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Some skin characteristics in British Friesian pedigree bulls and their correlation with relative breeding value

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SUMMARY. A possible relationship between the Relative Breeding Value (R.B.V.) and some skin characteristics has been investigated in 89 British Friesian pedigree bulls made available by the Milk Marketing Board of England and Wales.

It was found that there was a statistically significant correlation between the R.B.V. and the follicle diameter. There was also a correlation between the thickness of the papillary layer and the R.B.V. The possible value of the results for selection purposes is discussed.

INTRODUCTION

The possibility, however remote, that there may be some relationship between the structure of cutaneous glands and the structure of the mammary gland, induced many workers to investigate various skin characteristics and to relate them to milk yield. Raynaud (1961) reviewed and discussed various theories concerned with the homology of the mammary gland. He doubted that the mammary gland anlage can be derived from sweat gland anlagen, although sebaceous formations are sometimes associated with the mammary bud. Burcev (1937) reviewed reports of a correlation between the number of sweat glands per unit area of skin and milk yield and himself found a significant positive correlation between milk production and the numbers of sweat glands in the ear in Red German cows ($r = 0{\cdot}72$). The correlation between the numbers of sebaceous glands and milk yield was also positive ($r = 0{\cdot}297$).

Findlay, Goodall & Yang (1950) made a similar investigation in Ayrshire cows and found no correlation between milk yield and the number of sweat glands in the skin of the ear. They criticized the work of Burcev for certain technical inaccuracies, and also found no relation between age of cow and the numbers of sweat glands and sebaceous glands in his material. Finzi & Cenni (1962) in a study of Friesian cows, measured the thickness of the skin over 3 areas of the body with a gauge and then compared skin thickness with the milk yields of the animals. The skin thickness of one area of the mammary gland, gave a significant negative correlation with milk yield. Nay & McEwan Jenkinson (1964) examined various skin characteristics and milk yield in Ayrshire cows. Their results indicated that the cows with the thinnest papillary layer are potentially the best milk producing animals.

The purpose of the present work was to establish whether a relationship exists

between the skin characteristics of dairy bulls and the milk production of their offspring. Such a correlation could be of practical value in the selection of bulls for progeny testing.

MATERIALS AND METHODS

Animals

Skin samples from 90 British Friesian Pedigree bulls were obtained through the courtesy of the Milk Marketing Board of England and Wales. One sample was excluded from analysis because its hair follicles were unlike those in the rest of the group. They can best be described as exhibiting 'follicle gigantism'. The follicles were not only very large but also low in number to the square centimetre. The condition is relatively rare; this sample was the first of its kind out of 600 beef and dairy cattle examined. Such animals occur sporadically in some European breeds (unpublished data). Table 1 compares some characteristics of this sample with those of the remaining 89.

Table 1. *Mean values for skin measurements in* 89 *bulls, and in one bull showing 'follicle gigantism'*

Animals	Papillary layer thickness, μ	Follicle diam., μ	Sweat gland length/ diam. ratio	Sweat gland volume, $\mu^3 \times 10^{-6}$	Hair follicle density number/ cm^2
89	1648	52·5	8·34	18249	995
1	2520	93·7	19·25	14401	433

Skin measurement

Two skin samples, 1 cm in diam., were obtained by biopsy from the midside region of each animal, using the trephine biopsy technique as adapted for cattle by Carter & Dowling (1954). The samples were fixed in 5% formol-saline. Vertical sections 500–1000 μ in thickness, containing numbers of undamaged sweat glands, were cut parallel to the direction of hair growth and prepared for examination using a modification (Nay, 1959) of the method of Nay & Hayman (1956).

Horizontal sections were cut on a freezing microtome, about 30 μ thick, stained for a few minutes in a 1:100 solution of Toluidine Blue and mounted in an aqueous mountant. The skin sections were prestained overnight in Oil Blue N in order to stain the sebaceous glands selectively, a procedure which facilitates the cutting at the horizontal level.

The following measurements were made on the skin specimens from each animal:

Papillary layer depth

Papillary layer depth was defined as the mean of the shortest distances between the base of 10 active hair follicle bulbs and the surface of the skin. Giant follicles which are seated deep in the reticular layer and represent some 4–6% of the total present (Hayman & Nay, 1961) were ignored.

Hair follicle diameter in vertical sections

The diameter of hair follicles was measured in vertical and horizontal sections. The vertical sections were scanned under the microscope with a scale inserted in the eyepiece and the diameters of 10 active hair follicles containing a fibre were measured. Follicles in the telogen (resting) stage were not measured. Such follicles are easily recognizable as containing 'club hairs'. The shortest distance between the 2 opposite walls of the hair canal was considered as the estimation of the follicle diameter. Follicles which had just become active, recognized by their containing cone shaped fibres, were not measured. Giant follicles were ignored.

Since a plot of follicle diameter against the month in which skin sections were taken indicated that follicle diameter changes throughout the seasons, correction for seasonal differences in time of skin sampling was made in 65 animals for which the date of sampling was known. There was no certainty about the sampling time of the remaining 24 animals and they had to be left out of the analysis of corrected diameters. The correction was made by fitting a curve to the data, according to the formula:

$$y = c + a_1 \sin 2\pi(\tfrac{1}{12}n) + b_1 \cos 2\pi(\tfrac{1}{12}n) + a_2 \sin 4\pi(\tfrac{1}{12}n) + b_2 \cos 4\pi(\tfrac{1}{12}n),$$

where n is the number of months from the origin which was taken as July. As a_2 and b_2 did not contribute significantly to the regression, they were not included in the correction term. Both corrected and uncorrected values are given in Table 3.

Hair follicle diameter in horizontal sections

The sections used for density estimation were scanned under the microscope along 2 crossed red lines drawn on the slide underneath the section, using $\times$ 400 magnification. The diameters of the first 50 follicles which appeared in the field of vision were measured with the exception of the active follicles, which had just started to form fibres. The correlation coefficient between the estimates of diameter by the 2 different methods was $r = +0{\cdot}6670$ ($P < 0{\cdot}001$).

Hair fibre diameter

The diameters of 30 hair fibres/animal were measured on hair stubble protruding above the surface of the skin in vertical sections. Only fibres of active follicles were measured. The values were then compared with the follicle diameter measurements made on vertical sections. The correlation coefficient between the 2 sets of measurements was $r = +0{\cdot}844$, $P < 0{\cdot}001$.

Hair follicle density

The follicle density (i.e. the number of follicles/cm^2) was estimated as described by Turner, Nay & French (1962). Since in cattle every hair is of the primary type, and thus equipped with a sweat gland, the sweat gland density equals the hair follicle density.

Proportion of active and resting follicles

The vertical sections were scanned under the microscope and the proportions of active and resting follicles were determined in the first 100 follicles, thus giving their percentages.

Sweat glands

Measurements of sweat glands were carried out as described by Nay & Hayman (1956).

Milk yield data

The milk-yield data were supplied by the Milk Marketing Board of England and Wales. The bulls were given a rating by the method known as contemporary comparison. In this comparison, the mean yield of a bull's daughters is subtracted from the mean of their contemporaries in the same herds. The comparison is equivalent to that between 2 equal groups each containing $(2n_1n_2)/(n_1+n_2)$ individuals where n is the number of daughters and n_2 the number of contemporaries. The number, W, is the harmonic mean of n_1 and n_2. W can then be used to estimate the heritability of the difference in mean yield between daughters and contemporaries; if we call this difference C, its heritability h_C^2 is usually taken to be $W/(W+12{\cdot}3)$. This comes from the assumption that the heritability of yield based on a single lactation is about 30% and that

$$h_C^2 = \frac{W\frac{1}{4}h_2}{1+(n-1)\frac{1}{4}h^2}.$$

The genetic superiority of the bull tested can then be estimated by doubling the genetic superiority of his daughters and if this is divided by the mean yield of the group, both daughters and contemporaries, it is made proportional to herd production. This figure called the relative breeding value or R.B.V. is usually expressed as a percentage by multiplying by 100 and is equal to

$$\left[2\left(\frac{W}{W+12{\cdot}3}C\right)\Big/\bar{Y}\right]\times 100.$$

The advantage of this figure is that it is proportional to mean yield. Perhaps C, expressed directly or as a percentage, would be preferred but since R.B.V. was already calculated for us we used it. W, which averaged 243·7, ranged from 27·2 to 825·1. For further discussion see Robertson (1960).

RESULTS

The numerical results are given in Tables 2, 3, 4 and 6.

Table 3 gives the means of the various measurements, their standard deviations and the correlation coefficients between single skin characteristics and relative breeding value.

The relationship between papillary layer thickness and R.B.V. appears to be a curvilinear one. The regression statistics of R.B.V. on papillary layer thickness are shown in Table 4. In Table 5, the test of departure from linearity is shown and the calculated relationship is shown fitted to the points plotted in Fig. 1.

The regression of R.B.V. on follicle diameter, both before and after correction for season, was calculated and the regression coefficients and their standard errors are given in Table 6. The regression line after correction is presented in Fig. 2.

In addition, the correlation coefficient between R.B.V. and follicle diameter was

Table 2. *Correlations between the single skin characteristics*

Variate	Follicle diam.	Sweat gland length	Sweat gland diam.	Sweat gland length/diam.	Sweat gland volume
Follicle depth	+0·337**	+0·330**	−0·002	+0·361***	+0·128
Follicle diam.		+0·492***	−0·114	+0·542***	+0·180

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

Table 3. *Means and standard deviations of all measurements, and the correlation coefficients of skin measurements with* R.B.V.

Measurement	No. of animals	Mean	Standard deviation	Correlation coefficients with R.B.V.
Relative breeding value	89	103·6067	±7·9837	—
Papillary layer thickness, mm	89	1·6392	±0·2263	±0·2213*
Follicle diam., μ:				**±0·2951****
uncorrected	89	52·2200	±7·0043	+0·3304**
corrected for seasonal difference	65	48·8246	±5·4268	+0·3478**
horizontal sections	89	52·9790	±7·1042	+0·2470*
Fibre diam., μ	89	49·9148	±5·3834	+0·2509*
Sweat gland:				
length/diam.	89	8·2235	±1·8585	+0·1175 N.S.
volume, μ^3	89	18,249·79	±7,244·3	+0·0571 N.S.
number/cm^2	79	995·6962	±169·27	−0·0940 N.S.

±0·2951 recovered from the curvilinear regression equation.

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

Table 4. *The regression statistics of* R.B.V. *on the papillary layer thickness*

	Zero-order correlations		
Variate	X^2	Y	
X	0·9972	0·2213	$Sy^2 = 63{\cdot}7414$
X^2		0·2061	$P^2 = 0{\cdot}0871$
			$R = 0{\cdot}29513$

Remainder after linear regression $(1-rxy^2)\,(Sy^2) = 60{\cdot}6181$
Remainder after curved regression $(1-R^2)\,(Sy^2) = 58{\cdot}1895$

Table 5. *Test of significance of departure from linear regression*

Source of variation	Degrees of freedom	Sum of squares	Mean square
Deviations from regression	87	60·6181	—
Deviations from curved regression	86	58·1895	0·6766
Curvilinearity of regression	1	2·4286	2·4268

$F = 2{\cdot}4286/0{\cdot}6766 = 3{\cdot}5894$.

calculated in 22 animals sampled in June and July and these, presumably, had the follicles exclusively of the summer type. The correlation was $r = 0{\cdot}4652$, $P < 0{\cdot}05$.

There was no correlation between hair follicle density and R.B.V., but density estimations without correction for body weight are of little value (Turner *et al.* 1962).

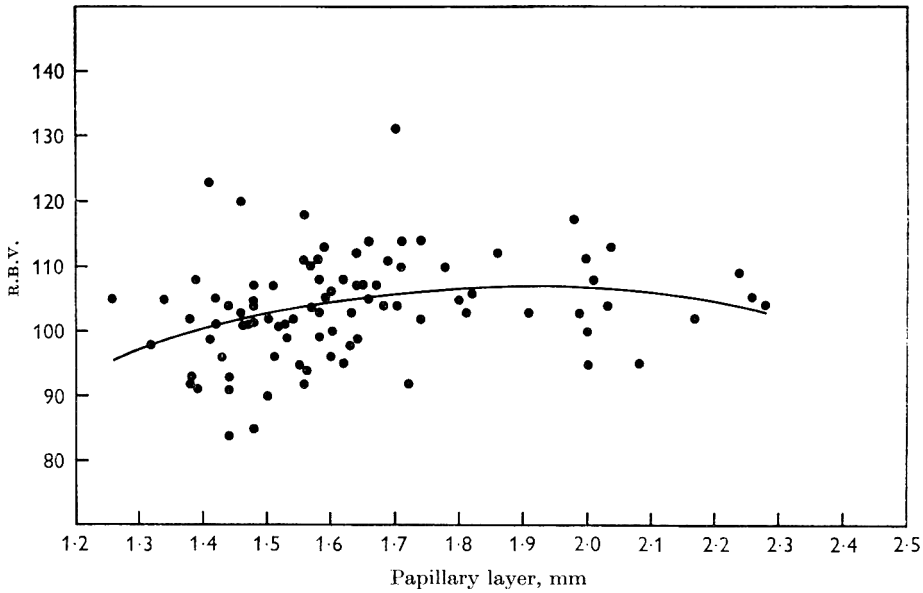

Fig. 1. Regression of R.B.V. on papillary layer thickness.

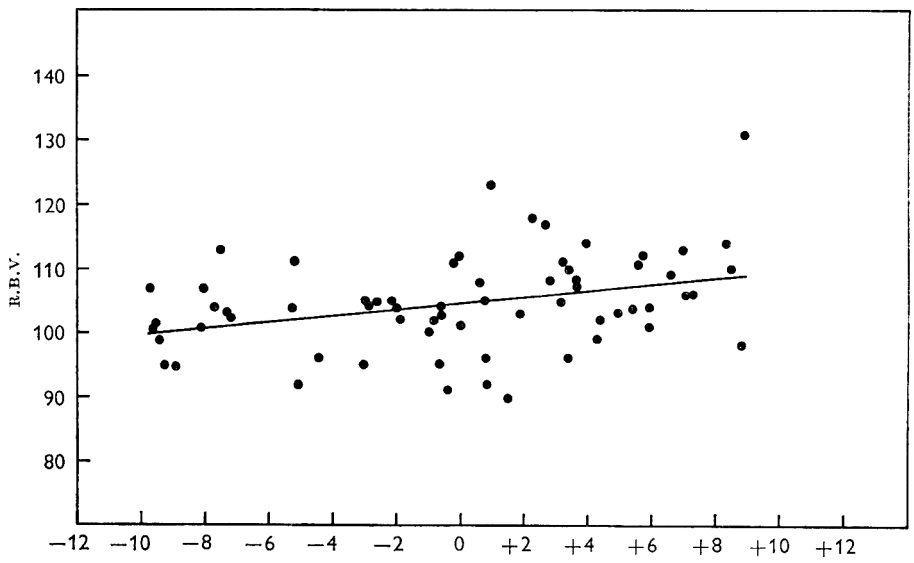

Fig. 2. Regression of R.B.V. on follicle diameter in μ corrected for seasonal difference, expressed in value above and below the mean.

Table 6. *Regression of* R.B.V. *on follicle diameter, uncorrected, and corrected for seasonal differences*

Measurement	No. of animals	Regression coefficient	Standard error
R.B.V.	89	—	—
Follicle diam., uncorrected	89	+0·3424	±0·1165
Follicle diam., corrected seasonal differences	65	+0·4826	+0·1638

The probability that either regression = 0 is $P < 0{\cdot}01$.

DISCUSSION

Since the follicle diameter and the thickness of the papillary layer appear to be of importance in the present work, these measurements will be discussed in detail.

Most of the observations on the skin and coat of cattle have been done in Australia. It has been demonstrated that there are 2 peaks of increased follicle activity in the skin of cattle: one in the spring, coinciding with the growth of the summer coat, and one in the autumn, coinciding with the growth of the winter coat. The greatest percentage of quiescent follicles, containing 'club hairs', was found at the height of the summer and winter seasons, although there was always a number of active follicles throughout the year (Dowling & Nay, 1960).

There is evidence that under the hot conditions that obtain in central Queensland, the summer coat is composed of fibres of greater diameter than are present in the winter coat. The differences were more pronounced in *Bos indicus* than in *Bos taurus* (Dowling & Nay, 1960). However, fibre diameter is not always greater in summer. Dowling (1958) found that in Herefords in the temperate climate of southern N.S.W., fibre diameter was slightly larger in winter.

Hayman & Nay (1961) found that in the comparatively temperate climate of the coastal area near Sydney (Badgery's Creek, N.S.W.), the fibre diameter was greater in summer in *Bos indicus*, but there was no difference between winter and summer values in various *Bos taurus* breeds (Jersey, Red Poll, Australian Illawarra Shorthorn and Friesian).

Similar studies on cattle in Britain are lacking and we do not know when the summer and winter coats begin to grow and whether there is a seasonal difference in the fibre diameter. Most of the bulls used in this study were sampled between May and October and only a few in April (3) and November (4).

When the follicle diameters of all bulls were plotted against the months between spring and autumn, it appeared that there was a seasonal difference in follicle diameter. The animals sampled in the spring and autumn generally had smaller follicle diameters than those sampled in June and July. This may indicate that the winter coat is not shed till late spring, and that the new winter coat is already present in November.

In order to check for pronounced peaks in follicle activity, the proportions of active and resting follicles were determined for each bull.

The results showed that there was a large percentage of active follicles in all

seasons. There was an indication of a peak beginning in June and lasting till October, and possibly another peak in late autumn. The data must be regarded as inconclusive since the months of April, August, September and November were represented by only a few animals. However, the large percentage of active follicles in nearly all animals suggests that the winter and summer coats may grow and shed gradually.

The number of follicles used to estimate diameter in vertical sections was limited to 10 because in some animals most follicles are in the resting stage. In such cases, it may be difficult to find many more than 10 active follicles, containing a full grown fibre, and observable in full length. However, such follicles represent the type of coat growing at the particular time of the year, whereas hair samples taken for measurements will contain fibres of 2 different coats in unknown proportion and are to this extent misleading.

Since duplicate skin samples were not taken and a test of repeatability on vertical sections was impossible, other measurements of diameter were made on horizontal sections as a check on the reliability of hair follicle measurements.

The second follicle measurement included all follicles, active and resting. It was assumed that the diameters of the resting follicles in an animal with larger follicles would be greater than those in an animal having smaller follicles. The disadvantage of this method is that the count probably included the follicles of both the winter and summer coats. The error may not be very great, since coat replacement in the animals in question was gradual as suggested by the proportions of active and resting follicles.

The third measurement, the fibre diameter above the skin, may also be biassed, although to a lesser degree, than the second follicle diameter measurement. It may contain fibres in the early growing stage, and therefore conical, and the fibres, thinning at the base just after the cessation of the follicle activity. Such fibres may not always be discriminated against with certainty.

The agreement between the 3 sets of observations was good, despite the use of 3 quite different methods of measurement. It was decided to use the first set of measurements in the analysis because it gave the best correlation with R.B.V.

The regression of R.B.V. on the corrected follicle diameter is 0·4826. The practical significance of this can be stated as follows:

When the best 32 animals out of 65 were selected on the basis of R.B.V., their mean value was found to be 110·3 (5·63 units above the overall mean). When the best 32 animals were selected, using as criterion the highest follicle diameter, their mean R.B.V. was found to be 107·0 (2·35 units above the overall mean). It follows that using follicle diameter as selection criterion, one would get $\frac{2}{5}$ of the way possible even before the progeny test is known. When, using the same criteria, only the top 10 animals were selected, the group selected for high R.B.V. had a value of 12·0 units above the mean, and the group selected for high follicle diameter had a value of 4·5 units above the mean. Therefore, when selecting the top 10 animals out of 65 one gets $\frac{3}{8}$ of the way possible before the progeny test is known.

In a properly co-ordinated scheme of bull breeding, all sires tested would be the sons of the best proven bulls of the previous generation, and one then has the task of picking from amongst them those which are most worth progeny testing. Dam's yield and sire's progeny test enable some choice to be made. Where there is still a surplus of bulls to choose from, or where this information is not readily available, skin

sections may well provide a means of making this choice. It is interesting to note that Robertson (1960) finds the value of the sires test about as powerful a guide to his sons' breeding value as was follicle diameter in this study. Before too much weight can be put on our findings, a check must be made of the regression of R.B.V. on follicle diameter within as well as between half sibs; for the sire's R.B.V. will usually be more readily available than the son's follicle diameter and it will usually be between sons of a particular set of sires that one wishes to make further selection.

The regression of R.B.V. on the papillary layer thickness was curvilinear. The multiple correlation coefficient between R.B.V. and papillary layer thickness, recovered from the curvilinear regression equation was 0·29513. It appears that there may be an optimum of papillary layer thickness in dairy bulls at about 1·7 mm, and after that, as the papillary layer increases and reaches that of beef bulls (about 2·3 mm, unpublished data), their value as dairy animals may decrease.

The results suggest that follicle diameter, and perhaps also follicle depth, may be of practical value in selecting dairy bulls for progeny testing.

It is difficult to explain why the increase in follicle size in sires should be correlated with the milking performance of their daughters. There is, however, one possibility which could be considered. Large follicles have, in general, large appendages, the sweat gland and the sebaceous gland. If the development of the mammary gland were related to the development of the cutaneous glands one would expect that the development of one reflects on the development of the other. In our material, however, there was no correlation between sweat gland size and the R.B.V.

At present, there is no way of describing the sebaceous glands in some numerical form. The size and the state of activity of the sebaceous glands change greatly according to the state of activity of the hair follicle. One cannot, at present, go beyond the general statement that the larger follicles tend to have larger sebaceous glands.

In the light of this investigation, the following points should be borne in mind for future investigations.

(i) All the animals should be sampled at the same season, preferably in June or July. Most of the uncertainties about seasonal fluctuations in follicle diameter would thus be eliminated.

(ii) Data on body weight of the animals are needed to discover if follicle density is of any importance.

(iii) If a method could be devised for giving a quantitative description of the sebaceous glands it might be very interesting.

(iv) The number of measurable follicles could be increased by cutting the sections under a binocular microscope which would give much better control of the plane of cutting.

(v) If hairs were left intact on the skin a better estimate of hair diameter could be made.

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Effects of repeated heat treatments on the levels of some vitamins of the B-complex in milk

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Summary. A study was made of the destructive effects of repeated heat processing of milk on its content of vitamin B_6, vitamin B_{12}, thiamine and folic acid. Losses of thiamine and vitamin B_6 were cumulative, and increased with frequency and severity of heating.

With folic acid, the first heat treatment potentiated the destructive effect of subsequent treatments; in contrast, the loss of vitamin B_{12} during in-bottle sterilization was markedly reduced by preliminary HTST pasteurization. These anomalous effects are possibly related to a specific interaction of ascorbic acid with each of these 2 vitamins.

