseinate while the viscous residue was reconstituted with distilled water. In later experiments, the casein solution or slurry was distilled under vacuum through a vertical condenser at 0 °C into a liquid nitrogen trap using essentially the method of Forss, Jacobsen & Ramshaw (1967).

Attempts were also made in this manner to remove the flavour by freeze-drying from 1, 5 and 10 % solutions of gluey sodium caseinate.

(iii) *Reprecipitation.* Gluey casein was dissolved to 1 % in water by addition of NaOH to pH 7, and then reprecipitated with H_2SO_4, washed, filtered, and freeze-dried.

(iv) *Hot-water washing.* Gluey casein was stirred into distilled water at 60–65 °C (about 2 g water/g casein), held for 5 min and then filtered. This washing was repeated 4 times and the final residue freeze-dried.

(v) *Treatment with activated carbon.* 500 ml 3 % gluey sodium caseinate was passed through a 5 in. × 1 in. column of activated carbon (E. Merck, AG, for gas chromatography, 0·5–0·75 mm particles). The eluant was evaluated as such and also precipitated, washed, filtered and freeze-dried as casein.

(vi) *Treatment with Sephadex.* Portions of 150 ml 5 % gluey sodium caseinate were passed through a 12 in. × 2·5 in. column containing 500 g Sephadex G25; the eluant was evaluated as such or precipitated, washed, filtered and freeze-dried as casein.

Results

The concentrations at which more than half the panel could perceive the gluey flavour (threshold concentations) of treated stored casein are shown in Table 4.

The effectiveness of treatments

Vacuum distillation or freeze-drying of gluey casein had little effect on its flavour; the threshold concentration remained at 0·3 %. It is noteworthy that the vacuum treatment almost entirely removed the gluey odour of the casein and this odour could be detected in the adjacent cold trap. Reprecipitation effected some improvement, but the casein was still noticeably gluey in flavour.

Table 4. *Threshold concentration of gluey flavour in treated and untreated casein*

Material and/or treatment	*Threshold concentration, %	Remarks
Gluey casein	0·3	Control
Gluey sodium caseinate	0·3	Control
Fresh freeze-dried casein or sodium caseinate	> 3·0	Control
Vacuum-treated casein or caseinate	0·3	Flavour not removed
Steam-distilled sodium caseinate	> 1·0	Flavour partially removed
Distillate from sodium caseinate	1·0	Distillate gluey
Freeze-dried sodium caseinate (1, 5, 10 %)	0·3	Flavour not removed
Reprecipitated casein	1·0	Flavour partially removed
Filtrate from reprecipitation	1·0	Filtrate gluey
Washed casein	> 1·0	Flavour partially removed
Wash water	1·0	Wash water gluey
Activated carbon-treated casein	> 3·0	Flavour removed
Sephadex G 25-treated casein	> 3·0	Flavour removed

* Concentration at which gluey flavour was first detectable.

In steam-distillation experiments, the threshold concentration of the residue rose from 0·3 to 1·0–3·0 % and the solutions were only slightly gluey. Moreover, when the distillate was reconstituted to 1 % protein, with fresh sodium caseinate, it was markedly gluey. A similar improvement in flavour could be achieved by hot water washes and the wash water became gluey.

The most effective removal of gluey flavour was obtained by passing a solution of sodium caseinate through a column of activated carbon or Sephadex. In both cases the eluted protein was organoleptically identical with a solution prepared from fresh freeze-dried sodium caseinate.

Accelerated storage of casein preparations

Preparations of casein low in fat or in lactose were tested in the accelerated storage trial together with samples of casein prepared by those of the treatments which showed good removal of off-flavour. The results are shown in Table 5.

Effect of fat removal. Although the low fat casein was considerably less susceptible than commercial casein to the development of gluey flavour, there was little, if any,

difference between the low fat casein and its control. It seems likely that the presence or absence of fat plays only a minor role in off-flavour development and that the apparent improvement was due to removal of some other component, probably lactose, during preparation.

The effect of lactose removal. Well-washed casein of low lactose content did not become gluey after heating for 80 h at 80 °C. Although this effect of washing might have been due to the removal of some other minor constituent, re-addition of lactose to a stable sample of casein has been shown (Part I) to increase its susceptibility to the development of gluey flavour.

Effect of treatments. Washing with hot water and treatment with carbon or Sephadex all gave a product of good flavour, but the treated caseins varied in their susceptibility to redevelopment of off-flavour. The Sephadex-treated casein was best—almost as good as the best casein preparation—while the carbon-treated casein was not quite so good and the washed casein was quite susceptible to redevelopment of gluey flavour.

Table 5. *Accelerated storage tests on casein preparations*

Preparation, Casein	Lactose, %	Flavour stability, heating at 80 °C
Fresh commercial	0·450	Gluey at 20 h
Fat extracted	0·061	Not gluey at 100 h
Non-extracted (control)	0·186	Not gluey at 100 h
Low lactose	0·067	Not gluey at 80 h
Washed	0·037	Gluey at 20 h
Activated carbon treated	0·051	Gluey at 40 h
Sephadex treated	0·026	Not gluey at 60 h

Discussion

The effects of treatment on the flavour and flavour stability of casein support the hypothesis that gluey off-flavour develops during the early stages of the non-enzymic browning reaction. It is clear that stored, stale casein has 2 sources of gluey flavour—lower molecular weight breakdown products of the browning reaction already present and their precursors, presumably of much higher molecular weight. Treatments, such as washing or steam distillation, effect a temporary improvement in flavour of the stored casein by removing the breakdown products but the precursors are still available and flavour stability is poor. On the other hand, carbon or Sephadex treatment remove much of the precursor as well as the breakdown products and improve flavour stability as well as the immediate flavour. However, both treatments suffer practical disadvantages—carbon treatment must be carefully performed to avoid contamination with minute carbon particles which are flavourless but not aesthetically pleasing, while Sephadex treatment dilutes the solution by a factor of about 4.

CONCLUSION

Practical applications. While methods are available for removing gluey off-flavour from stored casein and improving its flavour stability, the best remedy is to minimize the development of the off-flavours by careful preparation of the milk protein. Gluey off-flavour development can be minimized only if strenuous efforts are made to avoid

heat damage and possible browning at all stages. Processing should involve only low temperature preheat treatment, careful washing to low lactose level, drying at minimum temperature and cooling before storage, careful grinding to avoid high temperatures and cool storage at low moisture levels. Although the lowest obtainable lactose level, colour development and reducing power are desirable, a milk protein of lactose content below 0·3 %, colour difference less than 6 Agtron units and FRV below 0·60 mg/g can be expected to remain of good flavour for at least 6 months in normal shelf storage.

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Volatile compounds associated with the off-flavour in stored casein

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Summary. Compounds associated with the 'gluey' off-flavour in stored casein have been isolated and identified by a combination of gas chromatography and mass spectrometry. Most of the flavour was found in the non-acid steam-volatile fraction which contained many compounds including n-alkanals, n-alkan-2-ones, furfural and other furan derivatives, benzaldehyde, dimethyl disulphide and benzthiazole. No single compound had the typical gluey flavour, which must be a complex sensation in which the quantities of minor components may determine the specific gluey character. o-Aminoacetophenone was present in the casein in subthreshold amounts. This compound has some gluey flavour and may have a synergistic effect in enhancing the total flavour.

The wide use of casein as a food protein has been inhibited by an unpleasant stale off-flavour which is characteristic of the product. This flavour has been described as 'stale', 'gluey' or 'burnt feathers'. A similar stale flavour has been observed amongst the flavours developed on storage by other milk products such as dried skim-milk (Henry, Kon, Lea & White, 1948), dried whole milk (Whitney & Tracy, 1949) and sterilized concentrated milk (Arnold, Libbey & Day, 1966).

Investigations of the gluey off-flavour in stored casein have not been reported but in some other milk products the milk protein seems to contribute a similar storage off-flavour. Nawar, Lombard, Dall, Ganguly & Whitney (1963) and Kurtz (1965) found evidence that the stale flavour in whole-milk powder had 2 elements—one volatile in vacuum distillation and the other non-volatile and tightly bound to the milk powder. Parks & Patton (1961) identified a range of volatile carbonyl compounds including n-alkanals (about 1 ppm), n-alkanones, furfural and benzaldehyde in stale whole-milk powder and Bassette & Keeney (1960) isolated a similar range of carbonyl compounds from stored skim-milk powder including alkanals at a level of 5 parts in 10^8. Shtal'berg & Kretovich (1953) found 50 ppm aldehydes in dried milk which exhibited a darker colour and an unpleasant gluey flavour. These groups of workers all concluded that stale flavour was due largely to the increasing quantity of carbonyl compounds—particularly aldehydes—formed during storage of the milk powder. Parks, Schwartz & Keeney (1964) isolated a less volatile compound, o-aminoacetophenone, from skim-milk powder and showed that it was an important component of the stale flavour.

In the present investigation the compounds formed in gluey, stored casein have

been isolated and identified. The mode of their formation and their relevance to the flavour defect is discussed.

METHODS

Samples of commercial casein and sodium caseinate were examined fresh and after storage for at least 6 months under ambient conditions.

Isolation of flavour components

Preliminary experiments revealed that most of the off-flavour was not removed by high vacuum molecular distillation of 'dry' casein (as received, containing about 10 % moisture). Most of the flavour could be removed by washing with hot water or by steam distillation; the treated casein became less gluey and the wash water or distillate became gluey. Consequently, 3 methods of isolation were evolved.

Method A—hot washing. Casein was washed 3–5 times with about twice its weight of distilled water at 60 °C and the combined wash water was distilled under reduced pressure. The distillate was adjusted to pH 9 and extracted with diethyl ether in a separating funnel (non-acid extract) and the aqueous layer acidified to pH 2 and re-extracted (acid extract). The 2 ether extracts were concentrated separately by distillation to small volume using a short fractionating column.

Method B—steam distillation. Five hundred grams of casein was steam-distilled with 3 l distilled water at a temperature < 25 °C and pressure of 6–10 Torr. The distillate was concentrated by reflux through a vertical condenser at 0 °C and about 6 Torr into a liquid nitrogen trap using a method very similar to that of Forss, Jacobsen & Ramshaw (1967). Frequently the steam distillation and reflux concentration were combined in a single apparatus. The reflux concentrate and the residual distillate were extracted at pH 9 and again at pH 2, as described for method A above.

Method C—reflux concentration. The volatile components were concentrated from a solution of sodium caseinate at pH 7 by distillation under reflux through a vertical condenser at 0 °C and about 6 Torr into a liquid nitrogen trap. A non-acid extract was prepared as in method A. Negligible quantities of acid extract were obtained in this procedure.

Identification of components

Portions of the concentrated solvent extract were separated by gas chromatography using several columns with different stationary phases.

Column 1. A stainless steel column (4 ft × $\frac{1}{4}$ in. o.d.) packed with 7·0 g of 60–80 mesh Embacel coated with 15 % diethylene glycol succinate plus 2 % phosphoric acid. This was used for the acid fraction and operated at a constant temperature of 100 or 125 °C or was programmed from 50 to 150 °C at 2 deg C/min with the carrier gas flow rate 20 ml/min of helium.

Column 2. A stainless steel column (10 ft × $\frac{1}{8}$ in. o.d.) packed with 7·3 g of 60–80 mesh Chromosorb G-AW-DMCS coated with 1 % Carbowax 20 M-TPA. This column was programmed from 50 to 150 °C at 2 deg C/min and carrier flow rate 10 ml/min of helium.

Column 3. A stainless steel support-coated open tubular (SCOT) column (50 ft × 0·02 in. o.d.) coated with Apiezon L. The column was supplied by Perkin-Elmer.

Helium flow rate was 8 ml/min and the column temperature was programmed from 40 to 180 °C at 2 deg C/min.

Column 4. A SCOT column (50 ft × 0·02 in. i.d.) as above coated with Carbowax 1540. Helium flow rate was 8 ml/min and column temperature was programmed from 40 to 150 °C at 2 deg C/min.

Any of the columns could be operated in the combined gas chromatography–mass spectrometry (GC/MS) system described by Stark, Smith & Forss (1967). For further resolution of components, fractions trapped from column 2 were rechromatographed in the GC/MS combination using columns 3 or 4. Identification of components was made by comparison of their mass spectral fragmentation pattern with published spectra (Cornu & Massot, 1966) and spectra of authentic compounds. Correspondence of retention data on column 3 and MS data for an isolated component and a known compound was considered sufficient for identification. In most cases, retention data on a different stationary phase and odour appraisal were also available for confirmation of identity.

Flavour tests

All stages of the isolation and identification procedure were monitored by flavour tests in an attempt to assess the importance of various fractions in the gluey flavour. Fresh bland sodium caseinate was added to samples of the distillates, residues, and trapped fractions, and to authentic compounds, singly or admixed to make solutions containing 1 % sodium caseinate. The flavour of each solution was assessed by a laboratory panel of about 10 persons selected because of their experience in organoleptic evaluation of dairy products.

RESULTS

Evaluation of isolation techniques

For the isolation of flavour components by steam distillation (method B) slurries were prepared from distilled water and 30-mesh casein (1), with the addition of NaOH (2), by mixing with a Waring Blendor (3), and by passage through a colloid mill (4). Each slurry was steam distilled and the distillate extracted with ether and analysed by gas chromatography. All the slurries gave qualitatively similar non-acid extracts but the best qualitative recovery was obtained—particularly for the more volatile components—by distilling slurry (1) prepared from casein as received. Reflux concentration from a solution of sodium caseinate at pH 7 was evaluated at concentrations of 1, 5 and 10 %. The best quantitative recovery (per g of caseinate) was obtained from the most dilute solution, but larger quantities of components could be obtained most conveniently from the 5 % solution. Comparison of methods A, B and C for isolation of the more volatile fraction showed that reflux concentration (method C) was slightly better than 2-stage steam distillation (method B), while hot washing (method A) was very inefficient. On the other hand, isolation of the less volatile fraction or the acid fraction was best accomplished by hot washing (method A).

Flavour

Samples of the wash water, distillate and distillation residue were examined organoleptically. It was found that the gluey flavour was distillable—the distillate

was gluey while the residue was not significantly gluey. Further reflux concentration showed that most of the flavour resides in the more volatile fraction.

Separation into acid and non-acid fractions revealed that the gluey flavour was in the non-acid fraction—indeed, if anything, the non-acid fraction was more gluey than the total extract. Reaction with 2,4-dinitrophenylhydrazine in H_2SO_4 removed about 80% of the carbonyl compounds but there was a less marked effect on the gluey odour. There was some reduction in intensity but individual judges varied in their comments from 'little effect' to 'almost complete removal'. Odour appraisal of the effluent gases from an exit splitter during the gas chromatographic separation showed that no single peak or part of the chromatogram was associated with the typical gluey odour although odours were numerous and varied. Similarly, on examination of trapped broad fractions, none was found to have the typical gluey flavour, which was present only when fractions from all regions of the chromatogram were represented.

It was concluded that the main gluey flavour resides in the more volatile fraction and that no single compound or class of compound is typically gluey. Gluey flavour must be a complex sensation resulting from a mixture of several different compounds.

Identification of components of the acid fraction and their relevance to gluey flavour

Components of the acid fraction, prepared by method A, were separated by gas chromatography on column 1. Mass spectrometry and infra-red spectroscopy proved that all components were straight chain alkanoic acids. The approximate composition of the acid fraction is shown in Table 1. In fresh casein the total quantity of acids was negligible but on storage it increased to about 20 μg/g.

Table 1. *Composition of the acid extract* from stored casein*

Alkanoic acid, no. of carbons ...	2	3	4	5	6	7	8	9	10	11	12
% in extract	1	1	2	0·5	43	0·5	35	0	14	3	0
Approximate level in casein, ppm	0·2	0·2	0·4	0·1	8·6	0·1	7·0	0	2·8	0·6	0

* See p. 216.

After removal of acids the extract was still gluey, as was the reflux concentrate prepared by method C, which contained negligible quantities of acids, which were relatively non-volatile under these conditions. The flavour of the lower alkanoic acids is well-known as a sharp, bitter, rancid-fat taste with a threshold in water of the order of 5 ppm (Patton, 1964). These properties suggest that the acids have little relevance to gluey flavour although they are major components of the casein extract and several are present in amounts close to their flavour threshold values.

Identification of components of the volatile non-acid extract and their relevance to gluey flavour

Portions of the concentrated volatile non-acid extract prepared by method C were analysed by combined GC/MS. A typical chromatogram is shown in Fig. 1 and the

identity and approximate composition of the mixture (estimated from the gas chromatogram) is given in Table 2. In addition, the presence of trace amounts of other compounds such as methyl butanal, alk-1-en-3-ones, oct-1-en-3-ol, nona-2,6-dienal, nona-2,6-dienol and higher homologues of furfural and furyl ketones could be deduced from the appearance of their characteristic odours at the relevant retention time. However, insufficient quantities were available for positive identification. The total quantity of compounds was about 5 μg/g for stored casein while fresh casein yielded only very much smaller amounts of a similar range of compounds.

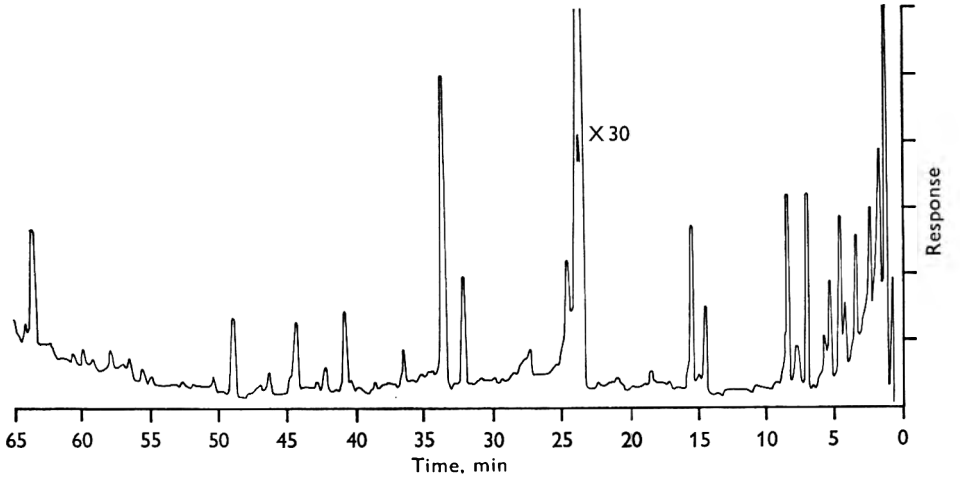

Fig. 1. Gas chromatogram of the steam-volatile non-acid fraction equivalent to approximately 40 g gluey sodium caseinate. Operating conditions described for column 3, p. 216.

The hydrocarbons and alkanols are unlikely to be of significance in the gluey flavour as both classes have weak flavours and relatively high flavour thresholds. The only components isolated at concentrations near or above their threshold concentrations (of the order of 0·005 ppm (Lea & Swoboda, 1958)) were the n-alkanals. These are well-reported components of oxidized and stale flavours, especially in systems containing lipids. They may be expected to have some significance in the overall gluey flavour even though their flavour character is not specifically gluey. The alkan-2-ones are also components of oxidized and stale flavours in lipids and could be important in gluey flavour but they have generally higher threshold values and were present in smaller amounts than the n-alkanals.

In organoleptic evaluation, benzaldehyde retained its almond, nutty flavour right down to its threshold and benzthiazole similarly retained its rubbery flavour. However, some judges thought furfural, methyl furfural and methyl furyl ketone were slightly gluey although most described them as weedy or bitter. All were isolated below their threshold value, even allowing for incomplete recovery, but they may well have had some additive effect on the flavour.

It was concluded that the flavour of the non-acid volatile fraction is due mainly to the n-alkanals with some contribution from the furan derivatives. Many of the minor components are strongly flavoured (e.g. diacetyl, dimethyl disulphide, methional,

phenylacetaldehyde) and could play some part by contributing to the specific gluey character of the flavour. Their importance cannot be assessed without laborious organoleptic evaluation of multi-component mixtures.

Table 2. *Composition of the volatile non-acid extract* from stored casein*

Compound	Retention time, min		MS	Odour confirmation	% in extract
	Ap L	C 1540			
Ethyl acetate	2·4	2·0	+	+	1·5
2,3-Butadione	2·4	.	+	+	1·5
3-Methyl butanal	4·3	3·2	+	+	1·5
n-Pentanal	4·6	3·5	+	+	1·0
Dimethyl disulphide	6·9	5·5	+	+	4·0
Butanol	.	14·0	+	.	0·5
Pentanol	7·6	18·5	+	.	4·5
Hexan-2-one	7·9	.	+	.	tr
Hexanal	8·3	12·5	+	+	5·5
Toluene	8·6	.	+	.	1·0
Heptan-2-one	14·5	34·2	+	+	1·4
Heptanal	15·0	14·8	+	+	4·2
Furfural	15·0	34·2	+	+	1·4
Hexanol	.	28·8	+	.	2·5
Ethyl benzene	15·0	.	†	.	tr
Dimethyl benzene	16·5	.	†	.	tr
Methional	18·5	.	†	+	tr
Methyl furyl ketone	20·2	37·0	+	.	0·5
Octan-2-one	23·6	23·0	+	+	1·4
Methyl furfural	24·0	.	+	.	1·4
Benzaldehyde	23·8	38·2	+	+	34·5
Octanal	24·6	23·5	+	+	2·1
Benzonitrile	24·8	.	+	.	0·5
Terpene	31·0	.	†	.	tr
Nonan-2-one	32·3	31·0	+	+	2·4
Acetophenone	33·0	46·0	+	.	0·5
Nonanal	33·7	31·3	+	+	8·3
Phenylacetaldehyde	34·7	.	+	+	0·5
Decan-2-one	40·7	.	+	.	1·4
Decanal	42·3	.	+	.	1·0
α-Terpineol	44·2	49·5	+	+	1·2
Naphthalene	46·3	50·3	+	.	1·3
Undecan-2-one	48·9	44·5	+	.	1·4
Undecanal	50·4	.	†	.	0·5
Methylnaphthalene	55·5	.	†	.	tr
Dodecan-2-one	56·5	.	+	.	0·5
Dodecanal	58·0	.	†	.	0·5
Tridecan-2-one	63·5	56·7	+	.	2·5
Tridecanal	65·0	.	†	.	tr
Trimethylnaphthalene	73·0	.	†	.	tr
Benzthiazole	.	63·0	+	+	2·5
Phenol	.	68·2	+	+	1·5

* See p. 216. † Not positively identified by MS but spectra similar.
+ Positive identification in MS.

Identification of components of the non-volatile non-acid fraction, and their relevance to gluey flavour

During the isolation of flavour components by method B, the residual steam-distillate, after removal of volatiles by reflux concentration, contains the non-volatile fraction. The non-acid non-volatile extract from stored casein was analysed

by combined GC/MS using column 2 and its composition is shown in Table 3. These components increased on storage, from negligible quantities in fresh casein to about 1·5 μg/g in badly deteriorated casein.

Only one of these components, o-aminoacetophenone, was examined organoleptically as Parks *et al.* (1964) found that it was an important constituent in the flavour of stale skim-milk powder. In 1 % sodium caseinate solution, it had a threshold of 3–10 parts in 10^9 (i.e. 0·3–1·0 ppm on the casein) and the panel were divided in their opinions—some thought it gluey while others described it as fruity, like grapes. It was also observed that the flavour of a solution of slightly gluey caseinate was intensified by subthreshold concentrations of o-aminoacetophenone (about 1 in 10^9).

Table 3. *Composition of the non-volatile non-acid extract* from stored casein*

Compound	% in extract	Approximate concentration in casein, ppm
Lower boiling compounds†	37	0·6
2-Furfurol	25	0·4
Benzyl alcohol	10	0·2
Cresol	4	0·1
Phenol	8	0·2
Benzthiazole	4	0·1
o-Aminoacetophenone	4	0·1

* See p. 216.

† Including benzaldehyde, furfural, alkanals, alkan-2-ones and terpenes.

Since this compound was not isolated in quantities above threshold and, moreover, since volatile fractions were isolated which contained none, it is unlikely to make an essential contribution to the gluey flavour. However, it is clear from the flavour trials that it has some gluey character and can exert a synergistic or potentiating effect on the other components of the flavour.

DISCUSSION

The origin of the flavour components

The range of compounds identified suggests that there are at least 2 degradative processes operating during the storage of casein—lipid oxidation and non-enzymic browning. The acids, alkanals, alkan-2-ones and alkanols are all common products of lipid oxidation and are likely to result from the degradation of the small amount (1–2 %) of milk fat remaining in commercial casein. Diacetyl and the furan derivatives are sugar fragmentation products occurring during non-enzymic browning while 3-methylbutanal, methional and phenylacetaldehyde can be formed during this reaction from leucine, methionine and phenylalanine.

The source of the major non-acid component, benzaldehyde, is unknown although it has been found as a product of the caramelization of sugars (Hodge, 1967). Small amounts of benzaldehyde are often found in dairy products but the present findings and those of Scanlan, Lindsay, Libbey & Day (1968) both suggest that it is formed during storage. Work in our laboratory has shown that benzaldehyde, as well as phenylacetaldehyde, is formed during reaction of phenylalanine/lactose mixtures.

Moreover, if the reaction is continued benzaldehyde becomes the major product and the amount of phenylacetaldehyde is reduced. This apparently indicates that benzaldehyde can be formed from the phenylalanine residue as well as the sugar molecule in non-enzymic browning.

o-Aminoacetophenone is likely to be formed by breakdown of tryptophan. Attempts to produce it by simple browning reaction of tryptophan and lactose have failed so far but tryptophan was easily degraded to o-aminoacetophenone in the presence of oxidizing agents such as periodate. Indole and other compounds were also produced. Thus, it is possible that the aminoacetophenone is produced by oxidation of the tryptophan coupled to the oxidizing lipid system, possibly in some free radical mechanism.

The results support the hypothesis put forward by Henry *et al.* (1948) and by Radema (1954) on chemical evidence that mechanisms of oxidation and browning are both operating in whole-milk powder and the final flavour depends on which reaction is dominant.

The involvement of the early stages of the non-enzymic browning reaction is supported by organoleptic evaluation of casein preparations of varying lactose content. Removal of most of the lactose inhibits the development of gluey flavour although Nakanishi & Itoh (1967) were able to form some carbonyl compounds by heating carefully purified casein, presumably by reaction of the protein with bound carbohydrate.

The flavour of gluey casein

Fresh commercial casein usually has a relatively bland, clean flavour and a steam distillate contains only very small amounts of flavour components. On storage, the flavour becomes gluey and the steam-distillate contains 25–30 μg of volatile components per g of casein. It is difficult to assess the relative amounts of the 3 fractions but an average breakdown might be 20 μg of acid fraction, 5 μg of volatile non-acid fraction and 1·5 μg of non-volatile non-acid fraction. The main efforts of identification have been concentrated on the volatile non-acid fraction as organoleptic evaluation indicated that most of the flavour was associated with this fraction. The acid fraction had no relevance but the non-volatile non-acids made some contribution to the overall flavour. This is in accord with the evidence (Nawar *et al.* 1963; Kurtz, 1965) that flavour components of high and low volatility are present in stored milk systems.

Most of the compounds identified have been isolated previously during one or other of the many investigations of dairy products and the specific gluey flavour seems more a result of a different quantitative balance of components than the effect of any one compound. The alkanals formed 20–25% of the non-acid volatile fraction—equivalent to about 1 ppm on the casein.

The results suggest that the overall gluey flavour is compounded of the flavour of n-alkanals and furan derivatives with some synergistic effect from o-aminoacetophenone. It is possible that other compounds isolated such as the higher alkan-2-ones, 2-furfurol, benzaldehyde and benzthiazole may make a contribution to the flavour. While more complex mixtures of these isolated compounds will, in time, be evaluated organoleptically, adding to our knowledge of the flavour, the work already done appears to point clearly enough to the chemical reactions which must be suppressed in practice if gluey flavour is to be eliminated from casein.

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Storage of chilled cream in relation to butter quality

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SUMMARY. The quality of fresh and stored butter made from chilled cream collected over 3 days and stored either by accumulation in a vat or by separate storage of each batch was compared with that of butter made from unchilled cream collected over one day.

Provided each batch of cream after separation at 85 °F was cooled to 55 °F before addition to the vat, accumulation of cream at either 40 or 48 °F gave butter similar in quality to the control butter. Butter from cream cooled to 55 °F and held at this temperature, however, was slightly inferior in quality. The addition of the cream to the vat with no cooling, or cooling only to 70 °F, after separation gave butter of inferior quality due to rancid or stale flavours.

Butter from cream cooled either to 70 or 50 °F before storage of each batch in separate containers at either 40 or 50 °F was only slightly inferior in quality and in keeping quality to the control butter.

Defects in the chilled cream butters appeared to be due to lipolysis caused either by activation of the natural lipase of the cream or by lipolytic bacteria or both.

Previous work at this Institute showed that the flavour of butter from chilled milk accumulated and stored over 3 days before separation and pasteurization of the cream was similar or even (with poor quality milk) superior to that of butter from unchilled milk collected only over one day before separation and cream pasteurization (McDowell, 1964*b*). Since under certain circumstances it might be preferable to separate the fresh milk and store the raw cream on the farm, the effect of holding chilled cream on the quality of the butter was investigated and the results are presented in this paper.

Several investigators (Fouts, 1940; Peters, Kester & Nelson, 1953; Crowe, 1955; Garrison, 1957) have shown that holding of cream for several days at temperatures between 38 and 55 °F causes lipolysis and that the extent of lipolysis usually is greatest at 38–40 °F and decreases with increasing temperature. Garrison (1957) found that the fat hydrolysis at 40 °F was due mainly to the action of the natural lipase of milk but that at 45–50 °F lipolytic bacteria also played a part. Fouts (1940) and Peters *et al.* (1953) considered, however, that even at 38–41 °F microbial lipases were of equal or greater importance than the milk lipase in promoting lipolysis.

If cold milk or cream is warmed and recooled, the activity of lipase is greatly stimulated (Krukovsky & Herrington, 1939; Krukovsky & Sharp, 1940). Thus, the

addition of warm cream to cream stored at refrigeration temperatures and recooling of the mixture would, presumably, have the same effect (McDowall, 1953). Garrison (1957), however, reported that lipolysis was no less after precooling cream to the storage temperature than it was after the addition of warm cream.

Garrison (1957) also showed that butter made from cream with a bitter or 'unclean' flavour from fat hydrolysis would itself also be graded as 'unclean'. He concluded that if the off-flavour from lipolysis was considered more objectionable than that from acid fermentation then the cream should be stored at 55–60 °F. At this temperature, not only is the action of milk lipase retarded but also acid production by other micro-organisms ensures that the growth of lipolytic bacteria is inhibited.

In a report on Australian research work (Gunnis & Loftus Hills, 1965) a temperature of 50 °F was recommended for farm storage of cream. In New Zealand, Burnett (1967) reported on a trial covering a number of farms where the cream from each milking over 2 days was held in separate cans in a trough at 40 °F. This treatment had no effect on the quality of the cream which was usually graded as 'finest'.

EXPERIMENTAL

Good-quality milk which had been water-cooled over a surface cooler was collected from one or more farms immediately after each milking over a period extending from the evening milking on one day to the morning milking 3 days later. The milk arrived at the Institute experimental factory at temperatures ranging from 65 to 80 °F and was at once warmed to 85 °F and separated. The cream, uncooled or cooled to a selected temperature, was divided into 2 or 3 portions and stored either by accumulation in a vat or in separate containers at 2 (or 3) different temperatures. After 3 days the bulk cream stored at each temperature was neutralized, pasteurized, held overnight and then churned under conditions described in a previous paper (McDowell, 1964*b*). Two 56-lb boxes of butter from each churning were stored at 14 °F and withdrawn for grading, one after 4 months and the other after 8 months. The fresh and the stored butters were scored and classified by Department of Agriculture butter graders in accordance with the official procedure, into: finest (93 points and over); first grade (90 points and under 93) and second grade (80 points and under 90).

Tests for peroxide value (cf. Holloway, 1966) and for free fat acidity (see below) were carried out in the laboratory on the butterfats obtained from the fresh and the stored butters after melting the butter, centrifuging, decanting the fat and filtering at 40 °C.

The free acidity of the fat in the cream was measured after extracting a mixture of 14 ml of diluted cream (10 ml diluted to 70 ml with water), and 14 ml of neutralized rectified spirit (90 % ethanol) with 20 ml of 2:3 diethyl-ether–light petroleum mixture. After centrifuging, 15 ml of the ether layer was withdrawn, 7·5 ml of neutralized rectified spirit added and the mixture titrated to the phenolphthalein end-point with 0·025 N-alcoholic potash. The acidity of the butterfat from the melted butter was obtained by bringing to the boil a mixture of 10 g butterfat and 50 ml neutralized rectified spirit and titrating to the phenolphthalein end point with 0·1 N caustic soda. All fat acidity results were recorded as ml of N alkali/100 g of fat.

Small samples for bacteriological analysis were taken from the bulk cream after

storage and also, when the cream was stored in separate containers (see project C below), from the first batch of cream just before it was mixed with the later batches to make up the bulk cream. Plate counts were determined on standard milk agar after incubation at 30 °C for 2 days. The milk agar contained (% w/v): yeast extract, 0·2; peptone, 0·5; agar, 1·0; fresh whole milk, 1·0. The constituents were dissolved in distilled water and the pH value adjusted to 7·0 before dispensing in 10 ml quantities and autoclaving at 15 lb/in.^{2} for 15 min. Methylene blue reductase tests on the cream were carried out according to the method described in the New Zealand Food and Drug Regulations (New Zealand Government, 1946). The concentration of methylene blue was 10 times that specified for milk.

Studies were made of the effects of cooling cream to various temperatures after separation, of different holding temperatures and of storage of all the cream collected over 3 days in one vat or in separate containers for each milking. The various projects are described in more detail below.

Project A (*4 trials*). The uncooled cream after separation was divided into 3 portions and transferred to refrigerated vats at 40, 48 and 55 °F. A 'control' cream was prepared by combining half the milk (held overnight) from the penultimate milking with half the milk from the final milking, pasteurizing at 162 °F for 15 sec, cooling to 85 °F for separation and then cooling the cream to 40 °F. The control cream and the 3 experimental creams were all held a further 24 h at their respective storage temperatures before neutralizing and pasteurizing.

Project B. The separated cream was cooled either to 70 °F (5 trials) or to 55 °F (5 trials) before dividing into 3 portions and transferring to refrigerated vats at 40, 48 and 55 °F. Soon after the addition of the final batch of cream, the bulk cream from each vat was neutralized, pasteurized, cooled and held for churning on the following day. A 'control' cream was obtained after separating the mixed (unchilled) milks from the evening milking on the fourth day and the morning milking on the fifth day of the trial. Pasteurization and churning of this cream was therefore carried out one day later than the same operations on the experimental creams.

Project C. After separation, each batch of cream was cooled either to 70 °F (4 trials) or to 55 °F (4 trials) and then divided into 2 portions and each portion run into an empty can held in a water tank maintained at either 40 or 50 °F. Thus, at the end of the collection period (cream received from the evening milking to the morning milking 3 days later) there were 6 cans in each tank. The cream from each tank was mixed in the neutralizing vat, neutralized, pasteurized and churned. A 'control' butter was obtained under the conditions described for project B.

RESULTS

All the butters were low—0·08 ppm or under—in copper content. Since, also, there were only small increases in peroxide value of the butterfat after 4 or 8 months' storage any defects in flavour of the stored butters were unlikely to have been due to fat oxidation (McDowell, 1964*a*).

Project A. The addition of the earlier batches of cream at 78–84 °F to the vat at 40 °F increased the temperature of the chilled cream already present by as much as 10–15 °F. The rise in temperature became smaller as further batches were added.

Corresponding but smaller rises also occurred in the vats at 48 and 55 °F. Average results for the bulk creams for titratable acidity (as lactic acid) and for flavour are shown in Table 1. The creams from the 40 and 48 °F-vats were low in acidity and were usually described as 'stale' in flavour, whereas the cream from the 55 °F-vat was high in acidity and 'acid and unclean' in flavour. Unfortunately, there are no results for free fat acidity nor for bacterial counts or reductase times of these creams. Results for the butterfats from the butters (see Table 1) indicate, however, that the fat acidities for all 3 vats would have been much higher than that of the control cream.

Average results for the butters are also shown in Table 1. The increase in fat acidity of butters from all 3 cream storage temperatures in comparison with the control butter apparently was great enough to cause stale and unclean flavours in all the fresh butters and rancid, stale and unclean flavours after storage.

Table 1. *Effect of accumulation and storage over 3 days (and storage for a further day) of cream (uncooled after separation) at 40, 48 and 55 °F on the quality of the bulk cream and of the fresh and the stored butter*

Cream temp. into holding vat, °F			Holding vat temp., °F			
			40	48	55	Control
78–84	Cream	Titratable acidity (as lactic acid %)	0·12	0·14	0·31	0·08
		Grading	Stale	Stale	Unclean and acid	Clean
	Butter	Free fat acidity of butterfat	1·16	1·31	0·99	0·43
		Average grade score	(1)	(1)	(1)	
		Fresh	92·6	92·1	92·0	93·5
			(2)	(2)	(2)	
		4 months	90·8	89·6	92·3	93·3
			(2)	(2)	(2)	
		8 months	90·9	89·3	89·9	93·1

(1) Stale or 'unclean' flavour
(2) Rancid, stale or 'unclean' flavour

Project B. With cooling of the cream to 70 °F after separation, the rise in temperature of the mixed cream vats at 40 and 48 °F after the addition of the earlier batches was 5–10 °F but only 3–5 °F in the vat at 55 °F. The average titratable acidity results for all 3 bulk creams were low but the reductase times decreased as the storage temperature rose (Table 2). The fat acidities were quite high for all 3 creams and 'stale' and 'slightly rancid' flavours were predominant.

Cooling of the separated cream to 55 °F reduced the rise in temperature of the vats to only 3–6 deg F in the 40 and 48 °F-vats and to nil in the 55 °F-vat. The average titratable acidities of the bulk creams once again were quite low but there were considerable increases in bacterial population and decreases in reductase times as the storage temperature rose (Table 2). Slight stale flavours were again detected in the creams stored at 40 and 48 °F and unclean, slightly acid flavours at 55 °F.