The exigencies of milk distribution in some countries may require that the milk be heated more than once before it reaches the consumer. For example, milk may be pasteurized at the point of production to prevent its spoilage during transport to a distant centre of distribution, where it may be repasteurized or sterilized before delivery to the consumer. As several of the nutrients in milk are unstable to heat, the cumulative effect of these heat treatments may impair the nutritive quality of the milk. Of the B-vitamins, in-bottle sterilization destroyed 40–50% of the thiamine and > 90% of the vitamin B_{12} (Ford, Kon & Thompson, 1959), and heat sterilization of canned evaporated milk destroyed 40–70% of its vitamin B_6 (Davies, Gregory & Henry, 1959). The folic acid in milk is also destroyed on heating, to an extent related to the initial level of reduced ascorbic acid, which has a marked protective action (Ford, 1967). The present paper reports the effects of repeated heat treatments on the 4 vitamins of the B-complex mentioned above. The other vitamins of the B-complex in milk, nicotinic acid, biotin, riboflavin and pantothenic acid, are comparatively stable during processing.

EXPERIMENTAL

Whole milk was taken from refrigerated bulked evening and morning milk of the Institute herd of Friesian cows and received its first heat treatment within 6 h.

Heat treatments

The heat treatments applied singly or in various combinations were: high temperature, short time (HTST) pasteurization according to British requirements (Regulations, 1963); 'flash' pasteurization as practised in some European countries,

and in-bottle sterilization. The order in which successive treatments were applied and the time intervals and the conditions of storage between them, were chosen as being similar to those which might obtain in practice. For comparison, the effects of single HTST pasteurization and in-bottle sterilization processes were determined. These various treatments and the time intervals between them are set out in Table 1. The whole experiment was done twice. In a third experiment the treatments that involved a preliminary flash pasteurization were omitted.

Table 1. *Summary of treatments applied*

Control milk; unheated
HTST
In-bottle
HTST—24-h interval—HTST
HTST—24-h interval—In-bottle
Flash—24-h interval—HTST
Flash—24-h interval—HTST—24-h interval—HTST
Flash—24-h interval—HTST—24-h interval—In-bottle

HTST = High temperature, short time pasteurization
Flash = 'Flash' pasteurization
In-bottle = In-bottle sterilization

HTST pasteurization

This was performed in the laboratory apparatus described by Franklin (1965), which gives a heat treatment equivalent to that given by a plate heat exchanger operating at a temperature of 161 °F (71·7 °C) with a holding time of 15 s. The treated milk was then cooled immediately to below 50 °F (10 °C) as required by the British pasteurization regulations (Regulations, 1963), and was stored at about 20 °C before receiving any further heat treatment.

'Flash' pasteurization

As applied in some countries, this involves the heating of milk to 85 °C with no specified minimum holding time: the holding time used in practice is, therefore, the shortest that can be conveniently achieved. The process was reproduced with a laboratory plate heater (Alfa Laval type PL1) intended for ultra-high-temperature milk treatment at a flow rate of about 70 l/h. The plant was operated with the final heating section heated by steam at atmospheric pressure, and controlled by hand to give a final milk temperature of 85 ± 0·5 °C. After a holding time of 2–3 s, which was the shortest time possible with the associated pipework, the milk was cooled in regenerator sections and with mains water to about 20 °C. The treated milk was stored at 3–4 °C before further heat treatment. This temperature was chosen as being about that of a refrigerated tanker, in order to simulate conditions which might obtain in practice.

In-bottle sterilization

One-pint quantities of milk were filled into narrow-necked (26-mm crown finish) bottles as used for commercial sterilized milk. The bottles were closed with crown seals and heated in a horizontal laboratory autoclave supplied with saturated steam from a supply at 30 lb/in^2 pressure. The atmosphere steam temperature within the autoclave

was raised to and held at 110° C for 20 min. After the autoclave had been vented to atmospheric pressure, the bottles were removed and allowed to cool naturally in air.

The autoclave used in the first experiment was no longer available for the later experiments, and a different autoclave was used. However, the indicated atmosphere temperatures and the length of the heating cycle were the same for all 3 experiments.

Vitamin assay methods

The methods of microbiological assay for thiamine, vitamin B_6 and vitamin B_{12} have been described fully in earlier publications (Chapman *et al.* 1957; Gregory, Ford & Kon, 1958; Gregory, 1959). Folic acid activity was measured with *Lactobacillus casei* by an adaptation of the procedure recommended by Herbert (1961) for the assay of folic acid in blood serum.

Table 2. *Levels of 4 vitamins in milk, unheated and after single and multiple heat treatments (percentages in parentheses)*

	Vitamin B_6, μg/ml		Vitamin B_{12}, mμg/ml		
Expt. no.	1	2	1	2	3
Control	0·39 (100)	0·36 (100)	4·0 (100)	2·8 (100)	3·1 (100)
HTST	0·37 (95)	0·35 (97)	3·8 (95)	2·7 (96)	3·0 (97)
In-bottle	0·27 (69)	0·17 (47)	0·34 (8·5)	0·28 (10)	0·40 (13)
HTST + HTST	0·35 (90)	0·25 (69)	3·8 (95)	2·3 (82)	3·0 (97)
HTST + in-bottle	0·26 (67)	0·19 (53)	1·8 (45)	1·0 (36)	1·2 (39)
Flash + HTST	0·37 (95)	0·35 (97)	3·8 (95)	2·5 (89)	—
Flash + HTST + HTST	0·37 (95)	0·30 (83)	3·6 (90)	2·7 (96)	—
Flash + HTST + in-bottle	0·30 (77)	0·19 (53)	1·8 (45)	0·56 (20)	—

	Folic acid, mμg/ml			Thiamine, μg/ml	
Expt. no.	1	2	3	1	2
Control	50 (100)	51 (100)	36 (100)	0·44 (100)	0·42 (100)
HTST	45 (90)	48 (94)	36 (100)	0·41 (93)	0·38 (91)
In-bottle	27 (54)	29 (57)	21 (58)	0·31 (70)	0·27 (64)
HTST + HTST	38 (76)	41 (80)	36 (100)	0·42 (95)	0·34 (81)
HTST + in-bottle	6·1 (12)	18 (35)	11 (29)	0·26 (59)	0·26 (62)
Flash + HTST	56 (100)	46 (90)	—	0·36 (82)	0·36 (88)
Flash + HTST + HTST	41 (82)	40 (78)	—	0·37 (84)	0·36 (88)
Flash + HTST + in-bottle	4·9 (10)	10 (20)	—	0·20 (46)	0·26 (62)

RESULTS AND DISCUSSION

The results of the vitamin assays are set out in Table 2. In general, they confirm the expectation that multiple heating would be more damaging than a single heat treatment.

For vitamin B_6, a single HTST pasteurization caused little loss, but repeated HTST treatment caused a mean loss of about 20%. In-bottle sterilization caused marked loss, amounting to 31% in the first experiment and 53% in the second. When sterilization was preceded by HTST pasteurization, the extent of the overall loss of vitamin B_6 was no greater than that found with the in-bottle sterilization alone. Preliminary flash pasteurization made no contribution to the overall losses caused by

the multiple heat treatments, and indeed seemed to reduce somewhat the destructive effect of subsequent treatments.

With thiamine, a single HTST treatment caused a mean loss of about 8 %; two HTST treatments caused a loss of only 5 % in the first experiment and of 19 % in the second, so with thiamine as with vitamin B_6, the second experiment showed greater losses resulting from repeated HTST treatments than were observed in the first experiment. In-bottle sterilization was more destructive than any combination of pasteurization treatments, and destroyed about 33 % of the vitamin. When the in-bottle sterilization was preceded by flash and HTST pasteurization, the mean loss of thiamine increased to about 46 %, suggesting a simple additive effect of the different processes.

With vitamin B_{12} and folic acid, however, the effects of the successive heat treatments were apparently not simply additive. Thus with folic acid, HTST pasteurization caused a mean loss of 5 %, and in-bottle sterilization a mean loss of 44 % whereas HTST treatment followed 24 h later by in-bottle sterilization caused a mean loss of 75 %. The first heat treatment appeared to magnify the effect on folic acid of subsequent treatments.

With vitamin B_{12}, a single HTST treatment caused a mean loss of 4 %, and the various combinations of pasteurization treatments caused in general not more than 10 % loss. An exception was a figure of 18 % loss found in one experiment for the milk twice subjected to HTST pasteurization. In-bottle sterilization destroyed about 90 % of the vitamin. The effect of pasteurization on the destruction of vitamin B_{12} during subsequent in-bottle sterilization was the opposite of that found for folic acid. Thus, whereas sterilization alone caused 90 % destruction, when preceded by pasteurization the overall destruction was considerably less.

The opposite effects on folic acid and vitamin B_{12} may be related to the interaction of both of these vitamins with ascorbic acid. Ford (1957) suggested that the destruction of vitamin B_{12} during sterilization comes about as a side reaction in the course of the oxidative destruction of ascorbic acid. He found that careful exclusion of oxygen from the milk prevented both the oxidation of ascorbic acid and the concomitant destruction of vitamin B_{12}. Conversely, the addition of ascorbic acid to oxygenated milk increased the destruction of vitamin B_{12} during sterilization. The loss of folic acid during sterilization is related to the content of reduced ascorbic acid in the milk immediately before heating: the higher the initial level of ascorbic acid the smaller is the loss of folic acid (Ford, 1967). There would have been a considerable loss of ascorbic acid during pasteurization and storage of the milk (Dr S. Y. Thompson, personal communication) and so it would be expected, as was found, that on subsequent sterilization the further loss of vitamin B_{12} would be smaller, and of folic acid greater, than that caused by the sterilization alone.

Except for vitamin B_{12}, where preliminary pasteurization reduced the losses on subsequent sterilization, the destructive effect of repeated heat treatment was generally greater than that of any single treatment. This adverse effect of multiple processing was especially marked with folic acid. To the extent that milk is an important dietary source of these vitamins, multiple heat treatments are clearly undesirable. Although with vitamin B_{12} an initial pasteurization treatment tended to preserve the vitamin during subsequent sterilization, the losses were still large. A

more practical way of preventing this loss would be the elimination of dissolved oxygen from the milk before heat treatment.

We acknowledge with thanks the helpful advice of Dr J. P. Greaves in the planning of this work.

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The effect on milk yield and composition of adding the calcium salts of acetic, propionic, butyric and lactic acids to the diets of dairy cows

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Summary. The effects, on the yield and composition of the milk of the cow, of additions to the diet of the calcium salts of acetic, propionic, butyric or lactic acids were determined in 3 change-over experiments. In all cases yield of milk was increased slightly and milk fat content was reduced; solids-not-fat (SNF) percentage was depressed by acetate and butyrate. The effects of these treatments differed markedly from those observed previously when dilute solutions of the corresponding acids were infused into the rumen, but the relative effects of the calcium salts were similar to those of the free acids. It was concluded that the specific effects of the acids were overshadowed by a more marked general effect arising from their addition to the diet as calcium salts. This was confirmed in a subsequent experiment with fistulated cows, in which the effect of an infusion of propionic acid into the rumen was compared with that of an infusion of calcium propionate and of calcium propionate given with the diet. The effect of the addition to the diet of calcium in the form of carbonate differed from that of calcium salts of the fatty acids. It was concluded that the salts were not likely to find practical use.

In the lactating cow, continuous addition to the rumen of dilute solutions of the short chain fatty acids or of lactic acid, causes changes in milk yield and composition: acetic acid increases milk yield and fat content, but has no effect on the content of milk SNF; propionic acid decreases milk fat and increases SNF; butyric acid increases milk fat, and lactic acid decreases milk fat and increases SNF though to a lesser extent than does propionic acid (Rook & Balch, 1961). The composition of the basal diet affects the extent, but does not change the nature, of the responses to additions of acetic or propionic acid (Rook, Balch & Johnson, 1965).

Under practical farming conditions, solutions of acids cannot be administered to cows in amounts sufficient to influence milk yield and composition. A possible alternative is to add to the diet salts of the acids. Sutton (1964) reviewed the more important experiments in which the effects on milk composition of additions of sodium acetate to the diet of milking cows have been examined, and concluded that the daily addition of up to 450 g of sodium acetate (equivalent to 200 g of acetic acid)

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to diets low in roughage usually, but not invariably, raised milk fat percentage. A similar conclusion was reached by Balch & Rowland (1959) after experiments in which they infused up to 1·5 kg of sodium acetate into the rumen of cows. More recently, Jorgensen, Schultz & Barr (1965) found that milk fat content, depressed by a diet high in maize and low in roughage, was not restored by a dietary addition of sodium acetate. The addition of larger quantities of the sodium salts to provide amounts of acids equivalent to those infused in the experiments of Rook & Balch (1961) causes harmful physiological effects. In our present study we have, therefore examined the practical possibility of including in the diet the calcium salts of the short chain fatty acids and of lactic acid.

The effects of dietary additions of the calcium salts of acetic, propionic, butyric and lactic acids on milk yield and composition were studied in 3 change-over experiments; in the first 2 experiments the basal diet contained a high proportion of roughage, including silage and hay, and would therefore be expected to give a comparatively high proportion of acetate in the contents of the reticulo-rumen, whereas in the third experiment the diet contained flaked maize and a low proportion of roughage and was designed to give a lower proportion of acetate. The results of the 3 experiments were consistent, but differed appreciably from those obtained previously when the free acids were infused into the rumen. A fourth experiment was accordingly undertaken, with cows with rumen fistulas, to compare the effects of propionic acid and calcium propionate when infused into the rumen, and of calcium propionate and calcium carbonate given with the diet.

EXPERIMENTAL

Animals

Lactating Friesian heifers were used for expts 1–3. The heifers in expts 1 and 2 had been collected from farms in the south of England and brought to the Institute before calving. Pre-partum, each heifer had received 1 of 2 levels of feeding, but after calving all the animals received a standard maintenance allowance and a commercial concentate mixture at a rate of 4 lb/10 lb milk produced. The animals had completed an average of 18 weeks of lactation at the beginning of the experiments. The heifers for expt 3 were selected from the Institute herd and were introduced to the experiment in their 13th week of lactation.

In expt 4, five lactating Friesian cows with permanent rumen fistulas were used.

Plan of experiments

Expts 1 *and* 2. In each experiment, 18 animals were blocked in groups of 3 in such a way that any carry-over effects from previous levels of feeding were balanced. Within each block, 3 treatments were imposed according to a Latin square design. Periods were of one month of which the first week constituted a change-over period. The treatments were: expt 1, basal diet, basal diet + calcium acetate, basal diet + calcium propionate; expt 2, basal diet, basal diet + calcium butyrate, basal diet + calcium lactate.

Expt 3. Twenty-four animals were blocked in groups of 3 according to level of feeding in late pregnancy, yield and date of calving and within each block 3 treat-

ments were imposed according to a Latin square design. Periods were of 6 weeks with a gradual change of diet over the first 2 weeks. The treatments were: basal diet, basal diet + calcium acetate, basal diet + calcium propionate.

Expt 4. Five animals were used. The design was a Latin square with periods of 24 days. The 5 treatments were: basal diet + a continuous intraruminal infusion of 70 l water daily; basal diet + a continuous intraruminal infusion of 725 g propionic acid in 70 l water daily; basal diet + a continuous intraruminal infusion of 1 kg calcium propionate in 70 l water daily; basal diet + 1 kg calcium propionate daily divided equally between the 2 daily meals of concentrates; basal diet + 490 g calcium carbonate daily divided equally between the 2 daily meals of concentrates. The 2 treatments including calcium propionate supplied propionate equivalent to 725 g propionic acid and calcium equivalent to 490 g calcium carbonate.

Table 1. *Composition of foods*

Expt no.	Food	Dry matter, %	Crude protein, % of dry matter	Ether extract, % of dry matter	Crude fibre, % of dry matter	Nitrogen-free-extract, % of dry matter	Ash % of dry matter
1 and 2	Hay	83·7	11·4	2·4	32·8	47·6	5·8
	Silage	18·6	12·1	4·8	38·1	34·3	10·7
	Concentrates	84·1	19·3	2·5	5·3	67·5	5·4
3	Hay	81·8	9·8	1·3	32·5	49·4	7·0
	Concentrates	83·9	19·0	2·0	4·2	70·2	4·6
	Flaked maize	83·5	9·3	2·1	1·5	86·0	1·1
4	Hay	85·1	4·7	1·4	33·3	54·2	6·4
	Concentrates	85·7	19·5	2·0	5·9	65·4	7·2

Diets

The composition of the hay, silage and concentrates is given in Table 1. The concentrates, which were pelleted, had the following composition: barley, 11·0 parts; wheat bran, 1·5 parts; maize, 1·5 parts; decorticated groundnut cake, 3·75 parts; minerals, 0·75 part; molasses, 1·5 parts in expts 1–3 and 1·0 part in expt 4.

The basal diets were as follows:

Expts 1 and 2: 7·5 lb hay, 30 lb grass silage, 0·85 lb concentrates/100 lb live weight in excess of 1000 lb together with 4 lb concentrates/10 lb milk produced in week 1 for the first 2 weeks, with a reduction at the end of each subsequent 2-weekly period based on an assumed weekly decline of 0·4 lb in mean daily milk yield.

Expt 3: 8 lb hay; 4 lb flaked maize with concentrates as in expts 1 and 2.

Expt 4: 15·5 lb hay; 19 lb concentrates and 2 lb decorticated groundnut cake in period 1; the amount of each dietary constituent was reduced by 5% at the beginning of periods 2–5.

Supplements

The supplements of calcium salts offered in expts 1–3 were mixed with the concentrates before they were pelleted. With the experimental treatments, up to 14 lb of the basal concentrates, or the entire allowance where less than 14 lb of concentrates was on offer, was replaced by the appropriate amount of concentrate/salt mixture.

Occasionally, cows did not consume all of the concentrate/salt mixture but any left uneaten was replaced by an equal weight of concentrates. The mean intake of salts, expressed as acids, was 430 g in expts 1 and 2 and 550 g in expt 3. The mean intake of calcium with the added salts was 125 g in expts 1 and 2 and 160 g in expt 3.

Sampling and methods of analysis

The milk was sampled at 2 consecutive milkings at the end of each week in expts 1 and 2 with additional samples at the 2 previous milkings in the last week of each period. In expt 3, the milk was sampled at 6 consecutive milkings at the end of each week. Weighted, bulk samples representative of the milk of individual cows were analysed for fat and total solids content. In expt 4, the milk was sampled daily and weighted subsamples representing the milk produced in each 4-day period were analysed for fat and total solids content.

In expt 4, samples of rumen contents were taken, at 2 and 7 h after feeding, from 3 of the cows in the last 2 days of each period; the pH of each sample was determined immediately. Total volatile fatty acids (VFA) were determined titrimetrically after steam-distillation and the individual acids were estimated by gas liquid chromatography (Storry & Millard, 1965).

Statistical analysis

Values obtained with each animal in the last 2 weeks of each experimental period were analysed for direct effects in expts 1 and 2 and for direct and residual effects in expt 3, but the residual effects were not significant. Treatment × block interactions were examined in expt 3. In expt 4, values for milk yield and composition for the last 12 days of each period were analysed for direct effects.

RESULTS

Expts 1 *and* 2 (Table 2). All the dietary supplements of calcium salts increased the mean daily milk yield by 0·5–0·7 lb, but in no instance was the effect significant ($P > 0·05$). There was a corresponding and significant fall in milk fat percentage, of 0·14 with acetate ($P < 0·01$), 0·08 with butyrate ($P < 0·05$), 0·27 with lactate ($P < 0·001$) and 0·45 with propionate ($P < 0·001$); the effect of propionate was significantly greater ($P < 0·001$) than that of lactate. The daily yield of milk fat was not affected significantly ($P > 0·05$) by acetate or butyrate, but was depressed significantly ($P < 0·05$) by 0·05 lb by lactate and by 0·10 lb with propionate, the difference between lactate and propionate again being significant ($P < 0·001$).

SNF percentage, calculated on a fat-free basis, was depressed by 0·16 with acetate ($P < 0·001$) and 0·10 with butyrate ($P < 0·01$). Lactate and propionate were without significant effect.

Expt 3 (Table 2). Calcium acetate and calcium propionate both increased daily milk yield, by 2·6 lb ($P < 0·001$). Milk fat percentage fell significantly, by 0·54 with acetate ($P < 0·001$) and 0·57 with propionate ($P < 0·001$). Propionate was without effect on SNF percentage, but acetate caused a reduction of 0·18 ($P < 0·001$). The yield of SNF was increased, by 0·19 lb with acetate and 0·22 lb with propionate ($P < 0·001$).

Significant treatment × block interactions were found for the effects on milk yield

Table 2. *Effect of feeding calcium salts of fatty acids to lactating cows on the daily yield and composition of milk*

Expt no.	Treatment	Milk yield,* lb	Milk fat		Milk SNF	
			Content, %	Yield,* lb	Content, %†	Yield,* lb
1 (18 cows)	Control	24·28	3·88	0·937	8·87	2·052
	Control + calcium acetate	24·91	3·74	0·923	8·71	2·084
	Control + calcium butyrate	24·97	3·80	0·957	8·77	2·124
	S.E. of differences of means	0·408	0·040	0·0173	0·029	0·0361
2 (18 cows)	Control	26·46	3·87	1·017	8·93	2·270
	Control + calcium lactate	27·00	3·60	0·964	8·89	2·308
	Control + calcium propionate	27·05	3·42	0·912	8·85	2·300
	S.E. of differences of means	0·282	0·027	0·0112	0·031	0·0269
3 (24 cows)	Control	32·51	3·30	1·073	8·91	2·828
	Control + calcium acetate	35·14	2·76	0·970	8·73	3·014
	Control + calcium propionate	35·11	2·73	0·959	8·85	3·048
	S.E. of differences of means	0·437	0·073	0·0249	0·035	0·0377

* The values for milk yield are means for 14 days. In determining the values for the yield of milk constituents, the yield of milk on the 1 or 3 days of milk sampling has been used.

† Calculated on a fat-free basis.

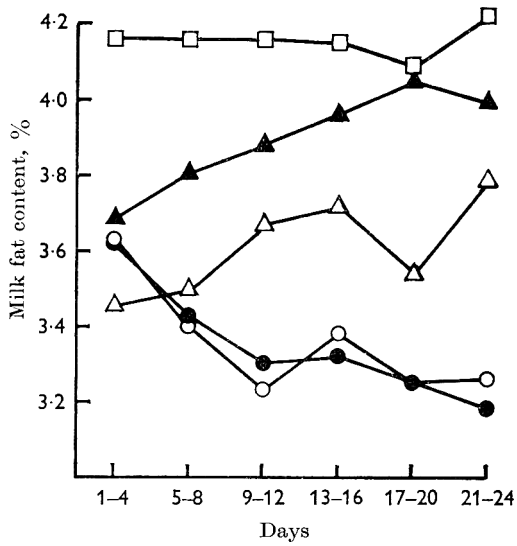

Fig. 1. Expt 4. Mean fat content in milk of cows receiving a basal diet of hay and concentrates with continuous intra-ruminal infusion of water, ▲; propionic acid, △; calcium propionate, ○. In 2 instances calcium propionate, ●, or calcium carbonate, □, were added to the concentrates. The 5 cows each received every treatment and values are means for periods of 4 days. Details of the diets and treatments are given on page 201.

and milk fat content. Block 1 included the highest yielding cows, and in this block propionate had little effect on milk yield and neither acetate nor propionate affected significantly ($P > 0·05$) the content of milk fat. The differences between the responses in this block and those from the other blocks may be related to the larger amounts of concentrate/salt mixture left uneaten by cows in this block.

Expt 4 (Table 3). Addition of calcium propionate to the food gave a non-significant ($P > 0{\cdot}05$) mean increase in milk yield of 2·6 lb/day, but with all the other treatments milk yield was within ±0·7 lb/day of the control value. There were significant ($P < 0{\cdot}05$) mean decreases in milk fat percentage of 0·32 with propionic acid, 0·75 with the dietary supplement of calcium propionate and 0·70 with the infusion of calcium propionate, but calcium carbonate was without significant ($P > 0{\cdot}05$) effect. Propionic acid and calcium carbonate increased by 0·16–0·18 and infused calcium propionate by 0·05 the percentage of SNF in the fat-free serum; these effects were

Table 3. *Effect of administering calcium and propionic acid in various ways to* 5 *cows, receiving a diet of hay and concentrates, on the daily yield and composition of milk*

Treatment*	Milk yield, lb	Milk content, %	SNF,† %
Control	34·04	4·00	9·04
Control + infused propionic acid	34·22	3·68	9·22
Control + calcium propionate in food	36·64	3·25	8·97
Control + infused calcium propionate	33·38	3·30	9·09
Control + calcium carbonate in food	34·48	4·10	9·20
S.E. of difference of means	2·054	0·085	0·078

* All cows received a daily intra-ruminal infusion of 70 l water. For further details of experimental treatments see p. 201.

† Calculated on a fat-free basis.

Table 4. *Effect of administering calcium and propionic acid in various ways to* 3 *cows, receiving a diet of hay and concentrates, on the pH and concentration of VFA in the rumen contents**

Treatment	pH	Total VFA m-equiv./l	Acetic	Propionic	Butyric (iso + n)	Valeric (iso + n)
			Molar % of total VFA			
Control	6·28	95·8	59·8	19·5	16·7	4·0
Control + infused propionic acid	6·23	95·9	50·5	31·0	15·1	3·4
Control + calcium propionate in food	6·53	109·6	48·3	33·2	14·7	3·8
Control + infused calcium propionate	6·63	97·0	49·6	33·5	13·1	3·8
Control + calcium carbonate in food	6·28	111·0	59·6	18·2	18·5	3·7

* See p. 202 for details of sampling routine.

not significant ($P > 0{\cdot}05$). Changes in milk fat content in successive 4-day periods are shown in Fig. 1. Because of the experimental design, treatments in which water only had been infused or calcium carbonate added to the diet were usually preceded by periods in which milk fat content had been depressed; with a change to calcium carbonate the recovery was almost immediate whereas with the infusion of water only it was progressive over a period of about 2 weeks. With both treatments containing calcium propionate, the falls in milk fat content were gradual; with propionic acid milk fat content fell immediately to about 0·3 % below the value with previous treatments but there appeared to be a partial recovery by the last 8 days of the experimental period (Fig. 1 and Table 3).

There was little effect of treatment on rumen pH as measured over the last week

of the experimental period (Table 4). The molar proportion of propionic acid in the total steam-VFA was increased with the administration of propionic acid or calcium propionate.

DISCUSSION

The changes in milk yield and composition on the addition to the diet of calcium salts of the volatile fatty acids observed in expts 1–3 were distinctively different from those obtained when dilute solutions of the corresponding acids were continuously infused into the rumen. Irrespective of the basal diet, all the salts gave an increase in milk yield, significant in only one experiment, and a decrease in milk fat content; acetate and butyrate decreased milk SNF content, whereas propionate and lactate were without effect. The relative effects of the various salts on fat and SNF contents (Table 5) tended, however, to be the same as for the free acids, but less marked, as

Table 5. *Expts* 1–3. *Effects, on milk fat and SNF contents, relative to those for calcium acetate, of adding to the diet the calcium salts of propionic, butyric and lactic acids*

(The number of cows in expts 1–3 is given in Table 1)

	Milk fat content, %			SNF content, %		
Salt added	Expts 1, 2	Expt 3	A*	Expts 1, 2	Expt 3	A*
Propionate	−0·32	−0·03	−0·59	+0·18	+0·12	+0·21
Butyrate	+0·08	—	+0·05	+0·06	—	+0·10
Lactate	−0·08	—	−0·30	+0·18	—	+0·30

* Effect observed by Rook & Balch (1961) and Rook, Balch & Johnson (1965) with intraruminal infusions of the appropriate acid.

would be expected because of smaller quantities of material given in the form of the salts. These results suggest that with additions of fatty acids as their calcium salts in the form of a dietary supplement, the specific effects of the acids are overshadowed by an effect resulting from the addition of calcium salts. Confirmation of this possibility was obtained in expt 4, in which with the infusion of propionic acid the expected changes, a decrease in fat content and an increase in SNF content, were observed. The addition of an equivalent amount of propionate, in the form of a twice-daily addition of calcium propionate to the concentrate part of the diet, was without effect on SNF content, but gave a more marked depression in fat content and increased milk yield. Calcium propionate given as a continuous intraruminal infusion gave no different effect from that of calcium propionate added to the concentrate part of the diet.

The reason for this general effect of the calcium salts of the fatty acids is not apparent. The incorporation of an equivalent amount of calcium in the form of the carbonate in expt 4 had little effect on milk yield or fat content, but increased the percentage of SNF in the fat-free serum by 0·16. There are a number of conflicting reports of the effect of adding buffers to the food of dairy cows on the composition of milk. Sutton (1964) concluded that in instances where the addition of buffers had prevented or corrected depressions in the milk fat content, the effect was due partly or wholly to alterations in the proportion of the VFA produced in the rumen. More recent reports have confirmed this conclusion and have stressed the variability of

changes in rumen concentration of the acids, following the addition of buffers (Davis, Brown & Beitz, 1964; Emery, Brown & Bell, 1965; Jorgensen & Schultz, 1965; Miller, Hemken, Waldo, Okamoto & Moore, 1965).

In our present experiments the addition of the calcium salts of short chain fatty acids and lactic acid to the diet of milking cows resulted in small increases in milk yield. Small, but characteristic changes in milk composition were observed, but these could be related only indirectly to those found when the free acids were infused into the rumen. It appears unlikely that the calcium salts of the short chain fatty acids will be of any practical value as supplements in the diet of milking cows.

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Factors affecting the design of milk total solids testing schemes

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Summary. An analysis of variance was applied to the 'within herd' variance of milk total solids in order to find the contribution due to sampling, testing and biological variances on a balanced 8×8 two factor (herds and days) experiment with replicate sampling and testing. Testing samples from every consignment from 10 herds over a 12-month period showed that the mean monthy 'within herd' variance was 0·039 and the yearly 'within herd' variance was 0·085. A formula is given showing how the variance of the mean for compositional quality testing can be obtained and on which the design of testing schemes can be based.

INTRODUCTION

When farm milk is paid for on the basis of compositional quality, the most accurate method of assessment is to test a sample from each consignment and to calculate the weighted average of the constituent on which the payment is based. As sampling and analysis of such frequency is rarely practicable, the monthly supply is usually judged to be the simple average of a small number of tests, each of which is subject to sampling and analytical errors.

On the recommendation of the Cook Committee, 1960, the 4 Milk Marketing Boards in Great Britain pay for farm milk on its total solids content. The Milk Marketing Board of England and Wales bases the monthly payment on the mean of 12 monthly samples. The mean is re-calculated every 6 months and payment for the subsequent 6 months is based on this value. The Scottish Milk Marketing Board and the Aberdeen and District Milk Marketing Board base the monthly payment on the mean total solids content of 4 samples at approximately weekly intervals each month. The North of Scotland Milk Marketing Board base the payment for each month on the average of the 12 immediately past months and make 1 test/month.

The purpose of the present work was to find the 'within herd' variance in total solids percentage and to separate it into its component parts, i.e. biological, sampling and testing variance. In this way it was hoped to consider the effect of testing accuracy on the different payment schemes, and by obtaining estimates of the monthly and yearly 'within herd' variances to find how different methods of rotal solids estimation for payment purposes compare and how they might be improved.

EXPERIMENTAL

Triplicate samples were taken from herd bulk tank consignments of mixed evening and morning milks for a period of 8 consecutive days. The total solids percentage of these samples was determined in duplicate on the Claesson milk testing machine, modified as described by Waite & McPhillips (1965). In addition, daily samples were drawn from the bulk tank milk of 10 herds, ranging in size from 28 to 74 cows, and in milk total solids percentage from 11·00 to 13·00%. These samples represented consignments which consisted of mixed evening and morning milks, and they were taken on each collection day for a period of 12 months. The samples were tested for total solids in duplicate by the modified Claesson method.

RESULTS AND DISCUSSION

An analysis of variance was carried out on the balanced 8×8 two-factor (herds and days) experiment with replicate sampling and testing in order to find the different components of the 'within herd' variance. This analysis of variance is given in Table 1.

Table 1. *Analysis of variance of the total solids percentage of bulk milk from 8 herds sampled daily for 8 days*

Source of variance	Sum of squares	Degrees of freedom	Mean square	F test	Expected mean square
Days	3·9633	7	0·5662	64·4***	$\sigma_T^2 + 2\sigma_S^2 + 48\sigma_D^2$
Herds	62·8923	7	8·9846	67·6***	$\sigma_T^2 + 2\sigma_S^2 + 6\sigma_{D(H)}^2 + 48[\sigma_H^2]$
Days/herds interaction	6 5191	49	0·1330	15·1***	$\sigma_T^2 + 2\sigma_S^2 + 6\sigma_{D(H)}^2$
Samples	1·1313	128	0·0088	3·76***	$\sigma_T^2 + 2\sigma_S^2$
Residual	0·4532	192	0·0024	—	σ_T^2
Total	74·9592	383	—	—	—

*** Highly significant: $P < 0{\cdot}001$.

Each cause of variation, days, herds, days/herds interaction, and samples gave a significant value ($P < 0{\cdot}001$); thus, each of these factors had a definite effect on the milk solids percentage. It follows from Table 1 that the variance due to days, days/herds, sampling and testing were:

Days	σ_D^2	= 0·0116
Days/herds	$\sigma_{D(H)}^2$	= 0·0207
Sampling	σ_S^2	= 0·0032
Testing	σ_T^2	= 0·0024

By algebraic calculations it can be shown that the variance of the mean of milk solids percentage for a particular herd V_H is given by

$$V_H = \frac{1}{d}\left(\sigma_D^2 + \sigma_{D(H)}^2 + \frac{\sigma_S^2}{s} + \frac{\sigma_T^2}{st}\right), \qquad (1)$$

where d is the number of days, s is the number of samples and t the number of tests within samples. Substituting the values of the variances obtained in this experiment:

$$V_H = \frac{1}{d}\left(0{\cdot}0323 + \frac{0{\cdot}0032}{s} + \frac{0{\cdot}0024}{st}\right),$$

with $d = 8$, $s = 3$, $t = 2$, i.e. $V_H = 0{\cdot}0042$ and $\sigma_H = 0{\cdot}0648$.

This experiment indicates that the variances due to samples and tests (σ_S^2, σ_T^2) do not have any large contribution to make to the mean milk total solids variance (V_H) especially if s and t are of any magnitude. It is apparent, therefore, that the most important factor in determining the mean milk total solids variance is the number of days on which consignments are sampled.

Table 2. *The 'between day' variance of total solids percentage for individual herds sampled daily for* 12 *months in* 1965–6

	Herd no.										
Month	1	2	3	4	5	6	7	8	9	10	Mean
Apr.	0·0230	0·0269	0·0171	0·0155	0·0892	0·0206	0·0712	0·0485	0·0410	0·0621	0·0415
May	0·0413	0·0297	0·0458	0·0628	0·0511	0·0361	0·0436	0·0470	0·0361	0·0712	0·0465
June	0·0390	0·0585	0·0472	0·0693	0·0573	0·0398	0·0384	0·0500	0·0297	0·0448	0·0474
July	0·0332	0·0179	0·0278	0·0546	0·0395	0·0503	0·0429	0·0211	0·0583	0·0496	0·0395
Aug.	0·0472	0·0288	0·0212	0·0727	0·0714	0·0769	—	0·0218	0·0670	0·0379	0·0494
Sept.	0·0409	0·0484	0·0495	0·0359	0·0505	0·0924	0·0603	0·0651	0·0465	0·0501	0·0540
Oct.	0·0295	0·0592	0·0324	0·0266	0·0660	0·1179	0·0216	0·0464	0·0619	0·0108	0·0472
Nov.	0·0506	0·0368	0·0325	0·0153	0·0506	0·0257	0·0254	0·0296	0·0305	0·0102	0·0307
Dec.	0·0161	0·0091	0·0138	0·0558	0·0346	0·0178	0·0216	0·0265	0·0067	0·0308	0·0233
Jan.	0·0275	0·0090	0·0069	0·0180	0·0679	0·0265	0·0310	0·0253	0·0332	0·0307	0·0276
Feb.	0·0178	0·0153	0·0362	0·0727	0·0590	0·0324	0·0340	0·0113	0·0184	0·0624	0·0359
Mar.	0·0164	0·0277	0·0079	0·0477	0·0619	0·0299	0·0119	0·0388	0·0084	0·0243	0·0275
Mean	0·0319	0·0306	0·0282	0·0456	0·0582	0·0472	0·0365	0·0359	0·0365	0·0404	—
12-month variance	0·0936	0·0354	0·0589	0·0700	0·1159	0·1271	0·1231	0·0588	0·0584	0·0821	—

Mean 'between day' variance: in a period of 1 month, 0·085; in a period of 1 year, 0·039.

The results of the experiment for determining the monthly and yearly 'within herd' variances are given in Table 2. The number of samples and tests carried out would make the contribution due to sampling and testing negligible, and the result of 0·039 for the mean monthly 'within herd' variance, and 0·085 for the mean yearly 'within herd' variance would, therefore, be an approximation of the biological variance ($\sigma_D^2 + \sigma_{D(H)}^2$). The S.E. of the mean of any number of samples and tests would approximately equal $\pm\sqrt{(0{\cdot}039/d)}$ for sampling carried out on d days within a period of a month and $\pm\sqrt{(0{\cdot}085/d)}$, if carried out on d days within a year, if the contribution due to sampling and testing are negligible.

These figures (0·039 and 0·085) as might be expected are larger than those obtained for the 8-day experiment. However, it is to be expected that there would be greater variation in the compositional quality over a period of a month, and greater still over a year, due to seasonal effects. Edwards (1956), concluded that generally composition of milk from large herds showed less variation than that from small herds. Against this, more recent work by Edwards & Donaldson (1966) showed that there was no significant reduction in the variability of chemical composition with increasing size of herd. The smallest herd in their work was of 26 cows. As the smallest herd in the present work was of 28 cows it has been assumed that the size did not affect variability.

The Scottish Milk Marketing Board and the Aberdeen and District Milk Marketing Board base their payments on the means of 4 samples/month, whereas the Milk Marketing Board of England and Wales, and that of the North of Scotland, base their

payments on the means of 12 samples, taken one each month for a year. It is apparent that in computing the variance of the mean yearly total solids content, the first named 2 Boards' estimates will be based on 48 samples, as against 12 samples for the last-named 2 Boards'. Consequently, it is to be expected that the greater number of samples will give a more accurate assessment of a producer's supply over a 12-month period, i.e. a smaller S.E. of the mean.

The variance of different methods for testing for the total solids content in milk can be substantial. In formula (1) (p. 208) these are generally small compared to the biological variance ($\sigma_D^2 + \sigma_{D(H)}^2$). It is clear that if d, s or t, is increased, then the variance of the mean can be reduced. Thus, in the example in p. 208 the variance V_H of 0·0042 would be the same if the number of analyses were doubled ($t = 4$), and 0·0041 if the number of samples taken were doubled ($s = 6$). However, if d were increased from 8 to 10 the variance would be 0·0034. Replicate testing is expensive and would make little contribution. The variance due to sampling could be reduced by taking more samples from the same consignment, by the use of smaller sampling dippers. The greatest reduction in variance could be obtained by the use of composite samples, i.e. by increasing d.

The accuracy of the methods of testing in the present schemes has been over emphasized, as it is clear from the present study that their contribution to the S.E. of the mean is very small. This should apply equally to individual cow testing schemes, as the biological variance of individual cows could be expected to be greater than that for herds. In the selection of a test method for the present compositional quality schemes, and for individual cow proving schemes, less importance may be attached to the accuracy of the method and greater emphasis should be placed on its ease of application and possible automation.

I am indebted to the staff and members of The Scottish Milk Marketing Board for assistance with this work and for the facilities provided. I also wish to thank Prof. M. O'Sé, University College, Cork, for his advice concerning this work, and Dr W. W. Muir, University of Strathclyde, for assistance with the statistical analysis of the results.

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A suggested correction to the British Standards hydrometric method for the estimation of total solids in milk

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Summary. 1082 drip samples of fresh milk were taken at a creamery over a period of 3 years and tested for total solids by the current gravimetric method (British Standards Institution, 1963) and the hydrometric method with the fat in the liquid state (British Standards Institution, 1959). On average, the hydrometric method over-estimated the total solids by 0·096. It is suggested that the formula which applied before 1 October 1957 is correct and should replace the present formula.

Introduction

Since the introduction of the Compositional Quality Payment Schemes in the U.K., doubts have been expressed concerning the accuracy of the hydrometric method for the estimations of total solids or solids-not-fat (SNF). G. Chambers and J. McDowell (personal communication) found that the density method gave a mean difference of 0·13 percentage units higher than the gravimetric method during the period May 1963–April 1966. In this comparison, preserved samples were used and an empirical correction factor was applied. Waite (1965) suggested that if a discrepancy did exist it may have arisen from the fact that the modified formula for use in the hydrometric procedure was established by using an open dish type of gravimetric method instead of the British Standards method. McGann (1967) found that the lactometer method over-estimated the SNF by 0·18 percentage units.

The Scottish Milk Marketing Board introduced their Compositional Quality Payment Scheme in April 1964. The scheme is based on the total solids content of milk estimated by the Claesson testing method, modified as described by Waite & McPhillips (1955). It was found that the results obtained were lower than those obtained at the Board Creameries, where testing was carried out by the density hydrometer method. Preliminary results have been given elsewhere (Scottish Milk Marketing Board, 1964).

The original British Standards method for the determination of total solids in milk was issued in 1937 (British Standards Institution, 1937). The formula given for the calculation of the total solids was:

$$T = 0{\cdot}25D + 1{\cdot}21F + 0{\cdot}66,$$

where T = percentage total solids by weight in the milk;

$$D = 1000(d - 1),$$

where d = density of milk in g/ml at 20 °C

F = milk fat percentage.

The formula was unchanged in British Standards No. 734 (British Standards Institution, 1955*b*). An amended formula was introduced on 1 October 1957 and incorporated in a revised British Standard (British Standards Institution, 1959). The new formula:

$$T = 0{\cdot}25D + 1{\cdot}22F + 0{\cdot}72$$

had the effect of increasing the total solids obtained by the density method by 0·1 percentage units.

This formula was based on the work of Rowland & Wagstaff (1959) and others representing scientific and industrial organizations on the British Standards Institution Committee entrusted with the preparation of the standard. Large numbers of samples of herd and bulk milk were tested. The formula takes into account the alteration in the volume of the Gerber milk pipette from 11·04 to 10·94 ml in the revised British Standards Institution (1955*a*). In some of these comparisons the gravimetric method used was British Standards Institution (1951) Method II. This method, employing 1 ml milk in open dishes (milk bottle caps), was not included in the current gravimetric standard (British Standards Institution, 1963).

Many workers have compared the results obtained by hydrometric and gravimetric methods. Boden & Campbell (1942) indicated the need for standardizing the evaporating technique before attempting any permanent revision of the density formula. Sharp & Hart (1936) pointed out that variations in the drying technique may in some instances account for lack of agreement between total solids as determined by drying and as calculated from the specific gravity.

The object of the present study was to compare the results obtained over a long period with the gravimetric method of British Standards Institution (1963) and those obtained by the use of the current density hydrometer method of British Standards Institution (1959).

EXPERIMENTAL

From April 1964 to March 1967, not less than 5 drip samples/week were taken from the Hogganfield Creamery of the Scottish Milk Marketing Board, where about $3\frac{1}{2}$ million gal milk are processed annually. In all, 1082 drip samples of fresh milk were tested for total solids by the density hydrometer method and by the gravimetric method and the results compared.

Analytical methods

Fat percentages were determined in duplicate analyses by the Gerber method as given in British Standards Institution (1955*a*); calibrated glassware was used.

Density at 20 °C was determined by the liquid fat method as described in British Standards Institution (1955*b*). No. 1 density hydrometers were used and were calibrated before use.

Total solids were determined gravimetrically in duplicate analyses, as described in British Standards Institution (1963). In addition, total solids in 56 of the samples were determined as described in British Standards Institution (1951) Method II.

RESULTS AND DISCUSSION

The results for the total solids obtained by the current British Standards Institutions' hydrometric and gravimetric methods were compared for a period of 3 years. The total solids results obtained gravimetrically were subtracted from those obtained with the density hydrometer method. The mean differences obtained each month during the 3-year period were used to construct Fig. 1. The differences were greatest in winter and early spring.

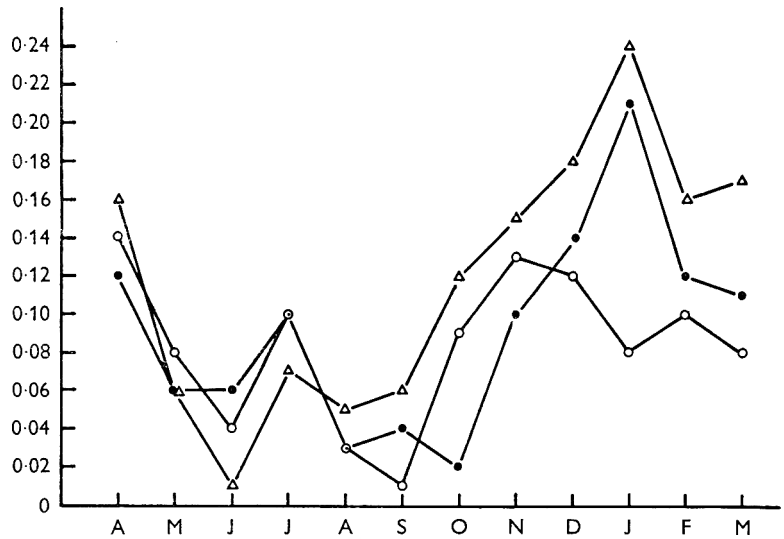

Fig. 1. Mean monthly differences between hydrometric and gravimetric total solids results. -○-○-, 1964–5; -●-●-, 1965–6; -△-△-, 1966–7.