Table 2. *Effect of accumulation and storage over 3 days of cream (cooled to either 70 or 55 °F after separation) at 40, 48 or 55 °F on the quality of the bulk cream and of the fresh and the stored butter*

Cream temp. into holding vat, °F			Holding vat temp., °F			
			40	48	55	Control
70	Cream	Titratable acidity, as lactic acid %	0·10	0·10	0·12	0·08
		Reductase time, h	6	5	2	6
		Free fat acidity of extracted fat	1·30	1·16	1·07	0·65
		Grading	Stale and slightly rancid	Stale and slightly rancid	Stale or unclean	Clean
	Butter	Free fat acidity of butterfat	0·90	0·74	0·64	0·43
		Average grade score: fresh	92·7 (1)	92·9 (1)	93·3	94·0
		4 months	92·3 (2)	92·4 (2)	92·9 (2)	93·6
		8 months	91·8 (2)	92·1 (2)	92·5 (2)	93·3
55	Cream	Titratable acidity, as lactic acid %	0·09	0·09	0·13	0·09
		Reductase time, h	6	4	1	6
		Plate count in millions/ml	0·04–0·6	0·5–17	13–415	0·13–1·5
		Free fat acidity of extracted fat	1·05	0·97	1·04	0·82
		Grading	Slightly stale	Slightly stale	Unclean and slightly acid	Clean
	Butter	Free fat acidity of butterfat	0·59	0·55	0·63	0·45
		Average grade score: fresh	93·7	93·7	92·9 (3)	93·7
		4 months	93·1 (4)	93·0 (4)	92·5 (3)	93·4
		8 months	93·2 (4)	93·0 (4)	92·3 (3)	93·5

(1) Slight rancid flavour.
(2) Rancid, stale or unclean flavour.
(3) Slight unclean or off-flavour.
(4) Less attractive flavour than control butter.

Results for the butters (Table 2) show that increases in fat acidity for the chilled cream butters from cream cooled to 70 °F after separation were not as great as in project A but rancid, stale and unclean flavours were still evident in the fresh and the stored butters with corresponding losses in grading points. Butter from cream cooled to 55 °F before storage at 40 or 48 °F showed only small increases in fat acidity and was graded 'finest' with a grading score only slightly below that of the control butter even after 8 months storage (see Table 2). The butter from cream stored at 55 °F, however, showed a slightly greater increase in fat acidity and was graded only as 'first' because of unclean or off-flavours.

Table 3. *Effect of collection and storage in separate containers over 3 days of cream (cooled to either 70 or 50 °F after separation) at 40 or 50 °F on the quality of the bulk cream and of the fresh and the stored butter*

Cream temp. into cans, °F			Holding temp. for cans, °F		
			40	50	Control
70	Cream	Titratable acidity, as lactic acid %	0·115	0·12	0·105
		Reduetase time, h	5	3	5
		Plate count in millions/ml	0·25–2	5–179	0·07–11
		Free fat acidity of extracted fat	1·65	1·55	1·00
		Grading	Fairly clean	Fairly clean	Clean
	Butter	Free fat acidity of butterfat	0·87	0·72	0·63
		Average grade score: fresh	93·5	93·6	93·7
		4 months	93·3	93·3	93·6
		8 months	93·2 (1)	93·0 (1)	93·5
50	Cream	Titratable acidity, as lactic acid %	0·11	0·125	0·10
		Reductase time, h	5	4	5
		Plate count in millions/ml	0·5–2	14–47	0·4–3
		Free fat acidity of extracted fat	1·80	1·58	1·32
		Grading	Clean	Clean	Clean
	Butter	Free fat acidity of butterfat	0·80	0·77	0·64
		Average grade score: fresh	93·2	93·4	93·7
		4 months	93·2	93·4	93·5
		8 months	93·1 (1)	93·2 (1)	93·5

(1) Less attractive flavour than control butter.

Project C. Irrespective of the temperature of cooling after separation, the bacterial counts for the bulk creams from storage at 50 °F were higher and reductase times were lower than those for the bulk creams from storage at 40 °F or for the control creams (Table 3). Free fat acidities were higher for the stored than for the control creams but the stored creams were all clean or fairly clean in flavour. It should be noted that the fat acidities of the control creams and of the butterfats from the control butters were considerably higher than those for the controls in projects A and B (compare Table 3 with Tables 1 and 2). One possible explanation is that in project C the proportion of Friesian compared with Jersey cows supplying the milk was much higher than in projects A or B.

Tests were also carried out after $2\frac{1}{2}$ days of storage on the first batch of cream received in each trial. Usually it was higher in bacterial count, lower in reductase

time and higher—often considerably higher—in fat acidity than the bulk cream of which it eventually formed a part.

The butters even though they were higher in fat acidity than the controls were all graded finest but were less attractive than the control butters (see Table 3).

DISCUSSION

Despite large increases in total bacterial population of the bulk creams after holding or accumulating cream at the higher temperatures (50 or 55 °F), the titratable acidities showed only small increases (0–0·06 %) compared with the control creams, with the exception of that of the 55 °F-vat in project A. The high acidity in this vat (0·31 %) could have been due to the fact that, in project A, after the addition of the final batch all the vats of cream were held at their respective temperatures for a further 24 h before pasteurizing.

Fat hydrolysis in cream stored at 38–60 °F is due partly to bacterial lipases and partly to natural lipase (Fouts, 1940; Peters *et al.* 1953; Garrison, 1957) and it appears probable that increases in free fat acidity in cream accumulated at 40, 48 and 55 °F over 3 days were due to both these causes. Since, also, the free fat acidities of the creams and butterfats (from butters) from cream cooled to 55 °F were lower than those from cream cooled only to 70 °F (project B), it may be inferred that stimulation of the natural lipase by the alternate warming and cooling resulting from the addition of each new batch of cream would also account for some fat hydrolysis in all the vats of accumulated cream, with the exception of that at 55 °F from cooling of cream to 55 °F.

The results indicate that provided each batch is cooled to 55 °F after separation, cream accumulated in one vat over 3 days at a temperature of either 40 or 48 °F will give a finest grade butter. Due possibly to large increases in total numbers of bacteria in addition to lipolysis, accumulated cream stored at 55 °F appears, however, to give a poorer quality butter.

With separate storage of each batch of cream, the bulk cream from storage at either 40 or 50 °F was still higher in fat acidity than the control cream. Since the first batch of cream received in each trial showed evidence of lipolysis after $2\frac{1}{2}$ days storage, it may be inferred that the second and possibly the third batch also showed increases, though to a lesser extent, in fat acidity so that the value for the bulk cream from all 6 batches was higher than that of the control cream held only for one day.

The temperature to which the cream is cooled before storage of each batch in separate containers appears, in contrast to that of the accumulated cream, to be unimportant. Butter from cream cooled to either 70 or 50 °F and held at either 40 or 50 °F was graded finest but was described as less attractive in flavour than the control butter—possibly because of slight lipolysis. Burnett (1967) already has shown that cream run into cans at temperatures as high as 84 °F after farm separation and stored at 40 °F was almost invariably graded finest after 2 days storage. In view, however, of the effect of slight hydrolysis of the fat on the quality of the cream and the butter reported in the present investigation, the extension to 3 days in the storage time of cream uncooled after separation should be approached with some caution.

Conditions in the trials described in the present paper were not, of course, identical with those for farm storage of cream since the milk was water-cooled on the farm and rewarmed, possibly with some stimulation of lipase, at the factory for separation. On the other hand, the bulk cream was neutralized and pasteurized soon after the separation of the final batch whereas with farm cream the delay between the time the cream leaves the farm and the commencement of factory treatment could be quite considerable with a consequent rise in temperature and a possible increase in fat acidity.

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Metabolism of [U-^{14}C]D-fructose by the isolated perfused udder

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SUMMARY. A lactating mammary gland of a sheep and a goat were perfused for several hours in the presence of [U-^{14}C]D-fructose and received adequate quantities of acetate, glucose and amino acids.

In both experiments, there was a small incorporation of ^{14}C in the expired CO_2. Smaller radioactivities were measured in milk citric acid, lactose, casein and fat, the activities decreasing in that order. The specific activities of the amino acids from one casein hydrolysate were determined. The highest radioactivities were found in alanine and serine; methionine, glutamic acid and aspartic acid showed a smaller incorporation of ^{14}C.

These results indicate that fructose is metabolized only to a very limited extent by the mammary gland. The relative distribution of ^{14}C observed among the different substances isolated may be explained by a direct splitting of fructose into two C_3-fragments, glycolysis and metabolism via the Krebs cycle.

Fructose is a minor component in the blood plasma of ruminants. Determinations carried out on samples of venous blood plasma from the mammary gland of lactating cows showed a mean concentration of 1·2 mg% fructose compared to 70 mg% glucose (Heyndrick & Peeters, 1960).

The metabolism of [U-^{14}C]fructose in intact dairy cows was studied by Luick, Kleiber, Lucas & Rogers (1957). After intravenous injection, [U-^{14}C]fructose disappeared rapidly from the plasma and there was a simultaneous rise in ^{14}C in the plasma glucose. From the relative ^{14}C incorporation in the different milk constituents and the more rapid appearance of ^{14}C in expired CO_2, as compared with similar experiments with [U-^{14}C]glucose, it was concluded that about 50% of the fructose carbon was transferred to CO_2 and milk products without passing through the plasma glucose pool.

Infusion of [U-^{14}C]fructose into the cistern of the mammary gland (Luick, Kleiber & Lucas, 1959) produced the highest specific activity in $^{14}CO_2$ of the milk followed by decreasing activities in citric acid, milk protein, lactose and milk fat. The specific activity of milk-CO_2, citrate and milk protein from the injected quarter was several times higher than the corresponding activities from the non-injected quarters. Since the specific activity of lactose was about the same in all quarters, it was concluded that fructose was not acting as a precursor of lactose within the gland.

These experiments suggested that fructose may be metabolized in the mammary gland by a mechanism different from that observed with the intact lactating cow. Since fructose metabolism may proceed via different pathways, depending on the mammalian tissue studied (Hers, 1955), perfusion experiments with [U-^{14}C]fructose on isolated udders were conducted to provide more information on the metabolism of this precursor in this organ.

EXPERIMENTAL

Two perfusion experiments were carried out. For the first experiment, a mammary gland was taken from a sheep, and for the second a mammary gland was taken from a goat. The techniques used for the preparation and perfusion have been described previously (Verbeke, Peeters, Massart-Leën & Cocquyt, 1968). The glands were perfused with heparinized sheep or goat blood containing 1 mg BOL (bromolysergic acid diethylamide; Sandoz, Switzerland)/l.

In the sheep experiment, 350 mg D-fructose + 50 μC [U-^{14}C]D-fructose (Commissariat à l'Energie Atomique, Saclay, France) were dissolved in 0·9 % (w/v) NaCl (specific activity: 355 nC/mg fructose-C). One hour after the onset of perfusion, 2·8 μC [U-^{14}C]fructose were added to the perfusion blood by way of the artificial lung and 25·8 μC to the fluid of the artificial kidney as a priming dose. 21·4 μC were added to 200 ml substrate solution containing glucose, acetate and amino acids. During the experiment, the substrate solution was infused continuously at a rate of 12–33 ml/h. Intestinal lymph, collected through a fistula from the intestinal lymph duct of a sheep, was infused at a rate twice that at which milk was produced. The purpose of the lymph infusion was to improve milk-fat synthesis (Lascelles, Hardwick, Linzell & Mepham, 1964) since lymph is very rich in chylomicra.

In the goat experiment, 100 μC [U-^{14}C]D-fructose (The Radiochemical Centre, Amersham, England) and 350 mg D-fructose were dissolved in 0·9 % NaCl (specific activity: 710 nC/mg of fructose-C). As compared to the sheep experiment, the priming dose was doubled and intestinal lymph was not added to the perfusate.

After administration of the isotope-labelled fructose the sheep experiment was continued for 8 h and the goat experiment for 10 h. The quantitative analysis of the milk components and the determination of the $^{14}CO_2$ production during the experiment were carried out as described by Verbeke *et al.* (1968). The conditions employed for hydrolysis of casein and chromatographic separation of the amino acids were as described by Verbeke, Lauryssens, Peeters & James (1959).

Triglycerides were obtained from crude milk fat after adsorption chromatography on Florisil (Hopkins and Williams Ltd.) (Carroll, 1961). After transesterification of the triglycerides, the fatty acid methyl esters were extracted with diethylether and the glycerol isolated as its trinitrobenzoate (Wood, Joffe, Gillespie, Hansen & Hardenbrook, 1958). Fructose and glucose were isolated from 10-ml samples of plasma collected from the artery and vein at different time intervals during perfusion. Following precipitation of the proteins (Somogyi, 1945) 95–99 % of the glucose was oxidized to gluconic acid by incubation with glucose oxidase for 2 h at 37 °C at pH 5·8 and in presence of O_2. After adsorption of the gluconic acid on a 1 × 3-cm column Dowex 2, HCO_3^- form (Fluka, A. G., Germany), the eluate was lyophilized. Fructose and residual glucose were isolated as their borate complexes

by column chromatography on Dowex 2 (Walborg, Christensson & Gardell, 1965).

The radioactivities of the water-soluble compounds were measured by liquid scintillation counting as described by Verbeke, Feteanu & Peeters (1967). The milk fat derivatives were measured by liquid scintillation counting after the addition of 15 ml toluene containing 0·5 % di-methyl POPOP and 5 % PPO.

RESULTS

Composition of milk. In its fat, lactose and protein fractions the composition of the milk obtained during the perfusions was not different from that observed for milk from intact animals in the week preceding the experiments. In both experiments the citric acid content was somewhat higher in the milk collected during perfusion.

Specific activities of plasma sugars. In the goat experiment, fructose was isolated

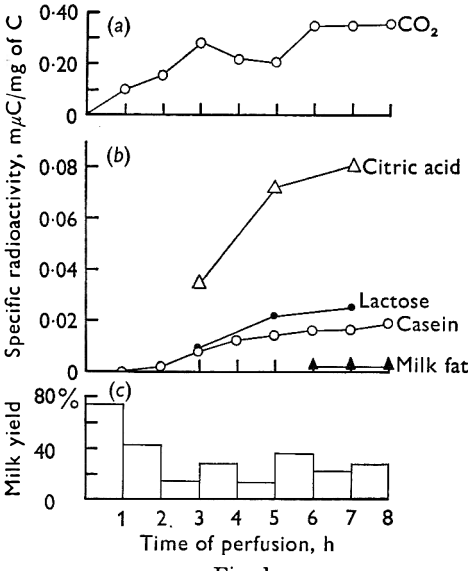

Fig. 1

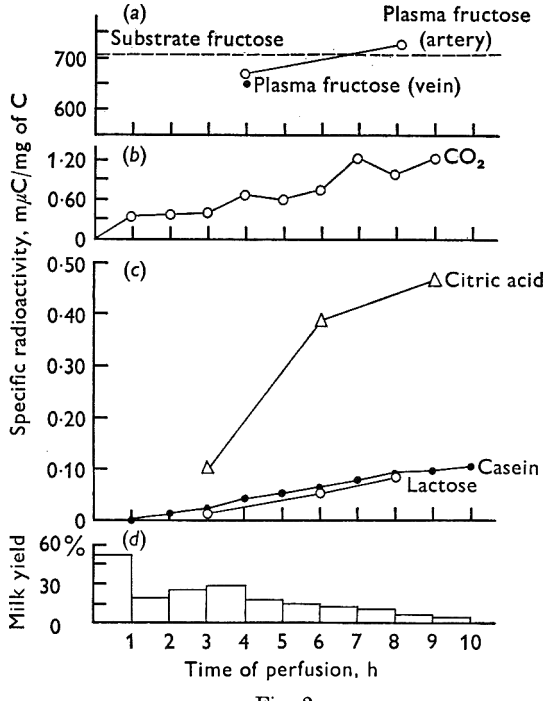

Fig. 2

Fig. 1. Perfusion of a sheep mammary gland with [U-^{14}C]D-fructose. The gland weighed 770 g and yielded 19·7 ml milk/h before the experiment (average for 8 days, shown as 100 %). The infusion of substrates in presence of [U-^{14}C]D-fructose was started at 0 h. The mean blood flow during perfusion was 29 ml per min per 100 g of tissue; the maximal O_2 consumption was about 1·3 ml per 100 g per min. (*a*) Specific activity of expired CO_2; (*b*) specific activities of milk components; (*c*) hourly milk yield (% of previous hourly yield).

Fig. 2. Perfusion of a goat mammary gland with [U-^{14}C]D-fructose. The gland weighed 1000 g and yielded 48·5 ml of milk/h before the experiment (average for 8 days, shown as 100 %). The infusion of substrates with [U-^{14}C]D-fructose was started at 0 h. The mean blood flow during perfusion was 24 ml per 100 g per min; the maximal O_2 consumption was about 1·1 ml per 100 g per min. Changes during the course of the perfusion in the specific activities of: (*a*) substrate fructose and plasma fructose; (*b*) expired CO_2; (*c*) milk components; (*d*) hourly milk yield as a percentage of the yield during the previous hour.

from 3 different collections of plasma. Samples obtained after 4 and 8 h of perfusion showed a variation of less than 7 % in the specific activity of the precursor (Fig. 2). The specific activity of fructose, isolated from venous plasma after 4 h of perfusion, did not differ significantly from that of arterial plasma. The specific activity of plasma glucose in this experiment varied between 0·10 and 0·15 nC/mg carbon.

Table 1. *Specific radioactivities of milk fat and triglyceride-components during perfusion*

(Specific radioactivity, pC/mg of C.)

Experiment	Sheep			Goat			
Time of perfusion, h ...	6	7	8	6	7	8	9
Milk fat	2·0	2·1	1·6	0·36	0·61	1·00	1·40
Triglycerides	0·21	0·24	0·35	0·43	0·77	1·00	1·36
Glycerol	3·2	N.D.	4·5	7·8	9·0	N.D.	N.D.
Fatty acids (methyl-esters)	0·20	0·25	0·30	0·17	0·40	0·79	0·90

N.D. = not determined.

Table 2. *Specific activities of amino acids isolated in casein from milk secreted by a sheep mammary gland after perfusion for 6 h with [U-^{14}C]D-fructose*

Isolated substance	Specific activity, pC/mg of C	^{14}C recovered in amino-acids, %
Casein	16·2 ± 0·2	98
Alanine	105 ± 0·8	16·7
Serine	83·6 ± 1·1	22·3
Methionine	44·5 ± 2·0	6·2
Glutamic acid	38·5 ± 0·2	43·5
Aspartic acid	27·5 ± 0·3	8·6
Glycine	2·9 ± 1·2	0·2
Proline	0·5 ± 0·5	0·4
Valine	2·9 ± 0·2	1·4
Histidine	3·9 ± 0·6	0·7

Carbon dioxide. In the sheep and goat experiment 0·62 and 2·3 % of the administered dose, respectively, were recovered as $^{14}CO_2$ indicating that [U-^{14}C]fructose is catabolized by the isolated mammary gland. The specific activity of $^{14}CO_2$ increased during both experiments, reaching a constant level in the final 3 h of perfusion (Figs 1, 2).

Synthesis of milk components. In both experiments, the highest specific activities were found in citric acid. The maximum specific activity measured in citric acid was about one-fifth to one-third of the maximum measured in $^{14}CO_2$ (Figs 1, 2).

The increase in time with the specific activities of lactose and casein was very similar in both experiments. However, the maximal specific activities of these milk components amounted to only one-fifteenth of the specific activity found in $^{14}CO_2$. The specific activity of the crude milk fat was very low in both experiments (Fig. 1 and Table 1).

Synthesis of fat. Very low specific activities were found in the triglyceride fraction (Table 1), the ^{14}C being mainly localized in the glycerol moiety. The radioactivity

measured in the glycerol fraction was only one-tenth of that observed in lactose. Similar results were obtained whether lymph was added to the perfusate, as in the sheep experiment, or omitted as in the goat experiment.

Synthesis of casein amino acids. Amino acids were isolated from a sample of casein obtained from milk secreted in the sheep experiment after 6 h of perfusion. The results are shown in Table 2. The highest specific activity was found in alanine, followed by serine. The ^{14}C incorporation in these non-essential amino acids was of the same order as that found in citric acid. A lesser incorporation of ^{14}C was found in methionine and glutamic acid, followed by aspartic acid. No appreciable labelling was found in any other amino acids.

DISCUSSION

Since the specific radioactivity of plasma fructose and most milk components reached a constant value in these experiments (Figs 1, 2), the ratio of the specific radioactivity of isolated product to precursor provides a measure of the percentage of the product which derived from the precursor. The product quotients, calculated from the results, are presented in Table 3.

Table 3. *Transfer of ^{14}C from [U-^{14}C]D-fructose to carbon dioxide and milk constituents*

	Product quotient (% of product formed from precursor)	
Experiment	Sheep	Goat
CO_2	0·11	0·16
Citric acid	0·023	0·051
Lactose	0·007	0·013
Casein	0·005	0·014
Milk fat	0·0007	0·0002
Glyceride glycerol	0·001	0·0012
Triglyceride fatty acids	0·0001	0·0001
Casein alanine	0·030	0·100
Casein serine	0·024	0·076
Casein methionine	0·012	0·040
Casein glutamic acid	0·011	0·035
Casein aspartic acid	0·008	0·025

A low product quotient for any milk constituent indicates that the perfused mammary gland did not readily convert fructose into that constituent. It should be noted, however, that the perfusions were carried out in the presence of physiological concentrations of blood sugars, since the amount of fructose in the perfusate was only about 1 % of that of the glucose present.

The relatively high specific activities found in expired CO_2, citric acid, alanine and serine as compared to lactose suggest a fructose metabolism in the mammary gland directed towards intermediary products favouring the formation of CO_2 and citrate rather than lactose. A similar pattern of labelling for milk constituents was observed by Luick *et al.* (1959) after infusion of [U-^{14}C]fructose into the udder cistern of a lactating cow. They suggested that fructose metabolism in the cow's udder proceeds by a pathway which was first described by Hers & Kusaka (1953) in rat liver,

according to which fructose is first phosphorylated to fructose-1-P and then broken down into glyceraldehyde and dihydroxy-acetone-P.

The relatively high specific activities found in alanine and serine, isolated from casein, as compared to lactose suggests that in the present experiments fructose was also metabolized through degradation via C_3-components. The participation of another more direct pathway for the conversion of fructose to hexose-6-P, not involving carbon chain fission (Muntz, 1968), would probably label lactose more than the other milk constituents.

If fructose were metabolized according to the proposed mechanism, lactose would acquire radioactivity as a result of gluconeogenesis from labelled C_3-components. The very low specific activities found in plasma glucose, which were of the same order as those in lactose at steady state, suggests that the isolated udder is a poor glucose former from fructose. However, the results show that the udder converts small quantities of fructose to lactose. Luick *et al.* (1959) failed to demonstrate this limited synthesis of lactose because their techniques lacked sensitivity.

By cleavage of fructose-1-P, arising from [U-^{14}C]fructose, radioactive glyceraldehyde and dihydroxyacetone-P would be formed. Conversion of dihydroxyacetone-P into glycerol probably explains the radioactivity found in triglyceride-glycerol. Following phosphorylation of glyceraldehyde, the triosephosphates may be metabolized by the glycolytic scheme giving 3-phosphoglyceric acid and pyruvic acid, which act as precursors of serine and alanine, respectively. Direct oxidation of D-glyceraldehyde to D-glyceric acid would provide an alternative route for serine synthesis (for a review, see Meister, 1965). By decarboxylation of pyruvic acid, radioactive CO_2 and acetyl-CoA would be formed. In contrast with the acetyl-CoA formed from acetate, the acetyl-CoA arising from pyruvate seems to be a poor precursor for fatty acid synthesis in the perfused udder preparation (Hardwick & Linzell, 1960). This would account for the relatively low ^{14}C activities found in the isolated triglyceride fatty acids during the present studies.

Since citrate is synthesized from oxaloacetate and acetyl-CoA, pyruvate may label citrate and intermediate products of the Krebs cycle via either or both of these substances. Transamination of α-ketoglutaric acid and oxaloacetic acid would result in the production of the corresponding amino acids. These reactions may explain the relative order of labelling found in the expired CO_2, citric acid, glutamic acid and aspartic acid.

In the present experiment, there was significant incorporation of ^{14}C into the essential casein amino acid methionine. Since the methionine residues of casein originate mainly from free methionine in plasma (Verbeke, Simeonov & Peeters, 1967), the relatively high ^{14}C level in this amino acid was probably the result of exchange processes between the methyl group of methionine and radioactive C_1 components. Radioactive C_1 groups, bound on tetrahydrofolic acid, may arise in the conversion of radioactive serine to glycine (cf. Meister, 1965). Radioactive glycine formed by this reaction will be considerably diluted by plasma glycine since the glycine residues of casein originate from free plasma glycine (Verbeke, Feteanu & Peeters, 1967). Moreover, in our experiments, inactive glycine was included in the substrate mixture.

Black & Kleiber (1962) injected [U-^{14}C]fructose into the udder cistern of a lactating

cow and observed an efficient incorporation of ^{14}C in some casein amino acids on the injected side. In agreement with our present results, the highest specific activity was found in alanine. However, they found considerably greater incorporation of ^{14}C into glycine than into serine. This was not observed in the present studies. The contradiction in results obtained may be due to the non-physiological route which Black & Kleiber (1962) used for the introduction of the metabolites in the mammary gland.

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The preparation of cheese curd by a continuous method: the effect of heating the curd in the absence of whey

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SUMMARY. Curd was made in a continuous curd-making apparatus (Berridge & Scurlock, 1969) and immediately separated from its whey. Its temperature was maintained at a high level (41–48 °C) as it was moved continuously in the form of a thin layer through a set of rollers. The curd then had a moisture content similar to that of normal curd at the end of scalding in the traditional process. After a suitable period of cheddaring the pH value and the moisture content of the continuously made curd were the same as those normally reached at the same stage in the traditional process.

In the traditional methods of making hard-pressed cheese such as Cheddar and Cheshire the curd is heated for periods of about 60–160 min while it is stirred in the whey. Besides its effect on the microflora of the curd this heating, or scalding, has the important function of accelerating the shrinkage of the curd particles and hence the drainage of whey from within them. In a continuous process such as that described by Berridge (1968) it would be advantageous to reduce this long heating period as much as possible, and Berridge & Scurlock (1969) have shown that it can be reduced to 4 min or less by using higher temperatures than are normal or even possible in the traditional process. Even a scalding period as short as 4 min constitutes an inconvenience in a continuous process partly because of the necessity of keeping the curd suspended in the whey, by stirring, for example, while at the same time ensuring 'plug flow' so that all the curd receives treatment at the required temperature for the required time. It would, therefore, be convenient if curd could be removed from the whey at an early stage and processed as a solid rather than as a suspension. This might also accelerate still more the rate of exudation of the whey.

It is well known that the rate of drainage of moisture from curd is sensitive to the mechanical conditions under which drainage takes place. The effectiveness of the traditional manner of cheddaring is evidence of this sensitivity. Another example of the importance of the mechanical conditions is provided by the procedure that Schulz & Kley (1956) found necessary in order to determine the shrinkage of curd in a reproducible manner. They allowed the cut curd to shrink for a period of 24 h, in presence of a preservative, in order to avoid errors resulting from the drainage of the

whey from within the particles under the influence of mechanical forces which begin to operate only when the curd is removed from the hydrostatic support of the surrounding whey. The effect of such mechanical forces was not significant with older, and therefore firmer, curd. Similarly, Thomé, Axelsson & Liljegren (1958) concluded that the removal of a curd cylinder from whey for the purpose of weighing affected the rate of whey drainage because a piece of curd which was weighed several times lost more whey than a similar piece which was weighed only once. The same effect was observed with a single weighing if the weighing time was prolonged. Lawrence (1959) observed that stirring accelerated the exudation of whey from cut curd, and Beeby (1959) noted that since stirring influenced the rate of syneresis, a constant speed of stirring was necessary during his measurements of syneresis.

In the traditional process of cheese manufacture the curd is kept suspended in the whey so that it does not mat together into a large mass and thus prevent the escape of the whey. The whey also acts as a heat-transfer medium while the temperature is being raised to that necessary for scalding. When curd is made by the continuous process it is already at a high temperature, and no heat-transfer medium is needed. The curd can be processed in a thin layer so that the escape of whey is not impeded. Experiments were therefore carried out to determine whether curd suitable for the making of hard cheese could be prepared by the immediate removal of the whey from continuously produced curds and whey. Preliminary batchwise experiments (described below) indicated that this could be done. Apparatus for doing it continuously was therefore designed. It was anticipated that something similar to a scalding process would be needed and as scalding for 4 min at 48 °C had been shown to be satisfactory, the apparatus was designed to retain the curd at approximately this temperature and in a thin layer for about this period.

METHODS

The methods of curd-making, cheddaring, and moisture and pH determinations were as previously described (Berridge & Scurlock, 1969). Moisture determinations were made in duplicate and mean values are reported, except where otherwise stated.

APPARATUS

The batchwise preliminary experiments were done with curd produced in the single membrane apparatus previously described (Berridge & Scurlock, 1969). The curd was received either on filter paper in a water-jacketed Buchner funnel or on fine nylon mesh or on expanded aluminium with diamond-shaped holes 7 mm × 3 mm. Scalding was simulated by keeping the curd in an incubator at 37 or 45 °C.

The apparatus used for the experiments in continuous processing was devised to produce and process curd to a stage equivalent to that at the end of scalding in the traditional procedure.

In its final form this apparatus contained 3 rotating rollers 8·5 in. diam. mounted one above the other and in contact, so that curd spread on the uppermost roller would descend with the rotation and at the lowest point be transferred to the intermediate roller, from which it would in turn be transferred to the lowest roller (see

Fig. 1). The speed of rotation of the rollers and the movements of the curd spreader were adjusted to give the required curd thickness and processing time, these being capable of variation above and below the preferred values. The preferred value for mean thickness was that obtained when the strands of curd originally 6 mm in diam. were deposited almost in contact with one another. Although the curd begins as a cylinder 6 mm in diam. it soon flattens and shrinks until it is much thinner. The

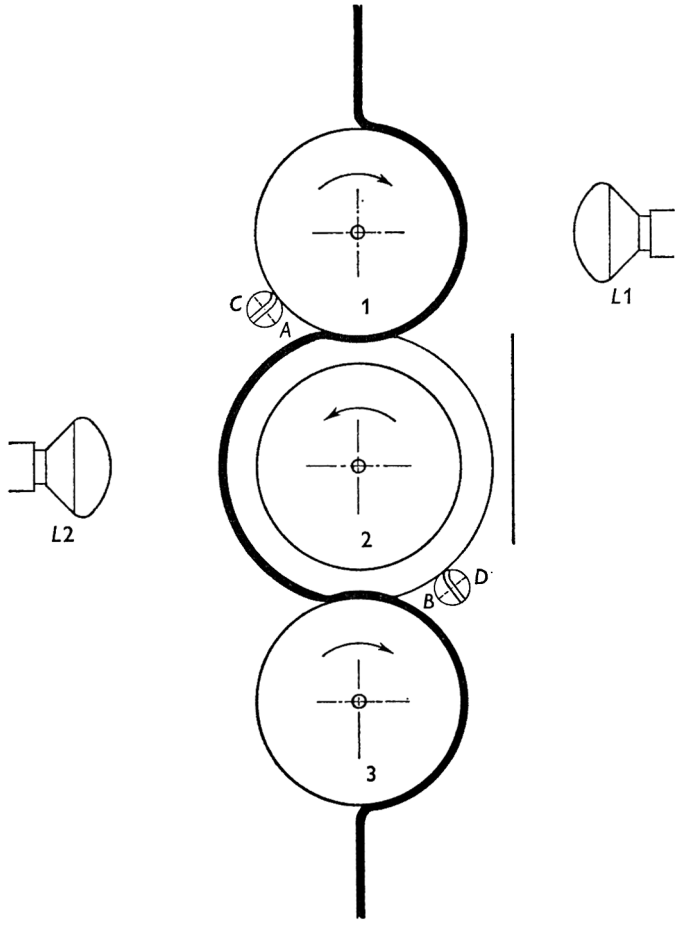

Fig. 1 (sectional). The relative positions of the rollers, curd spreader and curd removers. The route of the curd is shown by the thick line. The polyurethane foam layer is shown on roller no. 2 but the nylon mesh coverings are not indicated. *A* and *B* indicate positions in which the small heavy roller was used in some of the experiments. *L* 1 and *L* 2 are 250 watt reflector lamps. The vertical line by roller no. 2 represents a screen to prevent overheating of the cover of roller no. 2. *C* and *D* are curd removers (see text).

preferred time was 4 min. The apparatus is described in fuller detail under 3 headings as follows: (1) the curd-maker, (2) the curd spreader and (3) a set of rollers in an enclosure.

(1) *The curd-maker.* This was similar to that already described (Berridge & Scurlock, 1969) except that it had 4 membranes mounted in parallel inside a larger jacket and capillaries were used to control the rate of flow of milk into the membrane.

When relying on capillaries, i.e. on viscosity, to control a flow of milk it is of course necessary to control the temperature. In this apparatus, as in the first, it was necessary to maintain the lower end of the jacket at a low temperature and the milk itself was also kept cool. Both these controls were maintained with ice and water, so the use of capillaries was convenient. They were mounted in the bottom of the water jacket and opened directly into the membranes. Each capillary was 0·8 mm i.d. and approximately 5 cm long, the exact length being obtained by trial and error.

(2) *The curd spreader.* This was necessary because curd was extruded from the curd-maker at the rate of about 7 ft/min, and to receive it at this rate would have needed an inconveniently large apparatus. The spreader was, therefore, designed to move to and fro over a roller parallel to its axis. Four outlets on the spreader were connected to the curd-maker with PVC tubing. As the roller was rotating at the same time at a suitable speed, the start of the 4 strips of curd first laid down had moved away from the spreader by the total width of the 4 strips by the time it had completed its return stroke, giving, ideally, a criss-cross pattern of parallelograms. The ideal pattern was, however, seldom produced owing to the cylindrical shape of the curd strips which caused them to take up a parallel position for a proportion of the return stroke, leading to some parts of the curd layer being too thick while large holes remained elsewhere. The trouble was overcome by imparting a zig-zag motion to the spreader.

(3) *The rollers.* These were made from high density polythene bottles with a cylindrical portion 8·5 in. diam. and 8·5 in. long. A spherical glass joint was sealed into the opening to permit the use of vacuum or pressure in the first roller and a Meccano wheel was bolted on to the opposite end of each roller. Three such rollers were mounted with their axes horizontal in a large Meccano framework permitting ready variation in the relative positions of the rollers. Perforations about 1 in. apart were made in the first roller with a hot nail. The following coverings were tried singly or in combination on one or more of the rollers: Polyurethane foam $\frac{3}{16}$ in. thick, and $1\frac{1}{4}$ in. thick; nylon mesh 140 strands/linear in. (nylon sifting cloth grade 14N, aperture 90 μ, made by Henry Simon Ltd., Cheadle Heath, Stockport), nylon mesh 40 strands/linear in., stainless steel mesh, 80 strands/linear in. and 30 strands/linear in.

The rollers were electrically driven through suitable reduction gears at different speeds for different experiments. In some experiments a small extra roller was used to press the curd. The roller was made of brass, round which was cemented a layer of expanded polyurethane and a covering of thin rubber. This is referred to below as 'the small roller'. This roller was mounted at positions *A* or *B* in Fig. 1 and the curd remover (see immediately below) was not used.

In the later experiments a device for ensuring that curd parted from one roller to continue on the next was added. The device consisted of a number of 'fingers' cut from $\frac{1}{2}$ in. thick polyurethane foam and fixed spirally to a spindle. Two of these were used at the positions indicated by *C* and *D* in Fig. 1. Each was rotated in the same direction as the roller it was designed to wipe and at about the same peripheral speed, or slightly faster. As the foam fingers were soft there was no noticeable wear on the roller covering but the device was effective in preventing curd from adhering to the rising side of the roller.

In order to maintain the temperature of the curd a cabinet of timber (1 in. × 1 in.) and polythene film was constructed to contain the rollers and the spreader. The cabinet itself was not however sufficient to maintain the temperature of the curd. In the early experiments two 250 W reflector lamps were mounted to shine on the curd on the rollers, and in later experiments a thermostatically controlled 3 kW fan heater was installed in the cabinet. When the lamps were used the temperature of the curd at the bottom of the first roller and halfway down the third roller was 48 °C. Nevertheless, curd leaving the third roller was already down to 41 °C. Shields were inserted to prevent the lamps heating the uncovered surfaces of adjacent rollers since this was found to cause adhesion between curd and roller.

RESULTS

Batchwise experiments

Curd from milk with 2 % starter was deposited directly from the P.V.C. outlet tube on to nylon mesh 14N supported on expanded aluminium. The outlet tube was moved so that the cylinders of curd on the nylon were just touching one another but not overlapping. This was called standard thickness. A second sample of curd was deposited with the curd cylinders spaced at their own thickness from one another, and this was designated half thickness. A third sample was prepared at double thickness. These samples, while still on the nylon mesh and aluminium support, were wrapped loosely in polythene to prevent evaporation and scalded in the incubator at 37 °C for 30 min. Cheddaring for 90 min in tubes with weights as described by Berridge & Scurlock (1969) was then carried out and moisture contents determined. The results for standard, half and double thickness were 46·1, 44·8 and 49·6 %, respectively, and the pH values next day were 4·97, 4·97 and 4·90. Pimblett's (1962) maximum for moisture content at the end of cheddaring was 45 %. The low pH values recorded indicated a slightly high lactose, and therefore a high moisture content, in the curd, but as the moisture figures were only slightly above the acceptable level it appeared that early removal of whey from the curd would not have any deleterious effect.

The experiment was repeated with the temperature of the incubator for scalding raised to 44 °C. A curd layer of standard thickness was used. The pH values of the curd during cheddaring fell from 5·73 to 5·42 in 1 h and to 5·32 in 2 h. The moisture content of this curd was 42·9 %.

The experiment was repeated using different times of scalding in the incubator at 44 °C. The curd layers were made by superimposing 2 layers of half thickness at right angles to one another. This made a fabric of normal mean thickness which was easier to peel off the mesh. The curds were cheddared as usual, and then allowed to remain at 30 °C until 4 h had elapsed since the end of the scalding period. The scalding periods used were 7·5, 10, 20 and 30 min and the moisture contents of the curds at the end of the incubation period were, respectively, 44·4, 42·8, 40·2 and 40·3 %. The respective pH values were 5·38, 5·41, 5·33 and 5·38. These results indicated that scalding in the incubator was not inferior, as regards rate of loss of moisture, to scalding in whey. Although the curd samples were transferred to the incubator at 44 °C as quickly as possible it is likely that their temperature dropped considerably and did not regain

44 °C at once. The time actually spent at 44 °C was, therefore, less than that recorded as the scalding period.