The use of the density hydrometer formula pre-supposes that the relative proportions of the different components in the milk are constant. Waite (1959) has shown that the ratio of casein content to lactose content bore a linear relationship to the error in SNF content as measured by density, a high ratio leading to low SNF values by the density method. It is, therefore, possible that the greater differences in winter and early spring, when the protein content of milk could be expected to be low, are due to this factor.

The overall mean difference was +0·0963 for the period April 1964–March 1967. The mean yearly difference for the year 1964–5 was +0·080, for 1965–6 +0·096 and for 1966–7 +0·116. All these differences were statistically significant ($P < 0·001$). The annual average fat and SNF percentages were:

	Fat	SNF
1964–5	3·82	8·72
1965–6	3·83	8·69
1966–7	3·80	8·73

In a comparison of the results obtained for 56 samples by the gravimetric method (British Standards Institution, 1963) and by the method of British Standards Institution (1951) Method II, the latter method gave higher results by an average of 0·11. The

number of comparisons is too small to be conclusive, but the results indicate that the differences arise from the use of different gravimetric procedures.

The frequency distribution of the differences for the 3 years experiment in class intervals of 0·04 are given in Table 1. The standard deviation of the difference for each year was as follows: 1964–5, 0·089; 1965–6, 0·098; 1966–7, 0·086.

Table 1. *Frequency distribution of differences in total solids estimated by the density hydrometer and gravimetric methods*

	1964–5		1965–6			
Error	No. of samples	% of total no. of samples	No. of samples	% of total no. of samples	No.	% total
> 0·33	1	0·30	1	0·33	6	2·35
+0·32 to +0·28	4	1·22	3	1·00	8	3·14
+0·27 to +0·23	11	3·36	4	1·33	23	9·02
+0·22 to +0·18	24	7·34	15	5·00	32	12·55
+0·17 to +0·13	54	16·51	38	12·66	41	16·08
+0·12 to +0·08	74	22·63	57	19·00	53	20·78
+0·07 to +0·03	88	26·91	55	18·33	52	20·39
+0·02 to −0·02	45	13·76	48	16·00	21	8·23
−0·03 to −0·07	16	4·89	53	17·66	13	5·10
−0·08 to −0·12	3	0·92	15	5·00	4	1·57
−0·13 to −0·17	5	1·53	9	3·00	1	0·39
−0·18 to −0·22	2	0·61	—	—	1	0·39
−0·23 to −0·27	—	—	—	—	—	—
> −0·27	—	—	2	0·66	—	—
Total no. of samples	327		300		255	

It can be concluded from the results obtained that the density hydrometer method of determining the total solids content of milk over-estimates the results by 0·096.

I am indebted to the staff and members of The Scottish Milk Marketing Board for assistance with this work and for the facilities provided. I also wish to thank Prof. M. O'Sé, University College, Cork, for his advice concerning this work.

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A milking parlour for experimental work with lactating ewes

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Summary. A milking parlour and milking equipment are described which have been found suitable for experimental work on the nutrition and the physiology of lactating ewes.

Ewes are milked commercially in many Balkan and Mediterranean countries. Often the animals are part of a pattern of subsistence farming and are hand milked wherever they may be grazing. However, in France and Israel the milch ewe is part of intensive modern farming systems and large flocks are milked by machine. Various types of parlours are found, from simple abreast-type units to large rotolactors, capable of milking 600 ewes (Finci, 1957; Becker, 1958) and adapted herringbone parlours (Bosc, 1963).

The parlour described below was designed for experimental work on lactation in the ewe. It can be used either for milking up to 120 ewes when only milk yield and the food consumption need be recorded, or alternatively, for milking smaller numbers of ewes when more detailed records are required, as in physiological studies or milking experiments. The facilities allow the routine estimation, fractionation and sampling of total and half-udder milk yields; the rate of flow of milk and the effects of different mechanical milking procedures may also be determined. Easy access to both teats of the udder, and to the jugular vein for injection and blood sampling during milking, was necessary. It was also important to enable the ewes to be held in the parlour, either singly or in groups, without delaying the milking of the rest of the flock.

Design of the parlour

Design was guided by the generally accepted standards of milking practice for ewes, which would constitute the normal basic milking routine. Thus, teat cups were applied as soon as the ewe was in the milking position, and without udder washing or fore-milking; milk flow would start some 5–15 s later and last from 40 to 55 s. Machine-stripping, accompanied by udder massage, was practised for 10–15 s, since about 10% of the total yield is obtained through the stimulus of hand-stripping.

A 2-level, abreast parlour, with a slatted floor 110 cm above ground level (Fig. 1), was constructed in an existing standard farm building. The parlour was designed to allow the ewes to move forward from each stage of assembly and milking to the next, and to minimize work for the operators, and so allow large numbers of ewes to be dealt with quickly.

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Because the ewe's udder is most easily reached from the rear, between its hind legs, the abreast arrangement is most convenient; it also provides the most compact parlour layout for a given number of ewes. With a 2-level parlour, however, the abreast arrangement requires that the ewes, once in place and secured by a yoke, should be moved bodily backwards to bring their udders within reach of the operator. In the present design, which has standing for 4 ewes only on each side, the yoke was built on to a platform trolley (Plate 1, fig. 1), and the whole unit suspended on rollers from overhead tracks mounted 125 cm above the slatted floor.

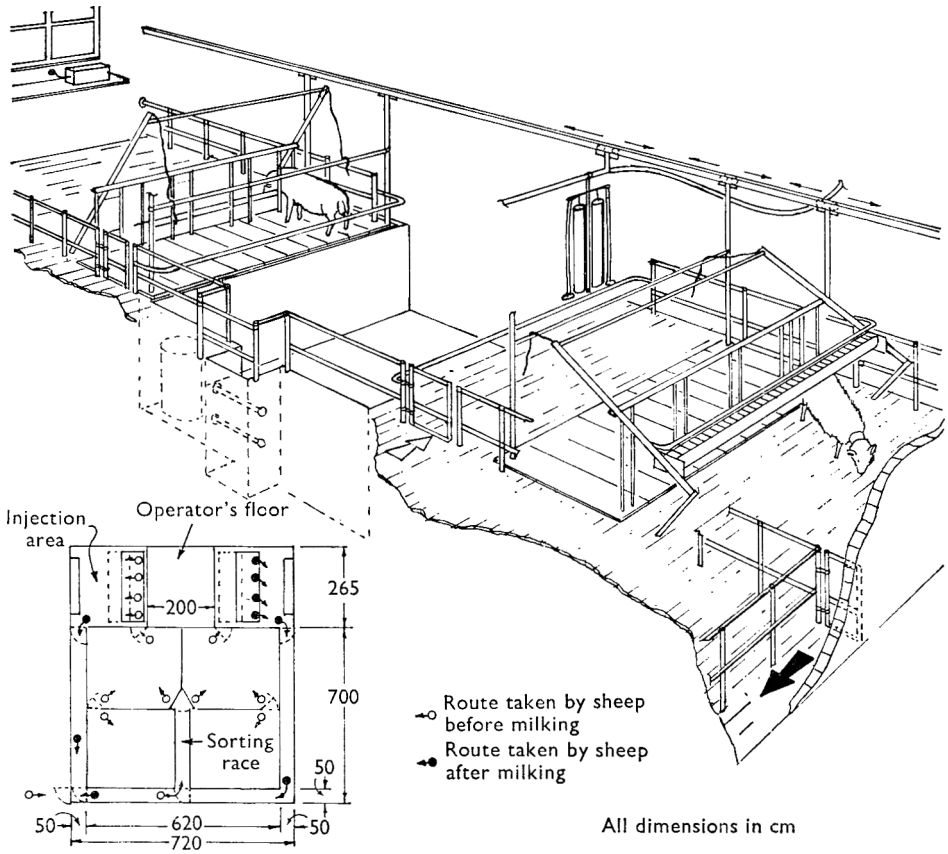


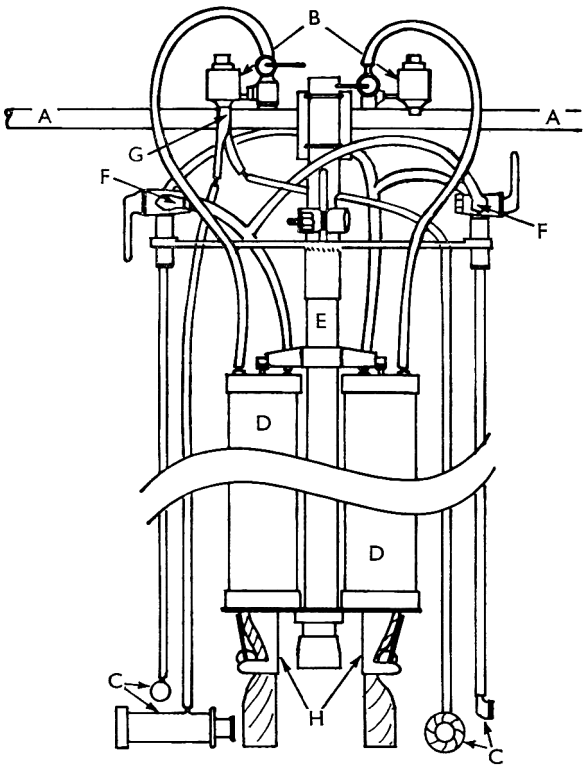
Fig. 1. Line drawing showing layout of the milking parlour and route taken by the sheep.

The yokes

The yoking device was designed to open sufficiently to allow the ewes to pass through when milking was completed. The yokes consisted of pairs of cranked vertical, bars of 12 mm mild steel, each held by removable pins at the top and bottom and located in holes in horizontal steel angle bars (Plate 1, figs. 1, 5, 6). The 2 upper bars, each carrying one member of each yoke, were supported on arms mounted on tufnol rollers running on a fixed angle bar which formed part of the main frame of the trolley (Plate 1, fig. 1). The 2 lower angle bars were supported only by the yoke bars. They were prevented from moving forwards by the lower extremities of the yoke bars

engaging with the web of the lower angle bar of the trolley main frame. Backward movement was prevented by restraining stops.

The opposite yoke bars and their supporting angle bars thus each made up a framework which could be moved laterally on rollers. By means of multi-strand stainless steel cables shackled to the ends of the angle bars, which at each corner passed through tubes bent through 90°, these frames were interconnected so that as one frame moved to the left, the other moved to the right. The cables were wound round, and anchored to, a wooden drum which was turned by a handle and crank from the operator's floor. Turning the handle in one direction increased the spaces between



A. Vacuum pipe
B. Pipeline valves
C. Teat cups
D. Milk holders
E. Vertical support for milk holders
F. 3-way valves controlling milk flow
G. Y-piece connections between pipeline valves and teat cups
H. Tube clip controlling milk release

Fig. 2. Line drawing showing construction of milk holders, and milk and vacuum flow control arrangements.

the yoke bars, opening up the whole width between the centre of one yoke and the centre of the adjacent one for an ewe to pass through. Turning in the other direction closed the yoke bars on the necks of the ewes, and an adjustable catch then held the yokes closed in the required position.

The food troughs formed an essential part of the ewe control system, as well as providing a convenient means of feeding the concentrate ration. As may be seen (Plate 1, figs. 1, 5, 7), they consisted of standard galvanized iron troughs mounted on 2 arms pivoted at the sides of the yoke main frames. The ends of these arms remote from the trough were connected by a rod which served as a handle (Plate 1, fig. 1).

Milking equipment (Fig. 2 and Plate 1, fig. 2)

A tubular frame suspended on rollers from the overhead tracks was constructed to support 4 milking units, so that they were easily moved to either side of the operator's floor. This movement allowed sufficient space between the units for the head and shoulders of the operator. The frame consisted of a $2\frac{1}{2}$ cm vacuum pipe (A) and it was connected by a flexible rubber tube to the main vacuum line from an Alfa-Laval VP. 18 vacuum pump. A sanitary trap and 33 cm Hg vacuum controller were fitted to the main vacuum line. On the $2\frac{1}{2}$-cm vacuum line on the frame were fitted 8 magnetic pulse line valves (B) (Alfa-Laval, 27097). The magnetic pulse line valves were connected to a 12-V transformer/rectifier unit and pulsation control unit so that 4 valves could be operated at one speed or ratio and the other 4 at a different speed or ratio. Alfa-Laval 26895 teat cups (C) and 25200 moulded liners were used, with 68 mm bore rubber tubes for milk and pulse connexions. The milk holders (D) consisted of 6 cm bore glass cylinders 60 cm long, with ebonite top and bottom covers. These were mounted in pairs on $2\frac{1}{2}$ cm vertical support spines (E) suspended from the vacuum pipe (A).

The vacuum feed nipple on each cover was connected to the vacuum cock of the magnetic pulse line valve. The milk nipple on each cover was connected via a Y piece to a special 3-way valve (F) and then to the milk nipple on the teat cup. The 3-way valves allowed milk to flow from both teat cups into either milk holder at the same time or from each teat cup into a single milk holder. Fractionation of the yield from one or both teats was also possible, giving facilities for half-udder work without any loss of vacuum. If the same pulsation speed or ratio was required at both teat cups one pulse valve only was utilized, the 2 pulse tubes being connected by a Y piece (G), but if different speeds or ratios were required both pulse valves were used and the pulse tubes connected to the teat cups separately. Rubber tubes of $1\frac{1}{4}$ cm bore fitted with tube clips (H) (Alfa-Laval, 27069) were attached to the outlet nipples at the bottom of the milk holders. After each ewe was milked the vacuum was turned off by the tap situated at the 27097 pulse line valve and the tube clip opened to allow the milk to be drained off into a suitable container.

A glass millimetre scale was baked on to the outside of each milk holder and the mm readings were then converted into g by calculation from the holder diameter. A suitable correction was made for the volume above the tube clip (H).

After each milking the equipment was washed by successively drawing cold water, hot detergent and hot water through the units. Air agitation was obtained by opening the tube clips (H) under vacuum.

Pulsation control (Plate 1, fig. 3)

The control unit was constructed to enable the pulsation rate and ratio to be varied within wide limits and also between the 2 udder halves. It employs a synchronous motor to drive a turntable carrying slotted discs, by means of which 2 beams of light directed on to photocells are interrupted for variable time periods. In practice, pulsation rates from 30 to 240 c/min, varying by 30-cycle steps, and with infinitely variable ratios, were easily obtained. The 2 concentric slot locations allowed

each teat cup to be separately timed for half-udder work. The provision of pre-programmed disks allowed rapid change of pulsation ratio and/or ratios during milking.

Operation of the parlour

The sequence of operation is as follows: the trough is filled with concentrates (Plate 1, fig. 4). This is easily done when the stalls are empty; the operator pulls the trolley towards the operator's floor, scoops the concentrates from bins on the floor into the troughs and then pushes the trolley away again. The 4 ewes are then let in (Plate 1, fig. 5), the yoke bars closed (Plate 1, fig. 6) and the trolley pulled back for milking to proceed. On completion of milking the yokes are opened and the trough handle pulled down to raise the feeding trough and to allow the ewes to walk out forwards underneath it (Plate 1, fig. 7). The troughs thus form barriers to the ewes which prevent them walking straight through the yokes before milking. Ropes were used for the initial downward pull on the handles.

The slatted floor area immediately adjacent to the milking unit was divided into before- and after-milking sections by means of wooden hurdles held by eyes and vertical rods. The before-milking area could be further subdivided according to experimental requirement—one such example is shown in Fig. 1.

Performance and general comments

The number of ewes milked/h varied with the milking routine. With a simple milking routine, 2 men could milk and hand strip 30–70 ewes/h. For this work the routine would include milk recording and milk sampling. If the yields were not sampled, 80 ewes could be milked/h. The provision of 2 jars/unit greatly speeded the milking process and enabled the yields of one group to be recorded and sampled whilst the second group was being milked. With more complicated milking routines employed in physiological investigations, the milking took far longer. The fractionation and half udder equipment described above worked well. Using 2 units/ewe, multiple fractionation of the milk of individual udder halves was easily and effectively done. Access to the jugular vein was easy and, injection through a cannula during milking caused no apparent distress to the ewe (Plate 1, fig. 8).

The rate of milk flow into the collecting cylinders was recorded by an additional operator equipped with a stop-watch. There was initial difficulty in reading the level of milk because of foam, and an attempt to overcome this was made by inserting an inner plastic tube into the collecting jars to act as a foam trap. These tubes presented cleaning problems, however, and were later discarded. With experience, the operators were able to take readings at 4-s intervals without the foam traps. It is of interest, in this connexion, that Labussiere & Martinet (1964) have described automatic mechanical and electronic devices for plotting milk flow rates in the ewe.

The simple cleaning routine described above proved quick and effective.

For the comfort of the milker it is recommended that an alternative type of silent pulsator be used and that the vacuum pump and motor be installed outside the building for projects involving long milking sessions. Access for injecting the ewes (see Fig. 1) and for any movement up and down the ladder from the operator's floor could have been facilitated by increasing the headroom under the main tracks above the slatted floor.

The construction of the parlour so that the operator's floor was at ground level had many advantages. Access to the floor for the operators and for manhandling the food and heavy experimental equipment was facilitated by the open end to the pit. Drainage and cleaning were also easy.

The parlour was constructed at Lane End Farm of the University of Reading during the spring of 1963.

We record our appreciation for the kind co-operation of Mr G. W. Pangbourne and Mr N. Corduroy of the Alfa-Laval Company Ltd. in the design and construction of the milking units.

The trolleys were built by Mr R. A. L. Hunt, the pulsation control by Mr E. G. Harvey and Mr H. Knight and the building was constructed by Mr F. Davis, all of the University of Reading. We are grateful to Mr C. S. Simmons for his suggestions on the design of the half udder equipment.

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EXPLANATION OF PLATE

Fig. 1. Platform trolley with yokes and food trough, before assembly into parlour.

Fig. 2. Milk and vacuum flow controls and pulse valves.

Fig. 3. 2-channel variable pulsation rate and ratio controller.

Fig. 4. Parlour operation (i): filling trough with concentrate food.

Fig. 5. Parlour operation (ii): ewes entering yokes.

Fig. 6. Parlour operation (iii): ewes held by yokes.

Fig. 7. Parlour operation (iv): ewes leaving opened yokes, passing underneath raised trough.

Fig. 8. Ewe continuing to feed during cannular injection.

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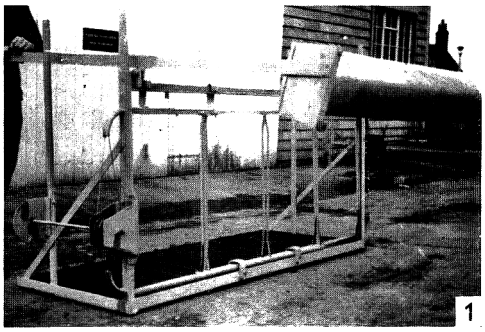
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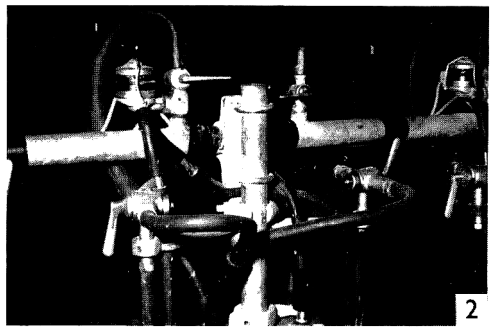
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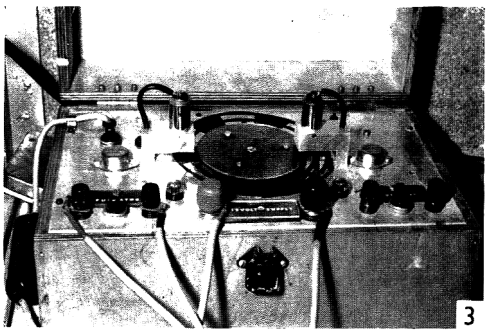
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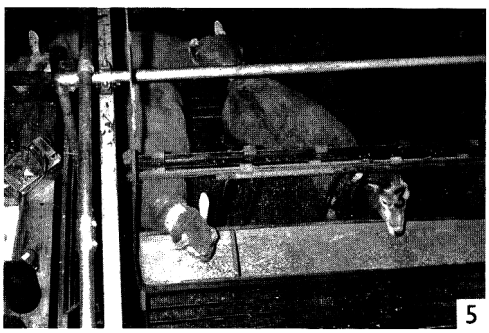
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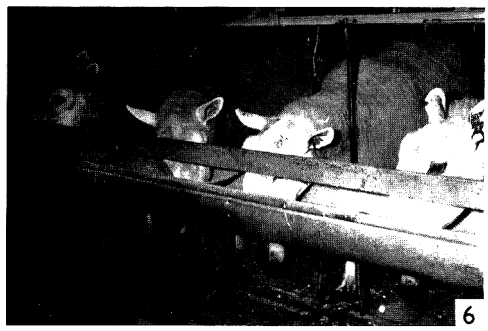
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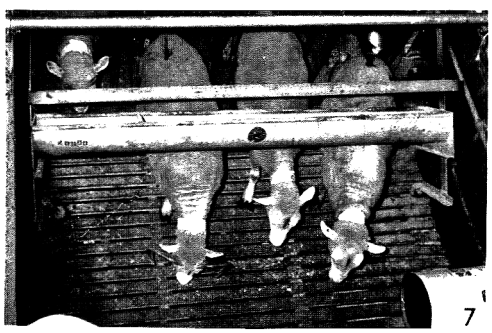
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Thermorésistance des spores de *Clostridium tyrobutyricum* et *Clostridium butyricum*

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RÉSUMÉ. Des suspensions de spores propres de 3 souches de *Clostridium tyrobutyricum* ont été préparées après culture en sac de dialyse. Les courbes de survie des spores chauffées à 90 °C en tampon phosphate 0·033 M à pH 7 ou dans du lait écrémé sont exponentielles. A partir des courbes des temps de destruction thermique (T.D.T.) on a calculé les valeurs suivantes: D à 121 °C = 0·003 à 0·012 min et z = 8·4 à 10 °C quand les spores ont été chauffées en tampon phosphate; D à 121 °C = 0·006 à 0·008 et z = 9·5 à 9·9 °C quand les spores ont été chauffées dans du lait.

Les courbes de survie des spores propres de 2 souches de *Clostridium butyricum* chauffées à 85 °C en tampon phosphate 0·005 M à pH 7 sont exponentielles, mais elles présentent une 'queue' quand les spores sont chauffées dans du lait écrémé. Les valeurs de D à 85 °C sont inférieures à celles obtenues avec les spores de *Cl. tyrobutyricum*.