Other similar experiments showed that: (1) a curd fabric could be collected on the expanded aluminium without the use of a nylon mesh and could be peeled off at the end of scalding; (2) the curd fabric so collected did not fall off the aluminium if the plane of the latter was raised to vertical 15 sec after collecting the curd; (3) with the use of nylon mesh it was possible to collect at least 6 layers of curd fabric, one above the other with nylon between them, without impeding the drainage, the resulting curd after scalding appearing firmer and dryer than when collected in a single layer; (4) curd fabric could be collected and drained by suction on filter paper (Whatman, no. 54) using a vacuum equivalent to 10 in. of water and the curd could then be peeled off the paper without first releasing the vacuum.

Continuous processing

The effect of modifications in the apparatus on the behaviour of the curd

The criteria by which the behaviour of the curd was assessed were that the flow of curd through the apparatus should be self-starting as soon as curd production began or after an interruption in the flow and that the flow should continue for several hours without hindrance due to the sticking of curd to the rollers or to its falling off the rollers.

The spreader was effective in producing a curd fabric which could often be passed over all 3 rollers without breaking. There was a natural tendency for the curd to fall off the surface of the first roller as it descended, but this actually happened only when the roller was rotating too slowly (so that the curd was too thick) or when an unperforated roller was used and the whey could not escape.

Relative positions of rollers. Various arrangements in which the curd had to leave a lower roller and climb up the rising side of a higher one were tried in the hope of obtaining a more compact apparatus, but for reliability in operation the arrangement shown in Fig. 1 was found to be necessary.

Use of suction. Suction prevented curd falling off the first roller but also tended to prevent it leaving and going on to the second. Whey could be sucked out of the bottom of the first roller if an adequate air intake were provided, but an advantage of not sucking out the whey was that if allowed to drain out of the bottom of roller 1 (Fig. 1) it assisted the transfer of curd.

Use of different coverings on rollers. A covering was necessary on the first 2 rollers to prevent the curd sliding off. Stainless steel mesh proved unsatisfactory because the curd adhered to it too tenaciously after the first trial followed by efficient cleaning. Coarse nylon mesh was also not very good because of curd adhesion; possibly curd was forced through the meshes. Fine nylon mesh was satisfactory but even this was not as good after cleaning with strong detergents as it had been when new. After the cleaning there was some tendency for the curd to stick to the nylon, but this was overcome by the curd removers. A layer of polyurethane foam beneath the nylon on the first or second roller (preferably the second) seemed to be an advantage, but this was probably due to the irregularities in the shape of the rollers after some weeks of use.

Perforations in the roller. These were necessary in the first roller since in their

absence some of the curd failed to cling to the roller until it reached the second roller. The curd behaved as though a layer of whey were retained beneath it.

Extra rollers pressing the curd. The effects on curd moisture of a heavy extra roller pressing against roller no. 3 (position *B* in Fig. 1) are given below under moisture content. As regards the behaviour of the curd with respect to ease of transfer from one roller to the next a heavy roller on no. 2 (position *A* in Fig. 1) seemed to have no effect when there was already some pressure between 1 and 2.

Function of the curd removers. Many experiments were made without the use of the curd removers and the curd often behaved in a satisfactory manner. Occasionally, however, there was light adhesion between curd and rollers but the curd was easily removed and the very soft foam fingers of the curd remover were adequate to maintain the regularity of transfer of the fabric between the rollers as required.

Moisture content of the curd

Curd was prepared in the normal way and the speed of the roller apparatus was adjusted so that the process time was about 4 min. The temperature of the curd was maintained by reflector lamps as indicated in Fig. 1 and described on p. 245.

The temperature of the curd as it left the lower roller was 41 °C. As whey was still dripping rapidly from the curd it was allowed to drain for 5 min in an incubator at 45 °C. Its moisture content was then 53·3 %. The small rubber-covered roller weighing 400 g was then placed in position *A* as marked on Fig. 1, the other conditions being maintained. The drained curd then had a moisture content of 51·5 %.

The effect of applying different pressures to the curd

The roller apparatus was modified so that the bearings of roller no. 2 (Fig. 1) were supported by nylon monofilament (fishing line) which passed over pulleys to a bar from which was hung a pan and weights. Enough weights were added to counterpoise the roller, and then additional weights totalling (*a*) 200, (*b*) 500 and (*c*) 1000 g were added.

Table 1. *Force between rollers and moisture content of curd*

Forces applied		Moisture content of curd	
Between rollers 1 and 2, g	Between the small roller and roller no. 3, g	Expt 1 (drained at 45 °C 5 min), %	Expt 2 (drained at 30 °C 5 min), %
0	400	48	54
0	600	46	49
0	900	48	54
0	1400	48	—
200	0 (no roller)	—	54
500	400	—	54
500	600	49	51
500	900	48	51
500	1400	45	—
1000	400	—	53
1000	600	47	53
1000	900	48	51
1000	1400	45	—

In a similar way the small roller at *B* (Fig. 1) was pulled downwards with (*a*) 200, (*b*) 500 and (*c*) 1000 g. As the roller weighed 400 g the total weights applied to the curd at this position were 400, 600, 900 and 1400 g. The pressure on the curd was not determined because the area over which the force was applied was unknown and with the small roller at *B* the area of contact increased as the force increased owing to the softness of the expanded polyurethane beneath the rubber surface.

The experiment was done on 2 different occasions. On the first the curd samples were drained for 5 min at 45 °C, and on the second for 5 min at 30 °C. Only single moisture determinations were made. The results are given in Table 1.

The effects of pressure on the moisture content of the curd were not very clear cut but the higher pressures did usually produce curd of lower moisture content. Thus, in expt 2 curd containing 51% of moisture was obtained only with the following combination of forces (force between large rollers given first), 500, 600; 500, 900; 1000, 900. The largest force on the small roller produced smearing of the curd and cloudy whey.

The effect of pressure was not, however, on moisture content only. After passing over all the rollers without pressure the curd fabric was weak; it broke easily under its own weight. A moderate force (e.g. 500 g) between only the first 2 rollers produced a notable increase in the tensile strength of the curd sheet; it broke less easily under its own weight and its passage over the remaining rollers was less liable to interruption by breakages.

The effect of different temperatures and different curd thicknesses

A small rise in the temperature of the curd when other conditions were maintained constant produced, as was expected, a diminution in moisture content. When curd was at 41 °C as it was received off the third roller its moisture content was 55·8%. This was reduced to 53·1% when the temperature of the curd moving through the apparatus had been raised to 43 °C by increasing the air temperature in the enclosure.

The thickness of the curd fabric was inversely proportional to the speed of rotation of the rollers. Thus, a thicker curd layer could be formed by reducing the speed of the rollers. Such thicker curd might be expected to retain more moisture but since it had longer to drain while it was on the rollers its moisture content was in fact not significantly different from that of the curd of normal thickness. Figures obtained in one of the experiments were 57·8% moisture for thin curd and 57·6% for thick curd. In another example both the thickness of the curd and its temperature were varied. The results were as follows:

Enclosure temp., °C	48	48	46
Curd temp., °C	—	47	45·4
Time of passage over rollers, min	3	4	7
Resulting thickness	Thin	Standard	Thick
Moisture content, %	60·1	57·2	59·4

In this experiment the curd sample was blotted lightly and rapidly (5–10 sec) on both sides with thick filter paper.

Moisture content of rolled curd after cheddaring

Cooled raw milk was inoculated with 2% of cooled neutralized starter (NCDO 1200) at the time of renneting and made into curd in the usual way (Berridge & Scurlock,

1969). It was cheddared in vertical tubes with weights as already described (vol. 36, p. 55) for 90 min and then allowed to remain for a further period at 30 °C for acid production before sampling for moisture content. The experiment was repeated to obtain lower pH values in the curd at the time of sampling for moisture. The results of both experiments are shown in Table 2.

Table 2. *The pH values and moisture contents after incubation of curd prepared at different roller speeds*

Time on rollers, min	Time at 30 °C including cheddaring, h	Final pH value	Final moisture content, %
4·5	5·5	5·60	42·5
9·5	5·0	5·70	43·2
2·0	7·0	5·46	44·3
4·0	6·5	5·57	43·4
6·0	6·25	5·61	44·0

DISCUSSION

Having shown that there might be advantages in removing the curd from the whey as soon as possible we felt it desirable to find out, with the minimum of delay, whether curd could conveniently be processed from such an early stage as a solid. Therefore, apparatus was constructed from materials at hand. This apparatus was adequate to show that it is indeed convenient to process curd in this way. What it failed to show was whether a more rapid exudation of whey could be achieved by this means, and what the quantitative relations were between time, temperature and pressure on the one hand and curd moisture on the other. This was partly because of inadequate control of the temperature of the curd. Later measurements with a thermocouple showed large differences between the temperature of the curd and that of the air surrounding it. However, the values given in this paper are temperatures of the curd itself measured with a thermometer. The measurement of the temperature of a thin layer of curd in this way is to be regarded only as a first approximation. Moreover, under the prevailing conditions the temperature of the curd fluctuated as it passed over the rollers and it was impossible to determine its average temperature. The general impression was one of difficulty in maintaining curd temperature. This is confirmed by the low values given in the results. The reasonable figures that were nevertheless obtained for curd moisture may have been due to the effects of early removal of whey and of the use of slight pressure. We hope shortly to be able to study the effect of temperature and pressure and possibly other variables on the rate of exudation of whey from continuously made curd.

The results of Table 1 show that the rate of passage through the rollers did not have a significant effect on the final moisture content of the curd. This was probably because the thicker curd layer formed when the rollers were rotating slowly had correspondingly longer to drain. However, a shorter time on the rollers would permit the use of higher temperatures without damaging the activity of the starter. The higher temperatures would cause more rapid drainage of whey and allow the time to be shortened further.

It remains to gain experience with the method described in this paper, applied on

a larger scale. A larger apparatus is in the course of construction and it is hoped that the regular production of cheese will be possible. Meanwhile, methods of shortening the time between the end of cheddaring and the addition of salt are being explored.

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The effect of temperature fluctuations on the bactericidal effectiveness of continuous heat-treatment processes

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SUMMARY. In correctly operated continuous heat-treatment plant the processing temperature is maintained at a sufficiently elevated level to give the desired thermal death rate for any micro-organisms present before treatment. This paper shows that if through imperfect operation of the plant, the processing temperature fluctuates about the required level, the chances of survival for bacteria and spores will theoretically be increased. Tabulated results show the magnitude of the effect under varying conditions and the compensating increase in processing temperature to counteract it.

Many continuous heat-treatment processes rely for a consistent bacteriological effectiveness on the maintenance of a constant temperature while the product passes through a holding tube at constant velocity or with a known distribution of velocities. The high-temperature, short-time pasteurization of milk and the ultra-high-temperature sterilization of liquids are examples of such systems. There is little difficulty in assessing the probable bactericidal or sporicidal effectiveness from bacteriological thermal death data, for any holding tube and product velocity, provided the processing temperature is constant. However, in some circumstances the temperature may not be constant.

The product is heated to the holding temperature either by indirect heating in plate, tubular or scraped-surface heat exchangers, or by direct heating through the injection of steam into the product or by spraying of the product into steam. The heating process is normally controlled automatically by a pneumatic or electrical control system designed to maintain a set temperature at the entrance to the holding tube. With indirect heating, changes in the processing temperature caused by variations in the operating conditions take place slowly because of the damping effect of the various thermal capacities, and the control system has no difficulty in acting at a comparable speed. With direct heating, however, particularly when steam is injected into the product, the processing temperature responds very much faster to any changes of input variables such as steam pressure. If the sensitivity of the control system is increased in a misguided attempt to counteract this speed of response, instability may instead develop and cause oscillatory temperature fluctuations. It is well known that such instability will give rise to sinusoidal oscillations provided

the excursion is not too great. It is, therefore, of interest to examine the effect of such sinusoidal variations in the treatment temperature on the performance of a continuous processing plant.

If the packaged units of the processed product are small, i.e. one unit volume passes the end of the heating section in a time that is small in relation to the period of the temperature oscillation, and no bulking takes place subsequently, each unit can be considered as processed at a constant temperature corresponding to one point on the temperature–time curve. The distribution of the effectiveness of sterilization among the accumulated units can then be determined, with the maximum and minimum effectivenesses corresponding to the extremes of temperature.

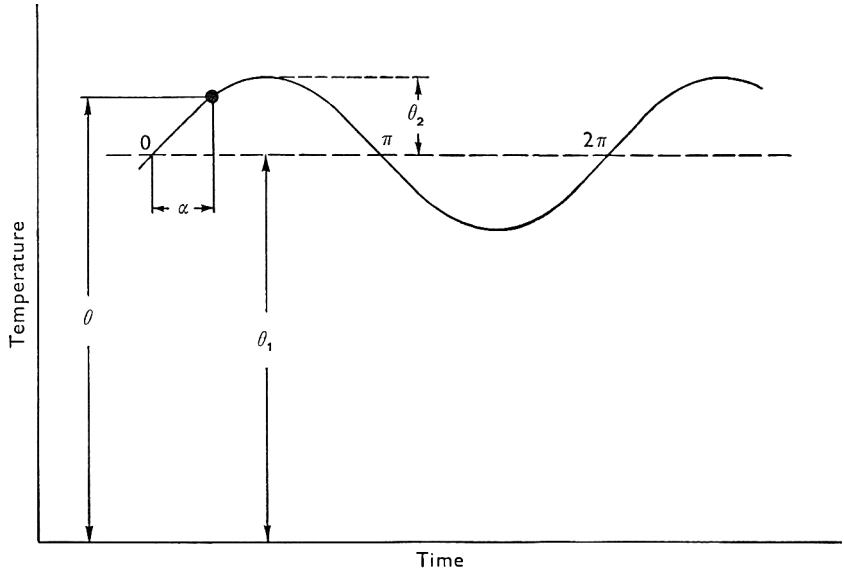

Fig. 1. Diagrammatic representation of the variation of processing temperature with time.

This is a somewhat artificial case, and in general the product will be bulked as it leaves the processing plant and before filling, so that the product then has an average bacteriological quality corresponding to the average effect of the temperature variation over many cycles of the sinusoidal oscillation. It is this average quality that we wish to calculate, in order to determine by how much it varies from that corresponding to a constant temperature equal to the mean of the oscillation.

CALCULATION OF THE AVERAGE BACTERIOLOGICAL EFFECTIVENESS

The temperature variation of the product with time is as shown in Fig. 1. Mathematically, the instantaneous temperature θ is given by

$$\theta = \theta_1 + \theta_2 \sin \alpha, \tag{1}$$

where θ_1 is the average temperature in °C, θ_2 is the peak value of the sine wave variation about the mean, in deg C and α is an angle representing the abscissa of the sine wave, in radians.

The thermal death curve of a population of micro-organisms can be considered to be semilogarithmic, or can be treated as a combination of 2 or more semilogarithmic curves. A semilogarithmic thermal death curve can be represented by the equation

$$L = -K_\theta t, \tag{2}$$

where L is the logarithm to base 10 of the proportion of surviving organisms, K_θ is the slope of the thermal death line at temperature θ and t is the time for which the temperature θ is applied (Burton, 1958).

Over a restricted range of temperatures, K_θ varies with temperature in such a way that

$$K_{\theta+1}/K_\theta = Q_1. \tag{3}$$

Q_1 represents the way in which the thermal death-rate varies with temperature, and is characteristic of a particular strain. Typical values for resistant spores in milk at processing temperatures of 110–140 °C lie between 1·25 and 1·45 (Franklin, Williams & Clegg, 1958; Franklin, Williams, Burton, Chapman & Clegg, 1959), although values as low as 1·1 have been reported (Miller & Kandler, 1967).

From (3) it follows that

$$K_\theta = K_{\theta_1} Q_1^{\theta-\theta_1}.$$

If this is now related to the temperature curve of Fig. 1, expressed in (1),

$$K_\theta = K_{\theta_1} Q_1^{\theta_2 \sin \alpha}.$$

For a small amount of product passing the beginning of the holding tube while the temperature can be considered to be constant at θ, the logarithm of the proportion of surviving organisms is given, from (2) by

$$L = -K_{\theta_1} Q_1^{\theta_2 \sin \alpha} t.$$

The proportion of surviving organisms in this small amount of product is $P = 10^L$. The average proportion of surviving organisms over one complete cycle of the temperature oscillation is therefore

$$\bar{P} = (1/2\pi) \int_0^{2\pi} 10^L d\alpha.$$

When there is no oscillation of the processing temperature

$$P_1 = 10^{-K_{\theta_1} t}. \tag{4}$$

The effect of the oscillatory temperature on the number of surviving organisms is expressed by the increased survivors ratio

$$\bar{P}/P_1 = \frac{1}{2\pi P_1} \int_0^{2\pi} 10^L d\alpha,$$

where $$L = -(K_{\theta_1} t) Q_1^{\theta_2 \sin \alpha}$$

and $$P_1 = 10^{-K_{\theta_1} t}.$$

This ratio depends on the values of Q_1 and θ_2, i.e. on the thermal death characteristics of the organism being considered and on the excursion in the operating

temperature. It also depends on the product $(K_{\theta_1} t)$, which is the number of log cycles reduction in the content of the organism under ideal non-varying conditions. For any fixed value of this product, the results will be the same whatever are the individual values of K_{θ_1} and t, and also whatever combinations of processing temperature θ_1 and thermal death characteristics of the micro-organisms concerned determine the value of K_{θ_1}.

As the definite integral which gives the increased survivors ratio, $\bar{P}/P_1$, is not easily evaluated analytically, it has been calculated by computer for a range of values of Q_1 and θ_2, and for several values of the product $(K_{\theta_1} t)$ representative of the reduction in spore count found in practical sterilizing processes.

CALCULATION OF THE APPARENT DROP IN OPERATING TEMPERATURE

The effective temperature θ_e is that temperature which, not being subject to the sinusoidal variations, would give the same average proportion of survivors $\bar{P}$.

Then $$\theta_e = \theta_1 - \Delta\theta$$

and $$\bar{P} = 10^{-K_{\theta_e} t},$$

therefore $$\log \bar{P} = -K_{\theta_e} t$$

$$= -K_{\theta_1} t Q_1^{-\Delta\theta} \quad \text{from (3)}$$

therefore $$Q_1^{\Delta\theta} = -K_{\theta_1} t / \log \bar{P},$$

therefore $$\Delta\theta = \log (-K_{\theta_1} t / \log \bar{P}) / \log Q_1.$$

This relationship can be written in terms of the increased survivors ratio $\bar{P}/P_1$ as

$$\Delta\theta = \log \frac{K_{\theta_1} t}{K_{\theta_1} t - \log \bar{P}/P_1} \Big/ \log Q_1.$$

The apparent drop in operating temperature, $\Delta\theta$, has been computed for each of the increased survivors ratios $\bar{P}/P_1$.

CALCULATION OF THE REQUIRED INCREASE IN THE SET OPERATING TEMPERATURE TO COMPENSATE FOR THE SINUSOIDAL VARIATION

It does not follow that an increase in the set point from θ_1 to $(\theta_1 + \Delta\theta)$ will correct for the loss in bacteriological performance. Because of the continuing effect of the sinusoidal variation, such an increase in temperature will fall short of what is required. To find the higher operating temperature it is necessary to use an iterative procedure in which the mean temperature is increased by successive small increments until a computed bacteriological effectiveness is obtained which is acceptably close to that given by the ideal processing conditions.

A computer program was written to carry out this process until the effective product (Kt) at the higher temperature but with oscillation about the mean was not less than the original ideal value $(K_{\theta_1} t)$, and was not more than 1 % above it. The compensating rise in the set point temperature was computed for each of the conditions studied in the previous sections.

RESULTS

Table 1 refers to a process with an ideal (Kt) value of 2, i.e. a process which reduces the count of micro-organisms by 2 log cycles under ideal non-varying conditions of temperature. For different values of Q_1 and of the temperature excursion θ_2, the computed values of the increased survivors ratio $\bar{P}/P_1$, the apparent drop in operating temperature $\Delta\theta$, and the upward adjustment of the set point to restore the thermal death to the ideal (Kt) value, are set out to 3 decimal places.

Table 1. *Computed values for increased survivors ratio, apparent drop in operating temperature, and upward adjustment of set point for* (Kt) = 2, *or 2 log cycles reduction in count of micro-organisms under ideal conditions*

θ_2	Q_1	Increased survivors ratio $\bar{P}/P_1$	Apparent drop in operating temp. deg C	Upward adjustment of set point deg C
1·0	1·1	1·037	0·084	0·093
2·0	1·1	1·150	0·325	0·357
3·0	1·1	1·340	0·689	0·757
4·0	1·1	1·605	1·137	1·302
5·0	1·1	1·946	1·639	1·913
1·0	1·2	1·138	0·156	0·187
2·0	1·2	1·553	0·551	0·636
3·0	1·2	2·247	1·061	1·273
4·0	1·2	3·217	1·605	2·044
5·0	1·2	4·447	2·147	2·845
1·0	1·3	1·286	0·214	0·247
2·0	1·3	2·148	0·692	0·831
3·0	1·3	3·579	1·235	1·606
4·0	1·3	5·533	1·770	2·442
5·0	1·3	7·906	2·271	3·309
1·0	1·4	1·471	0·260	0·298
2·0	1·4	2·888	0·778	0·986
3·0	1·4	5·205	1·318	1·800
4·0	1·4	8·237	1·819	2·663
5·0	1·4	11·695	2·269	3·560
1·0	1·5	1·684	0·296	0·343
2·0	1·5	3·736	0·831	1·086
3·0	1·5	7·003	1·354	1·933
4·0	1·5	11·056	1·819	2·825
5·0	1·5	15·362	2·218	3·728

The number of surviving organisms increases with both Q_1 and θ_2, until with $Q_1 = 1{\cdot}5$ and an excursion of 5 deg C, more than 15 times as many organisms survive as under steady-state conditions. However, it is only with relatively large values of Q_1 and θ_2 that the increase in survivors is likely to be detectable by conventional bacteriological techniques. Values of $\bar{P}/P_1$ which are below about 5 are likely to be insignificant bacteriologically. Peak temperature excursions of up to about $2\frac{1}{2}$ deg C over the range of values of Q_1 considered will therefore give acceptable results.

The upward adjustment of the set point to compensate for the effect of temperature oscillation correspondingly increases with Q_1 and θ_2. Under the most extreme conditions it is 50–75 % of θ_2, but under most conditions it is considerably less than half the peak temperature excursion.

Tables 2–4 present the corresponding data for ideal (Kt) values of 4, 6 and 8,

Table 2. *Computed values for increased survivors ratio, apparent drop in operating temperature, and upward adjustment in set point for* $(Kt) = 4$, *or 4 log cycles reduction in count of micro-organisms under ideal conditions*

θ_2	Q_1	Increased survivors ratio $\bar{P}/P_1$	Apparent drop in operating temp., deg C	Upward adjustment of set point, deg C
1·0	1·1	1·175	0·186	0·204
2·0	1·1	1·751	0·658	0·724
3·0	1·1	2·872	1·276	1·404
4·0	1·1	4·786	1·955	2·193
5·0	1·1	7·846	2·656	3·040
1·0	1·2	1·682	0·318	0·360
2·0	1·2	4·382	0·959	1·084
3·0	1·2	11·106	1·661	1·945
4·0	1·2	25·203	2·365	2·839
5·0	1·2	51·035	3·054	3·738
1·0	1·3	2·532	0·405	0·458
2·0	1·3	9·969	1·094	1·263
3·0	1·3	31·900	1·797	2·179
4·0	1·3	81·195	2·473	3·090
5·0	1·3	170·521	3·111	4·044
1·0	1·4	3·768	0·462	0·520
2·0	1·4	19·811	1·164	1·391
3·0	1·4	71·358	1·849	2·299
4·0	1·4	185·519	2·488	3·250
5·0	1·4	377·035	3·070	4·193
1·0	1·5	5·439	0·501	0·569
2·0	1·5	35·039	1·203	1·451
3·0	1·5	132·783	1·866	2·389
4·0	1·5	336·919	2·464	3·348
5·0	1·5	646·586	2·991	4·312

Table 3. *Computed values for increased survivors ratio, apparent drop in operating temperature, and upward adjustment in set point for* $(Kt) = 6$, *or 6 log cycles reduction in count of micro-organisms under ideal conditions*

θ_2	Q_1	Increased survivors ratio $\bar{P}/P_1$	Apparent drop in operating temp., deg C	Upward adjustment of set point, deg C
1·0	1·1	1·430	0·275	0·303
2·0	1·1	3·093	0·894	0·984
3·0	1·1	7·279	1·627	1·790
4·0	1·1	16·768	2·394	2·687
5·0	1·1	36·778	3·172	3·559
1·0	1·2	2·874	0·436	0·483
2·0	1·2	14·546	1·181	1·309
3·0	1·2	63·399	1·959	2·215
4·0	1·2	225·491	2·730	3·149
5·0	1·2	663·905	3·485	4·101
1·0	1·3	5·891	0·523	0·581
2·0	1·3	53·561	1·295	1·466
3·0	1·3	323·767	2·065	2·407
4·0	1·3	1344·499	2·808	3·356
5·0	1·3	4119·623	3·516	4·307
1·0	1·4	11·390	0·575	0·635
2·0	1·4	155·615	1·351	1·551
3·0	1·4	1105·331	2·103	2·500
4·0	1·4	4675·547	2·811	3·459
5·0	1·4	13486·631	3·464	4·436
1·0	1·5	20·577	0·609	0·679
2·0	1·5	373·839	1·381	1·601
3·0	1·5	2827·396	2·111	2·572
4·0	1·5	11408·734	2·781	3·542
5·0	1·5	29944·443	3·380	4·518

respectively, i.e. for processes which give survivor ratios of 1 in 10^4, 1 in 10^6 and 1 in 10^8. As the (Kt) value increases, the effect of temperature oscillation in increasing the proportion of survivors also becomes much greater. For example, a process which ideally gives 1 in 100 million surviving organisms with a Q_1 of 1·5, gives 1 in 1 million survivors for a peak temperature excursion of 1 deg C, and more than 1 in 100 survivors for an excursion of 5 deg C.

Table 4. *Computed values for increased survivors ratio, apparent drop in operating temperature, and upward adjustment in set point for* $(Kt) = 8$, *or 8 log cycles reduction in count of micro-organisms under ideal conditions*

θ_2	Q_1	Increased survivors ratio $\bar{P}/P_1$	Apparent drop in operating temp., deg C	Upward adjustment of set point, deg C
1·0	1·1	1·836	0·352	0·387
2·0	1·1	5·878	1·060	1·166
3·0	1·1	19·726	1·852	2·038
4·0	1·1	62·477	2·667	2·933
5·0	1·1	182·808	3·486	3·835
1·0	1·2	5·286	0·519	0·576
2·0	1·2	51·392	1·319	1·434
3·0	1·2	383·237	2·139	2·371
4·0	1·2	2130·798	2·950	3·336
5·0	1·2	9104·308	3·746	4·299
1·0	1·3	14·677	0·600	0·653
2·0	1·3	304·823	1·417	1·572
3·0	1·3	3468·460	2·227	2·520
4·0	1·3	23438·055	3·011	3·493
5·0	1·3	104527·328	3·762	4·474
1·0	1·4	36·691	0·646	0·713
2·0	1·4	1291·929	1·463	1·648
3·0	1·4	18031·692	2·256	2·604
4·0	1·4	123719·303	3·008	3·576
5·0	1·4	504964·807	3·708	4·565
1·0	1·5	82·720	0·675	0·738
2·0	1·5	4208·819	1·488	1·691
3·0	1·5	63284·859	2·260	2·660
4·0	1·5	404598·981	2·976	3·644
5·0	1·5	1446993·772	3·625	4·618

For a maximum excursion of θ_2 the required upward adjustment of the set point cannot exceed θ_2, but increases towards this limiting value with increasing (Kt). For high values of (Kt) and Q_1, the required adjustment is within 0·5 deg C of the peak excursion.

For the higher values of (Kt), and for Q_1 greater than 1·2, detectable changes in bacteriological results are likely to occur unless the temperature excursions are limited to less than about 1 deg C.

CONCLUSIONS

The calculations show that any oscillation of the processing temperature due to faulty control settings on a heat-treatment plant will reduce its bactericidal and sporicidal effectiveness. This is a theoretical conclusion. In practice, the increased survivor ratio will in most circumstances be difficult to detect by standard bacteriological counting methods if the maximum temperature excursion is less than 1 deg C.

Larger oscillations, however, can cause very pronounced changes in the effectiveness particularly at high values of (Kt).

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A fractionation of the α_s-casein complex of bovine milk

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Summary. The nature of the α_s-casein complex of bovine milk has been investigated by cation-exchange chromatography on columns of sulphoethyl Sephadex C-50 of purified preparations of α_s-casein and of whole acid-precipitated casein. Three main fractions were separated from each. Two behaved as single homogeneous proteins as judged by starch-gel electrophoresis; they were the main constituent of the complex, α_{s1}-casein, and a closely related phosphoprotein, designated α_{s0}-casein, which was present in small amounts only. The third fraction also constituted only a small part of the total complex. It was heterogeneous on starch gel electrophoresis and contained 2 major and 2 minor components. This fraction, while similar to other members of the α_s-casein complex and to β-caseins hitherto described in that it contained phosphorus, nevertheless differed significantly from these since 3 at least of its components contained sulphur either in the form of disulphide bonds or of sulphydryl groupings.

Information is presented on the composition of the 3 fractions including C-terminal end group analysis.

The essentially heterogeneous nature of α-casein isolated from bovine milk was first successfully demonstrated by Waugh & von Hippel (1956) who separated its 2 main components α_s- and κ-casein. Further investigation has shown that these components were themselves heterogeneous and that a major source of the heterogeneity of α-casein was the occurrence of a number of closely related genetically controlled variants of its main constituent (Thompson, Kiddy, Pepper & Zittle, 1962; Neelin, 1964). This heterogeneity of α_s-casein preparations is apparent from their behaviour when subjected to starch and polyacrylamide gel electrophoresis, and is not confined to that to be expected on the basis of genetic variation alone, since preparations obtained from the milk of homozygous cows have also been found to be heterogeneous (Thompson & Kiddy, 1964). Thus, while the major calcium-sensitive component of α-casein, when prepared from the milk of a homozygous cow, can be regarded as a single homogeneous genetic variant of α_{s1}-casein, it is invariably associated in the α_s-casein complex with small amounts of other proteins which have not hitherto been characterized. The present communication describes the fractionation of α_s-casein by cation-exchange chromatography on sulphoethyl Sephadex C-50 and the examination of certain of these minor constituents.

EXPERIMENTAL

Materials

Acid-precipitated whole casein. Milk was obtained from individual Ayrshire cows of the Institute herd which were selected as being free from mastitis, and centrifuged at 1500 *g* for 30 min at 15 °C. The skim-milk remaining after removal of the layer of fat was diluted with an equal volume of distilled water and the mixture at 25 °C was acidified to pH 4·6 by dropwise addition of N-HCl. The precipitated casein was separated by filtration and washed 3 times by suspension in distilled water before being dissolved by addition of N-NaOH in such a way that the pH value never exceeded 7·5. The solution was clarified by filtration through a thin layer of Celite 512 supported on glass-fibre paper (Whatman GF/A obtained from H. Reeve Angel and Co. Ltd., London). The filtrate contained approximately 1 % protein as determined by the method of Lowry, Rosebrough, Farr & Randall (1951) when compared with a standard solution of α_s-casein having a specific extinction coefficient of 10·6 at 278 nm. The acid-precipitated whole casein had a specific extinction coefficient of 8·1, measured at 278 nm.

α_s-Casein. α_s-Casein was prepared from acid-precipitated casein by electrophoresis in a column stabilized by a density gradient (Manson, 1965) and also by the procedure described by McMeekin, Hipp & Groves (1959) for the preparation of α_1-casein.

α-Lactalbumin. α-Lactalbumin was prepared by the procedure of Aschaffenburg & Drewry (1957). The purification process was checked by starch-gel electrophoresis of the product under the conditions described below, which were also employed for the examination of casein fractions.

Methods

Starch-gel electrophoresis. The vertical procedure described by Smithies (1959) was employed. The gel was prepared by dissolving 57 g starch in 343 ml buffer solution of pH 9·2 which had the following composition per litre: Tris 10·1g, EDTA.Na_2.$2H_2O$ 1·3 g, H_3BO_3 0·77 g (Aronsson & Grönwall, 1957). Urea was then added to the gel to a final concentration of approximately 5 M. Sufficient concentrated buffer solution and urea were added to solutions of casein preparations before electrophoresis to adjust their concentrations to be approximately equal to those in the gel. The total protein concentration in samples treated in this way was about 1 % immediately before application to the gel.

Electrophoresis was performed at 4 °C for 20–24 h with a potential gradient of 6–8 V/cm. In some determinations the samples were first reduced by treatment with 2-mercaptoethanol (ME) (0·2 %, v/v) for 1 h at room temperature. In these circumstances the gels used also contained 0·2 % (v/v) ME.

Chromatography on sulphoethyl Sephadex C-50. Casein preparations were fractionated on columns (2·0 × 44·5 cm) of sulphoethyl Sephadex C-50 (Pharmacia, Great Britain, Ltd., London) which were prepared in and equilibrated with sodium formate/formic acid buffer (4·0 g sodium formate, 11·5 g formic acid/l; I = 0·10) containing 8 M-urea. This had a pH value of 4·0.

Before application to the column the casein sample was first dialysed against this buffer. When the fractionation was performed with reduced casein the reduction

procedure of Anfinsen & Haber (1961), involving treatment of the protein with ME at pH 8·6, was first applied. The reduction was ended by addition of formic acid to pH 4·0 followed by dialysis of the mixture against the formate/urea buffer. In a typical fractionation, the casein sample (20 ml containing 0·5 g protein) was then applied to the top of the column followed by between 2- and 3-bed volumes of formate/urea buffer. Development was continued by washing the column with buffer solution incorporating a progressively increasing concentration of NaCl from zero to 0·2 M. No ME was present in this eluting buffer. The flow from the column was maintained at a constant rate of 17 ml/h and the effluent was collected in fractions of 10 ml. Their protein content was determined by measurement of their light absorption at 278 nm, and selected fractions were combined. Casein components were isolated by freeze-drying after removal of buffer salts by dialysis, and stored at −15 °C.

Determination of C-terminal sequences of α_s-casein components. α_s-Casein components were digested with carboxypeptidase A (EC 3.4.2.1; purchased from Sigma Chemical Co. Ltd., London) which had been treated with diisopropylfluorophosphate to eliminate possible activity arising from contamination with trypsin or chymotrypsin. A portion (0·030 ml) of an aqueous suspension of the enzyme was centrifuged and the solid material obtained was washed 3 times with 2 ml distilled water. After each washing the enzyme was again recovered by centrifugation. Finally, it was dissolved by addition of 0·167 M-NaCl solution (7·0 ml), 0·5 M-$NaHCO_3$ (1·0 ml) and 0·33 M-NaOH (0·5 ml). The pH value of the solution was reduced to 8·0 by dropwise addition of 0·33 M-HCl and the volume adjusted to 25 ml with distilled water. The more concentrated solution used in the later stages of the digestion of the casein samples was prepared in the same way from 0·187 ml of the enzyme suspension diluted finally to 30 ml.

For end-group analysis the samples of α_s-casein components, containing approximately 80 mg protein, were dissolved in 10 ml water and adjusted to pH 8·0 by addition of NaOH solution. The protein concentration in each sample was determined by the method of Lowry, *et al.* (1951). A 9-ml portion of each solution was incubated at 21 ± 1 °C with 2·0 ml of the dilute solution of carboxypeptidase A described above. Samples were withdrawn from the digestion mixture after 4 min and again after 3 h, and immediately acidified by addition of 3 ml of 0·06 M-HCl to stop further enzyme action. After 3·5 h, a 6·0 ml portion of the remaining digestion mixture was taken and treated with 8·0 ml of the more concentrated carboxypeptidase A solution and the digestion was continued. Samples (3·0 ml) were withdrawn after a further 10 min and after 4·5, 15·5 and 28·5 h, and acidified by addition of 1·0 ml 0·167 M-HCl. Two or three drops of toluene were added as preservative.

The amino acids released were separated from the protein remaining in the digestion mixture by passage of the samples through a column of Sephadex G-25 (fine grade particle size 20–80 μ) of diam. 1·8 cm and length 29 cm, prepared from a suspension in 0·001 M-HCl and 0·01 M-aeetic acid. The column was washed with this solution and the effluent was collected in 3 ml fractions. Typically, protein was eluted in fractions 10–12. Fractions 15–44 were combined, neutralized by addition of 3·0 ml 0·1 N-NaOH and concentrated in a rotary evaporator under reduced pressure at a temperature (external) of not more than 40 °C. The final volume was adjusted to 5·0 ml.

Quantitative amino acid analyses were performed on these extracts using an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex) following the procedure of Spackman, Stein & Moore (1958).

The possibility that some loss of tryptophan might occur during this isolation process was examined by subjecting solutions of tryptophan to the treatment described above. It was found that tryptophan was eluted from the Sephadex column in fractions 34–39 and that its recovery was 98–100 % of the calculated amount.

Reduction and alkylation of α_s-casein. Urea (2·25 g) was dissolved in 3·0 ml α_s-casein solution containing 0·21 g protein, and the pH value of the resulting solution adjusted to 8·6 by dropwise addition of 1 % methylamine solution. The final concentration of urea was 8 M. ME (0·21 ml) was added and the mixture allowed to stand for 4 h at room temperature before addition of 0·5 M-Tris/acetate buffer of pH 8·5 followed by 1·0 g iodoacetic acid or iodoacetamide dissolved in 5·4 ml N-NaOH. Sufficient Tris/acetate buffer was added to reduce the urea concentration to approximately 2 M. Under these concentrations it has been reported (Anfinsen & Haber, 1961; Haber & Anfinsen, 1961) that alkylation of groups other than SH groups can be expected to occur only to an insignificant extent. The solution was stirred for 13 min after which excess of alkylating agent was destroyed by treatment with 4·2 ml ME for 30 min at room temperature. The pH was then lowered to 3·0 by addition of glacial acetic acid and the urea removed by dialysis against HCl/NaCl buffer of pH 2·0 (I = 0·10). The precipitated casein derivative was redissolved by addition of 0·1 N-NaOH to pH 7·0 and the resulting solution concentrated 3-fold by treatment with Sephadex G-25. A portion (4·0 ml) of the resulting solution was fractionated on a column (1·6 cm diam. × 19 cm) of Sephadex G-25 using Tris/HCl buffer of pH 7·1 containing 6·5 g Tris/l. of 0·05 M-HCl, and the effluent collected in 2·5 ml fractions. The purified casein derivative was eluted in fractions 8–11 as judged by light absorption measurements at 255 nm. These were combined and concentrated until the protein content was approximately 2 % before being examined by starch-gel electrophoresis.

Phosphorus determination. Phosphorus was determined by the method of Allen (1940).

RESULTS AND DISCUSSION

Preparations of α_s-casein B when examined by starch-gel electrophoresis at pH 9·2 in the Aronsson & Grönwall (1957) buffer system in the presence of 5 M urea were found to contain 5 clearly distinguishable components in addition to the main constituent, α_{s1}-casein B, and also, occasionally, trace amounts of β- and κ-casein (Plate 1 (*a*)). Following the practice adopted by El-Negoumy (1967) and in accordance with the recommendations of the Committee on Milk Protein Nomenclature (Thompson *et al.* 1965) those constituents which migrated more slowly than the main component have been designated, in order of decreasing mobility, α_{s2}–α_{s5}. The remaining component which migrated immediately ahead of α_{s1}-casein has been arbitrarily designated α_{s0}-casein. If the relative mobility of the α_{s4} band is taken as 1·00 (El-Negoumy, 1967) the relative mobilities of the remaining components on starch-gels in this buffer system were α_{s0} (1·18), α_{s1} (1·14), α_{s2} (1·11), α_{s3} (1·06) and α_{s5} (0·88). A similar pattern was obtained when whole acid-precipitated casein was subjected to electrophoresis in the same starch-gel system (Plate 1 (*a*)) indicating that

none of these components arose as a result of procedures employed in the preparation of α_s-casein from acid-precipitated casein.

This general pattern, however, differed in one important respect from that produced (Plate 1 (*b*)) when the same electrophoretic procedure was applied to samples of α_s-casein and acid-precipitated casein which had previously been treated with ME, since in these circumstances the diffuse α_{s5} band was no longer discernible. A similar effect has been observed by MacKinlay & Wake (1965) and by Thompson (1966) under different experimental conditions. In addition, the disappearance of this component under the action of ME was accompanied by an apparent increase in the

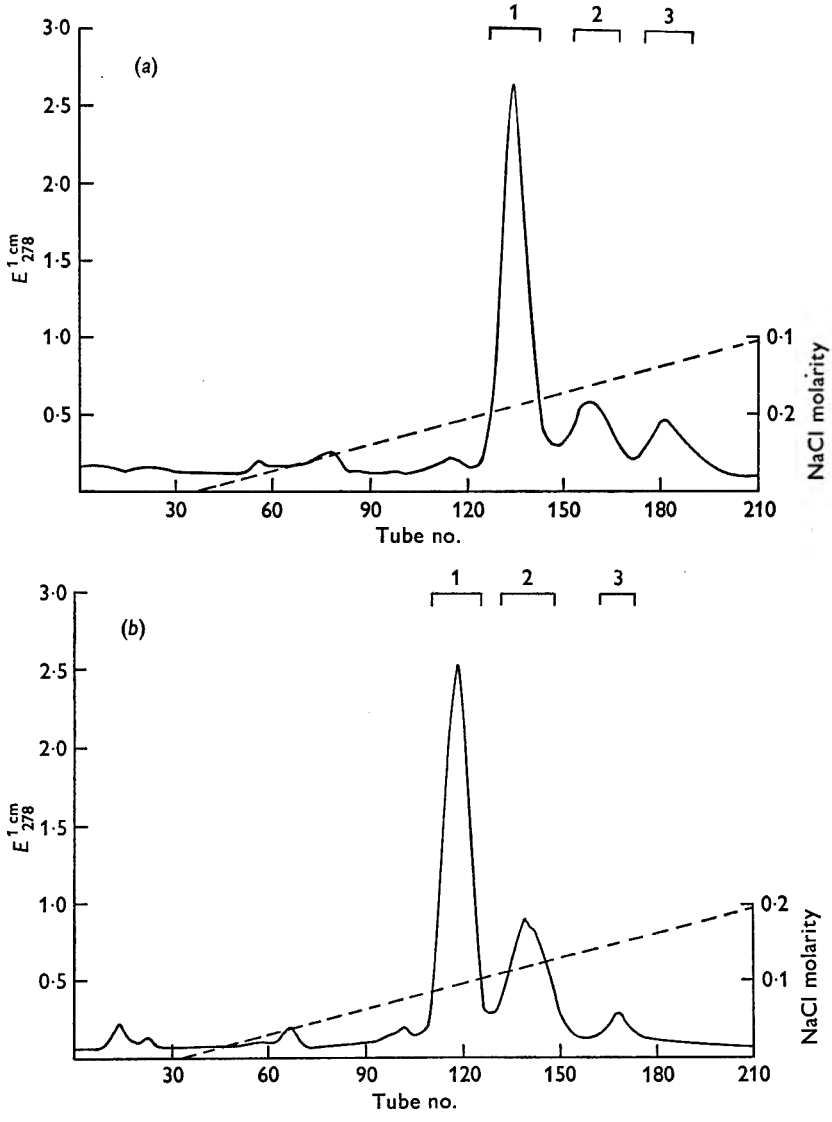

Fig. 1. Fractionation of α_s-casein (approx. 0·5 g) by chromatography at pH 4·0 on columns of sulphoethyl Sephadex C-50 in the presence of 8 M urea and a sodium chloride gradient. In (*b*) the α_s-casein applied to the column was first reduced by treatment with ME at pH 8·6. In (*a*) the α_s-casein received no such treatment.

amounts of the α_{s2}, α_{s3} and α_{s4} bands and the appearance of traces of a further band, designated α_{s6}, which had a mobility very close to that of the original diffuse α_{s5} band.

Further information concerning the constitution of these components and their interrelationships has been obtained from an examination of the products of the chromatographic fractionation of α_s-casein and of whole casein, on columns of sulphoethyl Sephadex C-50 in the presence of 8 M urea. Many of the methods hitherto used in fractionation experiments with casein have involved exposure of the casein to neutral or alkaline solutions of urea for considerable periods of time. In such conditions there is considerable risk of the formation of small amounts of cyanate and of its subsequent reaction with casein (Marier & Rose, 1964; Manson, 1962). The present fractionation was performed in acidic conditions and thus the disaggregating effect of strong solutions of urea has been utilized without risk of side effects due to the presence of cyanate.

When α_s-casein was subjected to chromatography at pH 4·0 on columns of sulphoethyl Sephadex C-50 in the presence both of 8 M urea and an increasing concentration of sodium chloride, 3 main fractions were obtained, as is shown in Fig. 1 (*a*). Chromatography of α_s-casein first treated with ME gave, in general, similar results with the exception that fraction 3 was much reduced in quantity and fraction 2 was proportionally increased (Fig. 1 (*b*)). Each of these 3 fractions was examined by starch-gel electrophoresis and compared with the patterns obtained, under identical conditions, from the unfractionated α_s-complex and from whole casein (Plate 1 (*a*) and (*b*)). Those fractions originating in α_s-casein which had not been treated with ME before chromatography behaved as follows.

Fraction 1 yielded bands corresponding to the α_{s0}- and α_{s1}-components of the starting material together with traces of κ-casein, the α_{s0}-casein being associated with that portion of the fraction eluted earliest. The remainder of the fraction was essentially pure α_{s1}-casein (Plate 1 (*a*)). The fraction 1 in gels incorporating ME (Plate 1 (*b*)) was similarly constituted. Fraction 2, when examined in a like manner, was found to be heterogeneous and to contain bands corresponding to those designated α_{s2}, α_{s3} and α_{s4} in the electropherogram of the unfractionated complex. This pattern was reproduced with the addition of traces of a fourth component α_{s6} when the electrophoresis was performed in the presence of ME (Plates 1 (*a*) and (*b*)). Fraction 3 clearly corresponded in behaviour to the α_{s5} component of unfractionated α_s-casein. In gels containing no ME it appeared as an ill-defined band in the α_{s5} position, whereas after treatment with ME this disappeared and was replaced by well-defined bands corresponding to the α_{s2}, α_{s3}, α_{s4} and α_{s6} components in a manner similar to that of unfractionated α_s-casein itself. This pattern was indistinguishable in its essential features from that of fraction 2, as shown in Plate 1 (*a*) and (*b*). It has consequently been concluded that the material constituting the α_{s5}-fraction of the α_s-complex is itself a complex from which simpler molecules characterized chiefly by the α_{s2}, α_{s3} and α_{s4} bands are released on reduction by ME.

The conditions under which fragmentation of this α_{s5}-fraction occurred are consistent with a reaction mechanism involving reduction of disulphide bonds in the complex and thus lend support to the contention of Waugh *et al.* (1962) that the α_s-casein complex contains cystine. Further evidence in support of this was obtained by reduction and alkylation of unfractionated α_s-casein followed by electrophoresis

of the products in a starch gel in the presence of 5 M urea at pH 9·2. The results are shown in Plate 2. It is apparent that the distances migrated by the α_{s3} and α_{s4} components after treatment with iodoacetic acid were greater than those of the untreated material, whereas the α_{s3} and α_{s4} bands obtained after treatment with iodoacetamide travelled more slowly than the corresponding bands before alkylation. This is consistent with the formation from free sulphydryl groups in both the α_{s3} and α_{s4} components of *s*-carboxymethyl derivatives on treatment with iodoacetic acid, and of the corresponding amides, *s*-carboxamidomethyl derivatives, from iodoacetamide. Examination of the original gel which was photographed to produce Plate 2 suggested that the α_{s2} component had behaved analogously to the α_{s3} and α_{s4} components, thus indicating that it too contained free sulphydryl groups.

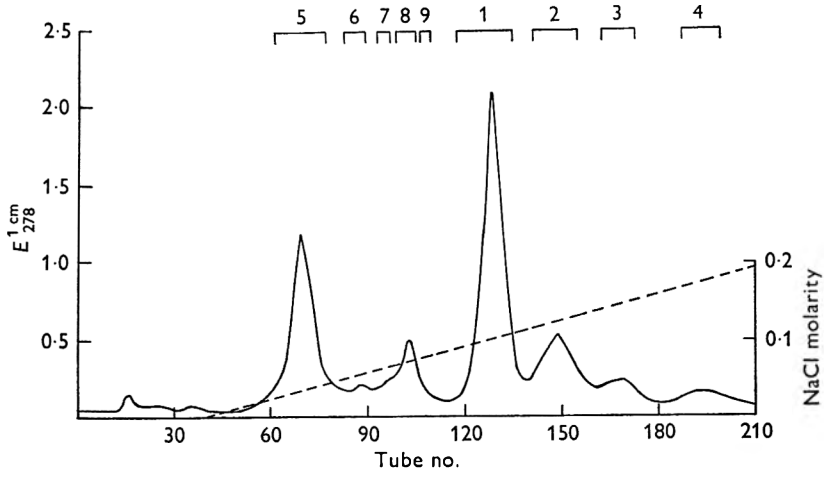

Fig. 2. Fractionation of whole acid-precipitated casein (approx. 0·5 g), after treatment with ME, by chromatography at pH 4·0 on a column of sulphoethyl Sephadex C-50 in the presence of 8 M urea and a sodium chloride gradient.

The chromatographic procedure was applied to reduced whole casein in place of the α_s-casein complex in order to confirm that the α_s-casein components separated were not artifacts of the preparation of α_s-casein from whole casein. The results from a typical separation are shown in Fig. 2. In appearance the elution pattern is qualitatively very similar to those obtained from α_s-casein preparations, particularly so in the region between tubes 100 and 180. However, starch-gel electrophoresis revealed that while fraction 1 behaved in a manner indistinguishable from the fraction 1 obtained from α_s-casein, fraction 2 contained only the α_{s2}, α_{s3} and α_{s4} components (Plate 3) and thus lacked the α_{s6} constituent of the fraction 2 from α_s-casein. In a like fashion fraction 3 contained the α_{s3}, α_{s4} and α_{s6} bands (Plate 4) and lacked the α_{s2} band of fraction 2 from α_s-casein. The final fraction of this group, fraction 4, contained all 4 components, α_{s2}, α_{s3}, α_{s4} and α_{s6} and could be considered as representing the unreduced aggregate containing these 4 components which was designated α_{s5}-casein in Plate 1(*a*). It may be further concluded that the fractions 2 and 3 isolated from whole casein together represent the fraction 2 obtained from α_s-casein.

In addition to these 4 fractions, a further 2 major and 3 minor fractions were discernible in Fig. 2. From their electrophoretic behaviour the 2 main fractions have been identified, fraction 5 as β-casein and fraction 8 as the major component of the κ-casein complex. The remaining fractions which were present only in very small amounts were discarded since they apparently formed no part of the α_s-casein complex.

Table 1. *Analyses of components isolated from the α_s-casein complex by chromatography on sulphoethyl Sephadex C-50*

	P, %	$E^{1\%}_{1\,cm}$ at 278 nm
α_{s0}-Casein	1·05	10·4
α_{s1}-Casein	1·02	10·7
Fraction 2*	1·03	10·1

* Prepared from α_s-casein as described in the Methods section and illustrated in Fig. 1 (*b*).

Table 2. *Amino acids released from α_s-casein components by the action of carboxypeptidase A**

(Amino acids released, moles/25000 g protein.)

	Incubation time, min					
	4	180	220	480	1140	1920
α_{s0}-Casein						
Tryptophan	0·84	0·91	0·90	0·86	0·99	0·85
Leucine	0·00	0·14	0·48	1·17	1·23	1·31
α_{s1}-Casein						
Tryptophan	0·84	0·91	0·97	1·00	0·92	1·00
Leucine	0·00	0·11	0·46	1·20	1·24	1·29
Fraction 2†						
Tyrosine	0·80	0·82	0·74	0·86	0·88	0·94
Leucine	0·82	0·82	0·79	0·97	0·85	0·90

* Experimental details are given in Methods section.

† Fraction 2 was obtained by chromatography of α_s-casein as described in the Methods section and illustrated in Fig. 1 (*b*).

The main components of the α_s-casein complex were further characterized on the basis of phosphorus content, light absorption at 278 nm and C-terminal end-group composition. By selection of portions of fraction 1, samples of α_{s0}- and α_{s1}-casein were obtained which behaved electrophoretically in starch gels as single homogeneous proteins. It was not, however, found possible to separate the remaining components completely on a preparative scale and consequently their aggregate, represented by fraction 2, was used for analytical comparison with α_{s0} and α_{s1}-casein.

The light absorption properties and phosphorus contents of these 3 constituents are recorded in Table 1. It is apparent that on this basis the 3 fractions were virtually indistinguishable from each other. The figures shown in Table 1 are also close to those of 1·03% phosphorus and $E^{1\%}_{1cm}$ = 10·6 reported earlier for unfractionated α_s-casein (Manson, 1965). Certain differences between the fractions were however apparent when each was digested with carboxypeptidase A. In these experiments the

same samples of α_{s0}- and α_{s1}-casein from which the figures in Table 1 were obtained were used, together with fraction 2 which consisted of roughly equal amounts of the α_{s3} and α_{s4} components accompanied by traces of α_{s2}- and α_{s6}-caseins. The amounts of amino acids liberated from the 3 preparations by the action of carboxypeptidase A are shown in Table 2.

The behaviour of α_{s0}-casein was clearly very similar to that of the α_{s1} component and is consistent with the belief that each protein contains a single stranded polypeptide chain terminating in the sequence Leu-TrpCOOH. The amount of leucine released from both proteins is, however, sufficiently in excess of one molar proportion in each case to suggest that the third position from the C-terminal is also occupied by a leucyl residue whose release by carboxypeptidase A is hindered by the nature of the fourth residue which is itself completely resistant to the action of this enzyme. From the results set out in Table 2 the minimum molecular weights of α_{s0}- and α_{s1}-casein can be estimated as approximately 28000 and 26000, respectively, in comparison with the value of 31000 obtained earlier for unfractionated α_s-casein (Manson, 1961). More recently, Schmidt, Payens, van Markwijk & Brinkhuis (1967), using physical techniques, have reported a subunit molecular weight of approximately 23000 for an α_{s1}-casein preparation comparable to that described here.

In contrast to these findings with α_{s0}- and α_{s1}-caseins, the amino acids tyrosine and leucine were liberated from reduced α_{s5}-casein by the action of carboxypeptidase A. The rates of release of these amino acids were apparently equal and it was not possible to determine with certainty whether they were released sequentially from a single protein or simultaneously from the ends of 2 polypeptide chains. In view of the known heterogeneity of fraction α_{s5} and of the speed of release of both the tyrosine and the leucine when compared with the rates of cleavage of the α_{s0} and α_{s1} components, it is not unlikely that the 2 main constituents of α_{s5}-casein, the α_{s3} and α_{s4} components, each contributed one of the liberated amino acids and that the penultimate residue in each case was resistant to attack by carboxypeptidase A. In this event, equal amounts of α_{s3} and α_{s4}-casein would yield one molar equivalent of tyrosine and leucine per molecular weight of each of approximately 14000, calculated on the results shown in Table 2. It is noteworthy that α-lactalbumin, whose primary unit has a molecular weight of approximately 15000 when subjected to starch-gel electrophoresis after reduction with ME and under the conditions employed with α_s-casein components, migrated in a manner indistinguishable from that of α_{s4}-casein. Furthermore, treatment of α-lactalbumin with carboxypeptidase A has been shown to liberate only one amino acid, leucine, per chain weight of 15000, the penultimate residue, lysine, being completely resistant to attack by this enzyme (Weil & Seibles, 1961). The possibility cannot therefore be excluded that the α_{s4} component of the α_s-casein complex is lactalbumin.

From the experiments described above it can be concluded that the main component of the α_s-casein complex of bovine milk, α_{s1}-casein, is accompanied in the complex by small amounts of a closely related component, α_{s0}-casein, together with a group of 4 components, 3 of which at least contain disulphide bonds or sulphydryl groups, and which themselves form a minor complex. In addition, all 3 divisions of the α_s-complex, the α_{s0} and α_{s1} components and the α_{s5} minor complex, contain phosphorus.

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EXPLANATION OF PLATES

PLATE 1

(*a*) Starch gel electrophoresis of casein components at pH 9·2. Whole acid-precipitated casein is represented by the pattern from slot 2 and the positions of individual constituents of the α_s-casein complex are indicated in the margin. Slot 5 was occupied by the α_s-casein complex and the remaining 4 slots by the 3 fractions obtained from column chromatography of α_s-casein, as illustrated in Fig. 1(*a*). Slot 1 contained the more quickly eluted material from fraction 1 and slot 3 the remainder of fraction 1. Fraction 2 is represented by the pattern from slot 4 and fraction 3 by that from slot 6.
(*b*) Starch gel electrophoresis of casein components at pH 9·2 after treatment with ME. The pattern obtained from whole acid-precipitated casein is shown in (2), and that from α_s-casein in (5). The pattern represented by (1) was obtained from that part of fraction 1, eluted earliest in the chromatographic fractionation of α_s-casein illustrated in Fig. 1 (*b*), and that represented by (3) was obtained from the remainder of fraction 1. Fractions 2 and 3 from the same separation migrated from slots 4 and 6, respectively.

PLATE 2

Starch gel electrophoresis of reduced α_s-casein at pH 9·2 before and after alkylation with iodoacetic acid and iodoacetamide. The unfractionated reduced α_s-casein occupied slot 2. The material obtained after treatment of α_s-casein with iodoacetic acid is represented by (1) and that obtained by alkylation with iodoacetamide by (3).

PLATE 3

Electrophoresis in a starch gel incorporating ME at pH 9·2 of major fractions obtained from chromatography of whole acid-precipitated casein. The fraction numbers are those employed in Fig. 2. Unfractionated casein is represented by (4), fraction 5 by (1), fraction 8 by (2) and fraction 2 by (6). Fraction 1 has been subdivided into quickly (3) and slowly (5) eluted portions.

PLATE 4

Electrophoresis in a starch gel incorporating ME of minor fractions obtained by chromatography of whole acid-precipitated casein. The fraction numbers refer to those employed in Fig. 2. Unfractionated casein is represented by (4), and fractions 6, 7, 9, 3 and 4 by the patterns from slots 1, 2, 3, 5 and 6, respectively.

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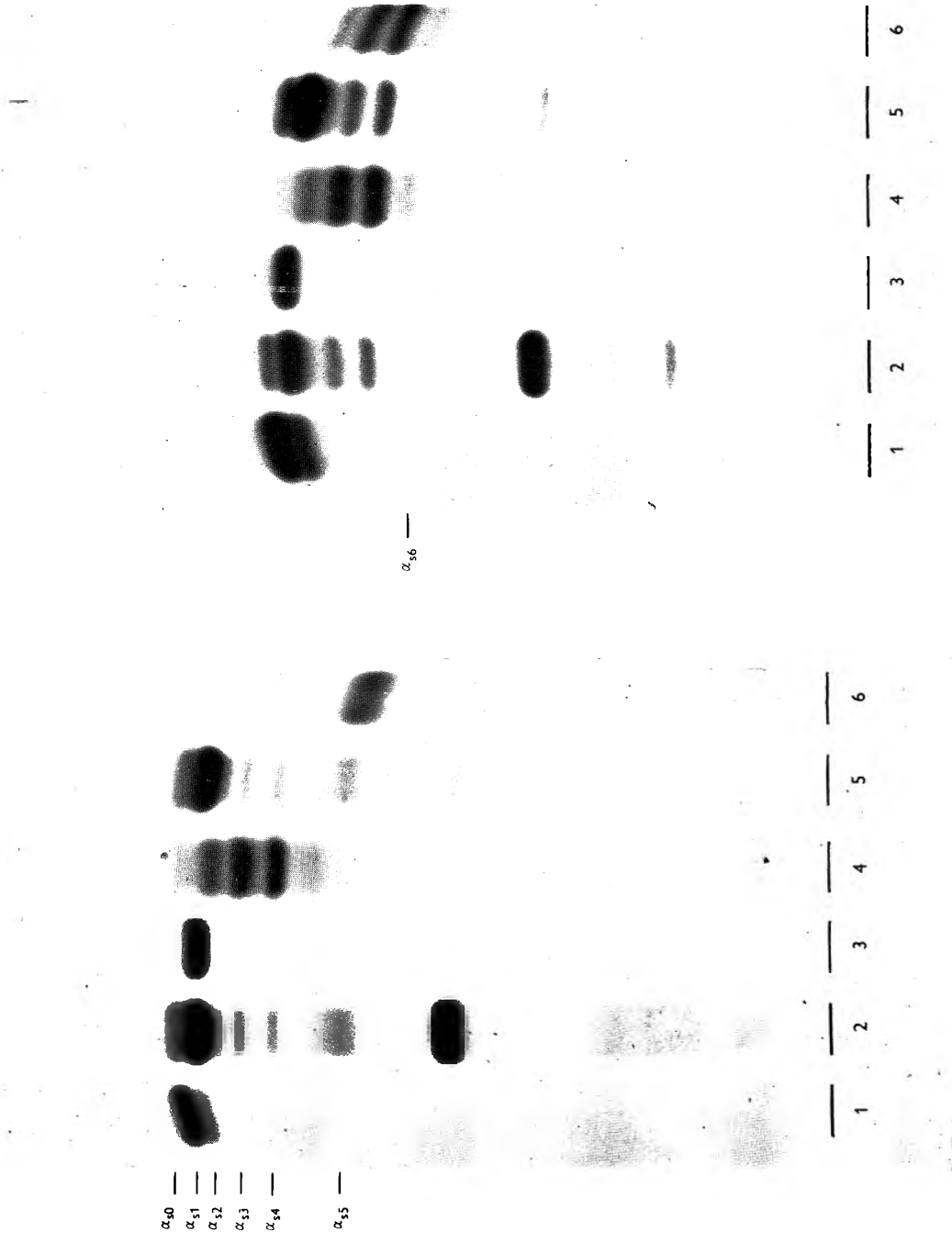
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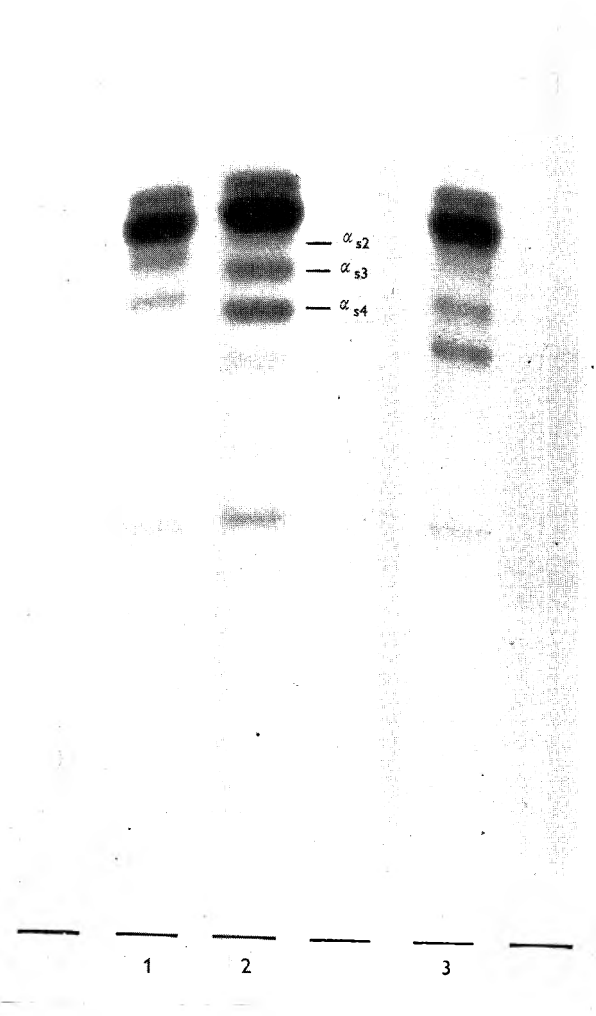
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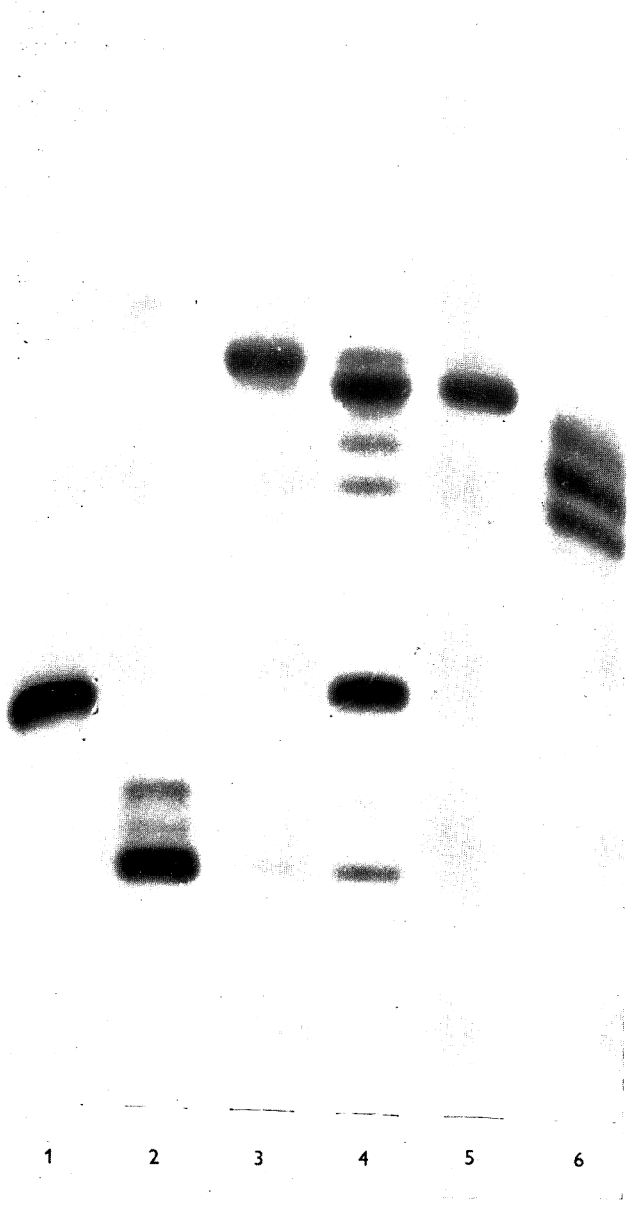
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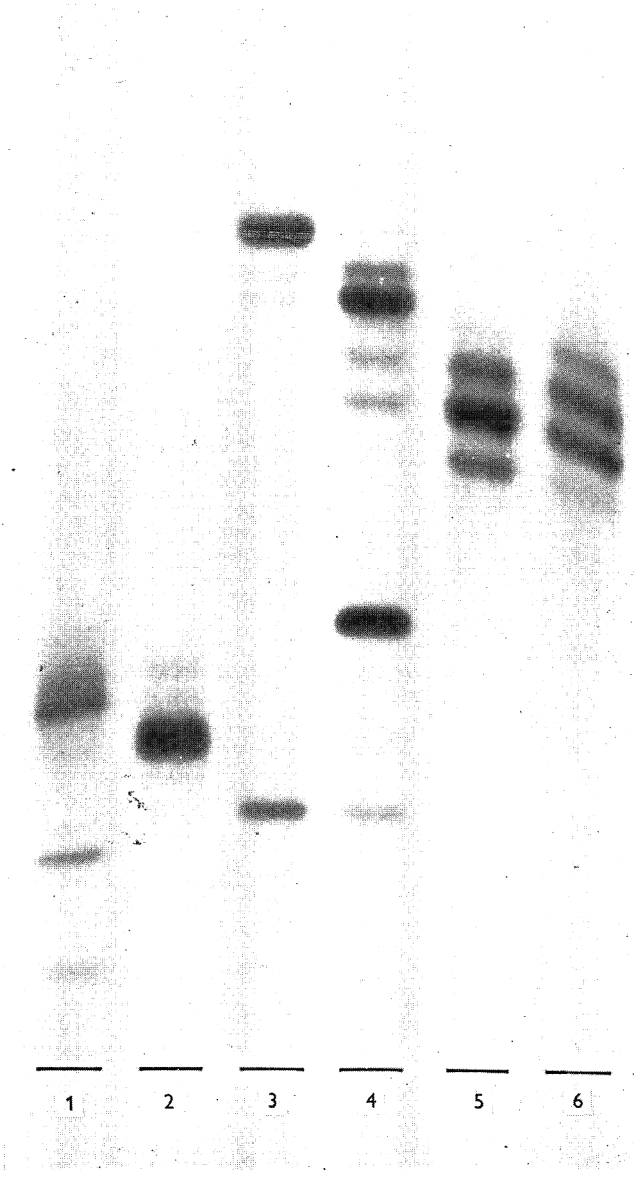
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Some effects of process treatment on the body of cream

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Summary. Effects of age and agitation of milk, temperature of separation, heat treatment and cooling, homogenization and ageing on the body of double cream were studied. Homogenization and temperature of heat treatment gave rise to the greatest effect. The effects of most treatments were inter-related to some extent, but this was most pronounced with cooling and ageing.

Cream containing 48% by weight of butterfat is produced commercially only in Great Britain, and few papers dealing specifically with the production process have been published. Earlier work has been comprehensively reviewed by Crossley & Rothwell (1954). Much of the present-day information about cream stems from the work of Babcock (1931) and of Skelton & Herreid (1941), who observed that "the body of cream is a temporary structural condition that can be altered by treatment". A paper by Rothwell (1962) summarized some of the effects of the processing treatments on the properties of the product, and more recently Rothwell (1966) described how processing affects the condition of the fat in cream. In addition, there is a considerable amount of 'know how' or technical expertise possessed by experienced individual technologists in the industry which has not been recorded.

An object of the present study was to establish what properties of cream could be ascribed to the physical processing conditions, and to determine to what extent the body of cream could be changed by changing the process. In particular, the experiments were designed to determine what factors affected the body of machine-separated cream under processing conditions that allowed interaction between several variables to be observed. They form part of an investigation to determine what processing variables and what characteristics of cream affect its body at the point of sale. It is assumed that the cream is processed throughout under good hygienic conditions so that the effects of bacterial contamination may be ignored.

Definition of the 'body' of cream

Cream is judged by its flavour, appearance, and consistency or body and it is only the body of the cream which has been considered in the present experiments. Body is a term which has not been precisely defined, but it is associated with the manner in which cream flows when it is poured slowly.

The flow of a liquid is described in terms of viscosity, but it is necessary to define what is meant by the viscosity of liquids such as cream. Viscosity is the coefficient connecting the applied stress with the induced rate of shear when a liquid is set in

steady flow and is independent of the method of measurement. Several workers, including Scott Blair, Hening & Wagstaff (1939), Crossley (1954), Rothwell (1966) and Scott Blair & Prentice (1966) have shown that cream does not follow such a simple relationship, but that the apparent viscosity, which is measured, depends upon the rate of flow at the time of measurement.

It has been shown by Prentice (1967) that within the limits of experimental error the flow of cream may be represented by an empirical formula $\tau = k\dot{\gamma}^{\alpha}$, where τ is the applied stress, $\dot{\gamma}$ is the ensuing rate of shear, and k and α are parameters characteristic of the cream sample. For the present purposes this is more conveniently written as $\eta' = \tau\dot{\gamma}^{-1} = k\dot{\gamma}^{-\beta}$, where η' is the apparent viscosity under the conditions of the measurement and $\beta\ (= 1 - \alpha)$ is an exponent defining the departure of the cream from the Newtonian behaviour of a true fluid. The value of k, which is numerically equal to the apparent viscosity when the shear rate $\dot{\gamma}$ is unity, is a convenient parameter for describing the body of the cream and will be referred to in this paper as the viscosity of the cream. Saunders (1961) called the departure of a fluid from Newtonian behaviour the abnormality of that fluid, and in keeping with his terminology the exponent β may be called the coefficient of abnormality. These 2 parameters, viscosity and abnormality, are sufficient to define the flow behaviour of cream. In another series of experiments (unpublished) it has been shown that there is a close correlation between the technologists' concept of body and the viscosity, as just defined.

EXPERIMENTAL

Cream samples used in the 3 experiments described here were produced from bulk milk from the Institute's herd at Church Farm. The milk was warmed in cans and then separated to a nominal 48 % fat in an Alfa Laval Separator Model 108 AE 120 gal/h. The cream was heat treated and cooled in a small Alfa Laval pasteurizing plant (throughput 10 gal/h).

Viscosity

Viscosity measurements were made in a concentric cylinder viscometer; this instrument, and the theory of its use for non-Newtonian fluids, has been described by Prentice (1967). Some creams are very readily modified irreversibly by shear and if the shear rates are high this may occur as viscosity is being measured (Rothwell, 1966). Since this thickening is in fact the onset of the churning process as in making butter, it is commonly referred to as churning. In order to reduce this possibility, the viscometer was designed to operate at low shear rates around 1 sec^{-1}, the highest shear rate used in this experiment being 3·67 sec^{-1}. The resistance of the creams to this type of irreversible change was determined by observing the lowest shear rate at which the sample thickened spontaneously. Only the most sensitive creams churned at the shear rates that were used in the experiment. A second viscometer, of similar dimensions, but capable of shear rates up to 180 sec^{-1}, was used to investigate churning of the remainder of the samples.

Some consideration was given to the timing of the viscosity measurements, since preliminary experiments had indicated a dependence of viscosity on age of the cream, and it was therefore necessary to ensure that all comparative measurements were

made on cream of the same age. From these earlier experiments it was known that cream which was undisturbed in the laboratory increased in viscosity by about 4 %/h. The age of the cream was measured from the time at which it issued from the separator, since this is when cream begins to exist as an entirely different substance from the milk from which it is derived. It took approximately 1 h to measure the viscosity and other properties of the cream that were determined in this experiment. With this in mind it was possible to adopt a strict timetable of production and measurement whereby all measurements began 1 h after the sample was taken from the separator.

Effects of temperature

In a first series of experiments, milk was warmed to 4 different temperatures: 90, 100, 110, 120 °F (32·2, 37·8, 43·3, and 48·9 °C) before separating, and these temperatures were maintained whilst the milk was in the separator bowl by circulating warm water through a heating coil immersed in the milk. The temperature at which the milk passed over the separator head was thus kept as near as possible to the nominal separating temperature. In every experiment the separator was allowed to run for a few minutes in order to settle down, then the cream sample for testing was drawn when the bowl was about half empty (i.e. during the third quarter of the separating run). Creams were separated over a range of fat contents, and each sample was analysed by the Gerber method.

The effect of varying both heat treatment and cooling temperatures was studied in a second series of experiments. In the light of results of the previous series of experiments 40 °C (104 °F) was taken as a satisfactory separating temperature, and one lower temperature, 35 °C (95 °F) and one higher temperature 45 °C (113 °F) were also used. The cream was then heat treated without delay. The proposed schedule (Milk and Milk Products Technical Advisory Committee 1967) for effective heat treatment of creams recommends as one treatment a holding time of 15 sec at 74 °C (165 °F). Accordingly, 15 sec was taken as the standard time throughout the experiments. Two other temperatures also were included in the experiment, 66 and 82 °C (151 and 180 °F), the lower temperature giving very inadequate heating, and the higher what we consider to be considerable over-heating. The cream was cooled in the plant, and the temperature at which it emerged was controlled at 10, 15 or 20 °C (50, 59, or 68 °F). It was possible on this occasion to complete 3 processing runs daily and this series of experiments conformed with a standard statistical pattern. As before, the order in which the samples were produced was completely randomized. The viscosities of all the creams were measured 1 h after processing and after storage for 24 h in a domestic refrigerator, at approximately 7 °C (45 °F).

Age and agitation

In the third series of experiments 2 more factors were controlled: the age of the milk and the amount of agitation it received between milking and separating. The effect of homogenization of the cream was also studied. Inclusion of these variables resulted in a 6-factor experiment and to have studied these factors in as great detail as in the previous series would have resulted in an unwieldy and time-consuming experiment. An indication of the magnitude and significance of the effect of any

process was obtained by studying each factor at only 2 levels, and a compact statistical design of half-replication was used.