SUMMARY. Clean spores suspensions of 3 strains of *Clostridium tyrobutyricum* were prepared after culture in dialysis sacs. The survivor curves of the spores heated at 90 °C in 0·033 M phosphate buffer (pH 7·0) or in skim-milk were logarithmic. From the thermal-death-time curves the following values were calculated: D at 121 °C = 0·003–0·012 min and z = 8·4–10 °C when spores were heated in phosphate buffer; D at 121 °C = 0·006–0·008 and z = 9·5–9·9 °C when spores were heated in milk.

When clean spores of 2 strains of *Clostridium butyricum* were heated at 85 °C in 0·005 M phosphate buffer (pH 7·0) the survivor curves were logarithmic, but 'tails' were observed after heating in skim-milk. The value of D at 85 °C was lower than that obtained with spores of *C. tyrobutyricum*.

INTRODUCTION

Certaines espèces de *Clostridium* du groupe butyrique jouent un rôle important dans l'industrie laitière car ils sont la cause de défauts redoutés dans les fromages de type Emmental, Gruyère ou Edam ainsi que dans les fromages fondus. Parmi les moyens de lutte contre ces accidents, on pourrait envisager la destruction des spores de ces *Clostridium* par chauffage du lait, à la condition que le traitement ait peu ou pas de répercussion sur la qualité du fromage affiné. Les données disponibles sur la thermorésistance des spores des *Clostridium* du groupe butyrique (Sjöström, 1943; Gibbs & Hirsch, 1956; Dorner, Demont & Chavannes, 1951; Prévot, 1961) ne permettent pas de calculer les traitements thermiques qui seraient nécessaires pour réduire la contamination initiale du lait en spores à une

teneur suffisamment faible pour écarter tout risque d'accident au cours de l'affinage.

C'est pourquoi nous avons étudié la thermorésistance des spores de *Cl. tyrobutyricum* et de *Cl. butyricum* qui sont les espèces incriminées dans les accidents de fromagerie. Dans le présent travail, nous présentons les résultats obtenus, ainsi que les techniques que nous avons mises au point pour produire des quantités suffisantes de spores de *Cl. tyrobutyricum* et pour nettoyer les spores des deux espèces étudiées.

Tableau 1. *Caractères d'identification des souches étudiées*

(CR = coagulé-rétracté; NC = non coagulé.)

	Souches			Souches	
	555	500		555	500
	556	514		556	415
		518			518
Lait cystéiné	CR	NC	Saccharose	+	−
Lait à pH 5·8	CR	NC	Salicine	+	−
SH_2	−	−	Mannitol	+	−
Gélatine	−	−	Glycérol	+	−
Nitrate	−	−	Amidon soluble	+	−
Glucose	+	+	Amidon de maïs	+	−
Lactose	+	−	Lactate	+	+

MATERIEL ET METHODES

Souches

Les 5 souches étudiées ont été isolées soit à partir de fromage fondu soit à partir de fromages d'Emmental d'origines diverses et présentant tous un gonflement tardif.

Les caractères culturaux des souches CNRZ 555 et 556 déterminés suivant la méthode de Beerens, Castel & Put (1962) sont identiques pour les 2 souches (Tableau 1). Elles fermentent non seulement le glycérol, mais aussi le lactate de sodium en présence d'acétate de sodium, ce qui ne permet pas de les faire entrer dans la classification de Beerens *et al.* Par ces caractères, les souches CNRZ 555 et 556 se rapprochent soit des *Cl. butyricum* de l'ATCC étudiés par Bryant & Burkey (1956), soit de *Cl. butyricum* var. *saccharobutyricum* de Schattenfroth & Grassberger (dans Prévot, 1961). Nous les classerons dans *Cl. butyricum*, comme l'ont fait Goudkov & Sharpe (1965) pour des souches qui présentaient les mêmes caractères que les nôtres.

Les 3 souches CNRZ 500, 514 et 518 montrent tous les caractères culturaux donnés par Beerens *et al.* (1962) pour *Cl. tyrobutyricum.*

Obtention des spores de Clostridium butyricum

La méthode de Stüssi (1961) a été utilisée avec quelques légères modifications pour produire des spores chez les 2 souches de *Cl. butyricum.* On procède à 4 cultures successives de 20 h à 37 °C. Pour la première, le milieu est inoculé à raison de 10 % avec une préculture effectuée à partir de la souche lyophilisée. Pour chacune des 3 autres, dont les volumes sont respectivement, de 10, 100 et 1000 ml, l'inoculum est constitué par la culture précédente, après réajustement du pH à 6·8 et chauffage à 78 °C pendant 5 min. La quatrième et dernière culture contient à la fin de l'incubation 3×10^8 cellules/ml, dont 90 % présentent une spore réfringente.

Le milieu de culture est le milieu EAH (Elliker, Anderson & Hannesson, 1956) dans

lequel on a supprimé la gélatine et la gélose et ajusté le pH à 6·8. La composition du milieu ainsi modifié est la suivante: extrait de levure Difco, 5 g; Bactotryptone Difco, 20 g; glucose, 5 g; lactose, 5 g; saccharose, 5 g; chlorure de sodium, 4 g; acétate de sodium, $3H_2O$, 1·5 g; acide ascorbique, 0·5 g; eau distillée, 1000 ml; pH 6·8.

Obtention des spores de Clostridium tyrobutyricum

Toutes les souches de *Cl. tyrobutyricum* isolées dans notre laboratoire sporulent très mal, quel que soit le milieu utilisé; moins de 1 % des cellules présentent des spores réfringentes. Au cours de différentes tentatives pour améliorer le taux de sporulation, la méthode de culture en boyau de dialyse suggérée par Schneider, Grecz & Anellis (1963) pour l'obtention de spores chez certaines espèces de *Clostridium* s'est révélée applicable au cas de *Cl. tyrobutyricum*.

Le milieu de culture est le TGE (Bergère & Hermier, 1965); sa composition est la suivante: glucose, 10 g; trypticase BBL, 10 g; extrait de levure Difco, 1 g; $MgSO_4$, $7H_2O$, 200 mg; $MnSO_4$, H_2O, 7 mg; $FeSO_4$, $7H_2O$, 10 mg; $CaCl_2$, $12H_2O$, 73·5 mg; acide ascorbique, 1 g; thioglycolate de sodium, 0·5 g; tampon phosphate 0·033 M en eau distillée, 1000 ml; pH 7·0.

Un boyau de dialyse ('regenerated cellulose' 36/32, Visking Co, Chicago, Ill.) contenant 100 ml de milieu TGE plongeant dans 900 ml du même milieu fraîchement autoclavé est inoculé avec 20 ml d'une culture de 20 h en phase de croissance, sur milieu RCM (Hirsch & Grinsted, 1954), puis l'ensemble est incubé à 37 °C pendant 3–4 jours suivant la souche, l'évolution de la sporulation étant suivie au microscope à contraste de phase. Le taux de sporulation atteint alors 50 %.

Nettoyage des spores

La culture, qui contient un mélange de spores encore incluses dans leur sporange et de cellules végétatives non sporulées, est centrifugée à 12000 ***g*** pendant 30 min à 4 °C. Les sporanges et les cellules non sporulées sont lysées par action du lysozyme et de la trypsine (Grecz, Anellis & Schneider, 1962). A cet effet, la culture est remise en suspension dans 100 ml de tampon phosphate 0·067 M à pH 7, contenant 30 mg de lysozyme, et le mélange est agité pendant 1 h à 37 °C. On ajoute alors 10 mg de trypsine sous forme de solution concentrée, et l'incubation est poursuivie dans les mêmes conditions pendant 90 min. Le lysozyme et la trypsine utilisés ont été stérilisés au préalable par filtration.

A la fin de l'incubation, la suspension contient des spores réfringentes, des spores non réfringentes et des débris cellulaires. A l'examen microscopique, les spores réfringentes paraissent dépourvues de débris cellulaires. Une série de centrifugations en eau distillée stérile à différentes vitesses permet de séparer les débris cellulaires, puis les spores non réfringentes qui se retrouvent à la partie supérieure du culot de centrifugation. La succession des centrifugations est la suivante: 5 centrifugations à 1000 ***g*** pendant 20 min, 4 centrifugations à 500 ***g*** pendant 45 min, 3 centrifugations à 100 ***g*** pendant 45 min, la température étant maintenue à 4 °C.

Après ces opérations, les suspensions de spores sont dépourvues de débris cellulaires visibles au microscope, et contiennent moins de 1 % de spores non réfringentes dans le cas de *Cl. butyricum* et environ 10 % dans le cas de *Cl. tyrobutyricum*. Le nombre de spores viables obtenu par numération en milieu liquide sans chauffage préalable

correspond au nombre de spores réfringentes dénombrées à la cellule de Petroff-Hausser, sauf dans le cas de *Cl. tyrobutyricum* 514 et 518 où le nombre de spores viables ne représente que la moitié du nombre de spores réfringentes.

Numération des spores viables

Malgré sa précision limitée, la méthode de numération en milieu liquide, dite méthode du nombre le plus probable (most probable number MPN), a été adoptée pour les raisons analogues à celles exposées par Gibbs & Freame (1965). Le milieu de dilution est l'eau distillée stérile et les numérations sont effectuées soit en milieu EAH pour *Cl. butyricum*, soit en milieu RCM à pH 6·8 pour *Cl. tyrobutyricum*. Les milieux de numération, répartis en tubes, sont régénérés au bain marie bouillant pendant 20 min puis ensemencés et recouverts d'un mélange en proportions égales de paraffine (t_f = 54 °C) et d'huile de paraffine stériles. Les tubes ensemencés à raison de 5 par dilution, sont incubés à 37 °C pendant un mois. Les tables utilisées pour la détermination du nombre le plus probable sont celles de Taylor (1962).

Détermination de la thermorésistance des spores

Les spores sont chauffées soit dans le tampon phosphate de potassium, soit dans du lait écrémé. La molarité et le pH du tampon sont indiqués dans le texte. Le lait écrémé, provenant du troupeau du Centre National de Recherches Zootechniques, est au préalable stérilisé par chauffage à 140 °C pendant 5 sec, dans un stérilisateur à ultra-haute température (Hermier, Vergé & Grosclaude, 1959).

Des tubes de verre ordinaire (100 × 5 mm) contenant 1·5 ml de suspension de spores dans le milieu de chauffage sont scellés à la flamme. Ils sont plongés dans un bain-marie de glycérine dont la température est maintenue à ± 0·02 °C près. Avant et après le chauffage, les ampoules sont plongées dans de l'eau maintenue à la température de la glace fondante. Pour le calcul de la durée du chauffage, il est tenu compte des temps de montée et de descente en température du milieu de chauffage. Après complet refroidissement, les ampoules sont ouvertes aseptiquement et le nombre de spores survivantes est établi pour chaque ampoule.

Pour établir une courbe de survie, la suspension de spores est chauffée pendant des temps variables à une température donnée à raison de 2 (ou de 3 dans le cas des spores de *Cl. tyrobutyricum* 514) ampoules par temps de chauffage. Les valeurs du temps de réduction décimale D sont calculées soit à partir des courbes de survie, soit à partir du nombre moyen de spores survivantes dans 6 ampoules chauffées dans les mêmes conditions de temps et de température.

RESULTATS

Choix du milieu de chauffage de référence

Le tampon phosphate de potassium, à la concentration 0·067 M est généralement utilisé comme milieu de chauffage de référence pour les études sur la thermorésistance de la spore bactérienne. Mais, comme le montre le tableau 2, il n'est pas possible d'utiliser le tampon phosphate à cette concentration 0·067 M pour les spores de *Cl. butyricum*, car aucune spore 'survivante' ne peut être dénombrée après chauffage dans un tel tampon. Le même effet inhibiteur se manifeste également, mais de façon beaucoup plus modérée, avec les spores de *Cl. tyrobutyricum*. En conséquence, nous

avons choisi comme milieu de chauffage de référence le tampon phosphate à pH 7 aux concentrations pour lesquelles on dénombre le maximum de survivants: 0·005 M pour *Cl. butyricum* et 0·003 M pour *Cl. tyrobutyricum*.

Tableau 2. *Pourcentage de spores survivant à un chauffage à* 85 °C *pendant* 30 *min dans des tampons phosphate à pH* 7 *de différentes molarités*

	0·067 M	0·033 M	0·005 M
Cl. butyricum CNRZ 555	0	0·3	2·2
Cl. tyrobutyricum CNRZ 514	14	26	6

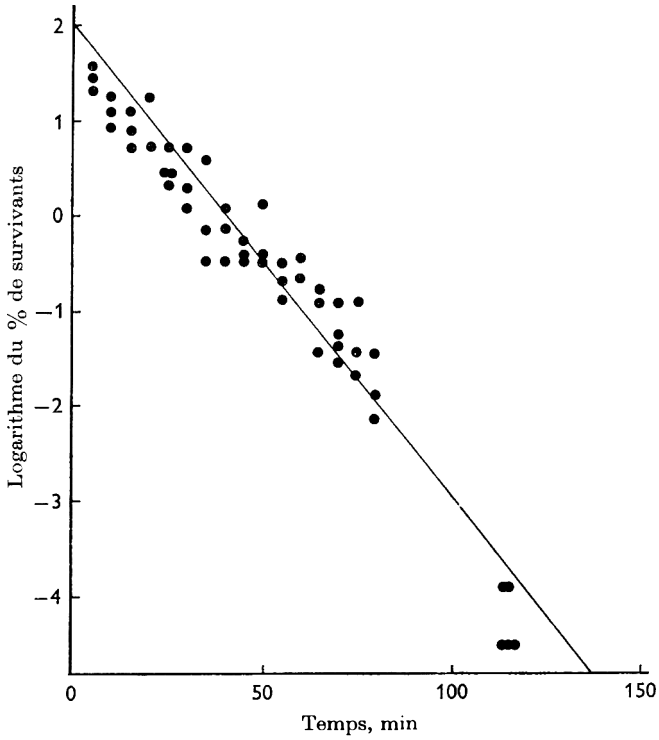

Fig. 1. Courbe de survie des spores de *Clostridium tyrobutyricum* souche 514 chauffées dans une solution de tampon phosphate 0·033 M à pH 7·0. A 90 °C, D = 20·9 min (coefficient de sécurité 99 %).

Thermorésistance des spores de Clostridium tyrobutyricum

L'étude de la thermorésistance de *Cl. tyrobutyricum* a porté principalement sur la souche CNRZ 514, et les résultats trouvés ont été vérifiés sur 2 autres souches CNRZ 500 et 518.

Sur la Fig. 1 on a reporté graphiquement le logarithme du pourcentage de spores survivantes de *Cl. tyrobutyricum* 514 en fonction du temps de chauffage à 90 °C, en tampon phosphate 0·033 M à pH 7. La droite de régression, calculée d'après ces données sur 6 réductions décimales, a été obtenue avec un coefficient de sécurité de 99 %. La valeur du temps de réduction décimale D, c'est-à-dire le temps nécessaire pour détruire 90 % de la population initiale de spores est de 20·9 min. Une relation

linéaire a été aussi obtenue après chauffage des spores dans du lait écrémé mais la valeur de D, 14·1 min, est plus faible que dans le cas du chauffage en tampon.

A partir des valeurs de D déterminées pour les températures comprises entre 85 et 105 °C, on a pu établir que les courbes du logarithme de D en fonction de la température sont linéaires (Fig. 2). Les droites de régression ont en effet été obtenues avec un coefficient de sécurité de 99 % pour le chauffage en tampon phosphate et de 95 % pour le chauffage dans le lait. Les valeurs de z — inverse de la pente des droites ainsi calculées — et la valeur de D à 121 °C sont données dans le Tableau 4. On remarquera que les valeurs de z sont indépendantes du milieu de chauffage.

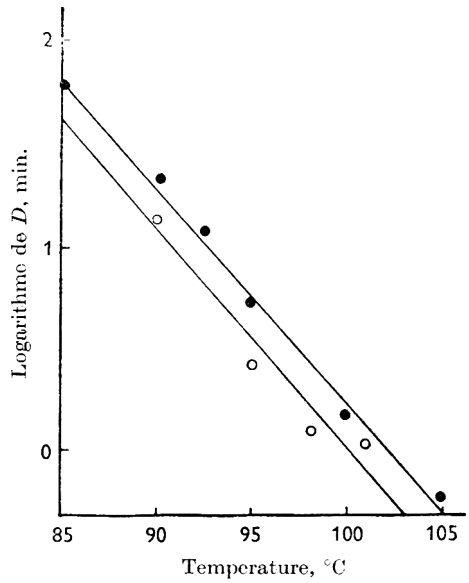

Fig. 2. Courbes fantômes de temps de destruction thermique des spores de *Clostridium tyrobutyricum* souche 514 chauffées dans une solution de tampon phosphate 0·033 M à pH 7·0 et dans du lait. ●——●, Chauffages en tampon: ○——○, chauffages en lait.

Tableau 3. *Temps de réduction décimale (D) à 90 °C pour les spores de* Clostridium tyrobutyricum

(Les spores sont chauffées soit en tampon phosphate 0·033 M à pH 7·0, soit dans du lait écrémé stérile.)

Souches		D, min
514	Tampon	20·9
	Lait	14·1
500	Tampon	14
	Lait	9
518	Tampon	18
	Lait	10

L'étude effectuée sur la souche CNRZ 514 a été répétée avec 2 autres souches de *Cl. tyrobutyricum*, CNRZ 500 et 518. Comme le montre le Tableau 3, il n'y a pas de grandes différences entre les valeurs de D à 90 °C obtenues pour les 3 souches; on notera toutefois, que pour une même souche, la valeur de D est plus élevée quand le

chauffage a lieu en tampon. Les valeurs de z et de D à 121 °C sont présentées dans le Tableau 4. On n'observe pas de différences entre les 3 souches quand le chauffage a lieu dans le lait. Par contre la souche CNRZ 518 est caractérisée par des valeurs plus faibles de z et de D à 121 °C dans le cas du chauffage en tampon.

Influence du pH sur la thermorésistance des spores de Clostridium tyrobutyricum

La différence observée entre les valeurs obtenues après chauffage en tampon à pH 7 ou dans le lait pourrait être expliquée par une influence du pH puisque le pH du lait était de 6·5. Tel semble bien être le cas puisque, comme le montre le Tableau 5, les valeurs de D quand les spores sont chauffées en tampon phosphate 0·033 M à pH 6·5 sont plus faibles que celles obtenues quand le pH du tampon est de 7·0, et du même ordre de grandeur que les valeurs de D des spores chauffées dans le lait. Comme le montre le Tableau 5, cette influence du pH n'est pas retrouvée quand le pH du tampon est de 7·5 au lieu de 7·0.

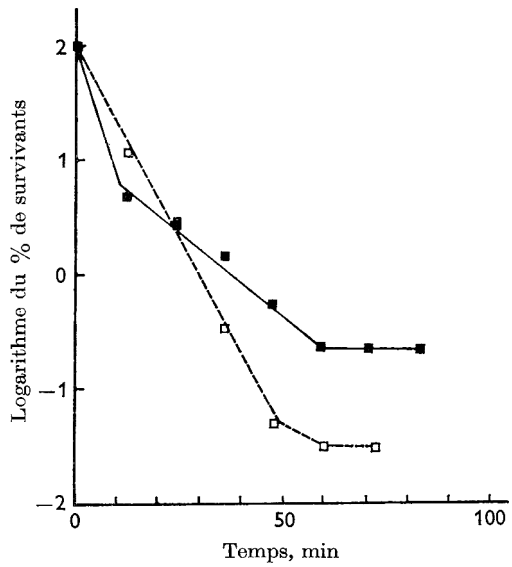

Fig. 3. Courbes de survie des spores de *Clostridium butyricum* souches 555 et 556 chauffées dans du lait à 85 °C. □ - - - □, Souche 555; ■——■, souche 556.

Tableau 4. *Valeurs de z et de D à* 121 *°C pour les spores de* Clostridium tyrobutyricum

(Les spores sont chauffées soit en tampon phosphate 0·033 M à pH 7·0, soit dans du lait écrémé stérile.)

Souches	Milieu de chauffage	z, °C	D_{121}, min
514	Tampon	9·8	0·012
	Lait	9·9	0·008
500	Tampon	10	0·012
	Lait	9·7	0·006
518	Tampon	8·4	0·003
	Lait	9·5	0·007

Thermorésistance des spores de Clostridium butyricum

Une étude préliminaire ayant montré que la thermorésistance des spores de *Cl. butyricum* était nettement inférieure à celle des spores de *Cl. tyrobutyricum*, l'étude des spores de *Cl. butyricum* s'est limitée à l'établissement de la courbe de survie à 85 °C.

Les courbes des logarithmes des nombres de spores survivantes après chauffage à 85 °C en tampon phosphate 0·005 M à pH 7 sont linéaires. Il n'en est pas de même quand le chauffage est effectué dans du lait écrémé (Fig. 3). Dans le cas de la souche CNRZ 555 la courbe est linéaire pendant les 50 premières min de chauffage, puis présente une 'queue'. La courbe de survie des spores de la souche CNRZ 556 est encore plus complexe puisqu'elle peut être décomposée en 3 droites. Dans ces conditions, la valeur de D ne peut plus être établie; celle présentée dans le Tableau 6 correspond arbitrairement aux 3 premières réductions décimales.

Tableau 5. *Influence du pH du tampon sur la thermorésistance des spores de* Clostridium tyrobutyricum

(Les spores sont chauffées en tampon phosphate 0·033 M au pH indiqué à 90 °C.)

	D, min à pH:		
Souches	6·5	7·0	7·5
500	6·5	13	13
514	15	20·9	18
518	11	18	21

Tableau 6. *Temps de réduction décimale* (D) *à* 85 °C *pour les spores de* Clostridium butyricum

	D, min		
Souches	Tampon pH 7·0	Tampon pH 6·5	Lait
555	23	23	14
556	12	18	21

DISCUSSION

La relation entre le logarithme du nombre de spores survivantes de *Cl. tyrobutyricum* et le temps de chauffage est linéaire. Il en est de même entre le logarithme du temps de réduction décimale (D) et la température de chauffage, dans la zone de température étudiée. Les lois de destruction des spores de *Cl. tyrobutyricum* en tampon et dans le lait sont identiques à celles qui servent de base aux calculs des traitements thermiques dans l'industrie de la conserve (Ball & Oslon, 1957). Les mêmes méthodes de calcul peuvent donc être utilisées pour la détermination de l'efficacité d'un traitement thermique du lait en prenant pour base les valeurs de z et de D à 121 °C obtenues pour les 3 souches étudiées. Ces trois souches servant de référence, il sera néanmoins nécessaire de confirmer les valeurs de z et de D sur un plus grand nombre de souches de *Cl. tyrobutyricum*.

L'absence de relation linéaire entre le logarithme du nombre de spores survivantes

en fonction du temps de chauffage, dans le cas de *Cl. butyricum*, exclut la possibilité de calculer l'efficacité d'un traitement thermique *vis-à-vis* des spores de cette espèce. Mais cela sera sans inconvénient, s'il est vérifié sur un plus grand nombre de souches que leur thermorésistance est inférieure à celle des spores de *Cl. tyrobutyricum*. Il suffira donc, dans la pratique, après avoir déterminé les caractéristiques d'un traitement thermique pour la destruction des spores de *Cl. tyrobutyricum*, de vérifier que ce traitement possède une efficacité au moins égale vis-à-vis des spores de *Cl. butyricum*.