To study the effect of age of the milk 2 ages were chosen to represent the intervals which occur in commercial practice between milk production at the farm and separation at the creamery. The fresher milk, under 24-h old, was drawn from bulked milk of the evening and morning milkings immediately before processing, and the older milk was drawn from bulked milk from the 2 milkings 24 h earlier. No really stale milk was used in these experiments.

Agitation, when required, was the normal intermittent stirring action provided by the stirrers in the bulk milk tank where the milk was held at 40 °F (4·4 °C). Unagitated milk was obtained by storing the required quantity of bulked milk in a separate tank also held at 40 °F (4·4 °C). Some agitation of the milk during milking, handling and transfer between tanks was unavoidable.

Separating, heat treatment and cooling temperatures were based on the results of the earlier experiments. The separating temperatures, 35 and 45 °C (95 and 113 °F), were used, followed by 2 heat treatments, at 66 and 74 °C (151 and 165 °F) and finally 2 cooling treatments, 10 or 20 °C (50 or 68 °F).

A Rannie homogenizer was included in the flow line between the heating and the holding sections of the heat-treatment plant so that homogenization was effected at the holding temperature. The homogenizing pressure was 35 kg/cm^2 (500 p.s.i.). When homogenized cream was not required, the homogenizer valve was released and the homogenizer then merely served to pump the cream through the plant.

It was not possible in this experiment to adhere to the previous time-table of measuring viscosity 1 h after production. It was more convenient to make the measurements some 3 h after production. There was thus an element of the ageing effect in all these values of viscosity but it was felt that any uncertainty this introduced would be small compared with the effect of the main factors. The viscosity of 1-day-old samples was measured after about 21-h storage in the refrigerator. A further subsample was retained and examined at the end of 7-day storage. The pH value of samples that were kept for 1 week was measured to give some indication of the effect of the different treatments on the keeping quality of the creams.

RESULTS

Viscosities of freshly separated cream obtained from the first experiment have been plotted against fat content in Fig. 1. There was a general increase of viscosity with concentration, the average values of η_1' at 20 °C being 0·65 Poise at 44 %, 1·1 Poise at 48 % and 2·2 Poise at 52 % fat. Not enough measurements were made at fat concentrations greater than 52 % for a reliable average to be calculated. There is another pronounced feature of this figure. All the points relating to separation at 120 °F lie near the bottom of the band, and the points for 110 °F are more or less scattered amongst them. On the other hand, there is a tendency for the points for 100 and 90 °F to lie above this band, greater with separation at 90 than at 100 °F, and this tendency was greater at higher fat concentration.

The results of the second series of experiments are collected in Table 1. The viscosity and the fat content of the creams varied considerably and were highly

correlated. In the statistical analysis, therefore, the fat content was treated as a covariate. Over and above the adventitious variation due to differences in fat content there was a highly systematic variation due to the heat treatment temperature. The effect of overheating (above 74 °C) was small but that of underheating was considerable, the adequately heat-treated cream being little more than half as thick as

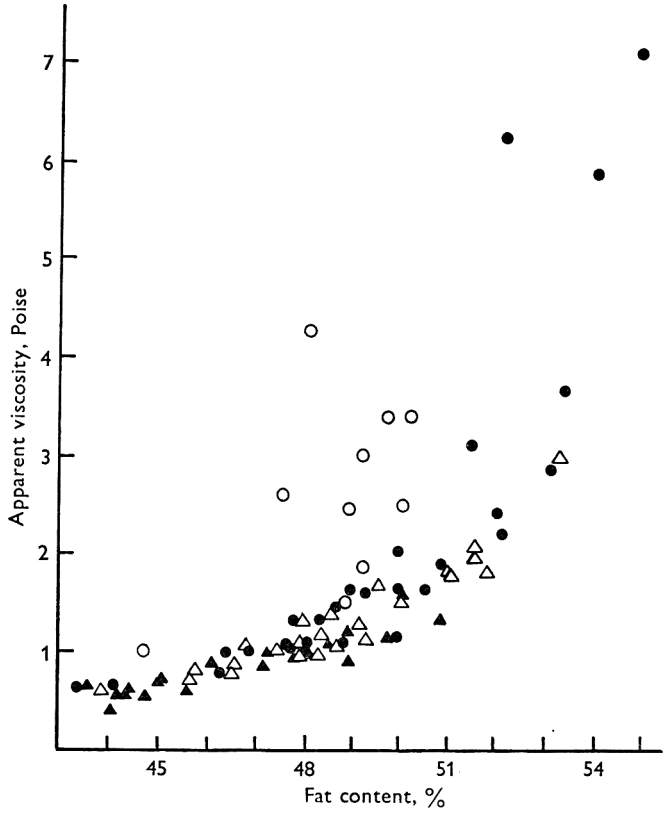

Fig. 1. Variation of apparent viscosity with fat content.
○, 90 °F; ●, 100 °F; △, 110 °F; ▲, 120 °F.

that heated to only 66 °C. There was also some tendency for the higher separating temperature to produce thinner cream, consistent with the findings of the earlier experiments, but this tendency was not large enough, compared with the residual variance due to uncontrolled factors, to be statistically significant.

The variation arising from differences in cooling temperatures was not linear, the most viscous cream, on average, being produced when cooled to 15 °C, the least viscous when cooled to 20 °C. A more important effect of cooling, however, is seen if the viscosities of the freshly produced cream and the day-old cream are compared. Cream cooled most quickly, that is down to 10 °C, increased in viscosity by only about 20 % during storage, whilst that which was originally cooled most slowly, down to only 20 °C in the plant, increased in viscosity by about 60 %. If the thickening had proceeded at the same rate for a further day the difference between the creams cooled to 15 and 20 °C would almost have disappeared, whilst that cooled to 10 °C

would have been appreciably the thinnest. Neither the temperature of separation nor the heat-treatment temperature had any significant effect on the rate of thickening. The average values of the viscosity (η_1') for each treatment are tabulated in Table 2, the values being corrected to a fat content of 50 % to facilitate comparison.

The residual variance in the analysis of this series was very large, giving rise to a standard deviation of approximately ± 18 % of the value of a single determination.

Table 1. *Effect of processing temperatures on viscosity of cream*

Temp., °C				Viscosity, Poise	
Separation	Pasteurization	Cooling	Fat content, %	Fresh	1 day old
35	66	10	51·3	3·52	4·33
		15	51·4	4·08	4·59
		20	51·3	1·64	1·88
	74	10	52·6	2·69	2·82
		15	50·6	1·32	3·46
		20	49·2	0·77	1·12
	82	10	51·0	1·61	1·76
		15	50·3	1·06	1·80
		20	50·5	0·77	1·45
40	66	10	50·1	2·40	2·36
		15	50·7	2·71	3·43
		20	49·1	1·50	2·27
	74	10	48·5	0·79	0·94
		15	49·3	1·07	1·36
		20	50·0	0·83	2·20
	82	10	48·8	0·64	0·91
		15	48·8	0·79	0·95
		20	49·4	0·71	0·95
45	66	10	50·5	2·50	2·73
		15	49·9	1·58	2·30
		20	50·0	1·92	3·24
	74	10	50·5	0·77	0·94
		15	47·3	0·77	0·97
		20	51·4	1·07	2·10
	82	10	50·9	1·20	1·41
		15	47·2	0·60	0·79
		20	47·6	0·54	0·74

Table 2. *Average effect of treatments on viscosity of cream*

Treatment	Temp., °C	Viscosity, Poise	
		Fresh	1 day old
Separation	35	1·37	1·87
	40	1·32	1·75
	45	1·19	1·64
Pasteurization	66	1·91	2·70
	74	1·10	1·52
	82	0·95	1·30
Cooling	10	1·28	1·56
	15	1·50	2·04
	20	1·05	1·69

This may be compared with the accuracy of the determination of the viscosity which is of the order of $\pm$ 1–2 %. The remainder of the variance, by far the largest portion, was due to differences between individual samples and to the effect of uncontrolled factors.

The compact statistical design of the third series of experiments resulted in some loss of precision since high order interactions were confounded with the main treatment

Table 3. *Average effect of treatments on some properties of cream*

	Viscosity, Poise			Churning index			pH		
Treatment	Fresh	1 day old	1 week	Fresh	1 day old	1 week	Fresh	1 day old	1 week
			(*a*) Unhomogenized cream						
Age of milk,									
24 h	1·17	2·08	1·92	½	2	2	6·66	6·69	6·69
48	1·00	1·88	1·97	⅜	2	4	6·71	6·71	6·62
Agitation of milk									
None	1·21	1·73	1·91	⅜	2	4	6·69	6·71	6·68
Some	0·97	2·25	1·98	½	2	3	6·69	6·68	6·64
Separation temp., °C									
35	1·08	2·46	2·24	¾	2	3	6·69	6·69	6·63
45	1·09	1·59	1·69	⅛	2	3	6·68	6·71	6·68
Pasteurization temp., °C									
66	1·36	2·86	2·63	½	2	3	6·69	6·72	6·64
74	0·86	1·37	1·43	⅜	2	3	6·68	6·68	6·67
Cooling temp., °C									
10	1·12	1·92	1·77	⅞	2	3	6·68	6·70	6·64
20	1·05	2·03	2·14	0	3	4	6·69	6·70	6·67
			(*b*) Homogenized cream						
Age of milk, h									
24	15·4	—	—	1	7	9	6·69	6·70	6·59
48	17·3	—	—	2	7	9	6·71	6·68	6·52
Agitation of milk									
None	15·5	—	—	1	5	7	6·71	6·67	6·55
Some	17·3	—	—	2	9	11	6·70	6·71	6·56
Separation temp., °C									
35	16·1	—	—	2	7	7	6·70	6·68	6·55
45	16·6	—	—	2	6	10	6·71	6·70	6·57
Pasteurization temp., °C									
66	22·3	—	—	2	8	10	6·69	6·67	6·45
74	12·0	—	—	2	6	7	6·71	6·71	6·66
Cooling temp., °C									
10	11·5	—	—	3	6	9	6·72	6·69	6·56
20	23·2	—	—	0	8	9	6·69	6·69	6·55

effects, and there was no replication of any measurements. Nevertheless, the analysis enabled the principal effects to be determined. Table 3 summarizes the average results, the viscosities being corrected to a standard fat content of 50 % as before. This table also contains a column headed 'churning index'. This is a figure on an arbitrary logarithmic scale, a churning index of zero indicating that the cream was not observed to churn in the viscometer at a shear rate of 180 sec^{-1}, the highest shear rate attainable. An index of 1 means that churning took place at the maximum shear rate, but not at a lower rate. Subsequent unit increments of the index correspond to reductions by a factor of 2 of the shear rate at which churning took place.

DISCUSSION

The principal findings may be classified according to the treatments:

(i) *Homogenization*

This had by far the greatest influence on all the properties of cream. Examination of photomicrographs of the cream samples showed that at the low pressure of 35 kg/cm^2 true homogenization had not taken place, but the mean globule diameter had been reduced from 3·04 μm, the average for the unhomogenized creams, to 2·27 μm, whilst the distribution of the globule sizes about the mean was not significantly altered. The average viscosity of the homogenized samples was almost 15 times that of the unhomogenized samples. The range of viscosities encountered was also somewhat increased, that is, the effect of other factors including both the controlled treatments and factors beyond our control, was greater. The homogenized samples also churned more readily than those that were not homogenized.

(ii) *Age of milk*

The age of the milk had no significant effect on the viscosity of the freshly prepared cream, nor on the rate at which it subsequently thickened. There was a slight tendency for the aged cream to churn more readily, but the difference was marginal. The pH value of the cream from the older milk fell more rapidly, and some of the homogenized creams from the older milk would have been too acid to be acceptable to a consumer at the end of one week.

(iii) *Agitation of the milk*

There was no significant difference between the viscosities of the cream from agitated and unagitated milk straight from the plant, but the agitated milk gave cream which thickened rather more rapidly at first. There was a very slight tendency for acidity to develop more rapidly in the cream from agitated milk, but not sufficient to affect its acceptability after one week. Homogenized creams from agitated milk showed a greater tendency to churn.

(iv) *Separation temperature*

Contrary to the findings of the earlier experiments, there was no significant difference between the viscosities of the fresh cream at the 2 separation temperatures on this occasion. After ageing, however, the difference was quite pronounced, the lower temperature giving rise to the thicker cream as expected. Moreover, cream separated at the lower temperature developed slightly more acidity on ageing, but this was only a marginal difference with the homogenized creams.

(v) *Heat-treatment temperature*

The effect of heat-treatment temperature was second only to homogenization in giving rise to differences in the viscosity of the creams. Creams which were inadequately heat-treated by heating to only 66 °C (151 °F) were almost twice as viscous as those heated to 74 °C (165 °F). After 7-day storage the relative viscosities were unchanged, but the under-heated cream had developed some acidity. The under-

heated homogenized cream was so acid after 7 days that it would have been unacceptable. The heat-treatment temperature had no effect on the stability of the unhomogenized creams, but the homogenized creams which were under-heated had a greater tendency to churn after ageing.

(iv) *Cooling temperature*

The cooling temperature had little effect on the viscosity of the unhomogenized creams immediately after production. As in the second series of experiments, the creams cooled to 10 °C had on average slightly higher viscosity than those cooled to 20 °C, but this difference was not sufficient to be statistically significant. However, as before, the thickening rates of cream cooled to 10 and 20 °C were appreciably different. Cream cooled to only 20 °C in the plant almost doubled its viscosity during ageing, whilst in the same time cream cooled to 10 °C increased in viscosity only by about 60 %. The behaviour of the homogenized creams was somewhat different. Three of the 16 samples were so unstable on the second day that measurements on them could not be made, so that a proper statistical treatment could not be undertaken. However, an inspection of the data showed that homogenized cream cooled to only 20 °C was approximately twice as viscous when fresh as that cooled to 10 °C, and that subsequently it continued to thicken faster, though less fast than the unhomogenized creams. The average for 6 creams which did not become too unstable was an increase of about 20 % after 1 day's ageing.

CONCLUSIONS

All 3 series of experiments bear out the fact that any prior treatment may affect the properties of the cream. Any heating results in a reduced viscosity of the cream when it emerges from the plant, but this may to some extent be overshadowed by a subsequent thickening during storage. In particular, the temperature at the heat-treatment stage has an important effect but it has not been established whether this is solely due to the temperature to which the cream is heated, or whether more prolonged heating at the lower temperature might also reduce the viscosity. This point remains to be clarified.

In general, those treatments which tend to produce a thick cream also result in one less satisfactory from other points of view. For example, a low separation temperature gives rise on average to a thicker cream but more variability in the product; homogenization produces a much thicker cream, but with much less resistance to churning, and a greater tendency to acid development. From the point of view of the cream producer some compromise is therefore necessary if he wishes to produce a thick cream without risk of the product being unsatisfactory in other ways.

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Impact force as a possible cause of mechanical transfer of bacteria to the interior of the cow's teat

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Summary. Using bacterial endotoxin as a tracer material, it has been shown that a jet of liquid impinging on the end of the teat for 1 min after milk flow had ceased during machine milking could force material past the barrier of the streak canal. Positive results were obtained when the maximum pressure at the centre of the impact area was about 0·25 bar, with a considerably higher frequency of positives at 0·3–0·5 bar. It seems possible that such impact pressures might occur during milking and cause transfer of infective material to the teat sinus. Impact force could arise when milk returned to the teatcup liner as the result of local or general conditions of instability in the milking machine vacuum.

Evidence in support of the contention that a fluctuating vacuum in the milking machine increases the incidence of new infections in mastitis has been considerably strengthened by recent work in Eire (Cowhig & Nyhan, 1965, 1966, 1967). An excellent review of the effect of vacuum fluctuation on udder disease was given by Nyhan (1969). He distinguished between cyclic vacuum fluctuations associated with liner movement and milk flow, and irregular vacuum fluctuations which result from reductions in plant vacuum or from localized vacuum fluctuations in milk tubes and pipelines. Almost all the evidence for a relationship between vacuum fluctuation and increased udder infection relates to irregular vacuum fluctuations. After discussing the possible ways in which vacuum fluctuation might influence infection he concluded that it must in some way be related to physical transport of bacteria.

The ways in which bacteria gain entry to the udder are largely unknown. However, it is not unlikely that the milking machine causes mechanical transfer of infective material to the teat sinus during the course of a milking. A possible mechanism of direct transfer would be impact force exerted on the teat orifice by small or large masses of milk returning to the liner as the result of local or more general reversal of pressure gradients. A study by McEwen & Samuel (1946) lends some support to this suggestion. They exposed teats of cows at the abbatoir to cultures of *Escherichia coli* (3×10^8/ml) during milking, after which the animals were immediately slaughtered and samples of milk, scrapings from the inner orifice of the teat canal, and scrapings from the teat cistern were taken aseptically. No organisms were detected in the milk samples following hand milking with the teats and the milker's hands contaminated with culture, or when the teats were dipped in culture at the end of milk flow and

machine milking continued for 5 min. However, when the ends of the teats were jetted with culture during machine milking, mainly after cessation of milk flow, from a tube pointing upwards from the base of the liner, 20 % of the milk samples taken aseptically from the teat sinus after slaughter contained the test organism. The purpose of the jetting was to ensure a continuous high concentration of organisms on the ends of the teats, but from the description given it appears that a fairly powerful jet was used. The diameter of the nozzle was 0·5 mm and the pressure drop from the surface of the culture in its container to the interior of the teatcup liner was over 0·8 bar. Thus, the positive results found with jetting might have been due not to increased supply of bacteria external to the teat but to impact force of the jet impinging on it.

The aim of the present work was to show whether impact of liquid on the end of the teat during milking could inject material into the teat sinus and if so to make some estimate of the force required.

Preliminary results with 2 cows have already been published (Thiel, 1969).

EXPERIMENTAL

Force was applied to the end of the teat by means of a jet of liquid from a nozzle as had been done by McEwen & Samuel (1946). Three intensities of jetting were used, the lowest just sufficing to keep the teat continuously wetted during treatment in an attempt to distinguish between the effect of mere presence of the test material and its effects when applied with increasing force. When milk flow ceases in machine milking, low pressure about equal to the vacuum in the pulsation chamber appears in the teat sinus. Jetting was therefore done at this stage in milking since pressure difference from the teat sinus to the space in the teatcup liner beneath the teat is then minimum (McDonald, 1969). Small amounts of bacterial endotoxin placed in the teat sinus cause an increase in leucocyte count in the milk (Carroll, Schalm & Lasmanis, 1964; Reiter & Oram, 1967), and this provided a convenient means, repeatable on the same animals at intervals, of detecting material entering the teat sinus during a particular milking. The response of individual animals to increasing dose levels of endotoxin placed in the teat sinus was also measured in an attempt to determine the quantity of endotoxin solution of known concentration entering the teat sinus on each treatment occasion.

Apparatus for jetting teats

Cows were milked one quarter at a time with a single transparent teatcup shell and liner (Bel-Mar Visa-flow shell M3; Bel-Mar Visa-flow inflation M2. The Bel-Mar Manufacturing Corp., Box 526 Siggelkow and Marsh Roads, McFarland, Wisconsin, U.S.A.) at a vacuum level of 0·5 bar and a pulsation rate of 60 c/min, with the liner more than half open for 60 % of each pulsation cycle. The claw milk tube (bore 11·5 mm) was cut short and attached to a nipple on the lid of a 150-ml metal chamber with a central vertical metal supply tube to a nozzle for jetting the teat (Fig. 1). With this arrangement it was possible, by holding the chamber with one hand and the teatcup with the other, to 'steer' the jet of liquid from the nozzle on to the end of the teat in the region of the teat orifice. The metal chamber was connected to a graduated jar by a 12·5-mm bore milk tube 75 cm long. To minimize fluctuations in

liner vacuum the milk tube sloped continuously to the milk jar, and air was admitted at the rate of 7 l/min of free air through a hole in the lid of the chamber. The graduated jar enabled data on cumulative yield against time to be collected, from which peak flow rate, machine rate, and machine yield could be taken.

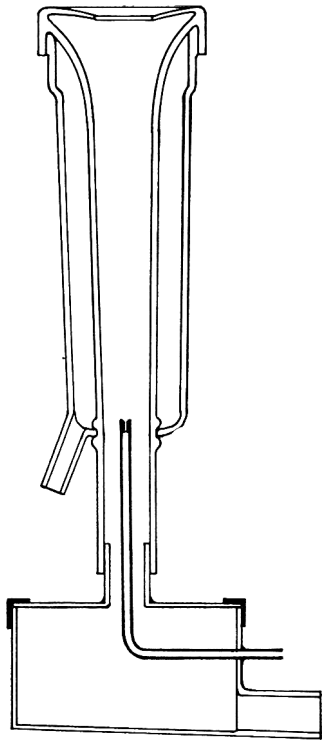

Fig. 1. Diagram of the jetting apparatus. The lid of the metal chamber was attached by means of a rubber band.

The bore of the nozzle, 0·50 mm, was the same as used by McEwen & Samuel (1946). The metal tube supporting the nozzle was connected by a nylon tube to a vessel containing the liquid to be jetted on to the teat. Air pressure in the space above the surface of the liquid in the supply vessel could be maintained automatically at any one of 3 preset values, which in conjunction with an almost constant vacuum of 0·5 bar inside the teatcup liner gave 3 reproducible rates of flow through the jetting nozzle. The calculated mean velocities through the bore of the nozzle, corresponding to low, medium and high intensities of jetting were 1·9, 6·2 and 9·8 m/s. The lowest velocity ensured a plentiful supply of liquid to the end of the teat, which was nominally 5 cm above the nozzle, but with a very low impact force. The highest velocity was probably somewhat lower than that used by McEwen & Samuel (1946) but this is uncertain as insufficient detail was given to enable their equipment to be reproduced exactly.

Characteristics of the jets used

A horizontal plate about 2·5 cm diam., capable of being moved by lead screws in 2 directions at right angles in the horizontal plane, was arranged above the jetting

nozzle. To measure localized pressures, a hole in the centre of the plate, 0·1 mm diam., was connected by a water-filled tube to a pressure transducer and a recorder. By adjusting the forward pressure of liquid to the nozzle, the mass flow rates corresponding to low, medium, and high intensities of jetting the teats of cows could be reproduced, and the pressure conditions in the area where the jet impinged on the

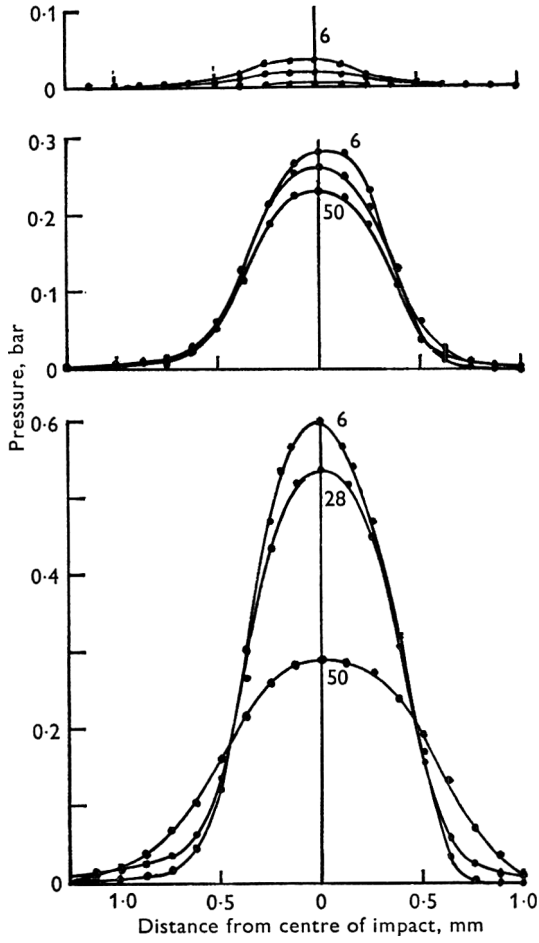

Fig. 2. Distribution of pressure across a diameter of the area of impact of the jets on a plane plate 6, 28 and 50 mm away from the nozzle. Upper curves: low intensity jet; middle curves: medium intensity; lower curves: high intensity.

plate explored with the plate at various distances above the nozzle. Once having found the point of maximum pressure at the centre of the pressure area by trial and error, pressure change across a diameter of the area of impact of the jet could then be explored by operating one lead screw. The results obtained are shown in Fig. 2. A summary of the main physical characteristics of the low, medium, and high intensity jets impinging on the horizontal plate when it was 6, 28, and 50 mm from the nozzle are given in Table 1. Since the pressure had in all instances fallen almost to zero at 1 mm from the centre of impact, total impact force was calculated from

Table 1. *Summary of data on jet characteristics*

	Jet intensity								
	Low			Medium			High		
Flow rate, ml/s ...	0·36			1·21			1·92		
Mean velocity in the nozzle, m/s ...	1·9			6·2			9·8		
Distance of impact plate from nozzle, mm	6	28	50	6	28	50	6	28	50
Max. pressure at centre of pressure area, bar	0·035	0·017	0·003	0·287	0·263	0·233	0·600	0·533	0·287
Total impact force, mN	2·2	1·3	0·1	16·7	15·7	16·8	36·3	37·9	37·5
Mean pressure on the 2 mm diam. pressure area, bar	0·007	0·004	0·001	0·054	0·051	0·054	0·118	0·123	0·122

the curves in Fig. 2 on a circular area 2 mm in diam. Mean pressure was then derived for the same area from the total force.

It may be seen from Fig. 2 and Table 1 that impact force with the low intensity jet was extremely low at all 3 distances of the plate from the nozzle. The medium intensity jet was regular in operation with little change in maximum pressure or total impact force at the 3 distances. However, the high intensity jet was more erratic, and appeared to the eye to become broader as the distance from the nozzle increased. The total impact force was similar at all 3 distances but the maximum pressure at the centre of impact declined sharply at the greatest distance. Since the teats in the liner were not always at the nominal distance of 50 mm, but sometimes closer, the maximum pressure within the impact area was variable with the high intensity jet, but much less so with the medium intensity jet. Force applied to teats might not have been the same as that measured with the horizontal plate. Roughness and convex curvature of the end of the teat would result in somewhat lower values. Teats shaped so that the orifice was in a conical depression would tend to generate rather higher pressures.

Detection of material entering the teat sinus

Endotoxin was extracted from peptone-broth cultures of *Esch. coli* 055-E5 by the ether–water method of Ribi, Haskins, Landy & Milner (1961).

It was known from preliminary work that 0·1 μg of the preparation of endotoxin placed in the teat sinus regularly resulted in a substantial increase in leucocyte count of the milk obtained subsequently, but 10 μg quantities placed 2–3 mm into the streak canal had no effect. The rise in cell count was accompanied by a striking increase in the proportion of polymorphonuclear leucocytes. Leucocyte counts rose to a maximum in 12–48 h after injection of endotoxin and declined to normal in 4–7 days. A concentration of 0·1 μg/mg in water was used for jetting teats. The procedure was to take 30 ml fore-milk samples aseptically from each udder quarter at 2 consecutive a.m. milkings, jetting taking place on the second occasion. Further milk samples were taken at the next 2 a.m. milkings. Smears were made for microscopic examination of the leucocytes, and leucocyte counts were made by means of a Coulter counter. The technique of Tolle, Zeidler & Heeschen (1966) was used, except that Lissapol NXP (I.C.I. Ltd.) was substituted as the surface active agent (Phipps, 1968). In addition, a duplicate set of fore-milk samples was taken aseptically for the detection of pathogens.

Other measurements

The length of the streak canal was measured by inserting a fine plastics catheter slowly into the canal after milk ejection was complete, and noting the length of catheter inserted at the moment milk began to flow from it. To measure the length of teat entering the liner, the position of the liner mouth was marked on the teat at the end of the milking, the teatcup removed, and the length of the teat below the mark immediately measured. Streak canal patency was measured by applying a teatcup to one teat at a time, equal vacuum within the liner and in the pulsation chamber then being steadily increased from zero until milk began to flow (Glover & Thomas, 1968). The pulse nipple of the teatcup shell and the claw tube of the liner were

attached to a small glass interceptor vessel. The readings were repeated until 3 consecutive results were identical. The values were recorded as patency (cmHg).

Cows and experimental design

Twelve Friesian cows of various lactation ages and stages of lactation were divided into groups of 3, one group only being milked at a time and treated with endotoxin solution. To minimize effects of the length of the intervals between milkings on leucocyte count of the milk, the cows in each group were milked in the same order on successive days.

Treatments were 3 intensities of jetting of the ends of teats for 1 min with the teatcup still in place on the teat following the cessation of milk flow. The experimental design used four 3×3 Latin squares, each square comprising 3 cows, 3 periods, and the 3 treatments. All 4 quarters of each cow received the same treatment on any one occasion.

In addition, the response in terms of increased leucocyte count to a known amount of endotoxin in 1 ml of pyrogen-free water introduced into each teat sinus of the 12 cows was determined by 2 identical assays, one preceding and the other following the jetting experiments. Four dose levels of endotoxin were used, 0·01, 0·05, 0·1 and 0·5 μg, and each cow received a different dose in each quarter. The basic design used in each assay was a 4×4 Latin square (4 cows, 4 quarter positions, 4 dose levels), the 12 cows providing 3 such Latin squares.

RESULTS

In all the comparisons the mean of the 2 pre-treatment leucocyte counts (n_b) was compared with the larger of the post-treatment counts (n_a) on the assumption that this count was the best available estimate of the maximum concentration of leucocytes attained in the individual quarters. Statistical analysis was based on the increase, x, in $\log_{10}$ (count), i.e. $x = \log(n_a) - \log(n_b) = \log(n_a/n_b)$. To make some estimate of the proportion of occasions on which endotoxin entered the teat sinus 2 arbitrary criteria defined a positive result. These were an increase of at least 5-fold in cell count, and a large increase in the proportion of polymorphs to at least 80%.

Summary of data on increase in cell count

Increase in log (cell count) in the milk from each of the 48 udder quarters at the 3 treatment intensities of jetting are given in Fig. 3. A line is drawn dividing the positive results according to the 2 arbitrary criteria from the results considered to be negative. On this basis, endotoxin solution did not enter a teat sinus on any occasion of 48 trials with the low intensity jet, but did so on 9 and 22 occasions with the medium and high intensity jets.

Cell counts before treatment ranged from 6×10^4/ml to 9×10^6/ml. Six of the 12 cows were free from udder infection throughout, and 6 were infected in 1 or more quarters throughout with micrococci or α-haemolytic staphylococci. There was no obvious indication that these infections interfered with the treatment effects.

Intensity of jetting and leucocyte count of the milk

Increases, x, in log (cell count) were calculated for each quarter and an arithmetic mean value obtained for each cow. Analysis of variance of cow values, following the Latin square design, revealed no significant period differences, so a simplified version of the analysis is given in Table 2. The error mean square is described as the interaction between cows and jet intensities.

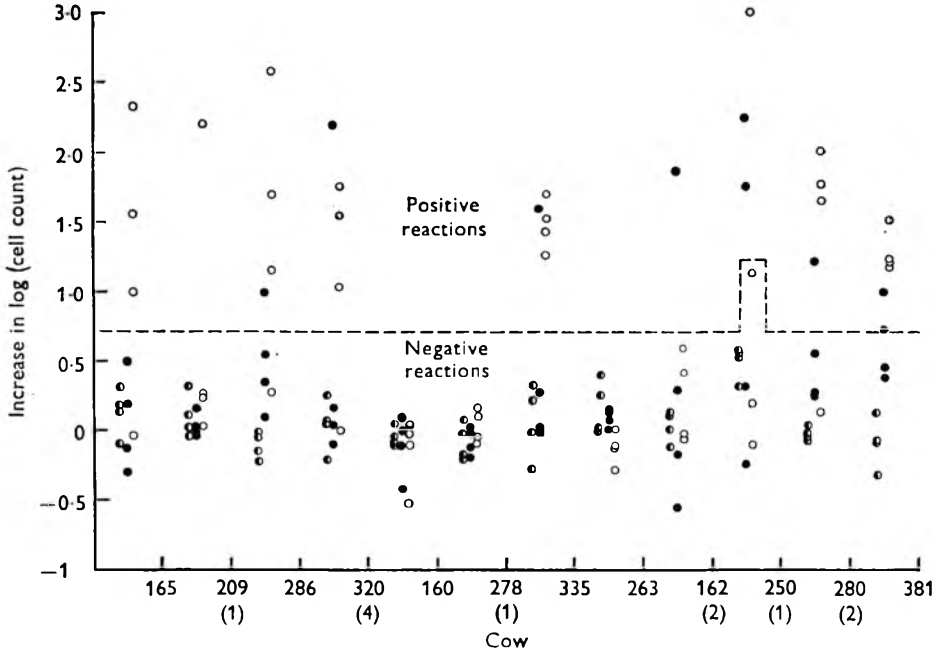

Fig. 3. Increase in log (cell count) when each teat of 12 cows was jetted with endotoxin solution at low, medium and high intensities. Intensity of jet: low, ◐; medium, ●; high, ○. Positive reactions defined as 5-fold increase in cell count with high % polymorphs after treatment. Positives: low, 0%; medium, 19%; high, 44%. Figures in parentheses under cow numbers indicate no. quarters infected throughout.

Table 2. *Analysis of variance of increase in log (cell count)*

Squares	D.F.	Mean square
Cows	11	0·2965*
Velocities	2	1·7021***
Cows × velocities	22	0·1285

Significance levels: * $P < 0{\cdot}05$; *** $P < 0{\cdot}001$.

The mean value of the increase in log (cell count) for each intensity of jetting is given in Table 3, together with the standard error of a mean. The effect of increased jet intensity on the increase in log (cell count) was highly significant ($P < 0{\cdot}001$). The mean value for the low jet intensity did not differ significantly from zero, even though the mean of the 2 pre-treatment leucocyte counts was compared with the larger of the post-treatment counts, a technique biassing the result in favour of a treatment effect.

Characteristics of quarters

The 48 quarters of the 12 cows were described in terms of peak flow rate of milking, average machine milking rate, machine yield, teat length, streak canal length, and patency. Analysis of variance for each characteristic is given in Table 4 and the mean values for each quarter position (averaged over cows) are shown in Table 5. One cow, no. 286, with a recognizable abnormality (fibrosis of the streak canals of the 2 front teats) had exceptional patency values of 20, 22, 13, 13 cmHg for the left front, right front, left hind and right hind quarters, respectively, and has been excluded from the patency results of Tables 4 and 5. Missing patency values for 3 quarters among the remaining 11 cows have also been estimated.

Table 3. *Mean values of increase in log (cell count) and n-fold increase*

Jet intensity	(*a*) Increase in log (cell count)	(*b*) n-Fold increase (antilog of (*a*))
Low	0·050	1·1
Medium	0·333	2·2
High	0·796	6·3
S.E. of a mean (22 D.F.)	±0·1035	—
5% L.S.D. between a mean and zero	0·215	—
5% L.S.D. between 2 means	0·303	—

Differences between quarter positions were not significant for machine rate, streak canal length or patency (although a significant patency difference between front and hind quarters was obtained when cow no. 286 was not excluded from the patency results). The mean peak flow rate of the left quarters was significantly greater than that of the right ($P < 0{\cdot}05$); the mean machine yield of hind quarters exceeded that of the front quarters ($P < 0{\cdot}001$), and the mean yield of the left quarters exceeded that of the right ($P < 0{\cdot}01$); the mean length of front teats exceeded that of the rear teats ($P < 0{\cdot}001$). Interaction between left *v.* right and front *v.* rear effects was not established for any characteristic.

Two of the effects, higher peak flow rate and higher machine yield of the left compared with the right quarters, were probably induced by the milking technique. The time taken to milk each cow one quarter at a time varied from about 10 min to as much as 30 min. To estimate the effect such protracted milking had on milking performance, the quarters of all cows were milked in a set order of left fore, left hind, right hind, right fore. The differences in peak flow rate and machine yield of the left and right udder halves show the adverse effects of prolonged milking, and to this extent the experimental milkings are known not to have been normal. A similar result was observed by Babcock (1889). It should also be mentioned that as a check on the milking performance of the transparent liner the 12 cows were milked 1 quarter at a time with the same apparatus but with a conventional liner. Peak flow rates, machine times, and yields were very similar with the 2 liners, indicating normal milking with the transparent liner.

Differences between cow means were significant ($P < 0{\cdot}01$, at least), except in

Table 4. *Analyses of variance of quarter characteristics*

	D.F.	Mean squares: Peak flow rate, kg/min	Machine rate, kg/min	Machine yield, kg	Teat length, cm	Streak canal length, cm	D.F.	Mean squares patency, cmHg
Cows, *C*	11	0·2105**	0·0596^{NS}	1·037**	4·482***	0·1449***	10	40·4409***
Quarters, *Q*	3	0·2209*	0·0397^{NS}	2·289***	1·777**	0·0069^{NS}	3	2·7273^{NS}
Front *v.* hind, F	1	0·0573^{NS}	0·0427^{NS}	4·132***	4·688***	0·0133^{NS}	1	2·2727^{NS}
Left *v.* right, *L*	1	0·4605*	0·0480^{NS}	2·090**	0·480^{NS}	0·0^{NS}	1	5·8182^{NS}
$F \times L$	1	0·1449^{NS}	0·0283^{NS}	0·643^{NS}	0·163^{NS}	0·0075^{NS}	1	0·0909^{NS}
$C \times Q$	33	0·0624	0·0386	0·276	0·333	0·0050	27	1·9377

Significance levels: NS $P > 0·05$; * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

Table 5. *Quarter means*

	Peak flow rate, kg/min			Machine rate, kg/min			Machine yield, kg			Teat length, cm			Streak canal, cm			Patency, cmHg		
	Front	Hind	Mean	Front	Hind	Mean	Front	Hind	Mean	Front	Hind	Mean	Front	Hind	Mean	Front	Hind	Mean
eft	0·77	0·95	0·86	0·58	0·69	0·63	1·41	2·22	1·81	5·81	5·30	5·56	0·91	0·90	0·90	7·4	7·0	7·2
ight	0·68	0·64	0·66	0·56	0·57	0·57	1·22	1·57	1·40	6·13	5·39	5·76	0·93	0·88	0·90	8·2	7·6	7·9
lean	0·73	0·79	0·76	0·57	0·63	0·60	1·31	1·90	1·61	5·97	5·35	5·66	0·92	0·89	0·90	7·8	7·3	7·5
E. of a marginal mean		±0·051			±0·040			±0·108			±0·118			±0·014			±0·31	
% L.S.D.		0·15			0·12			0·31			0·34			0·04			0·9	

terms of machine rate which revealed no significant between-cow variation (Table 4). For streak canal length and for patency (cmHg) the significant variation was associated solely with between-cow differences.