La forme complexe des courbes de survie des spores de *Cl. butyricum* dans le lait pourrait être attribuée à une hétérogénéité de la suspension de spores utilisée. Ceci paraît peu probable du fait que ces anomalies ne se manifestent pas quand le chauffage a lieu en tampon. Une autre interprétation serait plus satisfaisante, au moins pour expliquer la queue observée : le lait comme milieu de chauffage ne peut être considéré comme ayant une composition constante. Des changements de nature physico-chimique apparaissent progressivement au cours du chauffage et pourraient expliquer l'augmentation apparente de la thermorésistance, après un certain temps de chauffage.

Nous exprimons notre reconnaissance à M. Mocquot pour ses suggestions et critiques au cours de cette étude et de la rédaction du manuscrit.

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The effect of oxidation on the iodine values of phospholipid in milk, butter and washed-cream serum

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Summary. Phospholipid was isolated from milk, butter, and washed-cream serum by solvent extraction followed by simple counter-current distribution and thin-layer chromatography. Iodine values from fresh samples, determined by a micro-Wijs technique, ranged, for the cephalin fraction, from 70 to 86, for the lecithin fraction from 44 to 55, and for the sphingomyelin fraction from 36 to 44. In washed-cream serum, oxidation with copper and ascorbic acid led to reduction in extractable phosphorus, decreased chromatographic mobility of phospholipid and significant falls in the iodine values of the 3 phospholipid fractions. In milk, slight reductions in phospholipid iodine values were observed following copper-catalysed oxidation but they were not consistently significant. Iodine values of butter phospholipids remained unchanged even after gross oxidative quality deterioration.

In studies on the origin of storage defects in butter, the need has been felt in the authors' laboratory for a chemical method suitable for routine application in assessing phospholipid oxidation. Such a method should be compatible with thin-layer chromatography (TLC), which is the most satisfactory technique for separating phospholipids from neutral lipids. This rules out the possibility of measuring peroxide development, which in early oxidation requires samples of about 100 mg, which is inconveniently large for TLC techniques.

Consideration has been given to the use, for this purpose, of the iodine value, which requires only 1 mg or less of sample for precise determination. The iodine value is a very crude index of lipid oxidation as it is affected normally only under severe conditions of oxidation such as dairy products do not usually experience. There is evidence in the literature, however, that the iodine value of milk phospholipids is sensitive to moderate oxidation. Thus, Swanson & Sommer (1940), Koops (1957) and Smith & Dunkley (1959) have reported substantial falls in phospholipid iodine values during copper-catalysed oxidation in milk and cream.

Swanson & Sommer's (1940) results in particular have been widely quoted as evidence that phospholipid oxidation is the cause of oxidized flavour in milk. The earlier workers examining this problem separated phospholipids from milk fat by precipitation with acetone, a procedure which gives little certainty of the nature and identity of the fraction, and has now been superseded by chromatographic techniques. Because of its significance in this respect, and because of its possible use in the investigation of butter deterioration, a further study has been made of the effect

of oxidation on milk phospholipid iodine values using counter-current distribution and TLC techniques for the isolation of phospholipids. These methods were first developed and applied to the examination of phospholipids in washed-cream serum as it was felt that any changes in iodine value could most readily be demonstrated in this easily oxidized product. The investigation was then extended to include milk, and both neutral and acid salted butter.

MATERIALS AND METHODS

Pasteurized non-homogenized market milk was used. Washed cream was prepared by separating the milk at 40 °C, diluting the cream to the original milk volume with water, and reseparating. This process was repeated to give 4 washings.

To simplify subsequent extraction and separative procedures, the bulk of the fat was removed from the washed cream as follows. The cream was churned and the buttermilk collected. The butter grains were melted at 45 °C and the butter serum obtained by centrifuging and decanting the fat. The butter serum was then combined with the buttermilk. This mixture of buttermilk and butter serum, representing the total aqueous phase of the washed cream, was used in the investigation and will be referred to as washed-cream serum. Analyses have shown that only a negligible loss of phospholipid is involved in the procedure.

Butter was made by churning 4-kg lots of commercially pasteurized sweet cream, salting to 2 %, and working in a small sigma-blade blender. For acid butter, lactic acid was added to the cream before churning to give a pH of 5·0.

Copper was added as a solution of cupric chloride.

Isolation of lipids

Lipids were isolated by solvent extraction with chloroform and methanol. Milk and washed-cream serum were extracted directly, but with butter, extraction was carried out on the serum obtained by melting the butter at 45 °C and centrifuging it. Chloroform and methanol were used in the proportions stipulated by Bligh & Dyer (1959). The final emulsion in solvent containing chloroform, 20 parts; methanol, 20 parts and water, 18 parts was centrifuged in separating funnels, and the lipids removed with the chloroform layer. It was found an advantage to add 2 % NaCl to the milk samples beforehand as this gave a much more compact casein coagulum and facilitated separation of the chloroform layer.

The chloroform extracts were evaporated to dryness at 45 °C in a rotary-film evaporator under reduced pressure and in an atmosphere of nitrogen. In butter serum and washed-cream serum the ratio of neutral lipids to phospholipids is usually 5:1 or less and separation of the 2 groups can be effected conveniently on the thin-layer plate. The extracts from these products, therefore, were taken up immediately in chloroform containing 5 % (v/v) methanol, to give a phosphorus concentration of about 1 mg/ml, suitable for application to the thin-layer plate. Usually the volume of the final solution was from 3 to 5 ml, and to assist in the preparation and collection of this small volume of solution the flask used in the evaporator was fitted at its bottom with a small tube of about 2-ml capacity.

In milk the ratio of neutral lipid to phospholipid is about 100:1 and a preliminary

fractionation is necessary before application of the phospholipid fraction to the thin-layer plate. This was done by partitioning the extract between light petroleum (B.P. 40–60 °C) and 87 % (v/v) ethanol in the simple counter-current distribution system described by Galanos & Kapoulas (1962). The final ethanolic solution containing the phospholipids was evaporated under nitrogen and reduced pressure at 45 °C until only about 1 ml remained; this was taken up in a mixture of equal parts of chloroform and methanol, and 2 % sodium chloride solution was then added to adjust the composition of the fluid to: chloroform, 20 parts; methanol, 20 parts; sodium chloride solution, 18 parts (Bligh & Dyer, 1959). After separation, the chloroform layer was usually transferred to an open 11 × 2·5 cm tube and held overnight, packed in solid CO_2 in a Dewar flask (with butter serum and washed-cream serum, extraction and duplicate analyses could be completed in one day). Next morning the chloroform solution was evaporated to dryness and the crude phospholipids dissolved, as with the serum extracts, in chloroform containing 5 % (v/v) methanol to give a phosphorus concentration of about 1 mg/ml.

Thin-layer chromatography

Thin-layer plates, 20 × 20 cm, were coated with 0·5 mm silica gel G (Merck), air-dried and heated for 1 h at 110 °C 24 h before use. The lipid extract (0·5 ml, containing approximately 0·5 mg phosphorus or 12·5 mg phospholipid) was applied to the plate by spotting. The developing solution was: chloroform, 65 parts; methanol, 25 parts, and water, 4 parts. With this system, the neutral lipids run with the solvent front and the phospholipids divide into 3 well-defined bands corresponding, in descending order of R_F value, to the main phospholipid classes cephalin, lecithin and sphingomyelin. The bands were made visible by spraying the plate with a solution of bromthymol blue and were scraped off and transferred by suction to filter tubes made by letting 5 × 0·5 cm (I.D.) glass tubing into the bottom of a 12 × 1·5 cm-test tube and placing at the junction a small filter of cellulose powder supported on cotton wool.

Considerable difficulty was experienced in obtaining satisfactory recoveries of phospholipid from the thin-layer plates. The method finally adopted was as follows. The eluting solvent was: chloroform, 10 parts; methanol, 20 parts, and water, 8 parts —as stipulated by Bligh & Dyer (1959) for the first stage of their lipid extraction procedure. This solvent mixture was saturated with $CaCl_2$. It was added in 3 successive 5-ml portions to filter tubes containing the silica gel scraped from the plates. Each was gently stirred and passed through the filter under mild pressure. The filtrates were combined, made up to 19 ml with the solvent and further diluted in a separating funnel with 5 ml chloroform and 5 ml of 10 % aqueous NaCl. These additions brought the solvent ratio to: chloroform, 20 parts; methanol, 20 parts, and water, 18 parts—as in the second stage of the Bligh & Dyer (1959) extraction, and on shaking a chloroform layer of approximately 10 ml, containing the phospholipid, rapidly settled out. It was made up to 10·5 ml and duplicate 2- and 3-ml portions were taken from it for phosphorus and iodine value determinations, respectively.

Prevention of oxidation

To minimize lipid oxidation, all the chloroform used for extraction contained 0·005 % butylated hydroxytoluene (Wren & Szczepanowska, 1964). This antioxidant could not be used during development as it contaminated the phospholipid and, since it had an appreciable iodine uptake, it resulted in slightly higher iodine values. No significant differences in iodine values were observed when development was carried out with de-aerated solvents and in an atmosphere of commercial oxygen-free nitrogen, in equipment described by Badings (1964). It was concluded, therefore, that any oxidation during normal development in air could, for the purposes of the investigation, be disregarded.

Analytical procedures

To determine phosphorus, extracts were wet-ashed with H_2SO_4 and H_2O_2 and the phosphomolybdate blue colour developed with amidol (Allen, 1940). For very small quantities of phosphorus as in blanks from TLC plates, the method of Bartlett (1959) was used. Phospholipid values were calculated on the assumption that the cephalin band was oleyl-stearyl-phosphatidyl ethanolamine, M.W. 745, the lecithin band oleyl-stearyl-phosphatidyl choline, M.W. 788, and the third band sphingomyelin, with a C_{22} fatty acid and a M.W. of 787 (Sprecher, 1964).

Iodine values were determined by a micro-modification of the Wijs technique. The halogenating solution was standard Wijs solution, prepared from iodine monochloride according to the official method of the American Oil Chemists' Society (1956). It was diluted with acetic acid, to slightly weaker than N/50, so that 1 ml reacted with 9·5–10·0 ml N/500 $Na_2S_2O_3$. The 3-ml portions of chloroform solution, containing 1–1·5 mg phospholipid, were transferred to 50-ml glass-stoppered flasks, 1 ml halogenating solution added, and the mixture held for 1 h in the dark. After adding 5 ml 2 % iodine-free KI solution, the mixture was titrated with freshly prepared N/500 $Na_2S_2O_3$ solution using starch as an indicator. Flasks containing only 3 ml chloroform were treated similarly as blanks and the iodine values were calculated by difference in the usual way. This method was found to give results which did not differ significantly from those obtained with the macro-method for samples of butterfat, and of soyabean, peanut, maize and safflower oils. It was established by experiment that traces of bromthymol blue indicator accompanying the phospholipid fractions, and variations in the methanol and water contents of the chloroform solution, did not affect the iodine value determination.

Fat peroxide values were determined by the method of Loftus Hills & Thiel (1946). Thiobarbituric acid (TBA) tests were made on milk by the method of Dunkley & Jennings (1951) and on butter serum and washed-cream serum as described by Pont & Birtwistle (1966).

RESULTS

Treatments of samples

Milk, to which 5 ppm. copper had been added, was dispensed in 1-l quantities into clear glass flasks, which were then exposed to direct sunlight for 10 min and held for 7 days at 5 °C. At the end of this time the milk had developed an intense tallowy flavour.

Copper was added, at 0·6 ppm., to the cream used for making the butter. After 3 months' storage at 2 °C, the neutral butter had a strong tallowy flavour. The acid butter was extremely oxidized and had an intense fishy flavour. In both the milk and the butter the degrees of off-flavour and the chemical indices of oxidation were much higher than are encountered even in extremes of commercial practice. The products were clearly inedible.

Washed-cream serum had 2·0 ppm. copper and 50 ppm. ascorbic acid added to it, the latter as sodium ascorbate. The characteristic fishy flavour induced by this treatment developed within a few hours and was intense after 2 days.

Recoveries

The amounts of lipid-soluble phosphorus extracted from washed-cream serum dropped by 5–7 % following oxidation. There were no significant differences between fresh and oxidized samples in the amounts of phospholipid extracted from milk and butter.

Recoveries of phosphorus from thin-layer plates ranged from 95 to 100 %. No differences were observed between fresh and oxidized samples.

Table 1. *Effect of oxidation on the iodine values of phospholipid fractions from milk, butter and washed-cream serum*

	Washed-cream serum		Salted butter				Milk	
			Serum pH 7·0		Serum pH 5·0			
Copper added ...	2·0 ppm. plus 50 ppm ascorbic acid		0·6 ppm. to cream		0·6 ppm. to cream		5·0 ppm.	
Conditions of storage ...	2 days at 5 °C		3 months at 2 °C		3 months at 2 °C		7 days at 5 °C	
	Initial	After storage	Initial	After storage	Initial	After storage	Initial	After storage
Fat peroxide value	—	—	0·07	0·92	0·06	7·02	0·06	1·11
TBA value	0·08	0·70	0·21	0·83	0·20	15·80	0·06	0·13
Phospholipid fractions, percentage distribution								
Cephalin	33·8	29·1	29·1	28·8	26·0	25·2	32·6	29·1
Lecithin	42·0	42·3	43·0	43·1	41·7	41·5	33·2	37·0
Sphingomyelin	24·2	28·6	27·9	28·1	32·3	33·3	34·2	33·9
Iodine values								
Cephalin	86·6	73·0	82·6	83·2	70·5	71·6	82·3	80·0
Lecithin	54·9	44·2	49·9	47·8	44·8	44·7	47·2	45·3
Sphingomyelin	44·2	41·7	43·0	44·1	36·0	36·1	42·5	39·5
Combined iodine value	61·9	53·0	58·5	58·4	50·4	50·8	58·3	54·8

Phospholipid iodine values

The results are summarized in Table 1. The iodine values of the individual phospholipid fractions are the means of duplicate analyses on each of duplicate thin-layer plates. The values have been corrected also for a tendency, apparent from the statistical analysis, for the values to decrease slightly with increasing amounts of phospholipid taken for analysis.

As examination of the data suggested that the variability did not differ significantly from experiment to experiment, the results were combined to give a single

estimate of variability. The overall standard deviation for individual fractions was ± 0·73 and for the iodine values of the combined fractions it was ± 0·74. The least significant differences before and after storage, for the individual fractions, were 2·15 and 2·93 for the 0·05 and 0·01 probability levels, respectively. For the combined values, the least significant differences were 2·33 and 3·32 at the 0·05 and 0·01 probability levels.

In washed-cream serum there were substantial falls in phospholipid iodine values after oxidation, the decreases for the cephalin and lecithin fractions and in the combined values being significant at the 0·01 level. Similar results were obtained in other experiments with washed-cream serum.

In Table 1, the results for the milk sample showed small but significant falls after oxidation, both in the fractional and in the combined iodine values. This result contains an anomaly in that the sphingomyelin, which was the least unsaturated of the 3 fractions, showed a highly significant (P = 0·01) lowering of iodine value. This could be an artifact due to overlapping of the lecithin and sphingomyelin fractions which on some plates could not be distinguished with certainty from one another. A discrepancy, probably arising also from irregularities of distribution between the bands, was observed in another sample of milk treated similarly. The iodine values for the 3 fractions were initially: cephalin 77·5, lecithin 48·8 and sphingomyelin 37·9. After storage, the values were 75·1, 43·1 and 42·0, respectively, there being an apparent rise in the value of the sphingomyelin fraction. After storage there was a slight but non-significant fall from 54·7 to 53·4 in the combined values for the sample. It appears that changes in phospholipid iodine values for milk oxidized under these conditions are at the limit of detectability by this technique.

Both in neutral and acid salted butters, only negligible changes in iodine value were observed after storage.

Distribution of phospholipid fractions

The relative proportions of the phospholipid fractions from each sample are shown in Table 1. Significant falls in phospholipid iodine values during storage were accompanied by alterations in the distribution of phospholipid between the 3 bands on the TLC plates, the amount of material in the more polar fractions increasing at the expense of the less polar. In washed-cream serum a substantial decrease in the apparent cephalin and an increase in the apparent sphingomyelin fractions after oxidation was evident in this and other experiments; in some instances there was a decrease also in the apparent lecithin fraction. These changes in distribution could be due, in part at least, to the formation of lyso-compounds from cephalin and lecithin. Lyso-lecithin would be expected to run with, or near, sphingomyelin on TLC plates. Changes in the distribution of the fractions from milk followed the same pattern though the differences generally were less marked. In butter also, even though the phospholipid iodine values were unaltered, there was a tendency for the less polar fractions to decrease after storage. The differences were slight however.

DISCUSSION

In the present investigation care was taken to ensure that, as far as possible, results were not affected by failure to recover phospholipid from thin-layer plates or by oxidation during extraction, manipulation and isolation procedures. The only serious discrepancies in this respect were in the experiments with washed-cream serum in which, after oxidation, a fall in extractable phosphorus was observed, and an alteration in distribution due to increased polarity of the phospholipid fractions. Similar changes in the distribution on thin-layer plates of phospholipid from blood cells were observed by Dodge & Phillips (1966) who attributed them to oxidation of their lipid extracts. These changes are consistent with the significant decreases in the iodine values of the 2 more highly unsaturated phospholipid fractions, and taken together demonstrate the marked oxidative changes produced in the membrane phospholipids of washed-cream under the influence of the powerful copper-ascorbic acid catalyst system (Olson & Brown, 1942; Forss, Dunstone & Stark, 1960.)

In both milk and butter, there were no significant differences between fresh and oxidized samples in the amounts of phosphorus extracted and in the recoveries from thin-layer plates, though there was a tendency in the oxidized samples for the more polar fractions to increase and the cephalin fraction to decrease. These observations increase the significance of the main finding that phospholipid iodine values for milk were only slightly affected and those for butter showed no sign of change when oxidative deterioration had reached levels far beyond those experienced in practice. The phospholipid iodine value, therefore, appears to have little or no relevance for the study of phospholipid oxidation in these products.

The large alterations in phospholipid iodine values reported by Swanson & Sommer (1940) and Koops (1957) in oxidized milk and cream remain to be explained. It appears most likely that their results were influenced by difficulties in separating phospholipid from neutral lipid by precipitation with acetone. It is now recognized that with this technique fractionation may not be complete, since it is difficult to separate phospholipid from some of the higher melting-point neutral lipids. A significant proportion of the phospholipids may also remain in solution (Hanahan, 1960).

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The influence of the dissolved oxygen in milk on the stability of some vitamins towards heating and during subsequent exposure to sunlight

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Summary. Heat sterilization of milk destroyed about 50% of its folic acid activity, and subsequent exposure of the milk to sunlight caused a progressive further loss. This loss of folate, as of vitamin B_{12}, was a consequence of the oxidative destruction of the milk's ascorbic acid, and was largely prevented by thorough exclusion of oxygen from the milk during heat processing and subsequent storage. With thiamine, riboflavin, nicotinic acid, vitamin B_6 and biotin, stability towards heating and during exposure to sunlight was affected little, if at all, by the oxygen tension in the milk.

On leaving the udder milk quickly takes up oxygen from the air, and in the few hours' interval during which the milk is collected into a bulk tank and kept at about 5 °C, its oxygen content increases about fivefold. During subsequent processing and distribution, this dissolved oxygen promotes oxidative changes that degrade several important nutrients in the milk. Thus, though potentially milk could supply an important fraction of the daily dietary requirement for vitamin C, average market milk supplies relatively little. The loss of ascorbic acid is determined ultimately by the concentration of oxygen dissolved in the milk, and in milk from which this oxygen has been removed the vitamin is stable for considerable periods, even during exposure to full sunlight which, in the presence of oxygen, would greatly accelerate the rate of destruction (Kon & Watson, 1936; Hand, Guthrie & Sharp, 1938; Guthrie, 1946).

Similarly with vitamin B_{12}, much of which may be destroyed during heat processing, and particularly during sterilization 'in bottle' or 'in can'. The destruction is essentially oxidative and consequent upon the oxidative degradation of ascorbic acid (Ford, 1957). Thus, the removal of dissolved oxygen offers a practical means of preserving vitamin B_{12} as well as for stabilizing the ascorbic acid.

Until quite recently it was generally accepted that milk contained very little of another important vitamin, folic acid. It is now clear that previously published analyses were in error, and that fresh milk is in fact a rich source of a form of folic acid, probably N^5-methyltetrahydrofolate which, like the 'folic acid' in human blood serum, has little activity for the assay micro-organism *Streptococcus faecalis* Rogers but is highly active for an alternative assay micro-organism, *Lactobacillus casei* (Naiman & Oski, 1964). The present paper shows that, like vitamin B_{12} and ascorbic acid, the folic acid in milk is unstable to heating in the presence of dissolved oxygen. It can be preserved intact if measures are taken to exclude oxygen from the milk,

or to add more ascorbic acid to the milk, and so protect the labile folate from oxidative destruction. For comparative purposes the investigation was extended to include 7 vitamins of the B-complex, namely thiamine, riboflavin, vitamin B_6, vitamin B_{12}, nicotinic acid, biotin and folic acid. The influence of heat processing and subsequent exposure of milk to sunlight upon these vitamins has been examined in relation to the milk's content of ascorbic acid and of dissolved oxygen.

METHODS

Bulk milk. Except where otherwise indicated, the milk used was combined evening and morning milk from the Institute herd of Friesian cows. It was collected into a refrigerated stainless steel tank fitted with a mechanical agitator, and stored at about 5 °C. After thorough mixing, samples were withdrawn for test when the combined milk was about 3-h old.

Pasteurized milk. The milk was pasteurized at 72 °C (holding for 15 s) in a commercial A.P.V. plate-type heat exchanger, and cooled to about 5 °C before sampling.

Flushing with gas. For some experiments the milk was flushed with air or with nitrogen or with carbon dioxide before being bottled. The procedure for flushing with nitrogen and carbon dioxide was as follows. A brisk stream of oxygen-free gas passed through a sintered glass diffuser into 1 l of milk contained in a 2-l conical flask. After about 20 min, when the partial pressure of oxygen in the milk (see below) was below 5 mm Hg, the milk was carefully dispensed into 2-oz 'medical flat' clear glass bottles, which were filled to overflowing. A stream of gas was bubbled into each bottle in turn through a length of plastic cannula tubing, displacing about 5 ml milk with a froth of gas bubbles. As the tube was withdrawn the bottles were quickly sealed with rubber lined metal screw caps.

A similar procedure was employed for preparing the aerated milks. The raw and the pasteurized milks from which the test samples were taken were in equilibrium with atmospheric oxygen at 5 °C, and so the effect of this flushing with air at the laboratory temperature of about 20 °C was to reduce the P_{O_2} from about 190 mm Hg to about 155 mm Hg.

Sterilization. Some of these 2-oz bottles of milk were heated in a steam autoclave for 20 min at 111 °C, and on removal cooled to 5 °C.

Exposure to sunlight. Bottles of milk were placed outdoors on cloudless sunny days in March 1967, in full exposure to the sun.

Measurement of dissolved oxygen. Oxygen tension was determined with a Clark-type oxygen cathode (Clark, 1956). The electrode and its water jacket were manufactured by Radiometer A/S (Copenhagen) and were used in conjunction with a specialized pH meter, Radiometer model 27 GM. The apparatus was calibrated with water at 25 °C in equilibrium with atmospheric oxygen. The P_{O_2} value was calculated from the equation:

$$P_{O_2} = \frac{20{\cdot}93}{100} \times (\text{barometric pressure} - \text{partial pressure of water vapour at 25 °C}).$$

The scale zero was set with an oxygen-free solution, prepared by dissolving 1 g crystals of sodium sulphite in 50 ml freshly boiled 0·01 M-borax solution. All measurements were made at 25 °C.

Methods of vitamin assay. Ascorbic acid and dehydroascorbic acid were determined by the differential assay procedure of Kon & Watson (1936), which involves the titration of duplicate samples of the deproteinized milk before and after a treatment with H_2S designed to convert any dehydroascorbic acid to the reduced form. Dehydroascorbic acid was estimated by difference.

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

Vitamin B_{12} was assayed microbiologically with *Lactobacillus leichmannii*, as described by Gregory (1954).

Thiamine was assayed with *Lactobacillus viridescens*, as described by Deibel, Evans & Niven (1957). Vitamin B_6 was assayed with *Kloeckera brevis* as described by Barton-Wright (1963), except that the test samples were heated with 0·055 N-HCl for 30 min at 100 °C as recommended by Gregory (1959) and were not predigested with taka-diastase. Riboflavin, nicotinic acid and biotin were assayed by standard microbiological procedures described in earlier publications (Ford, Gregory, Porter & Thompson, 1953; Chapman *et al.* 1957).

RESULTS AND DISCUSSION

Oxygen content of milk in the udder. Three 2nd-lactation Friesian cows were selected from the Institute herd, each giving 40–50 lb milk/day of very low bacterial count. At the time of the afternoon milking, a forequarter of each cow's udder was drained with a teat cannula and milk samples taken at intervals for P_{O_2} measurement, representative of foremilk, main flow and strippings. The samples were collected into foil-wrapped $\frac{1}{2}$ oz glass bottles which were filled to overflowing and tightly stoppered to exclude air.

In the course of sampling, the stream of milk issuing from the cannula was briefly exposed to contamination with atmospheric oxygen. In a further series of samples taken from the same cows on a later date, this source of error was avoided by collecting the milk under medicinal paraffin. In addition to four $\frac{1}{2}$-oz samples taken from each cow, the remainder of the milk from the quarter—in total about 500–600 ml—was collected under paraffin and its P_{O_2} taken as representative of the average P_{O_2} of the milk within the quarter.

Besides these samples of milk from healthy cows, samples were also taken from 2 cows with subclinical mastitis; one (231) had a staphylococcal infection and the other (162) a coliform infection.

The P_{O_2} measurements were made within minutes of taking the samples. The milk samples were withdrawn from the sample bottles into a syringe fitted with a '0' gauge, $2\frac{1}{2}$-in. stainless steel needle and injected into the measurement cell. The results are shown in Table 1.

Table 1. P_{O_2} *(mm Hg) of milk taken from the udder with minimal exposure to the atmosphere*

	Cow no.				
	251	324	327	162*	231*
Sample 1 (foremilk)	42·4 (42·3)	47·8 (35·3)	43·4 (48·0)	(27·7)	(27·3)
Sample 2 (main flow)	42·3 (42·7)	44·7 (41·4)	41·2 (49·5)	(37·3)	(26·6)
Sample 3 (main flow)	40·2 (42·6)	40·3 (32·5)	40·3 (48·2)	(21·6)	(24·7)
Sample 4 (strippings)	37·3 (46·0)	36·1 (25·7)	29·5 (40·2)	(14·7)	(21·8)
Bulked milk (see p. 241)	(42·0)	(31·7)		(23·3)	

Values given in parentheses are for samples collected under paraffin. Other values are for samples that had been exposed briefly to air during collection.

* Cows with subclinical mastitis.

There was marked variation in P_{O_2} between foremilk and strippings, and between corresponding samples taken from different cows or from the same cow on different days. Some part of this variation was, no doubt, caused by the technical difficulty of ensuring complete exclusion of air during the collection and manipulation of the test samples. The P_{O_2} values tended to fall during emptying the quarter, and to be relatively low in the samples from the 2 mastitis-infected cows. Comment on the significance of these differences must await a more detailed study now in progress. For present purposes it is sufficient to record the oxygen tension of milk in the healthy bovine udder as being approximately 35 mm Hg.

Changes in oxygen content during processing. In samples taken during the afternoon milking, from the milk line at the point of entry into the refrigerated collection tank, the P_{O_2} measured about 107 mm Hg, and so was already considerably higher than that of milk in the udder. The milk was presumably near saturation with oxygen at the reduced pressure—nominally $\frac{1}{2}$ atmospheric—obtaining in the milk line and ancillary collecting vessels. During the period of cooling to 5 °C in the tank, assisted by the action of a paddle, the P_{O_2} rose further to 145 mm Hg at 30 min after the completion of milking, and to 192 mm Hg after storage overnight. About 2 h after the admission of the morning milk into the bulk tank and immediately before HTST pasteurization, the P_{O_2} was 210 mm Hg. It fell only a little during pasteurization and measured 204 mm Hg in the effluent from the cooler. Sterilization in-bottle under laboratory conditions (p. 240) caused a considerably greater fall in oxygen tension, from 210 to 42 mm Hg. In commercial milk, sterilized in-bottle by the batch process, there was similarly large fall in P_{O_2}, the change overall between the raw milk in the collecting tank and the freshly sterilized milk being from 166 to 44 mm Hg.

Changes in ascorbic acid and in oxygen content on exposure of milk to sunlight. Fig. 1 shows the rapid oxidation of ascorbic acid to dehydroascorbic acid under the action of sunlight on pasteurized milk, and the subsequent slower rate of disappearance of the dehydroascorbic acid. These results are much the same as those obtained over 30 years ago by Kon & Watson (1936), except that in the present study no greater loss

of dehydroascorbic acid was found during exposure for 120 min than during 30 min. Kon & Watson (1936) reported a continuing fall in dehydroascorbic acid, though at a slower rate than the initial rapid rate of oxidation of ascorbic acid to dehydroascorbic acid. They did not state, however, whether in their experiments the milk was exposed in vessels open to the air.

The oxygen tension in the pasteurized milk fell rapidly, from 187 to 13 mm Hg during 30-min illumination, and to 4 mm Hg after 60 min. By comparison, the residual free oxygen in the in-bottle sterilized milk fell more slowly, from the initially lower P_{O_2} of 65 to 30 mm Hg after 30 min, and to 29 mm Hg after 60 min. In pasteurized milk flushed with nitrogen, the P_{O_2} was initially 8 mm Hg and fell to zero during 60 min exposure to sunlight. There was no significant fall in ascorbic acid, thus again confirming the finding of Kon & Watson (1936) and others.

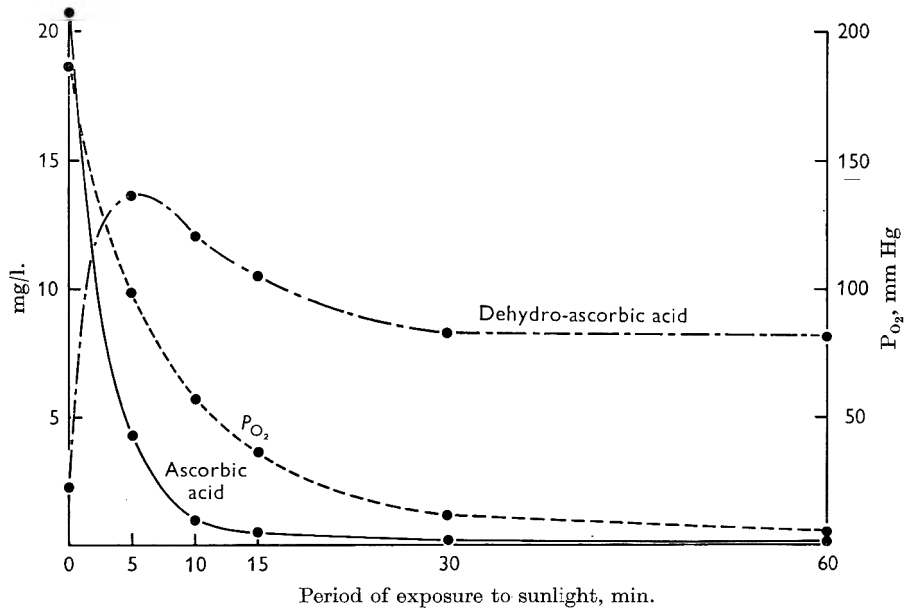

Fig. 1. Changes in ascorbic acid and in oxygen content of pasteurized milk on exposure to sunlight.

Changes in content of B-complex vitamins during in-bottle sterilization and subsequent exposure to sunlight. Fig. 2 shows the effects on 7 B-complex vitamins of in-bottle sterilization followed by storage for 3 days at 2 °C and at 21 °C, in total darkness and with exposure to sunlight for 1, 3 and 8 h. The different treatments were applied to milk flushed with oxygen-free nitrogen and to milk flushed with air before being bottled. In addition, for assays of vitamin B_{12} and folic acid, a third series of samples was prepared from milk that had been flushed with carbon dioxide.

Folic acid. In-bottle sterilization and subsequent storage in darkness caused no loss of folic acid from the nitrogen-gassed milk (Fig. 2). Sunlight caused a small loss amounting to about 10%. It was not established that this loss increased with increasing time of exposure. Of 3 experiments none showed significantly greater loss after 8 h than after 1 h.

In milk flushed with carbon dioxide, the sterilization process caused a somewhat

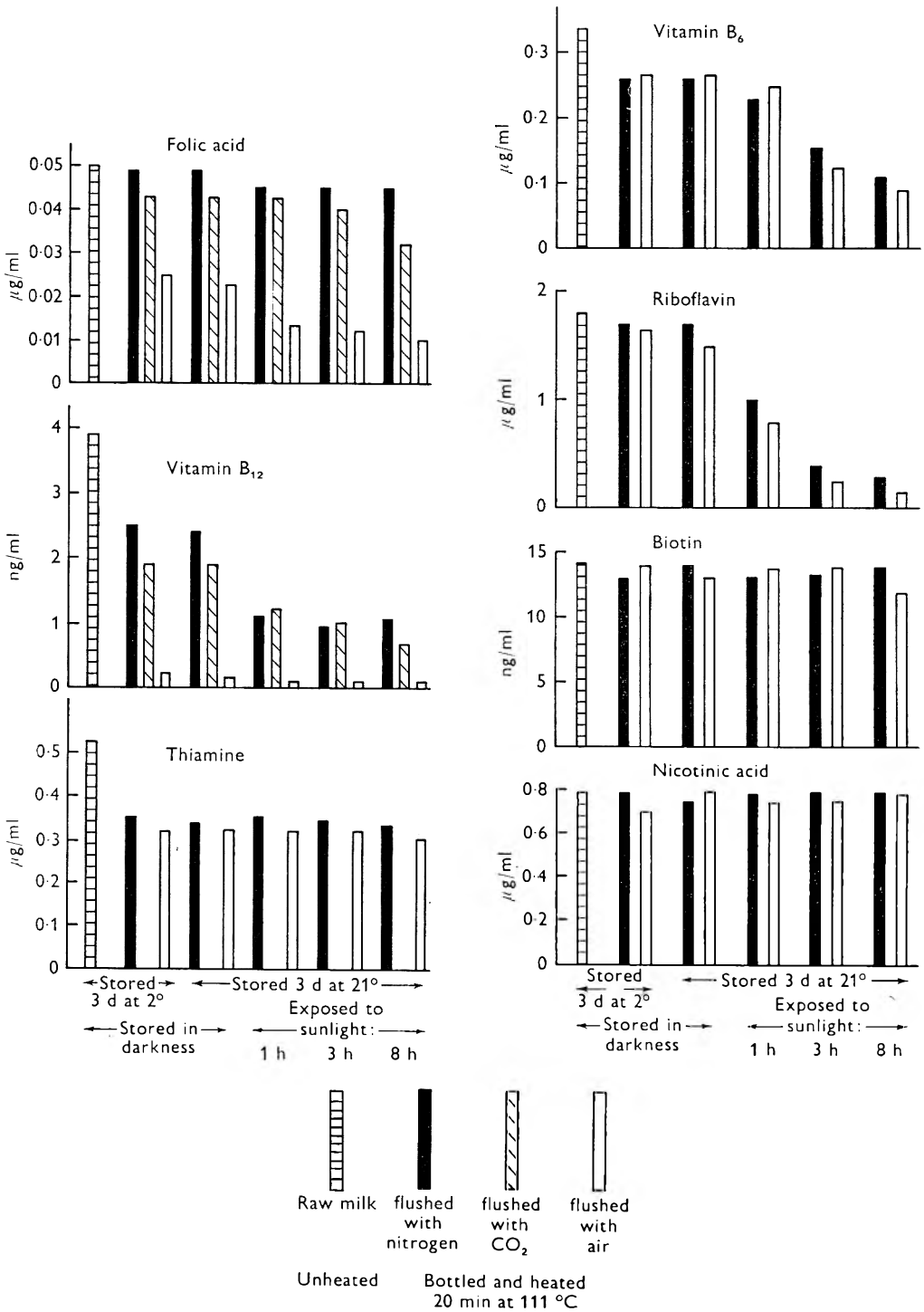

Fig. 2. Influence of the dissolved oxygen in milk on the stability of some vitamins of the B-complex towards heating and during subsequent storage and exposure to sunlight.

increased loss of folic acid of about 12%, increasing to 36% during the subsequent 8-h exposure to sunlight. This greater loss was possibly related to the initially lower pH value of the carbonated milk and not to any specific effect of carbon dioxide. The pH value of the carbonated milk was 6·0, as against 6·7 in the nitrogen-gassed milk.

Sterilization of the aerated milk destroyed about 50% of its folic acid, and subsequent exposure to sunlight caused a progressive further loss. After 8-h exposure the total loss was 80%.

The extent of the loss of folate during heat processing of bulk aerated milk is determined by the initial concentration of reduced ascorbic acid in the milk and by the severity of the heating. A comparison was made of the loss of folate from bulk raw milk containing 20 mg/l reduced ascorbic acid, during HTST pasteurization and during heating in sealed pint bottles for 20 min at 97 °C and at 115 °C. In 2 experiments the losses during HTST pasteurization were 0 and 12%; heating at 97 °C destroyed 23 and 30%, and sterilization at 115 °C destroyed 35 and 50%. When the unheated milk was supplemented with 1 mg ascorbic acid/ml, added as a neutral solution of sodium ascorbate, there was no loss of folate during subsequent heating.

Oxidation of the natural reduced ascorbate of the milk by addition of the calculated amount of indophenol reagent, or by minimum exposure to light, did not in itself cause loss of folate. However, on heating the oxidized milks for 20 min at 97 °C, 83% of the folate was destroyed, and after 20 min at 115 °C the loss was 94%. When the oxidized milks were supplemented with 1 mg ascorbic acid/ml, added as a neutral solution of sodium ascorbate, and then heated at 115 °C, no loss of folate was observed.

Vitamin B_{12}. As with folic acid, the destruction of vitamin B_{12} in milk undergoing sterilization is essentially oxidative and linked directly or indirectly with the oxidative degradation of ascorbic acid (Ford, 1957). In the present study, sterilization of aerated milk in-bottle caused almost complete destruction of vitamin B_{12}. In milk flushed with nitrogen or with carbon dioxide, the vitamin was comparatively stable, the losses on sterilization being respectively, 35 and 52%. Exposure to sunlight caused further loss, and in the course of 1 h the sterilized nitrogen-gassed milk lost more than half of its residual content of vitamin B_{12}. Thereafter the rate of loss was slower; in 2 experiments there was no significantly greater loss of vitamin B_{12} after exposure for 8 h than after 1 h. In the milk flushed with carbon dioxide, there was again a sharp fall in vitamin B_{12} during the first hour of exposure and thereafter a progressive slower fall.

Thiamine. Sterilization destroyed about 38% of the thiamine, and exposure to sunlight caused no further significant loss. The vitamin was marginally more stable towards heating in the nitrogen-gassed milk.

Vitamin B_6. Sterilization and storage for 3 days caused a 25% fall in vitamin B_6, and during 8 h exposure to sunlight 60% of the residual vitamin was destroyed. Removal of dissolved oxygen by flushing the milk with nitrogen made no significant difference to the extent of these losses.

Riboflavin. Sterilization and storage for 3 days at 2 °C caused a loss of about 7%; in the sterilized aerated milk stored at 21 °C the loss was 16%. Thereafter, the content of riboflavin fell steadily during exposure to sunlight, and after 8 h only about 10% remained. The vitamin seemed marginally more stable in the nitrogen-gassed

milk, but the small differences observed might perhaps be attributable to the influence of unequal exposure to subdued room daylight in the course of measurement.

Nicotinic acid and biotin. Both vitamins were stable towards heating and during exposure to sunlight.

CONCLUSIONS

The results underline yet again that in the presence of dissolved oxygen, several important nutrients in milk may suffer severe loss during heat processing and on exposure to daylight during distribution. In order to minimize these losses it is clearly essential to eliminate the dissolved oxygen from the milk before heat processing, and to protect the milk from light during distribution and storage. Ideally, atmospheric oxygen should be excluded during bottling or packaging; the milk should then be refrigerated without delay, and the time interval between processing and utilization should be kept short.

Hand *et al.* (1938) devised a practical procedure for freeing milk from dissolved oxygen. In this procedure, which they called vacuum cooling, the warmed milk was boiled under the action of a vacuum pump, and the evolution of water vapour flushed the dissolved oxygen from the milk. The milk was then protected from access of air during bottling and storage, and by this means it proved possible to avoid flavour defects caused by oxidation and to preserve the reduced ascorbic acid naturally present in the milk.

Some of the considerations relevant to the preservation of ascorbic acid in milk apply equally to folic acid. In presence of free oxygen both vitamins are unstable to heating. Again in the presence of free oxygen, riboflavin catalyses the photo-oxidation of folic acid as of ascorbic acid (Biamonte & Sehneller, 1951). Both vitamins may be of special importance to the bottle-fed infant, for whom milk constitutes the major source of nutriment. There is no precise information concerning the minimal folic acid requirement of the nursing child, but it seems likely that the folate content of milk formulas is important and may indeed be critical in the child's nutrition. In our experience (Ford & Scott, unpublished) mature human milk and cow's milk contain between 40 and 80 μg folate/l. Naiman & Oski (1964) gave a range 62–100 μg/l for cow's milk, and reported that preparations of the milk formula 'SMA' (John Wyeth & Brother Ltd.) contained substantially less, between 9 and 29 μg/l. They found wide variation in the folate content of autoclaved milk. Thus, the content in autoclaved evaporated milk ranged from 8 to 86 μg/l. Surprisingly, they found no significant correlation between the lability of the folate activity during autoclaving and the ascorbate content of the milk.

The same authors suggested that the megaloblastic anaemia associated with excessive consumption of goat's milk may be attributable to the low folate content of the milk—about 11 μg/l.

Matoth, Pinkas & Sroka (1965) measured the folate content of whole blood of breast fed and artificially fed infants, and found the levels in the artificially fed infants to be comparatively low, and positively correlated with the economic status of the mothers. The blood folate values in the breast fed infants were uniformly high, and unrelated to economic status. The authors also measured the folate content in 35 samples of breast milk and found values ranging from 7·4 to 61 μg/l, with a mean of 24 μg/l. For 5 samples of raw cow's milk they found values from 16·8 to 63·2 μg/l;

for 2 samples of sterilized milk they gave values of 8·6 and 10 μg/l, and for 2 samples of reconstituted whole milk powder they found 13·2 and 16·1 μg/l. They state categorically that in the artifically fed infant the dietary supply of folate is inadequate, and especially so in the infant being given re-heated pasteurized or sterilized milk.

The destruction of vitamin B_{12} during sterilization of milk 'in-bottle' or 'in-can' is related to the process of oxidation of the ascorbic acid and is probably a side reaction with one of the intermediate products (Ford, 1957). The addition of ascorbic acid to milk enhances the destruction of vitamin B_{12} caused by heating. With folic acid, on the other hand, added ascorbic acid preserves the vitamin during heating by maintaining it in its relatively stable reduced form. But though these different considerations apply, it is the oxidative destruction of the milk ascorbic acid that leads to the destruction of the vitamin B_{12} and folic acid, and it is this primary reaction that is effectively prevented by thorough removal of oxygen from the milk before heat processing.

It is an interesting question whether the loss of vitamin B_{12} and folic acid that took place during exposure of the sterilized nitrogen-gassed milk to sunlight was limited by the availability of oxygen. With both vitamins there was no significantly greater loss after 8 h exposure than after 1 h, and as the milk was not entirely free of oxygen at bottling it is possible that the extent of the losses was a measure of the amount of this residual oxygen present.

For the remainder of the B-complex vitamins examined in this study—thiamine, riboflavine, nicotinic acid, vitamin B_6 and biotin—stability towards heating and during exposure to sunlight was affected little, if at all, by the oxygen tension in the milk. It is of interest in this connexion that Lück & Schillinger (1959) heated milk under 8 atmospheres pressure of oxygen and found no loss of riboflavin, thiamine or vitamin B_6.

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The sensitivity of bovine staphylococci, streptococci and corynebacteria to cloxacillin and various other antibiotics

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Summary. The sensitivities of isolates of bovine staphylococci, corynebacteria and streptococci to various antibiotics were determined. The growth of various strains of *Staphylococcus aureus* was inhibited by the following range of concentrations of antibiotic: cloxacillin, 0·07 to 0·6 μg/ml; penicillin G, 0·018 to > 250 μg/ml; streptomycin, 1·25 to > 250 μg/ml; novobiocin, 0·15 to 25 μg/ml; chlortetracycline, 0·6 to 10 μg/ml. The concentrations of cloxacillin required to inhibit growth were between 0·15 and 1·25 μg/ml for strains of *Streptococcus agalactiae*; 0·07 to 0·3 μg/ml for strains of *Streptococcus dysgalactiae* and 0·15 to 0·6 μg/ml for strains of *Streptococcus uberis*.

The corynebacteria were generally sensitive to the penicillins (ampicillin, penicillin G, phenethicillin and cloxacillin), but showed least sensitivity to cloxacillin which had a similar activity to that of chlortetracycline, neomycin and oleandomycin. Chloramphenicol and streptomycin were less active than the penicillins and novobiocin showed little activity against *Corynebacterium ulcerans*.