The range of cow means for each characteristic is summarized in Table 6.

Table 6. *Range of cow means*

(Each cow value is the mean of the 4 quarters.)

	No. of cows	Range	Mean
Peak flow rate, kg/min	12	0·42–1·30	0·76
Machine rate, kg/min	12	0·42–0·84	0·60
Machine yield, kg	12	1·09–2·59	1·61
Teat length, cm	12	4·2–7·3	5·7
Streak canal length, cm	12	0·65–1·28	0·90
Patency, cmHg	11	3·2–13·2	7·5

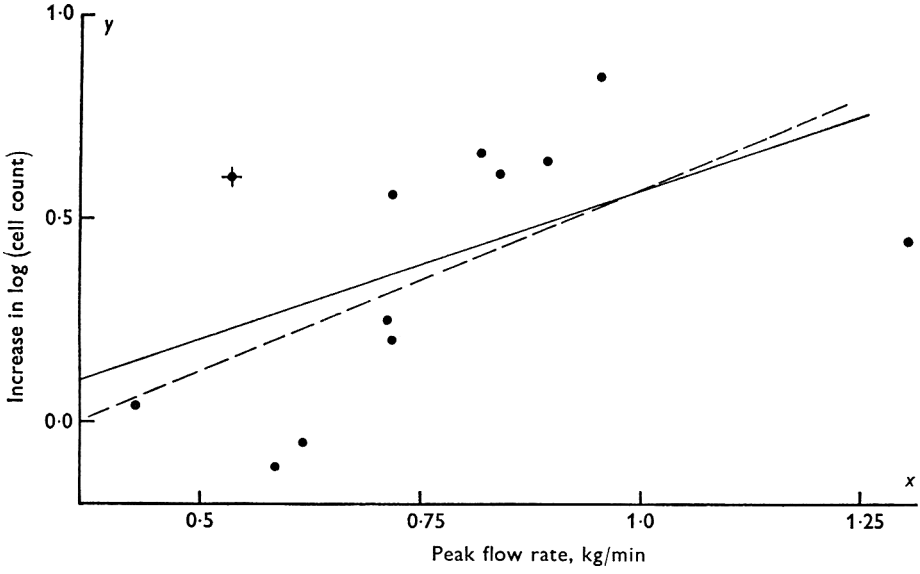

Fig. 4. Relationship between increase in log (cell count) and peak flow rate (kg/min). Each cow value is the mean of the quarter values. Regressions:

12 cows (full line) $y = -0{\cdot}153 + 0{\cdot}719^{\dagger}x$ S.E. slope: $\pm 0{\cdot}369$

11 cows (dotted line) (excluding 286, cross) $y = -0{\cdot}322 + 0{\cdot}894^{*}x$ S.E. slope: $\pm 0{\cdot}165$

† $P < 0{\cdot}1$; * $P < 0{\cdot}05$.

Relationships between cell count response and teat characteristics

Mean increases in log (cell count) were obtained for each cow by averaging over quarters and the 3 jetting velocities. The simple regressions of these cow means on the corresponding values for peak flow rate, machine rate, machine yield, teat length, streak canal length, and patency (cmHg) are shown in Figs 4–9.

The level of significance of these regressions depended on whether cow no. 286 was included, but the only major discrepancy arose with the regression on patency (Fig. 9) which was not significant with 12 cows and was very significant ($P < 0{\cdot}01$) with 11. Regressions on machine yield (Fig. 6) and teat length (Fig. 7) were not significant for either 11 or 12 cows, whereas regressions on peak flow rate (Fig. 4),

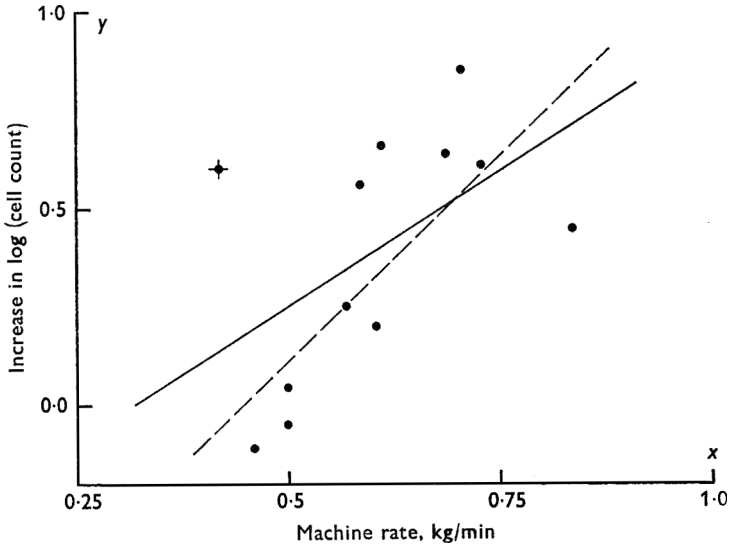

Fig. 5. Relationship between increase in log (cell count) and machine rate (kg/min). Each cow value is the mean of the quarter values. Regressions:

12 cows (full line) $y = -0{\cdot}431 + 1{\cdot}375^{\dagger}x$ S.E. slope: $\pm 0{\cdot}690$

11 cows (dotted line) (excluding 286, cross) $y = -0{\cdot}915 + 2{\cdot}090^{**}x$ S.E. slope: $\pm 0{\cdot}646$

† $P < 0{\cdot}1$; ** $P < 0{\cdot}01$.

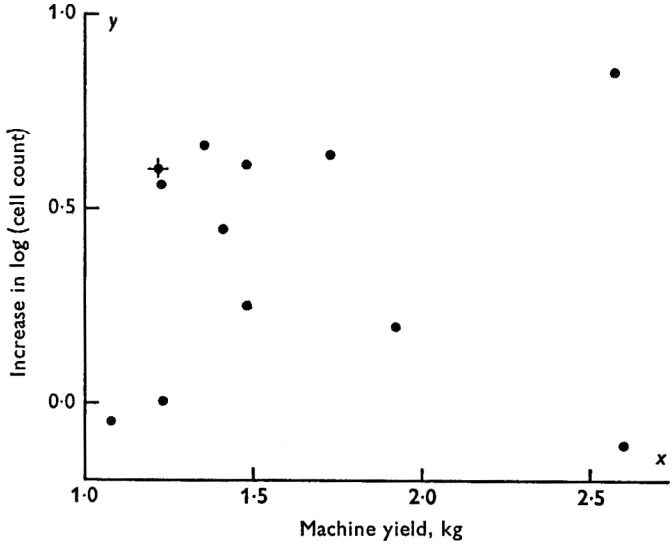

Fig. 6. Relationship between increase in log (cell count) and machine yield, kg. Regressions:

12 cows $y = 0{\cdot}370 + 0{\cdot}014^{NS}x$ S.E. slope: $\pm 0{\cdot}195$

11 cows (excluding 286, cross) $y = 0{\cdot}296 + 0{\cdot}048^{NS}x$ S.E. slope: $\pm 0{\cdot}207$

NS $P > 0{\cdot}1$.

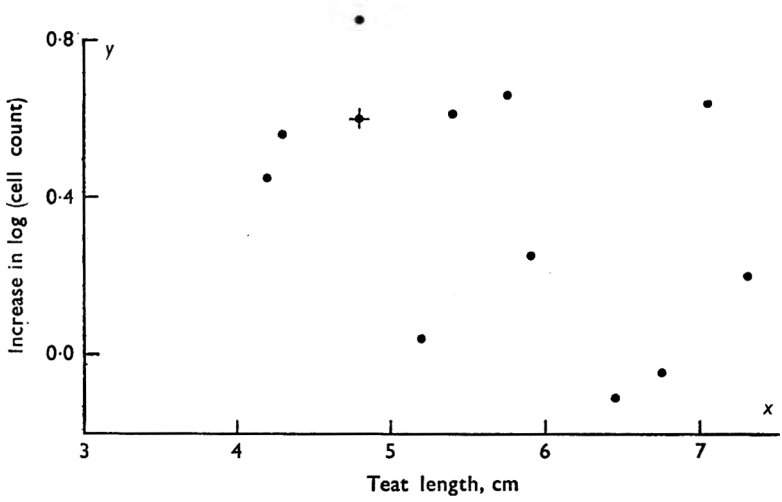

Fig. 7. Relationship between increase in log (cell count) and teat length (cm). Each cow value is the mean of the quarter values. Regressions:

12 cows	$y = 1{\cdot}139 - 0{\cdot}132^{NS}x$	S.E. slope: $\pm 0{\cdot}084$
11 cows (excluding 286, cross)	$y = 1{\cdot}087 - 0{\cdot}124^{NS}x$	S.E. slope: $\pm 0{\cdot}091$

NS $P > 0{\cdot}1$.

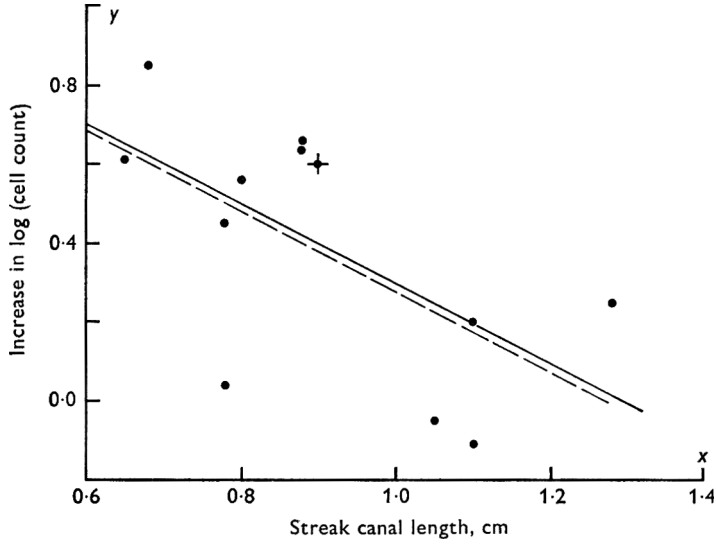

Fig. 8. Relationship between increase in log (cell count) and streak canal length (cm). Each cow value is the mean of the quarter values. Regressions:

12 cows (full line)	$y = 1{\cdot}316 - 1{\cdot}018^{*}x$	S.E. slope: $\pm 0{\cdot}411$
11 cows (dotted line) (excluding 286, cross)	$y = 1{\cdot}291 - 1{\cdot}011^{*}x$	S.E. slope: $\pm 0{\cdot}421$

* $P < 0{\cdot}05$.

machine rate (Fig. 5), and streak canal length (Fig. 8) were significant ($P < 0{\cdot}1$, at least) with 12 cows and were enhanced by excluding cow no. 286.

The simple regressions showed that the larger increases in cell count tended to occur in those cows with the greater peak flow rate or machine rate, and in cows with shorter streak canal lengths or (if cow no. 286 is excluded) smaller values of patency.

Multiple regressions have also been examined relating the increase in log (cell count) to all possible combinations of the 4 independent variates: peak flow rate, machine rate, streak canal length and patency. The other variates, machine yield and

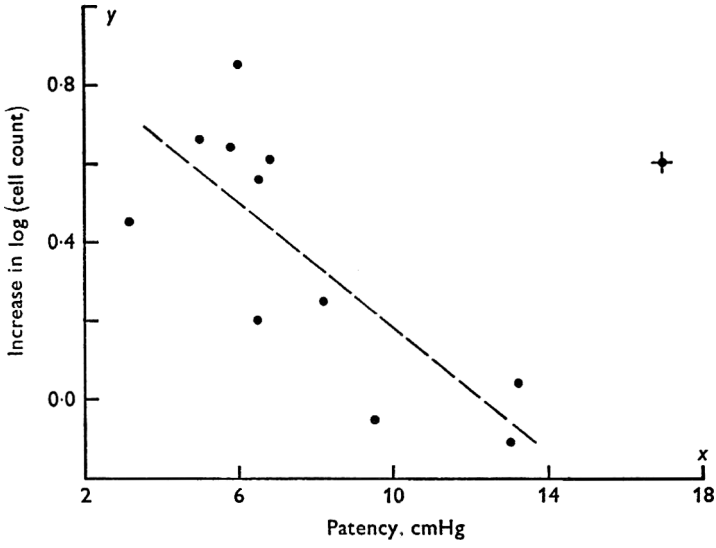

Fig. 9. Relationship between increase in log (cell count) and patency (cmHg). Each cow value is the mean of the quarter values. Regressions:

12 cows (full line) $y = 0{\cdot}670 - 0\ 033^{NS}x$ S.E. slope: $\pm 0{\cdot}022$

11 cows (dotted line) (excluding 286, cross) $y = 0{\cdot}978 - 0\ 079^{**}x$ S.E. slope: $\pm 0{\cdot}021$

NS $P > 0{\cdot}1$; ** $P < 0{\cdot}01$.

Table 7. *Simple correlation coefficients*

	Increase in log (cell count)	Peak flow	Machine rate	Streak canal	Patency
		(*a*) 12 Cows			
Increase in log (cell count)	1·00	—	—	—	—
Peak flow rate	$0{\cdot}52^{\dagger}$	1·00	—	—	—
Machine rate	$0{\cdot}53^{\dagger}$	$0{\cdot}93^{***}$	1·00	—	—
Streak canal length	$-0{\cdot}61^{*}$	$-0{\cdot}35^{NS}$	$-0{\cdot}49^{NS}$	1·00	—
Patency	$-0{\cdot}43^{NS}$	$-0{\cdot}81^{**}$	$-0{\cdot}87^{***}$	$0{\cdot}23^{NS}$	1·00
		(*b*) 11 Cows			
Increase in log (cell count)	1·00	—	—	—	—
Peak flow rate	$0{\cdot}63^{*}$	1·00	—	—	—
Machine rate	$0{\cdot}73^{*}$	$0{\cdot}93^{***}$	1·00	—	—
Streak canal length	$-0{\cdot}62^{*}$	$-0{\cdot}38^{NS}$	$-0{\cdot}56^{\dagger}$	1·00	—
Patency	$-0{\cdot}78^{**}$	$-0{\cdot}85^{***}$	$-0{\cdot}84^{**}$	$0{\cdot}32^{NS}$	1·00

Significance levels: NS $P > 0{\cdot}1$; † $P < 0{\cdot}1$; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

teat length, which failed to correlate significantly with the cell count response, were excluded from the multiple regressions. All the simple correlation coefficients relating the 5 selected variates are given in Table 7, and Table 8 summarizes the percentage inter-cow variation in cell count response accounted for by the various multiple regressions.

Table 8. *Percentage of inter-cow variation* in cell count response accounted for by multiple regressions on patency, streak canal length, peak flow rate, and machine rate*

Regressions calculated over ...	11 Cows	12 Cows
	% variation accounted for	
Independent variate(s)		
Patency (PAT)	56	10
Streak canal length (SCL)	32	31
Peak flow rate (PF)	33	20
Machine rate (MR)	49	21
PAT and SCL	70	34
PAT and PF	51	11
PAT and MR	53	13
SCL and PF	47	37
SCL and MR	50	32
PF and MR	45	13
PAT, SCL and PF	69	29
PAT, SCL and MR	68	26
PAT, PF and MR	56	3
SCL, PF and MR	43	30
PAT, SCL, PF and MR	64	24
Total sum of squares of increase in log (cell count):	1·0399 (10 D.F.)	1·0876 (11 D.F.)

* Percentage variation in total sum of squares accounted for by regression is p, where $p = 100r(b-s)/T$ %; b = mean square due to regression; s = residual mean square; r = regression degrees of freedom; T = total sum of squares of dependent variate, increase in log (cell count).

Table 8 demonstrates that when cow no. 286 was excluded, the single variate accounting for the most inter-cow variation in response was patency (50 %), but significance tests also showed that the variation accounted for by the multiple regression on streak canal length and patency (70 %) represented a significant improvement over patency alone. Jointly these 2 variates gave as good an account of response variation between cows as that provided by the multiple regression on all other combinations including 4 independent variates. These findings are supported by the correlation coefficients of Table 7 (11 cows) which show that patency was closely related to peak flow rate and machine rate but not to streak canal length.

Hence, the multiple regression equation selected to describe the response variation between the 11 cows is

$$y = 1{\cdot}491 - 0{\cdot}066^{**}x_1 - 0{\cdot}680^{*}x_2, \qquad (1)$$
$$(\pm 0{\cdot}019) \quad (\pm 0{\cdot}294)$$

where y = increase in log (cell count), x_1 = patency (cmHg), x_2 = streak canal length (cm).

When cow no. 286 was included, the simple regression on patency was no longer significant and the most effective single independent variate was streak canal length

which accounted for only 31 % of the variation in cell count response (Table 8). However, none of the multiple regressions improved significantly on the simple regression with streak canal length, and the selected equation describing the response variation between the 12 cows is

$$y = 1{\cdot}313 - 1{\cdot}015^{*}x_2. \quad (2)$$
$$(\pm 0{\cdot}414)$$

The failure to account for a substantial proportion of the variation among the 12 cows and the recognizable abnormality of cow no. 286 suggests that equation (1) provides the better general description.

Assay of endotoxin

A combined Latin square analysis of variance of increases in log (cell count) was calculated for each of the identical assays before and after the jetting experiments and showed no significant effect of positions of quarters. Hence, simplified versions of the analyses are given in Table 9 and show significant between-cow differences and dose effects. The mean response in the first assay did not differ significantly from that of the final assay nor was there evidence of significant interaction between assays and dose levels.

Table 9. *Assays: analyses of variance of increase in log (cell count)*

	D.F.	First assay, mean square	Final assay, mean square	Both assays, mean square
Cows, C	11	0·5836**	0·4396**	0·3771**
Doses, D	3	1·5000***	0·8358**	1·1359***
C × D	33	0·1667	0·1283	0·1078

Significance levels: ** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

Table 10. *Assays: mean responses*

Dose, μg endotoxin	First assay		Final assay		Both assays	
	Increase in log (cell count)	n-Fold increase	Increase in log (cell count)	n-Fold increase	Increase in log (cell count)	n-Fold increase
0·01	0·685	4·8	0·928	8·5	0·807	6·4
0·05	1·246	17·6	1·360	22·9	1·303	20·1
0·1	1·223	16·7	1·409	25·6	1·316	20·7
0·5	1·532	34·0	1·536	34·4	1·534	34·2
S.E. of a difference between 2 means	±0·1667		±0·1462		±0·1341	
5 % L.S.D.	0·339		0·298		0·273	

Mean responses to each dose are given in Table 10 for the first and final assays and for the pooled data of both assays. An increase in dose level from 0·01 to 0·05 μg was accompanied by a marked increase in response, but further increases in dose level show more gradual changes in response.

The relationship between the response, y, increase in log (cell count), and log (dose),

x, is illustrated in Fig. 10 and is approximately linear. The regression equation (calculated over the 4 dose levels) is

$$y = 1{\cdot}719 + 0{\cdot}416x$$

(S.E. $\pm 0{\cdot}093$, with 2 degrees of freedom)

and is significant ($P < 0{\cdot}05$).

Values for individual quarters are also shown in Fig. 10, and clearly indicate that estimation of the dose from the response in a single quarter would be subject to large error.

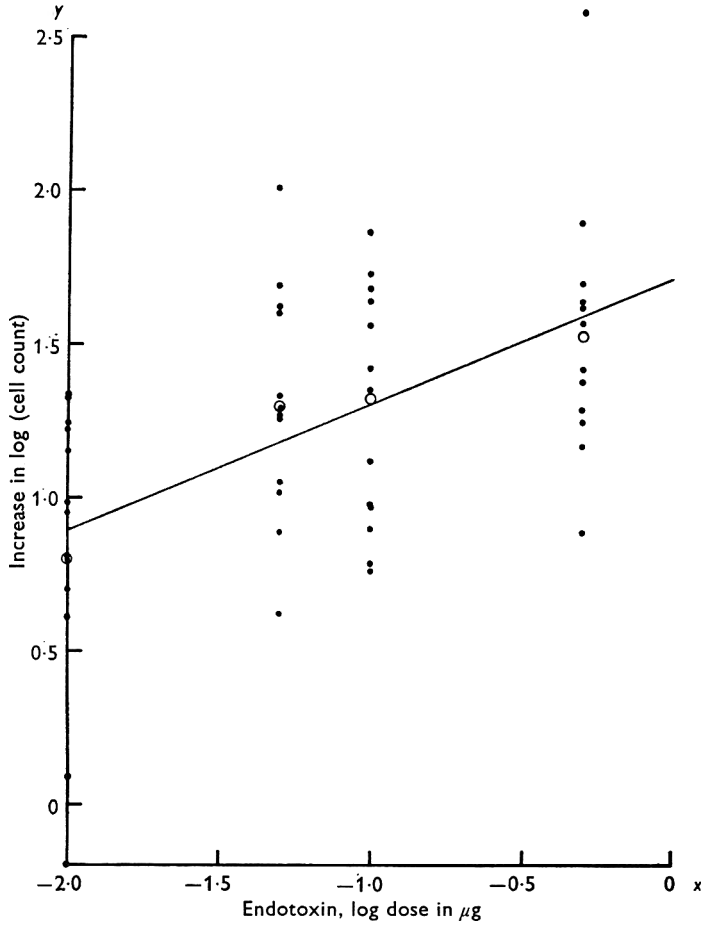

Fig. 10. Relationship between increase in log (cell count) and endotoxin (log dose). ●, Quarter values (mean of 2 assays); ○, mean response. Regression:

$$y = 1{\cdot}719 + 0{\cdot}416^{*}x$$
$$\pm 0{\cdot}093 \text{ (2 D.F.)}$$

* $P < 0{\cdot}05$.

DISCUSSION

The main conclusion from this work is that force of impact of the medium and high intensity jets caused penetration of fluid into the teat sinus (Tables 2, 3 and Fig. 3). Mere presence of the tracer material external to the teat from the low intensity jet

did not appear to cause penetration during 1 min of 'over milking'. Treatment with the high intensity jet was similar to that used by McEwen & Samuel (1946), whose bacteriological results are strikingly analogous to ours. It seems reasonable to conclude that force of the jet impinging on the end of the teat also led to most of the positive results they recorded. Both the present results and the data of McEwen & Samuel (1946) are consistent with the suggestion that the relationship between a fluctuating vacuum during machine milking and increased infection with bacteria causing mastitis may be associated with impact force of milk striking the end of the teat. It remains to be seen whether this mechanism operates during practical milking.

If the pattern of increased treatment effect with increased intensity of jetting is considered in relation to the jet characteristics shown in Fig. 2 and Table 1, it is evident that this information does not indicate the relative importance of maximum pressure at the centre of the 2-mm diam. area of pressure, and the mean pressure on this area. Also with the highest jetting intensity used, because distance from the nozzle to the point of impact became important (Fig. 2), the forces applied to the different teats were probably a great deal more variable than with the medium intensity jet. It seems, therefore, appropriate to consider the characteristics of the medium intensity jet as the best guide to force needed to cause penetration of the streak canal during the over milking period. Penetration, as arbitrarily defined, occurred about once in every 5 trials, each of 1 min duration, under the following conditions: diameter of the circular area in the region of the teat orifice where force was applied, about 2 mm; total force on this area, about 17 mN (mean pressure about 0·05 bar); and maximum pressure at the centre, about 0·25 bar. Impact forces of this nature do not seem unlikely in machine milking, although they would be transitory.

Response in terms of increased leucocyte count of milk to known amounts of endotoxin introduced into each quarter of the 12 cows indicated wide between-cow differences. A dose level as low as 0·01 μg of the particular preparation used increased the cell count significantly, but the response did not increase linearly with dose (Tables 9, 10, and Fig. 10). There was no evidence of change in response as a result of repeated exposure to endotoxin. Taking into account the widely variable responses between cows to known dose levels, most of the observed treatment effects in the jetting experiments could have been accounted for if on each occasion that endotoxin entered a teat the quantity had been 0·1 μg (i.e. the amount in 1 mg of solution). However, it seems more likely that widely differing quantities would have entered the teats on different occasions, the response depending partly on quantity of endotoxin and partly on the sensitivity of the animal or possibly of the particular quarter. Thus, the effect of increasing jet intensity giving higher mean values of increase in log (count), as shown in Table 2, would be expected to be a combined effect of increased number of occasions (as indicated in Fig. 3) and increased quantity of endotoxin on each occasion, but the assay responses were too imprecise to allow any separation of these factors to be attempted.

The cow and quarter differences described in Tables 4–6 show many of the usual characteristics for peak flow rate (Andreae, 1954), machine yield (Johansson & Korkman, 1952), teat length (Johansson, 1957) and streak canal length (McDonald, 1968), although machine rate, which is a less sensitive measure of cow and quarter differences than peak flow rate, showed no significant variation.

When increase in log (cell count) was related to the teat characteristics of the cows it is interesting that characteristics which might be expected to favour mechanical injection of material into the teat sinus—large bore of the streak canal as indicated by high peak flow rate and high machine rate, short length of the streak canal, and ease of opening as indicated by low values of patency (cmHg)—all appeared to do so.

These relationships may be associated with the observations of Dodd & Neave (1951) that with increasing milking rate there was an increase in amount of mastitis, and the results of Murphy (1944) who found a lower incidence of mastitis in quarters which were not patent. A correlation between teat patency, measured as stretchability of the teat orifice, and peak flow rate has been shown by Andreae (1958).

The results of the multiple regression analyses of increase in cell count on patency, streak canal length, peak flow rate, and machine rate are particularly informative. Most of the differences in increased cell count between cows, in response to jetting with endotoxin solution, were accounted for by the regressions on patency and streak canal length (Table 8). Adding peak flow rate and machine rate did not improve the proportion of the differences accounted for, as they themselves were closely correlated with patency (Table 7). Thus, most of the differences between cows could be adequately described by 2 direct measurements of the properties of teats.

We are deeply grateful for extensive assistance throughout this work by many colleagues at the National Institute for Research in Dairying, in particular Mr J. Brock for making the large quantity of endotoxin preparation used, Miss Susan D. Drought and Mr M. J. Gowing for undertaking all the leucocyte counts under the direction of Dr L. W. Phipps, Mr D. L. Simpkin for preparing smears for microscopic examination, Mrs A. T. Ford for statistical analyses and Dr F. H. Dodd for critical appraisal of the work at all stages.

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Effects of some protein modifying agents on the properties of rennin

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Summary. Loss of milk clotting activity when rennin was treated with urea at several concentrations and at different pH values was compared with the change in UV absorption of the protein. The change in absorption at 287 and 293 nm did not correlate with loss in activity, indicating that the loss of enzymic properties was not related to the extent of unfolding of the molecule but was related to the reaction of susceptible groups to the environmental conditions.

Reactions of modifying agents, for example *N*-bromoacetamide, 2-hydroxy-5-nitrobenzyl bromide and mercuric ions with tryptophan groups and the relationship of these changes to enzyme activity suggested that tryptophan was not specifically associated with enzyme action but was important in maintaining tertiary structure.

Rennin was not inhibited by the pepsin inhibitors *p*-bromophenacyl bromide and α-diazo-*p*-bromoacetophenone which suggested that the active site did not possess a reactive carboxyl group as in pepsin. However, the reversible inhibition with 1-cyclohexyl-3(2morpholinoethyl)-carbodiimide metho *p*-toluenesulphonate together with the results of oxidation by *N*-bromoacetamide indicated that a tyrosine group or groups was important in enzyme activity.

The enzyme rennin (E.C. 3.4.4.3) is a peptide peptidohydrolase having characteristics similar to, but not identical with, pepsin for the hydrolysis of peptide bonds (Fish, 1957; Bang-Jensen, Foltmann & Rombauts, 1964). It was first obtained as a crystalline protein by Berridge (1943). Its amino acid composition has been established (Foltmann, 1964; De Koning, 1967) and part of the amino acid sequence determined (Foltmann & Hartley, 1967). Little, however, is known of the mechanism of its catalytic action nor, apart from the work of Hill & Laing (1965, 1967) discussed below, of the identity of the amino acid groups involved in this activity.

The enzyme has a molecular weight of about 31 000 (Djurtoft, Foltmann & Johansen, 1964; De Koning, 1967). It contains a high proportion of acidic amino acids and has a low optimum pH (about 3·5) for proteolytic activity. It shows good stability in salt solutions at pH 5·4 but loses activity rapidly at pH values above 6·5 (Foltmann, 1959).

The enzyme is not inhibited by diisopropyl phosphorofluoridate, indicating that no reactive seryl hydroxyl is available (Cheeseman, 1963) nor are there free sulphydryl groups in the molecule capable of reaction (Cheeseman, 1965). Hill & Laing (1965) have reported that photo-oxidation of histidine groups results in the loss of enzyme activity while the oxidation of methionine or the modification of tryptophan

apparently has little effect. Modification of a lysine group in the molecule by treatment with dansyl chloride has been reported by Hill & Laing (1967) to result in loss of enzyme activity.

Enzyme catalysis depends upon the availability of the active or catalytic centre to react with the substrate. Interference with the properties of the amino acid residues comprising the active centre, either by chemical modification of these groups or by modification of groups—in regions away from the centre—which are involved in maintaining protein structure and in consequence the essential stereochemistry of the active centre, results in loss or modification of enzyme action.

The present paper reports the effects of some protein modifying agents on some properties of rennin and the relationship of these changes to enzyme activity.

Materials and Methods

All rennin preparations were made from crystallized rennin prepared in this laboratory. Urea, mercuric nitrate, mercuric chloride, mercuric acetate, hydroxylammonium chloride and buffer chemicals were of A.R. grade and obtained from B.D.H. Ltd., Poole, England. *p*-Bromophenacyl bromide (PBB), 2-hydroxy-5-nitrobenzyl bromide (HNB) and *p*-bromobenzoyl chloride were also obtained from the same source. *N*-bromoacetamide (NBA) and *p*-bromophenone were obtained from Koch-Light Laboratories Ltd., Colnbrook, England. 1-Cyclohexyl-3(2 morpholinoethyl)-carbodiimide metho *p*-toluene sulphonate (CMC) was obtained from Aldrich Chem. Co., Milwaukee, U.S.A. α-Diazo-*p*-bromoacetophenone (DBP) was prepared from *p*-bromobenzoyl chloride by the method of Erlanger, Vratsanos, Wassermann & Cooper (1967). Yellow crystals, yield 11 %, which were obtained on recrystallization from n-butanol gave a melting point of 123–124 °C, identical with that reported by Erlanger *et al.* (1967).

Enzyme activity was determined by the milk-clotting technique of Berridge (1952). Portions (0·1 ml) of the solutions containing rennin were added to 10 ml of 0·02 M phosphate buffer of pH 5·4 and 1 ml of this solution added to 9 ml of reconstituted milk powder (12 g in 100 ml of 0·01 M calcium chloride) and the time taken to clot the milk at 30 °C recorded.

Effect of urea

A solution of rennin (0·5 %, w/v) was prepared in 0·01 M phosphate buffer of pH 5·4. The difference spectrum of rennin in the presence of various concentrations of urea and at different pH values was measured using the Unicam SP 700. The range of wavelength scanned was from 220 to 320 nm. Four 1 cm spectrosil cells were used, one pair in the reference beam and the other pair in the sample beam. The base line was obtained with buffer in all 4 cells and the instrument was adjusted so as to set the base line in the centre of the chart to allow for positive and negative shift.

For each experiment 1 ml of the rennin solution was added to 4 ml of the appropriate buffer containing urea. The buffers used were 0·02 M phosphate buffers of pH 7·0 and 6·0, and 0·02 M acetate buffers of pH 5·0 and 4·0. The rennin and urea mixture was placed in the first cell in the sample beam and the second cell in this beam was filled with buffer. A further 1 ml of rennin solution was added to 4 ml of the buffer without urea and the solution placed in the first cell in the reference beam. The second cell in this beam was filled with buffer containing the appropriate urea con-

centration. The solutions were kept at room temperature and scanned over the wavelengths specified at time intervals during a 6-h period and once again after 24 h.

Methods used to modify the enzyme

(*a*) The effect of NBA on rennin in solution at several pH values was examined by preparing solutions of rennin containing 15 mg protein/ml in 0·1 M citrate, acetate and Tris/acetate buffers at pH values from 2·3 to 7·0. A concentrated aqueous solution of the reagent was added to each rennin solution to give a final concentration of 0·2 mg NBA/ml of reaction mixture. The mixture was incubated at room temperature, samples were taken at intervals and the enzyme activity was compared with that of untreated rennin. The effect of different concentrations of NBA on enzyme activity was examined by using 1 % (w/v) solutions of rennin in 0·1 M acetate buffers at pH 4·0 and 4·5. Mixtures were prepared containing 1–10 M excess of reagent, incubated for 3–5 h at 30 °C and the activity of the treated enzyme determined. The rate of oxidation of tryptophan and tyrosine was followed by measuring the change in absorption at 280 nm.

(*b*) The effect of PBB and DBP on rennin was examined under the same experimental conditions as those described by Erlanger, Vratsanos, Wassermann & Cooper (1965, 1967) for the inactivation of pepsin. The method was checked with a pepsin solution and the inhibitory properties of both reagents on pepsin activity were confirmed. The reaction mixtures contained 7 ml of 0·006 M acetate buffer of pH 5·0, 3 ml of methyl alcohol, 0·1 ml of 1 % rennin solution and 0·1 ml 1 % (w/v) reagent solution. In experiments using DBP, 0·2 ml 0·01 M cupric chloride was added to the reaction mixture before addition of the reagent. The mixtures were incubated for 30 min at 40 °C and the activity of the enzyme compared with that of untreated controls. Some other pH values, and alcohol concentrations, were also examined as was the effect of the addition to the reaction mixtures of sodium dodecyl sulphate to 0·002 M and urea to 4 M.

(*c*) The effect of HNB on enzyme activity was examined by adding solutions of the reagent in dry acetone to a 0·25 % (w/v) solution of rennin in 1 % acetic acid. Mixtures were prepared containing 5–200 M excess of reagent; the reagent (0·1 ml) was added to 4 ml of a rapidly stirred rennin solution at room temperature. The enzyme was separated from excess reagent by filtration in Sephadex gel G. 25 using the acetic acid as eluting buffer. Enzyme activity was determined and the yellow colour obtained after solutions of modified enzyme were made alkaline was measured at 410 nm using the Unicam SP 500 and 1-cm cells. Total tryptophan in the enzyme molecule was determined by the method of Barman & Koshland (1967). The enzyme was carboxymethylated by the method of Canfield & Anfinsen (1963) and dissolved in 8 M urea before treatment with HNB.

(*d*) The effect of mercuric salts on enzyme activity was examined by the addition of solutions of 10 % (w/v) mercuric nitrate, 6 % (w/v) mercuric chloride and 0·48 % (w/v) mercuric acetate in 1 % acetic acid to an equal volume of 0·05 % (w/v) rennin solution in 1 % acetic acid. The mixtures (1–4 ml) were incubated at room temperature for 10 min, 0·1 ml portions were taken, diluted to 2 ml with 0·006 M acetate buffer of pH 5·0 and the activity of 1 ml of the solution compared with that of untreated rennin.

The change in spectrum which occurred when mercuric ions reacted with the protein (cf. Ramachandran & Witkop, 1964) was compared with loss of enzyme activity. Eight ml of a solution of rennin ($1{\cdot}5 \times 10^{-5}$ M) in 1 % acetic acid was mixed with 8 ml of a solution of mercuric acetate ($1{\cdot}5 \times 10^{-2}$ M) in 1 % acetic acid and the spectrum compared with that of the same concentration of rennin mixed with 1 % acetic acid. The difference spectrum having peaks at 288 and 298 nm was measured using the Unicam SP 700 with conditions as for the urea perturbation described above except that the base-line was set in the normal position. Portions of the mixture were taken at intervals and the activity determined as described above.

(*e*) The effect of CMC on the activity of rennin was examined by adding aqueous solutions of the reagent (4·3 %, w/v) to rennin solutions (0·05 %, w/v) at pH 5·0 ± 1·0, to give a protein to reagent ratio w/w of 1:10 or 20. Glycine methyl ester (GME) (1 % w/v) in water was added to act as a suitable nucleophile to promote the reaction. The mixtures were incubated at room temperature and enzyme activity determined as already described.

Measurement of the difference spectrum of CMC-treated rennin was as described above. The solution in the reference beam contained 2 ml of the rennin solution, 0·2 ml of GME solution and 1·8 ml water, and that in the sample beam 2 ml rennin solution, 0·2 ml GME solution, 0·2 ml CMC solution and 1·6 ml water. The other cell in the reference beam contained 0·2 ml carbodiimide solution and 3·8 ml water and that in the sample beam 4 ml water.

Molecular weights were determined by analytical ultracentrifugation using the Spinco Model E ultracentrifuge and the combined schlieren and interference method of Chervenka (1966). The 8-channel cell (Yphantis, 1960) was used and the molecular weight determined when sedimentation equilibrium was obtained. Determinations were made in buffers of 4 different pH values at 2 concentrations of buffer and at 2 concentrations of enzyme. The conditions used for the attainment of equilibrium are given in Table 2. To obtain suitable masking of the schlieren pattern before recording the interference pattern, an adjustable bar (4·2 cm × 0·47 cm) was fitted to the swinging gate mechanism. When the gate was in the light path the bar masked the region of the photographic plate which corresponded to that required for the interference pattern. Movement of the swinging gate removed the bar from the light path and enabled the interference pattern to be recorded on the same frame.

Sedimentation velocity determinations were made at 59780 rev/min with 2·6 % (w/v) solutions of rennin in 0·08 M phosphate buffer of pH 5 containing 0·02 M-NaCl. In some experiments urea at a concentration of 4 M was also present.

Amino acid analysis was carried out by ion exchange resin chromatography according to Spackman, Stein & Moore (1958). Samples of protein were hydrolysed at 105–110 °C for 24 h in 6 N-HCl in ampoules which had been pumped free of air and sealed.

RESULTS

The effect of urea

The action of urea resulted in spectral changes at 238, 287 and 293 nm. These changes were initially positive but after a period, the length of which depended both on the pH value and concentration of urea, they became negative. An example of

the difference spectrum obtained in 8 M urea at pH 7·0 after about 1 h is shown in Fig. 1. The change in absorption obtained after 24 h was about twice as great at 238 nm as at 287 and 293 nm. There was no difference in rate of appearance of the 287 and 293 nm spectral change.

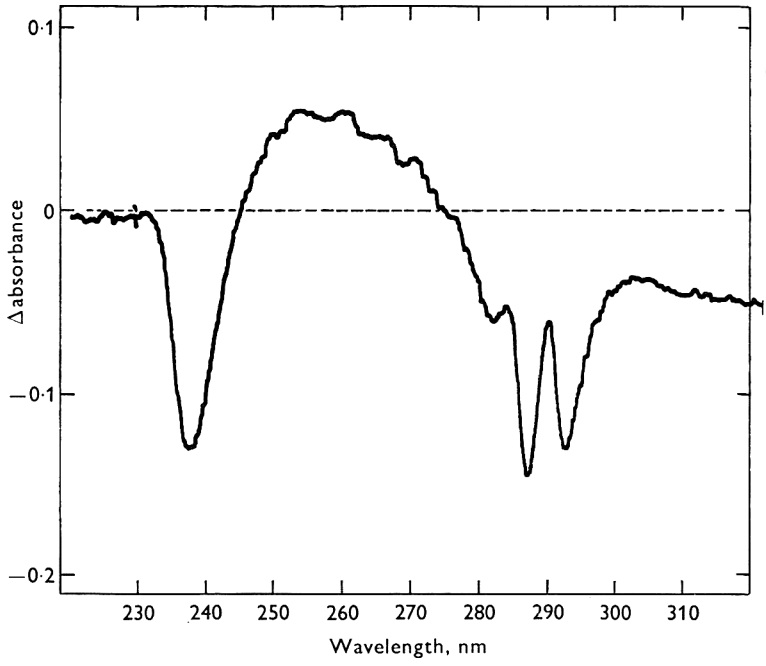

Fig. 1. Example of the difference spectrum obtained when rennin is treated with urea. Rennin 0·1 % (w/v) in 0·02 M phosphate buffer of pH 7·0 compared with rennin under the same conditions but in buffer containing 8 M urea. Reaction time 1 h at room temperature. Troughs at 238, 287 and 293 nm.

The relationship between urea concentration and pH value to the maximum change in absorption is shown in Fig. 2, where it can be seen that the greatest change at 238 nm was obtained at 6 M urea. In experiments with 4 and 8 M urea there was a maximum change at pH 6·0 but there was little difference between the values obtained at pH 5·0, 6·0 and 7·0 with 6 M urea. The changes at 293 nm appear to be less dependent upon the changes in pH. The enzyme was not soluble in the presence of 4 M urea at pH 4, but the activity was fully recoverable when the precipitate which formed was dissolved in 0·02 M phosphate buffer of pH 5·4.

The rate at which these changes occurred depended both upon the urea concentration and upon the pH value of the system. In general, the rate increased as the urea concentration and pH value increased. Two examples of the relationship of activity and change in absorption are shown in Fig. 3(*a*) and (*b*), for solutions of rennin at pH 4 containing 6 M urea and at pH 5 containing 8 M urea, respectively. In both experiments loss of activity occurred as the absorption became negative.

The amount of unfolding of the rennin molecule which was coincident with 50 % loss of activity at the various urea concentrations and pH values examined has been summarized in Table 1, where the amount of change in absorption at 287 nm,

expressed as Δ A 287/A 280 at this activity value, and the amount of total change that is involved are given.

The increase in absorption in the region of 280 and 290 nm which occurred at the beginning of each experiment (see Fig. 3 (*a*) and (*b*)) may have been due to perturbation of chromophore groups occurring before the added urea affected the molecular

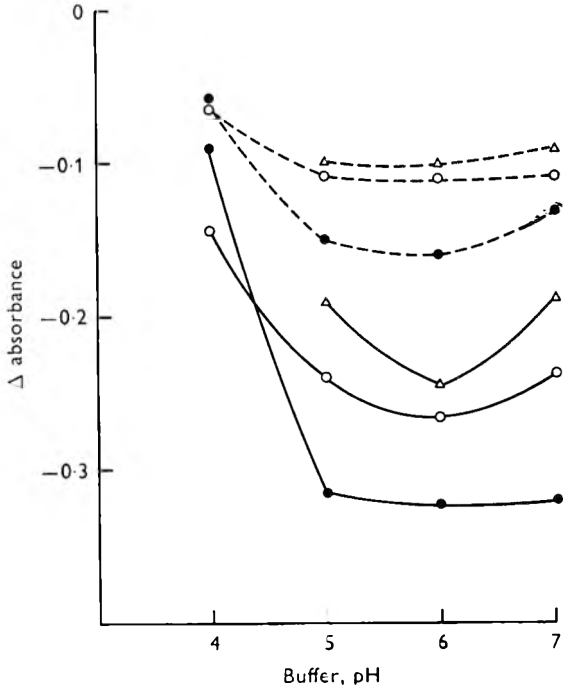

Fig. 2. Relationship of the pH of the reaction mixture and the urea concentration with the amount of change at 238 and 293 nm in rennin–urea solutions. Absorbance recorded after 6 h at room temperature. Continuous line, 238 nm; broken line, 293 nm; ○, 8 M urea; ●, 6 M urea; △, 4 M urea.

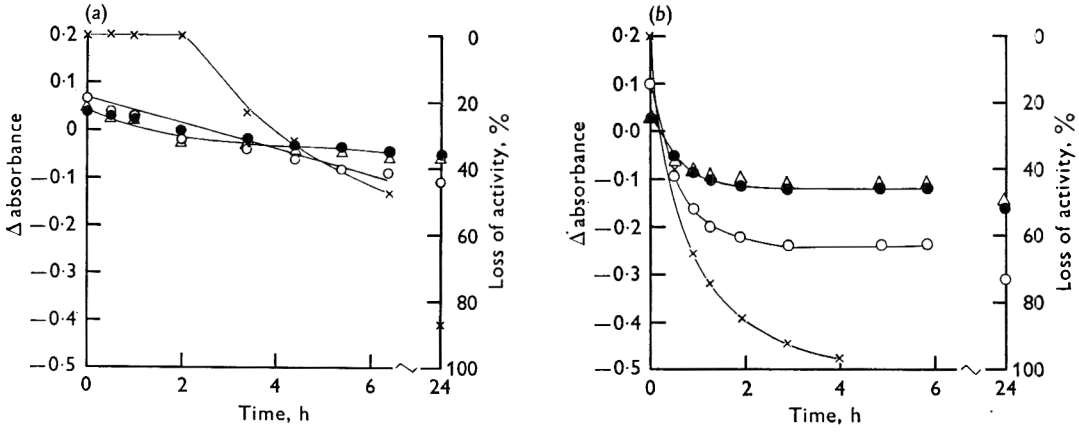

Fig. 3. Examples of the relationship of enzyme activity to spectral change of the rennin–urea solutions. (*a*) Reaction at pH 4 with 6 M urea. (*b*) Reaction at pH 5 with 8 M urea. ×, Milk clotting activity; ○, 238 nm; ●, 287 nm; △, 293 nm.

structure. To find whether dissociation of polymers could have been an important factor in this phenomenon the apparent molecular weight of rennin was determined by analytical ultracentrifugation at several pH values and electrolyte concentrations covering the range of experimental conditions used. The results of this investigation are summarized in Table 2 and show that a dimer probably existed initially at most of

Table 1. *The ratio Δ A287/A280 at 50 % loss of rennin activity*

	Urea concentration		
pH of solution	4 M	6 M	8 M
4·0	—*	0·35 (94)	0·31 (64)
5·0	0·84 (56)	0·63 (49)	0·38 (40)
6·0	0·60 (52)	0·42 (34)	0·35 (27)
7·0	0·56 (89)	0·89 (80)†	—‡

* Enzyme not soluble in this system.

† Rapid change in absorption and activity made measurement difficult. Value given is probably too large.

‡ Loss of activity was too rapid to allow it to be related to change in absorption.

Figures in parentheses give the percentage of the total absorption change that the data for 50 % inactivation represent.

Table 2. *Molecular weight of rennin determined by analytical ultracentrifugation showing the relationships of molecular weight with pH and ionic concentration of buffer*

Buffer	NaCl added, M	pH	Approx. % (w/v) concentration of rennin	Speed, rev/min	Temp. of run, °C	Molecular weight ($M_{app.}$)	Mean ($M_{app.}$)
0·02 M acetic acid	0·08	3·1	1·0	16 200	23	29 930	29 930
			0·6			29 930	
	0·38	3·1	1·0	24 630	23	30 500	30 950
			0·6			31 390	
0·02 M Tris/HCl	0·08	5·8	0·5	12 590	23	65 190	63 620
			0·3			62 040	
	0·38	5·7	1·0	12 590	23	61 510	60 160
			0·6			58 810	
0·02 M Tris/HCl	0·08	6·5	1·0	12 590	23	65 820	67 345
			0·6			68 870	
	0·38	6·5	1·0	12 590	23	65 290	63 400
			0·6			61 510	
0·02 M Tris/HCl	0·38	8·1	1·0	6 166	26	970 000	960 000
			0·8			950 000	

the pH values examined in the urea experiments and that the monomer occurred only at low pH. At the highest pH value examined more extensive polymerization occurred. The slight effect of increase in ionic strength of the buffer in giving a slightly lower molecular weight may not be significant. Dissociation by urea at concentrations of 4 M and above was also confirmed by sedimentation velocity and it is suggested that the first effect of urea was to cause dissociation into monomers which have slightly more absorption by their chromophore groups than have the corresponding dimers.

The effect of NBA

After treatment of rennin with a 5–10-fold molar excess of NBA about 50 % loss of activity was obtained. Inactivation was pH-dependent but not time-dependent as is shown in Fig. 4. Two regions of more extensive inactivation occurred, one commencing about pH 5·5 and increasing to higher pH values and the other with a maximum around pH 4. The relationship of concentration of NBA to enzyme inactivation at pH 4·0 and 4·5 is shown in Fig. 5. The plot of inactivation was not linear and showed a slower inactivation after a reagent to enzyme molar ratio of

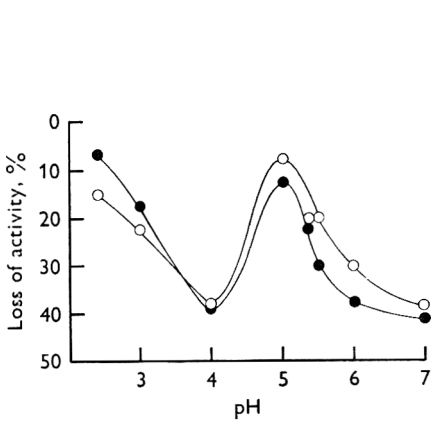

Fig. 4

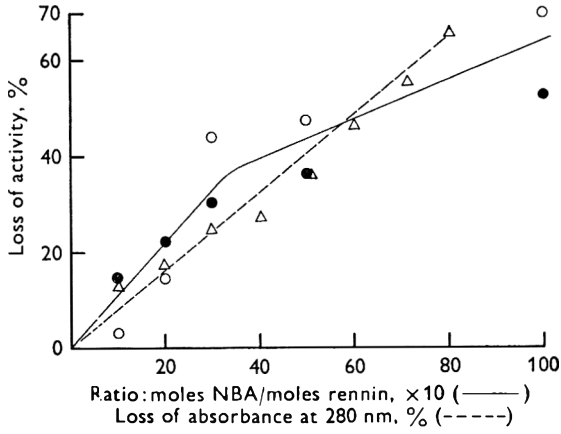

Fig. 5

Fig. 4. The relationship between loss of activity and the pH of the reaction when rennin is treated with *N*-bromoacetamide. NBA, 0·2 mg/ml, was added to solutions of enzyme, 15 mg/ml, in the appropriate buffer. The reaction mixture was incubated at room temperature and portions taken for determination of activity at 15 min, ○, and 30 min, ●.

Fig. 5. The effect of the concentration of NBA on loss of enzyme activity and the relationship of the loss of absorption at 280 nm to the loss of activity. The experiment was carried out at pH 4·5, ○, and pH 4·0, ●. The continuous line indicates the slope estimated from these experimental results; △, loss of absorption at 280 nm at pH 4·0.

about 3–1 is exceeded. The relationship of loss of absorption at 280 nm and loss of activity is shown in the same figure. The results of an amino acid analysis of rennin which had been completely inhibited by treatment with a 60-fold molar excess of the reagent showed that tyrosine and histidine had been oxidized and that methionine had been converted to the sulphone. When small amounts of reagent were used so as to give about 40 % loss of enzyme activity the only significant loss was in tyrosine. Under these conditions about 20 % of the tyrosine was oxidized. Tryptophan was not determined in either experiment, but from the reduction in absorption at 280 nm it is apparent that most of the tryptophan would have been oxidized under conditions which resulted in 40 % loss of activity.

The effect of PBB and DBP

Both of these compounds were shown by Erlanger *et al.* (1965, 1967) to be effective inhibitors of pepsin. However, neither of them inhibited rennin when used under the same conditions and at the same molar ratios as used to inhibit pepsin. A parallel

experiment with pepsin confirmed that both these compounds inhibited pepsin action. To see if conditions could be obtained which would facilitate a reaction between rennin and these compounds, the reaction was carried out at higher and lower pH values and also in presence of 0·002 M sodium dodecyl sulphate or 4 M urea. However, neither the change in pH value nor the presence of the dissociating agents enhanced the reaction.

The effect of HNB

The addition of increasing quantities of HNB to the enzyme resulted in modification of the tryptophan residues in parallel with loss of enzyme activity. The result of this experiment is shown in Fig. 6. A 200-fold molar excess of HNB gave a 40 % loss

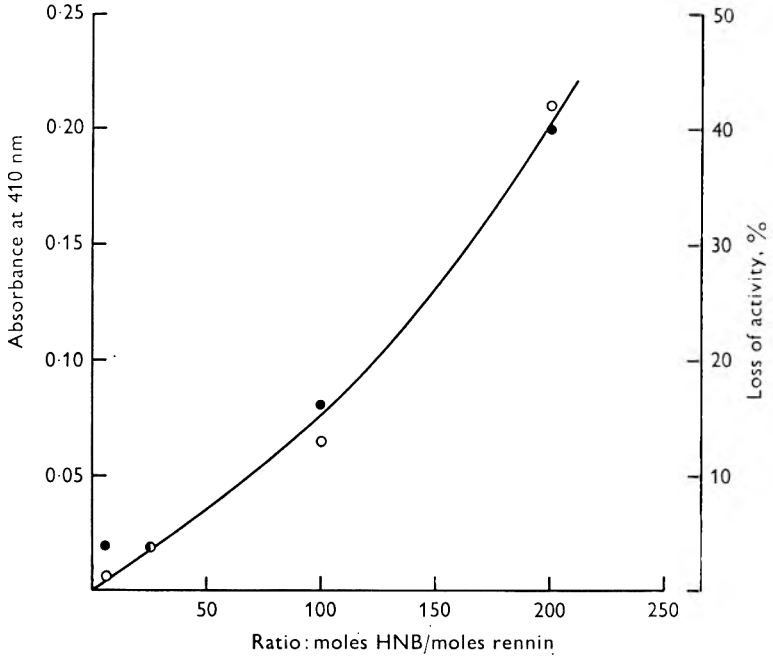

Fig. 6. The effect of HNB on loss of enzyme activity, ●, and modification of tryptophan groups, ○. Rennin 0·25 % (w/v) in 1 % acetic acid was treated with increasing concentrations of HNB. The modified enzyme, after separation from reagent on Sephadex gel G 25 using 1 % acetic acid as eluting buffer, was measured in alkaline solution at 410 nm.

of enzyme activity. The amount of absorption at 410 nm would indicate that the equivalent of 1·5 tryptophan residues were modified per rennin molecule at the 40 % inactivation level. The determination of the total tryptophan by the method of Barman & Koshland (1967) using a 50-fold molar excess of HNB indicated the presence of 2 tryptophan residues/molecule.

The effect of mercuric salts

Mercuric nitrate, chloride and acetate at concentrations of 5, 3 and 0·24 % respectively, inactivated the enzyme; concentrations about a tenth of these values did not. The nitrate and chloride salts gave an insoluble precipitate of rennin, which redissolved on addition of a little solid cysteine to the suspension, but the loss of

Fig. 7. Example of the difference spectrum obtained when rennin was treated with mercuric acetate. Rennin, $7{\cdot}5 \times 10^{-6}$ M, in 1 % acetic acid compared with rennin solution under the same conditions but containing $7{\cdot}5 \times 10^{-3}$ M mercuric acetate. Reaction time 2 h at room temperature. Peaks of absorbance occurred at 288 and 298 nm.

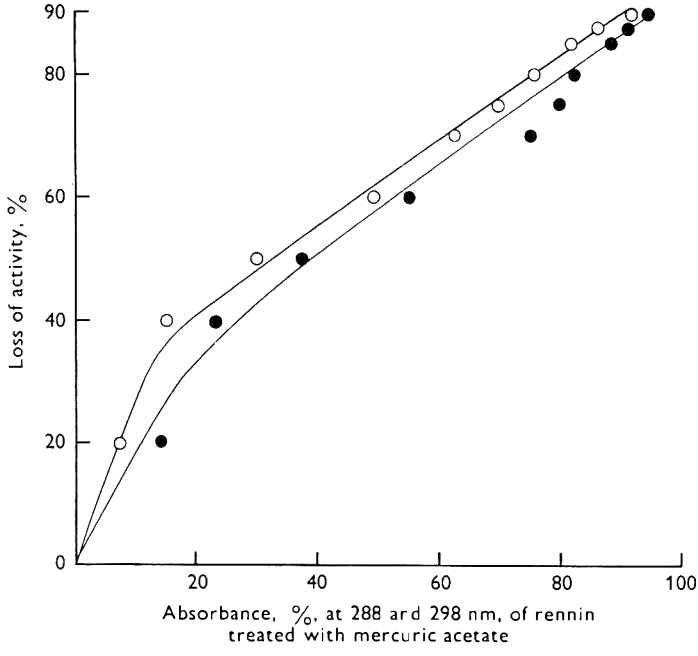

Fig. 8. The relationship of activity to the change in spectrum at 288 and 298 nm of rennin modified by mercuric ions. Conditions as for Fig. 7. Spectra and activity measured at intervals until maximum spectral change was obtained. ○, 288 nm; ●, 298 nm.

activity was not reversible. The difference spectrum obtained when the enzyme was treated with mercuric acetate is shown in Fig. 7. The increase in absorption at values below 280 nm was probably due to the denaturation and aggregation of the enzyme resulting in some light scattering by the solution. Maximum extinction was obtained at 288 and 298 nm and the relationship of loss of activity to the change in absorption at these 2 wavelengths is plotted in Fig. 8.

The effect of CMC

This compound, when allowed to react with rennin at about a 750-fold molar excess, produced complete inhibition of enzyme activity and a soluble inactivated enzyme. There was no change in the spectrum of the inactivated enzyme nor did a 5-fold molar excess of HNB modify any more tryptophan groups than in the active enzyme, thus suggesting that there was no unfolding of the molecule. This inhibitor apparently also inactivates the enzyme in the absence of a suitable nucleophile because the presence of GME did not enhance the rate of inactivation; indeed at higher concentrations, e.g. 25-fold molar excess of GME to CMC, there was a significant reduction in the rate of inactivation. Amino acid analysis of rennin inactivated by CMC in the presence of GME did not indicate the presence of extra glycine residues. The reversal of inactivation by CMC was obtained by adding hydroxylamine to the solution of the reactants. When a mixture (1·2 ml) containing 0·24 mg rennin and 17·4 mg CMC was allowed to react for 1 h at 40 °C there was a 50–60 % loss of activity. When hydroxylamine (288 mg in 0·8 ml) was added to the mixture full recovery of activity was observed within a few minutes.

DISCUSSION

The earlier observation (Cheeseman, 1965) that rennin is rapidly inactivated in solutions containing urea was confirmed. Denaturation by treatment with urea was shown to result in large troughs in the difference spectrum at 238, 287 and 293 nm which suggests that considerable unfolding of the rennin molecule occurred giving rise to change in the environment of the chromophore groups associated with the simultaneous irreversible change in enzymic properties. The spectral changes which paralleled loss of activity were probably due to the increased exposure of tryptophan and tyrosine residues to the solvent environment. The results shown in Table 1 suggest that the amount of unfolding of the molecule required to give 50 % inactivation varied, and that inactivation and also the total spectral change were related both to the pH value at which the reaction occurred, and to urea concentration. The smallest perturbation (0·3–0·4, $\Delta A\,287/A\,280$) which resulted in 50 % inactivation occurred in 8 M urea at all pH values. However, as the pH values increased this amount of change became a smaller proportion of the total spectral change. With decreased urea concentration a greater change was required to give the same degree of inactivation. According to the experimental conditions, 50 % inactivation was obtained with changes varying from 27 to 94 % of the total spectral change which would suggest that there is no correlation between degree of unfolding and the loss of activity. Factors other than the availability of the chromophore groups by the unfolding of the molecule must influence the loss of enzyme activity.

There also appeared to be, at least under some conditions, 2 different rates of spectral change, for if the data were plotted as a first-order reaction (Fig. 9) the slopes of values obtained from experiments at pH 5 and 6 in the presence of 8 M urea suggested 2 different rates of unfolding. There was no correlation between spectral change and the loss of activity.

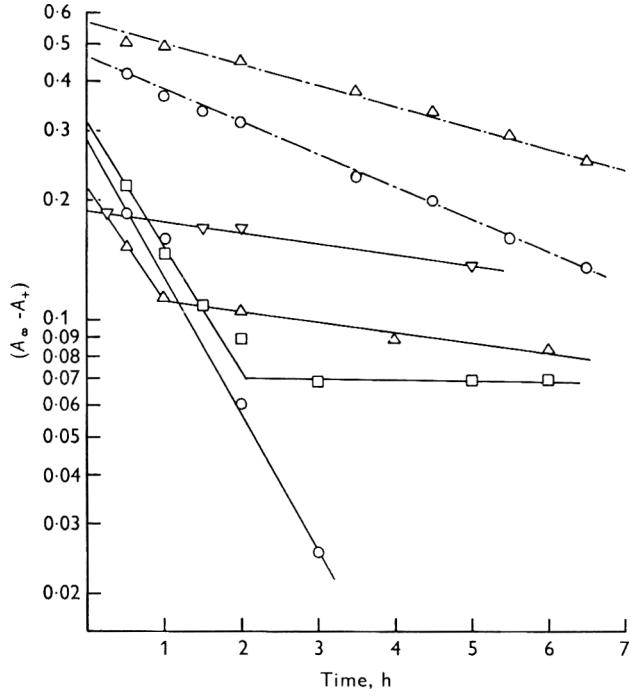

Fig. 9. Data of spectral change at 293 nm obtained from experiments on the effect of urea on rennin plotted as first order reactions. Continuous lines, 8 M urea: ○, pH 4; □, pH 5; △, pH 6; ▽, pH 7. Broken lines, 4 M urea: △, pH 5; ○, pH 6.

Inactivation of rennin by NBA appeared to be due to oxidation of one or several residues. When the enzyme was completely inhibited by treatment with a large excess of NBA, tryptophan, tyrosine, histidine and methionine were modified. The more extensive inactivation at pH 4·0 (Fig. 4) and the apparent difference in rate of inactivation between the first 40 % loss of activity and subsequent loss (Fig. 5) may indicate that some particular residue or residues are more susceptible to attack than others. The tryptophan residues are usually the most susceptible to selective oxidation by *N*-bromosuccinimide and NBA (Spande & Witkop, 1968). The activity of enzymes treated with the reagent will be affected according to the relative importance of the readily oxidized groups in maintaining enzyme activity. For example, 90 % of trypsin activity is lost when 60 % of the tryptophan groups are oxidized (Viswanatha, Lawson & Witkop, 1960). Analysis of rennin treated with only sufficient NBA to give about 40 % inactivation showed that under these conditions of inactivation about 20 % of the tyrosine residues were modified, whereas most of the tryptophan was oxidized. This result suggests that some tyrosine groups may be specifically involved in maintaining enzyme activity.

Treatment with HNB at low concentrations did not result in any appreciable modification of the tryptophan groups nor loss of activity. It therefore appears unlikely that there is very much exposure of these groups in the native molecule. Higher concentrations of HNB reacted with tryptophan groups causing loss of activity, aggregation and precipitation of the enzyme. The content of total tryptophan, as determined by the method of Barman & Koshland (1967), indicated 2 residues/molecule. This value is about half that reported by Foltmann (1966). The reason for this discrepancy is not known.

When mercuric ions react with tryptophan in proteins the appearance of the modified tryptophan can be followed by the spectral change (Ramachandran & Witkop, 1964). The results with rennin suggest that modification of the tryptophan residues is coincident with loss of activity (Fig. 8) and that complete loss of activity occurred when the maximum spectral change was obtained. The difference spectrum showed peaks at 288 and 298 nm analogous to the spectral changes reported by Ramachandran & Witkop for proteins containing tryptophan.

Erlanger *et al.* (1966) have shown that modification of the β-carboxylic group of a reactive aspartic acid residue of pepsin by PBB caused loss of pepsin activity. When rennin was tested under the conditions which inactivated pepsin no loss of activity was observed. This result, however, appeared to be contrary to the results obtained with CMC, as Hoare & Koshland (1966) have shown that carbodiimides will react with free carboxylic groups. That inactivation with CMC was not primarily due to modication of a carboxylic group was suggested from the observation that GME added to the mixture to promote the formation of a —CO—NH linkage did not enhance the reaction and indeed at high concentrations inhibited it. This observation suggested that CMC was reacting with a group other than the carboxyl for which it would normally be expected to have a strong preference. Carraway & Koshland (1968) have shown that carbodiimides can also react with phenolic compounds and it is, therefore, possible that the amino acid involved in the reaction is tyrosine. Amino acid analysis of the CMC-treated rennin did not indicate any significant difference in the tyrosine content from that of untreated material. However, as a total of 16 residues of tyrosine/molecule are involved, detection of modification of one residue would require further analysis. The inhibition obtained with CMC was reversed by the addition of hydroxylamine. Carraway & Koshland (1968) have suggested that carbodiimides react with tyrosine to form the *O*-aryl isourea, which when treated with a powerful nucleophile such as hydroxylamine is converted back to tyrosine.

Hill & Laing (1965, 1967) have reported that histidine and lysine groups are involved in enzyme activity. The work reported here suggests that a tyrosine residue or residues is also likely to be involved in the activity of rennin. The apparent lack of structural change in the molecule when rennin is inactivated with CMC, and the lack of extensive modification of tyrosine groups may in fact indicate that the group involved forms part of the active centre. However, the action of NBA on tyrosine residues did not suggest that any one particular residue was more sensitive to attack than the others. The position with regard to tryptophan involvement has yet to be clarified, but the results may indicate that modification of tryptophan and subsequent loss of enzyme activity is related to loss of structural integrity, since a considerable proportion of the tryptophan can be modified by NBA without total loss of activity,

and also since the extent of modification with mercuric ions parallels the degree of inactivation. This would be in agreement with Hill & Laing (1965) who suggest that photoxidation of tryptophan does not result in loss of activity.

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Reviews of the progress of Dairy Science

Section G. General. Nitrogen metabolism and the rumen

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CONTENTS

INTRODUCTION

The fact that rumen micro-organisms have a modifying effect on the utilization of food nitrogen by ruminants has been recognized for very many years (references to early work are given in Hungate, 1966) but until about 1947, when this subject was reviewed by McNaught & Smith (1947), many of the processes involved were not well understood. Since that time a large amount of work has been done in this field and more recent reviews (Chalmers & Synge, 1954; Blackburn, 1965; Hungate, 1966; Waldo, 1968) present a much clearer picture. Most of the work covered by these authors will be mentioned only briefly in the present review and attention will be directed mainly to developments reported in the last few years. Few references to work published before about 1964 will be given as they may readily be found in later publications.

Essentially the processes in the rumen consist, first, of the conversion, by microbial action, of part of the dietary and endogenously secreted nitrogenous compounds into degradation products. Some of these products may be absorbed directly by the animal, either in the rumen itself or in the lower alimentary tract, but most are normally used in the formation of the bodies of micro-organisms. Thus, the nitrogenous substances presented to the abomasum and intestines of the ruminant consist, usually to a considerable extent, of those present in micro-organisms (bacteria and protozoa) rather than those in the diet. In general, the changes occurring in the rumen are advantageous to the animal when its diet contains mainly poor quality protein or a non-protein nitrogenous material such as urea, but may be disadvantageous when good quality protein is eaten. Clearly the relative importance of the different routes and of the sizes of the pools of different nitrogenous substances

can vary with different conditions. The merits and demerits of such variations, as they affect the nitrogen economy of the animal as a whole, have been extensively studied and attempts have been made to modify the processes for the benefit of the animal. From the practical point of view most research has been directed at one of 2 main objectives: (1) to make it possible to use, effectively and safely, a high proportion of inexpensive non-protein nitrogen (usually urea) in the diet, or (2) to avoid, as far as possible, the degradation of good quality food protein in the rumen.

NITROGENOUS DEGRADATION PRODUCTS IN THE RUMEN

Ammonia

Most of the nitrogenous material ingested by the ruminant receiving natural feeds consists of proteins. A considerable amount of work (reviewed in detail by Blackburn (1965) and Hungate (1966)) shows that these are generally broken down by the rumen bacteria to yield, first, free amino acids, the rate of proteolysis being closely related to the solubility of the protein in rumen fluid, and then ammonia (unless otherwise stated the term ammonia will be used to mean both un-ionized ammonia and ammonium ions). Many individual species of rumen bacteria use ammonia as a nitrogen source in preference to amino acids or other more elaborate compounds and some species have an absolute requirement for it. The mixed bacterial population in rumen contents appears to digest starch more efficiently in the presence of ammonia than in the presence of amino acids (Acord, Mitchell, Little & Karr, 1966; Acord, Mitchell & Little, 1968). It seems probable, in fact, that ammonia is the main nitrogenous nutrient for bacterial growth in the rumen. On the other hand, the protozoa in the rumen are mainly ciliates (Hungate, 1966) and ciliates in general can rarely use ammonia as a major source of nitrogen. They usually have an absolute requirement for many of the amino acids essential to higher animals and for preformed purine and pyrimidine bases (Kidder, 1967). There is little reason to suppose that the rumen protozoa are any exception. It seems likely that these organisms obtain their nitrogenous nutrients by engulfing and digesting bacteria or small particles derived from the food such as chloroplasts and by actively taking up free amino acids, purine bases and pyrimidine bases from the medium. All these processes have been demonstrated (Mangan & Pryor, 1968; Coleman, 1967*a*, *b*, 1968) and it has been shown by Coleman (1967*a*, *b*, 1968) that the rumen ciliate, *Entodinium caudatum*, incorporates amino acids into its protein without inter-conversion and incorporates nucleotides, derived from bacterial nucleic acids, directly into its own nucleic acids. The finding of Ulbrich & Scholz (1966) that ^{15}N, labelling rumen ammonia, accumulated rapidly in the bacteria and more slowly in the protozoa, is consistent with the view that the protozoa derive some of their nutrients by ingesting bacteria but the relative importance of bacteria, food particles and soluble nutrients in protozoal nutrition is likely to vary with the diet. Chloroplast ingestion may, for example, be of particular importance when plant leaves are fed (Mangan, 1968).

The diets received by ruminants normally contain appreciable amounts of nitrogenous materials other than proteins; pasture plants, for example, contain about 20–30% of their total nitrogen as non-protein nitrogen (NPN) (Ferguson & Terry, 1954; Hogan, 1964) and silage contains a much greater proportion (McDonald,

Edwards & Greenhalgh, 1966). Most of the compounds in the NPN fraction such as amino acids and peptides (Lewis, 1955; Lewis & Emery, 1962; Vaz Portugal & Sutherland, 1966; Wright & Hungate, 1967*b*), nucleic acids (McAllan & Smith, 1968), nitrate (Li Chuan Wang, Garcia-Rivera & Burris, 1961) and various amines (Neumark, 1962; Sjaastad, 1967*b*) are rapidly degraded in the rumen and some, at least, form ammonia. Apart from NPN derived from natural foods, ammonium compounds or other nitrogenous materials, of which the most frequently used is urea, may be added to the diet. The additives may provide the whole of the dietary nitrogen (Virtanen, 1967), although animal performance then usually deteriorates and, in practice, not more than about 40 % is normally used. The use of urea in ruminant diets and its metabolism in the rumen has recently been reviewed (Briggs, 1967). The rapid conversion of urea to ammonia in the rumen has been demonstrated many times; the powerful urease activity is apparently restricted to the rumen bacteria (Jones, MacLeod & Blackwood, 1964; Abdel Rahman & Decker, 1966).

Thus, for a wide variety of diets it appears that ammonia forms an important intermediate in the conversion of food nitrogen to microbial nitrogen. Excessively high rates of ammonia production can occur, particularly if large amounts of urea, or proteins, such as casein, which are very soluble in rumen fluid, are eaten rapidly. The ingestion of spring grass, particularly the leafy parts grazed naturally, also tends to cause rapid ammonia production in the rumen. If the rate of production exceeds the rate at which the bacteria can utilize the ammonia, the concentration of ammonia in the rumen increases. This becomes most apparent if the diet is deficient in readily available carbohydrate such as starch; cellulose is of little value in enabling bacteria to make use of ammonia (Lewis & McDonald, 1958; Lewis, 1962; Hogan, 1964; Robertson & Hawke, 1965; Christian & Williams, 1966; Oltjen & Putnam, 1966; Purser & Moir, 1966*a*; Williams & Christian, 1966; Davis & Stallcup, 1967; Deif, el-Shazly & Abou Akkada, 1968). Ammonia accumulation is also influenced by the composition of the microbial population. A number of experiments have shown that, when the protozoa are suppressed, ammonia concentrations are reduced (Abou Akkada & el-Shazly, 1964; Christiansen, Kawashima & Burroughs, 1965; Luther, Trenkle & Burroughs, 1966; Purser & Moir, 1966*b*; Chalmers, Davidson, Eadie & Gill, 1968). This is probably associated with a concomitant increase in the bacterial population (Eadie & Hobson, 1962; Giesecke, Lawlor & Walser-Kärst, 1966) and more efficient utilization of ammonia. The adaptation which occurs with the continued feeding of diets containing large amounts of urea (Holzschuh & Wetterau, 1965; Schaadt, Johnson & McClure, 1966; Clifford & Tillman, 1968) has been shown to be related to the suppression of protozoa (Virtanen, 1967) although changes in the properties, and therefore probably the composition, of the bacterial population have also been implicated (Caffrey, Hatfield, Norton & Garrigus, 1967; Wortham, Wilson, Knight, Holland & Scheirer, 1968).

The accumulation of ammonia in the rumen generally has an adverse effect on the animal's nutrition, mainly because some of the ammonia passes directly into the blood. Toxic effects occur only in extreme cases and are apparently rare in practice although inhibition of rumen motility has been reported with ammonia concentrations in the rumen fluid of about 100 mg/100 ml (Juhász, 1965). The extent of nitrogen disappearance from the digesta above the duodenum has been demonstrated in

experiments with sheep (Hogan, 1965; Clarke, Ellinger & Phillipson, 1966; Hogan & Weston, 1967*a*, *b*; Beever, Thomson, Pfeffer & Armstrong, 1969; Nicholson & Sutton, 1969) and buffaloes (Aliev, 1967) cannulated at that site. With low nitrogen intakes, and therefore low concentrations of ammonia in the rumen, there was usually a net increase of nitrogen between the diet and the duodenal digesta; with high nitrogen intakes, on the other hand, there was usually a loss of nitrogen in the stomachs which clearly varied with the composition of the diet but which was sometimes as much as 50 % of the nitrogen intake. It can reasonably be supposed that most of the nitrogen lost was absorbed as ammonia, and other experiments (Lewis, 1957; Ide, Shimbayashi & Yonemura, 1967; Weston & Hogan, 1967) have demonstrated a close correlation between the concentrations of ammonia in rumen digesta and concentrations of urea in blood under some conditions. Ammonia entering the blood is rapidly converted to urea (probably mainly in the liver although the results of Aliev & Košarov (1967) suggest that some conversion may take place in the rumen mucosa) and this correlation suggests, therefore, that the concentration of ammonia in the rumen digesta is an important factor governing the loss of nitrogen from the stomachs. A high nitrogen intake probably leads to such loss only in so far as it encourages ammonia accumulation. If energy intake is sufficiently high to prevent ammonia accumulation then no loss ensues (Nicholson & Sutton, 1969). However, anomalies in the relationship between rumen ammonia and blood urea have been reported (Abou Akkada & el-Shazly, 1965) and other factors such as rumen pH (Hogan, 1961) undoubtedly also influence the absorption of ammonia. Furthermore, the situation is complicated by the fact that, although much ammonia undoubtedly passes directly across the rumen wall into the blood as long as the rumen pH is not much below normal (Hogan, 1961), not all leaves in this way. Hogan & Weston (1967*a*) found that, in sheep, up to 25 % of the nitrogen lost between the diet and duodenal digesta was absorbed as ammonia between the rumen and duodenum and we have found (R. H. Smith & A. B. McAllan, unpublished), in ruminating calves, that there is a very great reduction in ammonia concentration in digesta between the rumen and duodenum when rumen concentrations of ammonia are high. Ammonia which remains in duodenal digesta is largely absorbed in the small intestine. Thus, the relative importance of the different routes of ammonia absorption and the effect of different conditions upon them are not yet fully understood.

It is well established that urea can move from the blood to the rumen both in the saliva and by direct transfer across the rumen wall, the latter process, apparently, being of greater quantitative importance (Houpt, 1959). Recent work has confirmed that an increase in urea concentration in the blood, achieved either by intravenous infusion of urea or by infusion of urea, or sometimes casein, into the abomasum, leads to an appreciable increase of ammonia in the rumen of animals receiving low nitrogen intakes (Egan, 1965; Egan & Moir, 1965; Gutowski, Barej & Kulasek, 1966; Weston & Hogan, 1967). This ammonia can be used as a microbial nutrient and McCune (1967) showed the incorporation into microbial protein of the ^{15}N from an intravenously injected tracer dose of labelled urea. Thus, there are 2 main pathways available for the disposal of blood urea: excretion in the urine or return to the rumen. The factors involved in controlling this partition and the quantitative nutritional significance of the return to the rumen are however not well understood. Blaxter (1964) has esti-

mated that about 20 % of the nitrogen absorbed as ammonia is recycled but this was presumably for sheep on normal nitrogen intakes. The process would be of the greatest value to animals on low nitrogen intakes. Under these conditions, and particularly when the water intake is low, ruminants show a low urea clearance by the kidney (Schmidt Nielsen & Osaki, 1958; Gans, 1966) but there is little evidence to suggest that this is accompanied by an increase in the proportion of urea entering the rumen. Such animals have low concentrations of urea in the blood (in one experiment sheep receiving a diet containing 6·2 % crude protein had blood urea-nitrogen concentrations of only 2·7 mg/100 ml (Preston, Schnakenberg & Pfander, 1965)) and Weston & Hogan (1967) showed that movement of urea from blood to rumen decreased in direct proportion to blood urea-nitrogen concentration below about 18 mg/100 ml. It is still to be established whether urea re-cycling is of much benefit to a ruminant under conditions of nitrogen deficiency.

Apart from being converted to urea it is possible that ammonia absorbed into the blood may also be used in the liver for the synthesis of non-essential amino acids. Although this process has not apparently been demonstrated in ruminants it has been shown to occur to an important extent in man and the rat (Frost & Sandy, 1951; Richards, Metcalfe-Gibson, Ward, Wrong & Houghton, 1967; Wrong, 1967).

Other products

Although ammonia appears to be the main end product of most degradation processes occurring in the rumen, other non-protein nitrogenous compounds accumulate in rumen fluid to a relatively small extent, particularly in the first 1 or 2 h after feeding (Moore & King, 1958; Blackburn & Hobson, 1960; Stallcup & Davis, 1966). Amino acids, arising partly directly from the diet and partly as intermediates in protein degradation, are probably the most important and some consideration has been given to the possible nutritional importance of their uptake across the rumen wall. It has been shown that certain amino acids added in fairly large amounts to the rumen can enter the blood in this way (Demaux, Le Bars, Mollé, Rérat & Simonnet, 1961; Cook, Brown & Davis, 1965) but several workers (Vaz Portugal, 1963; Leibholz, 1965; Wright & Hungate, 1967*a*), using both cattle and sheep, have found that, after normal feeds, the concentrations of individual free amino acids in rumen fluid rarely exceed about 0·3 m-mole/l. Leibholz (1965) compared the concentrations of individual amino acids in deproteinized rumen fluid with those in samples of blood plasma taken at the same time. She concluded that only lysine, histidine, cystine and alanine were ever in appreciably greater concentrations in the rumen than in the blood and then usually for only a fairly short time after feeding. Bearing in mind that these rumen values were obtained after acidification of rumen fluid, which has been shown by Wright & Hungate (1967*a*) to release some intracellular amino acids from the micro-organisms, it seems unlikely that appreciable amounts of amino acids could normally pass from the rumen to blood by passive diffusion, and a mechanism for active transport has yet to be unequivocally demonstrated. Chand, Varma & Kushwaha (1968) have shown removal of glycine from media incubated with rumen mucosal tissue which they regarded as evidence of active uptake, but it seems that the results could equally well be explained by the glycine having been metabolized (cf. Cook *et al.* 1965).

Amines, other than the common amino acids, have been identified in rumen fluid. Some of these appear to originate only in the diet but some may be formed in the rumen by the decarboxylation of amino acids or in other ways (e.g. cadaverine, putrescine, histamine, phenylethylamine, δ-amino valeric acid, β-amino piperidone and α-amino isobutyric acid) (Sanford, 1963; van der Horst, 1965; Clarke *et al.* 1966; Hendriks, Preston, Prins & van Rheenan, 1966; Sjaastad, 1967*a*). With normal feeding these substances are present in only small amounts in the rumen and clearly have no appreciable influence on the animal's nitrogen economy. Some of them would be expected to have marked pharmacological effects if they entered the blood, even in very small amounts, but there is no evidence that they do so.

Nucleic acids (RNA or DNA), added in large amounts to the calf rumen, are rapidly degraded (McAllan & Smith, 1968). The degradation was shown (R. H. Smith & A. B. McAllan, unpublished) to lead to temporary accumulations of low molecular weight polynucleotides (from DNA only), mono- and/or oligo-nucleotides, guanosine, adenosine, pyrimidine nucleosides and pyrimidine (but not purine) bases. These substances disappeared from the rumen much more rapidly than polyethylene glycol added as a marker and were not present in appreciable quantities in the rumen fluid of calves receiving normal diets. Van der Horst (1965) has shown that the incubation of thymine or uracil with rumen contents from adult cattle leads to the formation of β-amino isobutyric acid and β-alanine, respectively.

FORMATION OF MICROBIAL NITROGEN COMPOUNDS IN THE RUMEN

Nucleic acids

The bacteria and protozoa in the rumen acquire their nitrogenous nutrients in different ways but the net result of their activity is a conversion of much dietary nitrogen to microbial nitrogen. It is well recognized that excessive production of ammonia during the microbial attack can result in a waste of nitrogen resources but another disadvantageous feature of the process, the production of quite large amounts of nucleic acids in association with the microbial protein, has received much less attention. One reason for this has been the lack of a satisfactory method for determining nucleic acids in digesta. McDonald (1954*b*) concluded that established methods used for determining nucleic acids in animal tissues were unsuitable for digesta and this has been confirmed by McAllan & Smith (1968, 1969). Such methods have sometimes been used (Topps & Elliott, 1965; Radin, 1965), but their accuracy must be open to question in the absence of data to the contrary. In an attempt to circumvent the difficulties McDonald (1954*b*) suggested using purine base determination as an estimate of nucleic acids, and Gaussères & Fauconneau (1965) developed a method involving elaborate separations of these bases and other nucleic acid breakdown products. More recently, the sources of error in applying standard methods of nucleic acid analysis to digesta samples have been examined in detail and satisfactory modifications of the methods introduced (McAllan & Smith, 1968, 1969).

It was pointed out by Blaxter (1961) that starvation produced a bigger decrease in the excretion of allantoin in the urine of ruminants than of non-ruminants. This suggested that much of the allantoin in ruminant urine was usually of dietary origin. Allantoin is the major end product of nucleic acid metabolism in most mammals so

the results suggested also the formation of appreciable amounts of nucleic acids in the rumen. This was confirmed by Topps & Elliott (1965) and Radin (1965) who reported estimates of total nucleic acids in the rumen fluid of sheep of up to about 50 mg/100 ml. Topps & Elliott (1965) found some dependence on diet and a significant correlation between concentrations of nucleic acids in the rumen and the amount of allantoin excreted in the urine. Smith, McAllan & Hill (1968 and unpublished) found much higher maximum concentrations of nucleic acids (250 mg/100 ml) in the rumen fluid of calves, with marked differences between different diets which appeared to be related to nitrogen intake (Table 1). There were also marked diurnal variations, particularly in the first few hours after feeding diets containing urea when nucleic acid concentration approximately doubled.

Table 1. *Mean nucleic acid concentrations (mg/100 g water* ± S.E.) *in strained rumen fluid from calves given a variety of diets (R. H. Smith, A. B. McAllan & W. B. Hill, unpublished)*

Diet							
	Main constituents	Approximate nitrogen content, g/100 g dry matter	No. calves	No. samples	RNA	DNA	Total nucleic acid
A.	Cellulose, starch, glucose + straw	0·2	2	8	7 ± 1	3 ± 0	10 ± 1
B.	Barley, fishmeal, starch + straw	1·5	2	24	26 ± 2	14 ± 2	40 ± 3
C.	Flaked maize + hay	1·5	4	40	32 ± 3	19 ± 2	51 ± 5
D.	Flaked maize, decorticated ground-nut meal + hay	4·0	4	39	67 ± 6	34 ± 3	101 ± 9
	Pasture (spring grass)	4·0	4	35	116 ± 8	61 ± 3	177 ± 12

The probable quantitative importance of nucleic acids in ruminant nutrition was first demonstrated by Ellis & Pfander (1965) who showed that RNA and DNA increased in samples of rumen fluid incubated *in vitro* and together accounted for about 15 % of the microbial nitrogenous compounds formed. Their results, and other values for the nucleic acid composition of rumen bacteria and certain other micro-organisms, are shown in Table 2. Rumen bacteria contain about 75–85 % of their total nitrogen in the form of proteins, peptides or free amino acids (Weller, 1957; Purser & Buechler, 1966) so that these substances and nucleic acids together appear to account for nearly all the bacterial nitrogen. It appears that for every 4 parts of dietary nitrogen converted to bacterial protein or amino acids approximately one part is converted to nucleic acids. The situation is less clear with respect to the protozoa; no nucleic acid analyses of rumen organisms have been reported but by analogy with results on other protozoa (Table 2) it seems likely that they contain something like half as much total nucleic acid as the bacteria per unit of total nitrogen. This is consistent with the fact that the mixed bacterial and protozoal samples examined by Ellis & Pfander (1965) contained rather less total nucleic acid per unit of total nitrogen than did our bacterial samples.

Table 2. *Nucleic acid nitrogen to total nitrogen ratios in different micro-organisms*

(Samples of mixed organisms were separated from the rumen contents of animals receiving different diets. The numbers of samples taken on each diet are shown by figures in parentheses. The calf diets were as described in Table 1.)

Derivation		Type	RNA-N/ total-N	DNA-N/ total-N	Total nucleic acid-N/total-N	Reference
Sheep rumen (urea or zein containing diets)		Mixed bacteria and protozoa (8)	0·10–0·15	0·02–0·04	0·14–0·18	Ellis & Pfander (1965)
Calf rumen	Diet A	Mixed bacteria (2)	0·13–0·15	0·05–0·08	0·18–0·22	R. H. Smith & A. B. McAllan (unpublished)
	Diet C	Mixed bacteria (4)	0·11–0·13	0·03–0·09	0·14–0·22	
	Diet D	Mixed bacteria (4)	0·08–0·13	0·06–0·11	0·14–0·25	
	Pasture	Mixed bacteria (2)	0·11–0·11	0·05–0·09	0·16–0·20	
Pure culture: Rumen bacteria		*Bacteroides ruminicola*	0·12	0·12	0·23	
		Streptococcus bovis 597	0·13	0·05	0·17	
		Str. bovis SB39	0·10	0·06	0·16	
		Succinivibrio dextrinosolvens	0·12	0·13	0·25	
		Selenomonas	0·12	0·05	0·17	
Pure culture: Non-rumen protozoa		*Trichomonas gallinae* JB	0·09	0·006	0·10	Mandel & Honigberg (1964)
		Tr. gallinae YG	0·09	0·008	0·10	
		Euglena gracilis Z*	0·08	0·012	0·09	Brawerman, Pogo & Chargaff (1962)

* Calculated assuming total nitrogen = protein + nucleic acid nitrogen.

It can be expected therefore that nucleic acids would make a substantial contribution to the nitrogenous compounds in the rumen and, in fact, samples of strained rumen fluid taken from calves about 4 h after feeding a variety of diets usually contained about 10–16 % of their total non-ammonia nitrogen in the form of nucleic acids (Smith *et al.* 1968 and Fig. 1(*a*)). Strained rumen fluid probably corresponds fairly closely to material passing from the rumen to the abomasum and there was no marked destruction of nucleic acids between the rumen and duodenum. Samples of digesta entering the duodenum usually contained only about 8–13 % of their total non-ammonia nitrogen in the form of nucleic acids (Fig. 1(*b*)), the lower percentages presumably being due to addition of endogenous nitrogen mainly in the abomasum. It appeared from these results that, under the conditions used (adequate readily

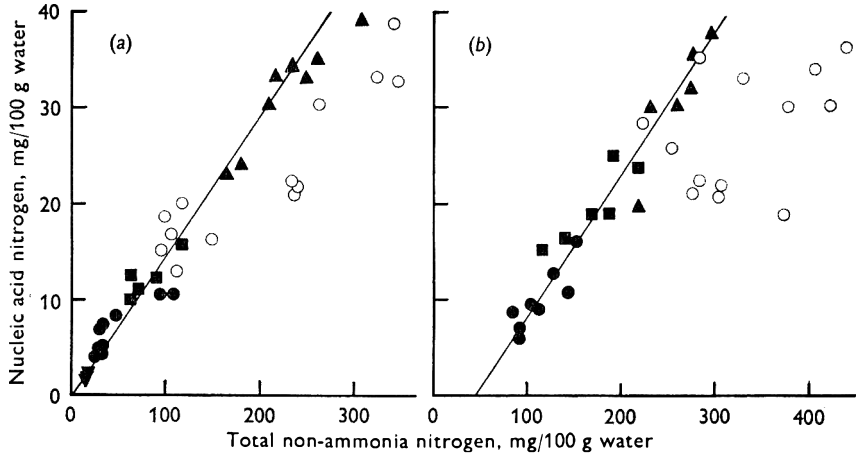

Fig. 1. Relationship between nucleic acid nitrogen and total non-ammonia nitrogen in strained rumen fluid (*a*) and abomasal effluent (*b*) of a calf receiving different diets. The diets were as described in Table 1 and samples were taken about 4 h after feeding diet A, ▼; diet B, ■; diet C, ●; diet D, ○ or while the calf was at pasture, ▲ (R. H. Smith, A. B. McAllan & W. B. Hill, unpublished).

available energy but different nitrogen intakes), the production of microbial nucleic acids, and presumably, therefore, the growth of the microbial population, was controlled by the total nitrogen concentration in the rumen and broadly, therefore, by the nitrogen intake (see also Table 1). Exceptions to the relationships were, however, often seen when large amounts of decorticated ground-nut meal were added to the diet (Fig. 1) implying a relatively smaller response in microbial growth when the dietary nitrogen was in this form.

Extent of the conversion of food to microbial nitrogen

Some of the foregoing conclusions depend upon there being a fairly constant proportionality between total nucleic acids and total microbial nitrogen in the digesta. It is probably reasonable to assume this as an approximation, in view of the fact that nucleic acids entering the rumen are rapidly degraded (McAllan & Smith, 1968) so that little interference from dietary nucleic acids would be expected. However, it is known that the amount of DNA in a pure bacterial culture tends to reflect the number of organisms present, irrespective of their stage of growth as individuals,

whereas the amount of RNA is much more closely associated with protein synthesis (Price, 1952). Moreover, it appears from the results given in Table 2 that the DNA to total nitrogen ratio varies markedly between different organisms and particularly between bacteria and protozoa. It is apparent that changes in the microbial composition and stage of growth of the organisms could be responsible for considerable changes in DNA to total nitrogen ratios. On the other hand, it appears that RNA, as might be expected, represents a much more constant proportion of total microbial nitrogen and should consequently be a much more reliable microbial marker in digesta than either DNA or total nucleic acid. By comparing a mean value for RNA/total nitrogen ratio in rumen micro-organisms with mean values for this ratio in samples of calf digesta obtained 4 h after feeding, it was estimated that, for most diets, about 70 and 60 % of the total non-ammonia nitrogen was microbial in ruminal and duodenal digesta samples, respectively. With decorticated ground-nut meal in the diet these values sometimes decreased by about one-third.

Other methods of assessing the microbial contribution to the nitrogenous materials in ruminant digesta have been used. McDonald (1954*a*) and Ely, Little, Woolfolk & Mitchell (1967) used the solubility of zein in alcohol and McDonald & Hall (1957) used the phosphorus content of casein to estimate the amounts of these proteins remaining in the duodenal digesta of sheep after they had been given in the diet. McDonald (1954*a*), Temler-Kucharski & Gaussères (1965) and Ely *et al.* (1967) measured the lysine contents of the diet and duodenal digesta of sheep and calves and based estimates of microbial nitrogen in the digesta on differences in lysine content between dietary and microbial proteins. Temler-Kucharski & Gaussères (1965) made similar estimates based on DNA contents. A number of workers have studied the formation of microbial protein in the rumen of sheep and cows using the rate of incorporation of ^{35}S, added in an inorganic form, as an indicator (Conrad, Miles & Butdorf, 1967; Walker & Nader, 1968; Roberts & Miller, 1969). Others have measured the concentrations of α,ϵ-diaminopimelic acid (an amino acid apparently peculiar to bacteria) in the rumen contents of sheep and cows and used this to estimate bacterial growth (Weller, Pilgrim & Gray, 1962; el-Shazly & Hungate, 1966), although Synge (1953) showed that the amounts of this substance, relative to total nitrogen, varied considerably between different rumen bacteria. Diaminopimelic acid has been used to distinguish between bacterial and protozoal growth since it is absent from protozoa (Virtanen, 1967).

Precise comparisons between the results of different workers are not possible because of different techniques and sites of study. However, there is general agreement that, with most normal diets of hay and/or concentrates, in which ammonia accumulation is not quantitatively important, about 50–80 % of the food nitrogen is converted to microbial nitrogenous compounds by the time the digesta enter the small intestine. Decorticated ground-nut meal and, more particularly, the very insoluble protein, zein, appear to be more resistant and the latter is converted to an extent of only 20–40 %. At the other extreme, casein was shown to be almost completely degraded before the duodenum was reached but it is probable that much of its nitrogen was lost as ammonia. Conrad *et al.* (1967) concluded, from experiments in which fishmeal was suspended in the rumen in a net bag, that the protein in this material was degraded only to an extent of about 10 % before passing out of the

rumen. However, in experiments with calves, using RNA as an indicator (R. H. Smith, A. B. McAllan & W. B. Hill, unpublished) we have found fishmeal to be converted to microbial nitrogenous substances to an extent of at least 60–70 %. It seems possible that, in the experiments of Conrad *et al.* (1967), microbial attack was limited by the fishmeal not being distributed throughout the rumen.

NUTRITIONAL VALUE OF NITROGENOUS COMPOUNDS IN DIGESTA PASSING INTO THE SMALL INTESTINE

Fate of microbial nucleic acid

Apart from ammonia, which is not generally present in very great amount, the nitrogenous compounds entering the duodenum consist mainly of proteins, or products of protein digestion in the abomasum, which derive partly from the food (except where the diet consists solely of NPN), partly from microbial protein and partly from endogenous secretion into the abomasum. However, nucleic acids also account for an appreciable proportion of the nitrogen in duodenal digesta and their subsequent fate has received little attention.

Smith, McAllan & Hill (1969) found that the nucleic acids entering the duodenum of the ruminating calf (comprising 8–13 % of the total nitrogen on many diets) were digested and absorbed to an extent of about 80 % in the small intestine. It would be of interest to know if the absorbed products have any value for the animal. It is known that conversion of adenine and guanine to allantoin, cytosine and uracil to β-alanine and thymine to β-aminoisobutyric acid can occur in most mammals and that these products are excreted to a greater or lesser extent in the urine (Davidson, 1965). The quantitative aspects of these and other possible metabolic processes, as applied to nucleic acid digestion, utilization and excretion have not, however, been extensively studied. In experiments in which pure RNA or DNA was fed to rats and pre-ruminant calves, only about 25–30 % of the nucleic acid nitrogen appeared in the urine as allantoin to augment the fairly considerable endogenous secretion of this substance (Smith *et al.* 1969). The extent of the increase in allantoin excretion, observed when milk-fed calves were weaned, was consistent with a similar excretion pattern for nucleic acids produced in the rumen and confirmed the relationship between nucleic acid concentration in the rumen and allantoin excretion demonstrated by Topps & Elliott (1965). In rats, some of the dietary nucleic acid nitrogen additional to that excreted as allantoin was apparently retained but the remainder (about 50 %) was excreted in the urine almost entirely as urea. The limited evidence suggests that about 40–50 % of the microbial nucleic acid nitrogen produced in the rumen is not absorbed from the gut or is absorbed but excreted in the urine as allantoin and that most of the remainder enters the urea pool. It appears, therefore, to be of limited value to the animal.

Digestibility of microbial protein

In general, ruminants show a greater relative loss of nitrogen in the faeces than do simple-stomached animals. This is particularly apparent with low-nitrogen intakes. Blaxter (1964) concluded that this high faecal excretion is caused by trapping of nitrogen in the digesta as a result of microbial activity in the rumen, a view which is

supported by the relatively low true digestibility (about 75 %) of the nitrogen in air-dried rumen bacteria shown in experiments with rats (McNaught, Owen, Henry & Kon, 1954; Bergen, Purser & Cline, 1968*a*). Such experiments can only be an approximation to the situation in the ruminant digestive tract since the bacterial cells entering the ruminant abomasum are largely intact and, indeed, viable (Pounden, Ferguson & Hibbs, 1950). Intact cells are probably less digestible than preparations damaged by air-drying; it was shown by Tannenbaum & Miller (1967) that the nitrogen of intact cells of *Bacillus megaterium* was digested less well by rats than that of broken cells. Moreover, *in vitro* digestion tests (pepsin followed by pancreatic enzymes) of different rumen bacteria, grown in pure culture, have shown wide differences between different organisms so that variations in the composition of the microflora could lead to differences in digestibility (Bergen, Purser & Cline, 1967) The most probable explanation of the low availability of nitrogen in bacteria is that the proteins are partially protected by surviving organized structures such as indigestible cell walls. This view is supported by the finding that the digestibility of microbial nucleic acids which enter the duodenum in ruminant digesta is also relatively low (Smith *et al.* 1969). Rumen protozoa appear to be better digested than bacteria (about 89 % true digestibility for rats (McNaught *et al.* 1954; Bergen *et al.* 1968*a*)) presumably because they are rapidly and completely destroyed in the abomasum (Pounden *et al.* 1950). It might be supposed that, in the absence of protozoa, the digestion and utilization of nitrogenous substances in the ruminant small intestine would be adversely affected. Decreased overall nitrogen digestibility in defaunated lambs has, in fact, been demonstrated (Klopfenstein, Purser & Tyznik, 1966) but it is difficult to separate effects in the small intestine from changes in digestion in the rumen.

Biological value of protein in digesta

Various experimental approaches have been used in attempts to assess the amino acid requirements of sheep and cattle and to see to what extent these requirements are met by the digesta presented to their small intestines. Estimates have been made of the amino acid composition of the microbial contribution to these digesta by examining the rumen contents of animals fed diets in which the only nitrogen source was urea (Duncan, Agrawala, Huffman & Luecke, 1953; Schelling, Hinds & Hatfield, 1967), mixed micro-organisms separated from rumen contents (Bergen, Purser & Cline, 1968*a*, *b*) or rumen micro-organisms grown in pure culture (Purser & Buechler, 1966). There are some considerable differences between the different sets of data; glutamic acid results for example show a 2-fold variation and whereas Schelling *et al.* (1967) concluded that histidine was low in mixed microbial protein compared to whole-egg protein, the values reported by Bergen *et al.* (1968*a*) do not show this. There is, however, agreement that lysine values in microbial protein are as high as, or higher than, those in egg protein. The differences were presumably due to variations in the composition of the microbial population although Purser & Buechler (1966) failed to find marked differences between a number of pure species of rumen bacteria.

The results, in general, show no obvious consistent deficiency in any one of the essential amino acids. However, amino acid composition after acid hydrolysis is not necessarily a good indicator of the biological value of a protein and a biological assay

would be preferable. McNaught *et al.* (1954) fed separated, air-dried, preparations of rumen bacteria and protozoa to rats and found that both had fairly high biological values (85 and 82, respectively), for this animal. Since the microbial preparations must have contained about 10–25 % of their nitrogen in the form of nucleic acids, the biological values of the microbial proteins must have been considerably higher than this. The findings of McNaught *et al.* (1954) were confirmed by Bergen *et al.* (1968*a*) who also concluded, from changes in plasma amino acid concentrations between fasted and fed rats, that the first limiting amino acids in bacterial and protozoal protein were cystine and histidine, respectively. It is, however, doubtful what relevance these results with rats have to the situation in the ruminant. The results of Bergen *et al.* (1967) and Tannenbaum & Miller (1967) suggest that the biological values, as well as the digestibilities of intact micro-organisms may differ from those of the dried organisms fed to rats and, furthermore, the amino acid requirements of the ruminant probably differ from those of the rat. This is particularly likely in sheep growing a lot of wool, which has a high content of sulphur-containing amino acids, and in lactating cows. Casein contains a high proportion of lysine which, like most of the other amino acids needed for milk formation, must be supplied, preformed, from the blood (Linzell, 1968). Thus, it would be desirable to be able to assess, in the ruminant itself, the adequacy or otherwise of amino acid absorption from the gut.

Since most ruminant diets contain some protein, it is first necessary to consider to what extent the dietary protein influences the amino acid composition of the digesta passing into the duodenum. This has been examined in sheep by relating dietary composition to the composition of digesta in the duodenum (Clarke *et al.* 1966) and the rumen (Bigwood, 1964). Results were essentially similar and showed, fairly consistently for the diets studied, that the rumen processes led to a decrease in proline, arginine and glutamic acid and an increase in lysine, tyrosine, threonine and isoleucine. The net effect on these and the other amino acids was to make the amino acid compositions of the different digesta samples very similar to each other even when the dietary compositions were markedly divergent. It appeared that, although the diet had some influence, the digesta compositions tended to approach that of microbial protein and the results were broadly consistent with the conversion of some 70–80 % of the food nitrogen to microbial nitrogen. Bigwood (1964) used these results with sheep to calculate, by analogy, the amino acids supplied in the digesta presented to the duodenum of lactating cows and related these supplies to estimated requirements. The figures obtained can be only very rough approximations in view of the many assumptions made but they do bring out the importance of the relatively high lysine content of microbial protein. It is not known at present whether or not lysine is the limiting amino acid for the lactating cow but it seems certain that, without the net increase in this amino acid due to the formation of microbial protein in the rumen, some diets would be markedly lysine deficient for this animal.

A number of workers have investigated the relationships between the composition of the diet, or of the microbial population in the rumen, and the concentrations of amino acids in the blood plasma of ruminants, but the results are difficult to interpret. It is well known that, even with simple stomached animals, it is difficult to determine the limiting amino acids in any particular diet from its effect on plasma amino acid concentrations. Techniques which have been devised for the purpose (Longenecker &

Hause, 1959; McLaughlan, 1964; McLaughlan, Rao, Noel & Morrison, 1967) are not readily applicable to ruminants since they depend upon comparisons with fasting plasma amino acid concentrations and a fasting condition cannot be achieved in the ruminant without prolonged starvation. Moreover, interpretation is further complicated by the absorption of ammonia and perhaps other nitrogenous compounds from the rumen. Differences in such absorption may have been partly responsible for the fact that in most experiments (Oltjen & Putnam, 1966; Schelling *et al.* 1967; Freitag, Smith & Beeson, 1968) sheep and cattle receiving diets in which the only nitrogen source was urea showed higher concentrations of glycine and serine and lower concentrations of many essential amino acids in the plasma than animals receiving diets containing good quality plant protein. Clifford & Tillman (1968) reported quite different results however, finding that the only appreciable differences in plasma amino acid concentrations between the 2 diets were higher glutamic and aspartic acid concentrations in sheep given the urea-containing diet. The reason for this discrepancy is not known but it is not altogether surprising in view of the complexity and variability of the overall processes of digestion and inter-conversion of nitrogenous compounds within the alimentary tract of the ruminant. In other experiments (Purser, Klopfenstein & Cline, 1966) changes in plasma amino acid concentrations were caused when the pattern of digestion was altered by adding starch and glucose to the rumen. Schelling *et al.* (1967) found that increases in the amounts of soya protein given to lambs were associated with an upward trend in plasma amino acid concentrations except for lysine and histidine, which did not change greatly and methionine, which fell significantly. They interpreted this as giving a possible indication that these 3 amino acids, and particularly methionine, were limiting under the conditions studied. On the other hand, Purser *et al.* (1966) and Klopfenstein *et al.* (1966), on the basis of changes in plasma amino acid concentrations after feeding soya protein diets, found no evidence that any particular amino acids were limiting in normal sheep but concluded that lysine was probably limiting in defaunated animals. The importance of lactation in determining the amino acid requirements of cows is shown in the results of Halfpenny & Rook (1968) who found that the onset of lactation was usually accompanied by falls in the plasma concentrations of lysine, arginine, threonine, histidine, glutamic acid and leucine. It has also been shown (Halfpenny, Smith & Rook, 1969) that the concentrations of many essential amino acids in the plasma of lactating cows are depressed by conditions (increased energy intake or infusion of propionic acid into the rumen) which lead to an increase in milk protein content.

The values for plasma amino acid concentrations obtained so far have given no clear-cut evidence concerning the adequacy of the amino acid mixtures absorbed from the ruminant small intestine. It appears that a more productive approach might be an investigation of the effects of supplementing digesta with specific amino acids. Although some improvements in the nitrogen utilization or growth of sheep given supplementary methionine, tryptophan, or lysine in their diets have been reported (Hale, Sherman, Reynolds & Appel, 1959; McLaren, Anderson & Barth, 1965) most experiments of this type have failed to show such an effect (Harbers, Oltjen & Tillman, 1961; Gossett, Perry, Mohler, Plumlee & Beeson, 1962; Oltjen, Robbins & Davis, 1964; McDonald, 1967; Schelling *et al.* 1967). On the other hand, Schelling

& Hatfield (1968) found that the infusion into the abomasum of certain mixtures of essential amino acids, particularly those containing lysine, improved nitrogen utilization by sheep. The same was shown, with lysine infusion into the abomasum, for cattle (Devlin, 1966). Reis (1967) demonstrated a dramatic improvement in wool growth in sheep following abomasal infusion of methionine or compounds which could be metabolized to methionine. The general failure of orally administered amino acids to affect nitrogen utilization is, therefore, usually attributed to their being degraded in the rumen. Such degradation occurs and has been discussed previously (p. 315) but there is evidence that, when present singly, some amino acids are relatively resistant to microbial attack (Lewis, 1955; Lewis & Emery, 1962). Chalmers & Hughes (1969) recently reported that considerable amounts of the glycine, alanine and lysine fed with a cereal ration to sheep passed out of the rumen undegraded. The amino acids were, however, given in very large doses and only 14 days were allowed for the rumen microflora to adapt to the new nutrient. It is uncertain what relevance the findings have to the feeding of potentially useful doses of individual amino acids for a protracted period but they do show that extensive degradation cannot be automatically assumed.

To summarize: it appears that our present knowledge of the amino acid requirements of ruminants and the extent to which these requirements are met is disappointingly meagre. It seems that microbial protein, which provides much of the amino acid supply, is of good quality for rat growth and probably also for ruminant growth but there is good evidence that methionine may be deficient for maximum wool production in sheep and some evidence that methionine, lysine and perhaps histidine might sometimes be limiting for tissue growth. The demand for essential amino acids as a result of milk production in the lactating cow seems likely to alter the pattern of any limitations which might exist, and to exacerbate limitations imposed in respect of lysine and methionine, but this has yet to be demonstrated in terms of animal performance.

CONTROL OF PROTEIN DEGRADATION IN THE RUMEN

For ruminants receiving diets containing protein, the change in amino acid composition which results from the conversion to microbial protein in the rumen can be an advantage or a disadvantage depending on the composition of the food protein. Provided, however, that the latter is of reasonably good quality, any apparent advantage is likely to be more than compensated in terms of overall nitrogen utilization, by possible wastage of nitrogen as ammonia and by the facts that the microbial protein formed is of relatively low digestibility and is accompanied by nucleic acids. Thus, the nitrogen of several different proteins was retained more efficiently by sheep when the proteins were added to the abomasum than when they were given orally or added to the rumen (Chalmers, Cuthbertson & Synge, 1954; Blaxter & Martin, 1962; Little & Mitchell, 1967). It appears, therefore, that a practical advantage might be gained by limiting the degradation of dietary protein, although it should be borne in mind that this would probably lead to a reduced microbial population and the possibility of secondary effects, on cellulolytic activity and volatile fatty acid production, for example.

Protein degradation can be reduced by giving a natural protein which is very insoluble in rumen fluid. Zein is relatively little attacked in the rumen (McDonald, 1954*a*; Ely *et al.* 1967) but it also appears to be poorly digested in the small intestine (Little & Mitchell, 1967) so that its use would not be expected to offer any advantage. It has been shown that dietary protein in liquid solution or suspension can be induced to by-pass the rumen in adult sheep by encouraging closure of the oesophageal groove (Ørskov & Benzie, 1969) but this, although perhaps of use for research purposes, is unlikely to be of practical value. The most promising approach appears to be the chemical modification of good quality protein in such a way as to render it resistant to microbial attack without, at the same time, greatly reducing its nutritive value in the small intestine. Heating protein does reduce its nutritive value for rats (Cama & Morton, 1950; Buraczewski, Buraczewska & Ford, 1967) but nevertheless Chalmers *et al.* (1954) and Chalmers, Jayasinghe & Marshall (1964), respectively, found that heat-treated casein in diets for sheep and heat-treated groundnut meal in diets for goats gave better overall nitrogen utilization than did the untreated proteins. Lower ammonia concentrations in the rumen with the heated proteins suggested that this was due to decreased degradation in the rumen, the advantages of which presumably outweighed any reduction in orthodox nutritive value.

Similar improvements in nitrogen retention, sometimes with reduced production of rumen ammonia, on feeding heated rather than unheated proteins, have been reported for sheep given soya meals (Tagari, Ascarelli & Bondi, 1962), calves given groundnut meal (Whitelaw, Preston & Dawson, 1961) and steers given fishmeal (Broster, 1966). Other protein modifications examined have been those brought about by treatment with formaldehyde (Ferguson, Hemsley & Reis, 1967) or tannins (Leroy, Zelter & François, 1964). Both of these treatments have been shown to reduce the solubility of the protein and its susceptibility to microbial attack. Formaldehyde-treated casein appears to be well utilized by sheep and its inclusion in sheep diets has resulted in marked improvement in wool growth, suggesting improved utilization of the sulphur-containing amino acids. A possible disadvantage of the use of tannin has been suggested by the finding of Tagari, Henis, Tamir & Volcani (1965) that certain tannins interfere with the cellulolytic activity of rumen micro-organisms.

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DIRECTIONS TO CONTRIBUTORS

GENERAL

Papers submitted for publication should be sent to Dr J. E. Ford (*The Journal of Dairy Research*), National Institute for Research in Dairying, Shinfield, Reading. England. Submission of a paper will be held to imply that it reports unpublished original work, that it is not under consideration for publication elsewhere, and that if accepted for the *Journal* it will not be published elsewhere in English or in any other language, without the consent of the Editors.

FORM OF PAPERS

The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author who, in his own interests, should follow these directions carefully, and consult a current issue of the *Journal* for guidance on details of typographical and other conventions.

Every paper should be headed with its title, the names and initials of the authors (women supplying one given name) and the name and address of the laboratory where the work was done.

Papers should be in English, the spelling being that of the *Shorter Oxford English Dictionary*. They should be typed with double spacing, on one side only of the sheets, and with ample margins for editorial annotations.

Papers should in general be divided into the following parts in the order indicated: (*a*) Summary, brief and self-contained; (*b*) Introductory paragraphs, briefly explaining the object of the work but without giving an extensive account of the literature; (c) Experimental or Methods; (*d*) Results; (*e*) Discussion and Conclusions; (*f*) Acknowledgements without a heading; (*g*) References. Only with some exceptional types of material will headings different from (*c*), (*d*) and (*e*) be necessary.

The use of footnotes should be avoided if possible. Underlining should be used only to indicate italics. Proper nouns, including trade names, should be given a capital initial letter. Wherever possible numerals should be used unless this leads to ambiguity. The typescript should carry the name and address of the person to whom the proofs are to be sent, and give a shortened version of the paper's title, not exceeding 45 letters and spaces, suitable for a running title in the published pages of the work.

TABLES

Tables should be numbered and should carry headings describing their content. They should be comprehensible without reference to the text. They should be typed on separate sheets and their approximate positions in the text indicated.

ILLUSTRATIONS

Line drawings, which must be originals, should be numbered as Figures and photographs as Plates, in Arabic numerals. Drawings should be in indian ink, on Bristol board or cartridge paper. However, a technique which may be more convenient to authors is to use a double-sized piece of tracing paper, or translucent graph paper faintly lined in *blue* or *grey*, folded down the centre with the drawing on one half and the other half acting as a flyleaf.

Attached to every figure and plate there should be a translucent flyleaf cover on the outside of which should be written legibly: (*a*) title of paper and name of author; (*b*) figure or plate number and explanatory legend; (*c*) the figures and lettering, which are intended to appear on the finished block, in the correct positions relative to the drawing underneath. For each paper there should be also a separate typed sheet listing figure and plate numbers with their legends, and the approximate positions of illustrations should be indicated in the text.

As a rule the photographs and diagrams should be about twice the size of the finished block and not larger over-all than the sheets on which the paper itself is typed. For general guidance in preparing diagrams, it is suggested that for a figure measuring 9 in. × 6 in. all lines, axes and curves, should have a thickness of 0·4 mm, thus ————, Graph symbols in order of preference should be ○ ●, △ ▲, □ ■, × +, and for a 9 in. × 6 in. graph the open circles should be $\frac{1}{8}$ in. in diam. The open triangles should be large enough to contain circles of $\frac{3}{32}$ in. diam. and the open square circles of $\frac{1}{8}$ in. diam. The crosses should have lines $\frac{1}{8}$ in. long. The black symbols should be slightly smaller than the corresponding open symbols. Scale marks on the axes should be on the inner side of each axis and should be $\frac{1}{8}$ in. long.

REFERENCES

In the text, references should be quoted by whichever of the following ways is appropriate: Arnold & Barnard (1900); Arnold & Barnard (1900*a*); Arnold & Barnard (1900*a*, *b*); (Arnold & Barnard, 1900). Where there are more than 2 authors all the surnames should be quoted at the first mention, but in subsequent citations only the first surname should be given thus, Brown *et al.* (1901). If there are 6 or more names *et al.* should be used in first instance. Also, if the combinations of names are similar, e.g. Brown, Smith & Allen (1954); Brown, Allen & Smith (1954), the names should be repeated each time. Reference to anonymous sources is not acceptable.

References should be listed alphabetically at the end of the paper, titles of journals being abbreviated as in the *World List of Scientific Periodicals*. Authors' initials should be included, and each reference should be punctuated in the typescript thus: Arnold, T. B., Barnard, R. N. & Compound, P. J. (1900). *J. Dairy Res.* **18**, 158. References to books should include names of authors, names of editors, year of publication, title, town of publication and name of publisher in that order, thus, Arnold, T. B. (1900). *Dairying*. London: Brown and Chester.

It is the duty of the author *to check all references* and to ensure that the correct abbreviations are used.

SYMBOLS AND ABBREVIATIONS

The symbols and abbreviations used are those of British Standard 1991: Part 1: 1954, *Letter Symbols, Signs and Abbreviations*.

DESCRIPTIONS OF SOLUTIONS

Normality and molarity should be indicated thus: N-HCl, 0·1 M-NaH_2PO_4. The term '%' means g/100 g solution. For ml/100 ml solution the term '% (v/v)' should be used and for g/100 ml solution the correct abbreviation is '% (w/v)'.

REPRINTS

Order forms giving quotations for reprints are sent to authors with their proofs.

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ORIGINAL ARTICLES

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