A clear relationship was confirmed between the sensitivity of staphylococci to cloxacillin and penicillin G. Some indication was found of an association between the sensitivity of staphylococci to cloxacillin and the success of cloxacillin intramammary therapy in the non-lactating udder. There was no evidence of a change in the sensitivity of *Staph. aureus* following exposure to cloxacillin in the non-lactating udder.

In most respects penicillin G has been the ideal antibiotic for mastitis therapy. Its outstanding characteristic is a high antibacterial action, yet it remains almost non-toxic to humans and animals, but with the increasing resistance of strains of *Staph. aureus* to penicillin G, the antibiotic has lost much of its attraction. The increase of penicillin G resistant staphylococci is mainly a process of substitution, the sensitive strains having been eliminated by treatment, thereby allowing the penicillin-resistant strains to proliferate. The strains of *Staph. aureus* which produce penicillinase are generally distinct from penicillin-sensitive strains, and according to Barber,

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penicillinase negative strains have not been observed to become penicillinase-producing strains (Barber, 1947, 1962). Since the isolation of the penicillin nucleus (Batchelor, Doyle, Nayler & Rolinson, 1959), rapid progress has been made in developing new semi-synthetic penicillins with specific desirable properties. One of these, cloxacillin, which was used in this study, inhibits growth of most bovine staphylococci and streptococci at a concentration of 1·0 μg/ml (Jones, Bagley & Cleverly, 1962). However, it has been reported that strains of staphylococci can become tolerant to increased levels of cloxacillin, but single step mutants of high resistance have not been encountered. It is, therefore, possible that slight resistance of staphylococci to cloxacillin may develop (Jones *et al.* 1962; Nayler *et al.* 1962).

The object of this study was to determine the sensitivity to cloxacillin and to various other antibiotics of the staphylococci, streptococci and corynebacteria isolated during a field experiment and also (*a*) to relate the sensitivity of staphylococci to cloxacillin with the sensitivity to penicillin G and with the success of therapy, and (*b*) to determine whether the resistance of staphylococci to cloxacillin increased following antibiotic therapy at drying-off.

EXPERIMENTAL

Bacteria were isolated from the milk of cows in 35 herds during 1965–6. Bacteriological examinations were made on the fresh aseptically taken samples of secretions from each quarter collected 7 days before the animals were dried off, and also at calving and within 7 days after calving. The cows in each herd were allocated at random to one of the following experimental treatments:

(1) No antibiotic therapy (control).

(2) Each quarter infused with a preparation containing 0·2 g of cloxacillin as the sodium salt in a 3 % aluminium monostearate base (Orbenin, L.A., Beecham Research Laboratories, Brentford, Middlesex, England) after the last milking of the lactation.

(3) Each quarter infused with a preparation containing 1 g of cloxacillin as the benzathine salt in a 3 % aluminium monostearate base after the last milking of the lactation (Smith, Neave, Dodd & Brander, 1966; Smith, Neave, Dodd, Jones & Gore, 1967).

METHOD

The bacteria tested for sensitivity to cloxacillin and other antibiotics were isolated on aesculin ox-blood agar plates and subcultured once before sensitivity testing.

The sensitivity of *Staph. aureus* was determined by the growth on nutrient agar (Oxoid CM 55) containing serial dilutions of the test antibiotics. Bovine serum was added (10 %, v/v) to the medium for streptococci, and bovine serum (10 %, v/v) and glucose (5 %, w/v) for corynebacteria. In all cases, a loopful (2 mm diam.) of an undiluted overnight broth culture was used as inoculum. Plates were examined for bacterial growth after incubation for 24 h at 37 °C and again after 48 h incubation. The minimum inhibitory concentration (M.I.C.) of an antibiotic was taken as the lowest concentration of the antibiotic which completely inhibited growth of the test organism.

Of the 863 isolates of *Staph. aureus* tested for sensitivity to cloxacillin and penicillin

G (benzylpenicillin), 767 of them were also tested for sensitivity to streptomycin, 666 to novobiocin and 258 to chlortetracycline.

A selection of streptococci and corynebacteria was also tested for sensitivity to cloxacillin. Also, since quarters infected with *C. ulcerans* did not in general respond well to antibiotic therapy, the sensitivity of corynebacteria to the following antibiotics was determined: ampicillin, chloramphenicol, chlortetracycline, neomycin, novobiocin, oleandomycin, penicillin G, phenethicillin and streptomycin.

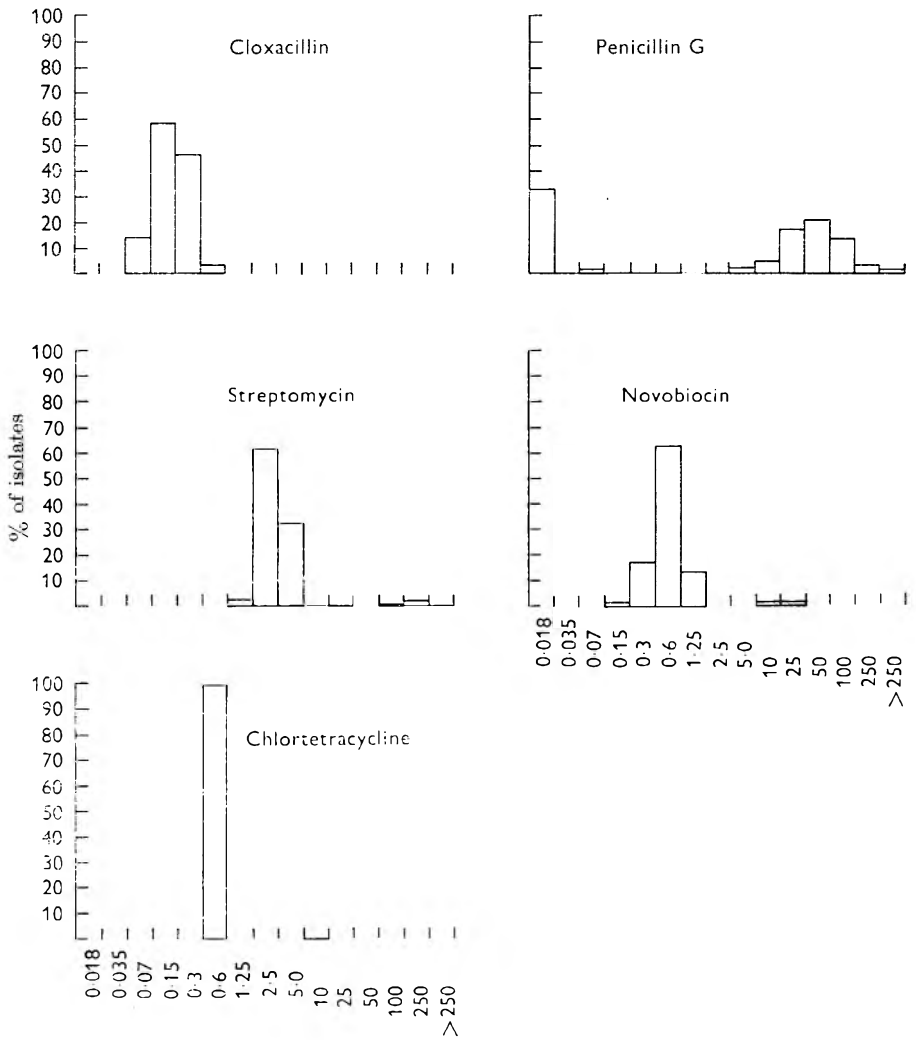

Fig. 1. Frequency distributions of the sensitivity of isolates of *Staph. aureus* to various antibodies.

RESULTS

Inhibition of Staph. aureus. The minimum concentration of various antibiotics required to inhibit growth of isolates of *Staph. aureus* are shown in Fig. 1.

The isolates of *Staph. aureus* fell, as expected, into 2 distinct groups in respect of

their sensitivity to penicillin G. The division between these 2 groups occurred at a penicillin G concentration about 0·3 μg/ml. 35·5 % of the isolates were in the more sensitive group and their growth was inhibited at 0·3 μg/ml or less. In the less sensitive group the inhibitory concentrations ranged from about 3 to > 7250 μg/ml; growth of about half of the isolates was inhibited by penicillin G at less than 10 μg/ml and growth of only 5 % of the isolates required 250 μg/ml or higher for inhibition.

Growth of all the isolates was inhibited by cloxacillin at concentrations of 0·07–0·6 μg/ml.

Of the 767 isolates tested 96·5 % was sensitive to streptomycin at concentrations of 1·25–5 μg/ml whereas only 2·9 % required at least 100 μg/ml for inhibition.

Table 1. *The sensitivity of* C. ulcerans *and* C. bovis *to antibiotics*

	C. ulcerans		*C. bovis*	
Antibiotic	No. of isolates	Range of M.I.C.* μg/ml	No. of isolates	Range of M.I.C. μg/ml
Ampicillin	17	0·15–0·6	18	0·07–0·15
Cloxacillin	22	0·3–5	18	0·3–2·5
Penicillin G	22	0·018–0·15	18	0·035–0·15
Phenethicillin	17	< 0·07–0·3	18	0·07–0·6
Chloramphenicol	7	< 5–10	18	< 2·5–5
Chlortetracycline	7	< 1·25–5	18	< 0·6–1·25
Neomycin	7	1·25–2·5	18	< 0·6
Novobiocin	7	10–25	18	< 0·3–0·6
Oleandomycin	7	< 0·3–5	18	0·3–0·6
Streptomycin	7	5–10	18	< 2·5–5

* Minimum inhibitory concentration.

With the exception of one isolate which required 10 μg/ml of chlortetracycline to inhibit growth, all were inhibited at 0·6 μg/ml.

Most (95 %) of the 666 isolates tested were inhibited by novobiocin at a concentration of 0·15–1·25 μg/ml. For 11 isolates the concentration required to inhibit growth was 25 μg/ml and for 22 isolates it was between 2·5 and 25 μg/ml.

Inhibition of Corynebacteria. The minimum concentrations of the various antibiotics required to inhibit growth of *Corynebacterium bovis* and *C. ulcerans* are indicated in Table 1. All corynebacteria tested were sensitive to penicillin G; *C. bovis* showed a similar sensitivity to ampicillin and penicillin G, but was less sensitive to cloxacillin. *C. ulcerans* was most sensitive to penicillin G, less so to ampicillin and considerably less so to cloxacillin. The corynebacteria tested were almost as sensitive to phenethicillin as to penicillin G.

The sensitivities of the corynebacteria to chlortetracyline, neomycin and oleandomycin were similar to that to cloxacillin. Chloramphenicol and streptomycin were less active than the penicillins. Novobiocin showed little activity against *C. ulcerans*, but inhibited *C. bovis* at 0·6 μg/ml or less.

Inhibition of Streptococci. The sensitivity of 40 isolates of streptococci to cloxacillin was measured. The strains comprised 20 isolates of *Str. agalactiae*, 12 of *Str. dysgalactiae* and 8 of *Str. uberis*. The minimum concentrations of cloxacillin required to inhibit growth were within the ranges 0·15–1·25, 0·07–0·3 and 0·15–0·6 μg/ml, respectively.

The relationship between the sensitivity of staphylococci to cloxacillin and to penicillin G

In Table 2, the 863 isolates of *Staph. aureus* have been grouped according to their sensitivity to cloxacillin, and in each of the groups is shown the number which was sensitive to 0·3 μg/ml or less of penicillin G.

Of all the staphylococci isolated 35·5 % were sensitive to penicillin G; these sensitive strains included 92·6 % of the isolates which were very sensitive to cloxacillin (growth inhibited by 0·07 μg/ml cloxacillin). Of the isolates least sensitive to cloxacillin (growth inhibited at minimum cloxacillin concentration 0·3 μg/ml), most were to some degree resistant to penicillin G; only 6·1 % of these isolates were classed as sensitive to penicillin G. The relationship between the resistance of isolates of *Staph. aureus* to cloxacillin and their resistance to penicillin G, illustrated in Table 2, was found to be statistically significant by χ^2 analysis ($P > 0{\cdot}001$).

Streptomycin resistance, found only in the isolates from a few herds, was almost invariably associated with some degree of resistance to penicillin G. Similarly, resistance to novobiocin was accompanied by resistance to penicillin G for the staphylococci isolated from 2 herds.

Table 2. *The relationship between the sensitivity of strains of staphylococci to cloxacillin and their sensitivity to penicillin G*

M.I.C.* Cloxacillin, μg/ml	No. of isolates	% isolates sensitive to 0·3 μg/ml or less of penicillin G
0·07	121	92·6
0·15	508	35·4
0·3	231	6·1
0·6	3	0
Total	863	35·5

* Minimum inhibitory concentration.

The response of Staph. aureus *infections to therapy in relation to the sensitivity of the strains of staphylococci to cloxacillin*

During the course of the trial, 214 of the 287 quarters which were infected with *Staph aureus* at drying-off were cleared from infection by cloxacillin therapy at drying-off. The sensitivity to cloxacillin of 278 of the 287 isolates was measured.

76·5 % of the quarters which were infected with staphylococci sensitive to cloxacillin at a concentration of 0·07 μg/ml were freed from infection by the 0·2 g cloxacillin preparation which tests showed persisted for about 1 week in the dry udder (Smith *et al.* 1967). However, only 48·6 % of the infections were eliminated when the staphylococcal isolates were sensitive to cloxacillin at a minimum inhibitory concentration of 0·3 μg/ml. The results of infusions of 1 g cloxacillin as the benzathine salt which persisted for about 3 weeks, showed a similar trend, but the percentage of cures was much higher. In this study, the response to therapy, as measured by the

proportion of quarters cleared from infection, decreased when the sensitivity of the staphylococci to cloxacillin decreased (Table 3). The correlation was not, however, statistically significant.

Table 3. *The relationship between the sensitivity of* Staph. aureus *to cloxacillin and the response to therapy*

Antibiotic therapy	Isolates sensitive to cloxacillin at μg/ml		
	0·07	0·15	0·3–0·6
0·2 g cloxacillin as sodium salt at drying-off			
No. of infections treated	17	82	35
No. of infections eliminated	13	55	17
Percentage infections eliminated	76·5	67·1	48·6
1 g cloxacillin as benzathine salt at drying-off			
No. of infections treated	16	91	37
No. of infections eliminated	15	77	30
Percentage infections eliminated	93·7	84·6	81·1

Table 4. *The sensitivity to cloxacillin of* Staph. aureus *isolated at drying-off and at calving from quarters with infection persisting through the dry period*

	Antibiotic therapy at drying-off (combined experimental groups 2 and 3)	Untreated control
No. of persistent infections	71	159
Proportion of isolates showing the same sensitivity at calving and at drying-off	56·3 %	48·0 %
Proportion of isolates with M.I.C.* one serial dilution higher at calving than at drying-off (less sensitive)	11·3 %	23·2 %
Proportion of isolates with M.I.C.* one serial dilution lower at calving than at drying-off (more sensitive)	32·4 %	28·8 %

* Minimum inhibitory concentration.

The sensitivity to cloxacillin of Staph. aureus, *isolated at drying-off and at calving*

Staphylococci, isolated from quarters in which infection persisted throughout the dry period despite the infusion of cloxacillin at the time of drying-off, showed no apparent difference in their sensitivity to cloxacillin from those isolated at the time of drying-off (Table 4). Of the 287 quarters infected with staphylococci, 73 infections were not eliminated by therapy. For 71 of these infections, the sensitivity of the relevant isolates of staphylococci to cloxacillin was determined at drying-off and at calving. Similar measurements were made for the staphylococcal isolates from 159 quarters which did not receive antibiotic therapy. Both for treated and untreated quarters, the sensitivities of staphylococci isolated at drying-off and at calving did not differ by more than one serial dilution in the M.I.C. tests. There was no evidence of a systematic change in the sensitivity of the isolates.

DISCUSSION

This study confirms previous reports for the sensitivity of *Staph. aureus* and streptococci to cloxacillin (Nayler *et al.* 1962; Jones *et al.* 1962; Sidell, Bulger, Brodie & Kirby, 1964). More than 99% of the staphylococci isolated from infected quarters were sensitive to cloxacillin at concentrations of 0·3 μg/ml or less, and all were sensitive at 0·6 μg/ml. All isolates of streptococci were sensitive to cloxacillin at a concentration of 1·25 μg/ml or less. Cloxacillin is therefore potentially a highly effective antibiotic for the treatment of staphylococcal and streptococcal infections of the udder. About a third of the staphylococcal isolates showed a high degree of resistance to penicillin G. Some resistance to streptomycin and novobiocin were also found, but only one isolate showed some resistance to tetracycline. Similar resistance patterns for bovine staphylococci were reported by Wilson (1961) and Jones *et al.* (1962).

The relationship between the sensitivity of human strains of staphylococci to cloxacillin and to penicillin G was also confirmed for bovine strains in this present series. The use of one antibiotic may therefore result in bacterial resistance to other antibiotics (Nayler *et al.* 1962).

There was no evidence of a systematic change in the sensitivity of *Staph. aureus* isolated from infected quarters to cloxacillin following antibiotic therapy at drying-off; but there was an indication that even though the range in sensitivity of staphylococci to cloxacillin was small, it was related to the success of the therapy in eliminating infection. Of the infections caused by the most sensitive staphylococci, 76% were eliminated by the 0·2 g cloxacillin preparation compared with only 48% of the infections caused by the more resistant staphylococci. The relationship which was evident to a lesser degree with the 1 g cloxacillin preparation was, however, not statistically significant; because of its importance it requires further investigation.

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The effect of the microbial flora on the flavour and free fatty acid composition of Cheddar cheese*

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Summary. Comparisons were made of the flavour, free fatty acids and bacterial flora of commercial cheese made at different factories and experimental cheese made under aseptic conditions: (i) with δ-gluconic acid lactone instead of starter, (ii) with starter only, (iii) with starter and added floras derived from the curd of the commercial cheeses (reference flora cheeses).

Comparison of the bacterial flora of commercial and reference flora cheeses showed that replication of organisms was better with some reference floras than with others. In all the cheeses the lactobacilli increased in numbers during maturation, whilst other groups of organisms died out.

The amount of acetic acid present was influenced by the starter and by the lactobacilli. Single-strain starters produced some acetic acid, most of which was lost in the whey; commercial starters produced considerably more, due to the presence in them of *Streptococcus diacetilactis*. Later in maturation lactobacilli increased the acetic acid content, a greater increase being observed with homo- than with heterofermentative strains.

The initial levels of butyric and higher fatty acids in the milk varied with source of the milk and with the season, summer milk having higher levels than winter milk. During cheese-making a slight increase of these acids occurred in every cheese made with starter and a further small increase occurred during ripening. However, there was no increase in the content of these acids in the cheese made with δ-gluconic acid lactone, indicating that lactic acid bacteria were weakly hydrolysing the milk fat.

Flavour trials showed that Cheddar flavour was present not only in the reference flora and commercial cheese, but also in the cheese made with starter only. Different starters produced different intensities of flavour; one strain produced an intense fruity off-flavour. Cheeses made with δ-gluconic acid lactone were devoid of cheese flavour.

Because of the variation and complexity of the microflora of raw milk and of cheese made from either raw or heat-treated milk, it is a difficult problem to identify the bacteria responsible for the development of the characteristic flavour of Cheddar

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cheese. A logical approach was to make cheese from heat-treated milk and to prevent recontamination by adventitious bacteria during the cheesemaking process. This was achieved successfully by means of aseptically operated cheese vats (Mabbitt, Chapman & Sharpe, 1959; Chapman, Mabbitt & Sharpe, 1966). A second step was to add to the heat-treated cheese-milk a raw milk flora which permitted a good replication of a raw milk flora throughout many experiments (Reiter, Fryer & Sharpe, 1965; Fryer, Sharpe & Reiter, 1966). A further step was to utilize the technique developed by Mabbitt, Chapman & Berridge (1955) for producing cheese without a starter, by the use of δ-gluconic acid lactone. These techniques provided a means for assessing the contribution of the starter and other bacteria to the cheese flavour, and for determining overall chemical changes in the maturing cheese caused by bacteria and by native milk enzymes. These methods of investigation have obvious advantages over those used in earlier studies, in which attempts were made to correlate flavour in randomly chosen cheeses with the composition of their microfloras, or to analyse the effects on the flavour of the cheese of the addition of micro-organisms to cheese milk (Sherwood 1939; Robertson & Perry, 1961).

In the present investigation, the microflora of cheese curds isolated from curds at different creameries (reference floras) were used in the preparation of experimental cheeses instead of using microfloras from raw milk. The flavours of cheeses were compared with those of their commercial counterparts and with those of cheeses made with starter only or with δ-gluconic acid lactone. In addition, the effect of the bacterial flora on the hydrolysis of fat was determined.

METHODS

Cheese-making

The 5 commercial cheeses were manufactured in 5 cheese factories in the West of England according to standard factory practice. All the cheese-milks were preheated to at least 155 °F for 15–20 s.

The experimental cheeses were made from single-herd milk, treated at 161 °F for 15–20 s (A.P.V. HX type, plate heat-exchanger). Cheeses were made under bacteriologically controlled conditions in an aseptic vat (Chapman *et al.* 1966).

Control cheeses were made without aseptic precautions in an open twin vat according to the standard routine practised in our Experimental Dairy (Chapman & Harrison, 1963).

δ-gluconic acid lactone cheeses were made aseptically by the method of Mabbitt *et al.* (1955). In early experiments, cheeses made by this method became contaminated by the growth of heat-resistant microflora present in the milk and in later experiments a mixture of Streptopen (Glaxo Ltd., England) and Nisin (Aplin and Barrett, Yeovil, England) in dilution giving, per ml milk, 1·2 units procaine penicillin G, 1·2 μg dihydrostreptomycin sulphate, and 100 units Nisin, were added to suppress bacterial growth.

All the cheeses were waxed and matured at 55 °F and 80% R.H. To achieve a reasonably uniform chemical composition in cheeses made throughout the year the milk was adjusted to a fat:casein ratio of 1:0·68–0·72. In cheeses made during June–July 1965 and November–December 1965 the following ranges in composition

were obtained: fat in dry matter, 50–52 and 51–54 %; moisture in fat free solids, 52–55 and 54–56 %, and salt 1·4–1·7 and 1·4–1·6 %.

Commercial cheeses are hereinafter denoted by the prefix Co. Experimental cheeses containing the reference flora of the respective Co-cheese are denoted by RF, those made with single-strain starters by S, those with multiple-strain starter by M, and those with δ-gluconic acid lactone instead of starter by GAL. Cheeses made aseptically with starter and no other organisms added are referred to as starter-only cheeses.

Microflora of cheese

Starter. The Co-cheeses were made with multiple-strain starters, containing *Str. cremoris* and varying proportions of *Str. diacetilactis* and leuconostocs. The M-cheeses were made with one multiple-strain starter (CH), composed of the same groups of organisms as above and the S-cheese with the following single-strain starters: *Str. cremoris* 924 (National Collection of Dairy Organisms 924), TR (NCDO 1200), HP (NCDO 607), K (NCDO 1218): *Str. lactis* ML_3 (NCDO 763), C_{10} (NCDO 509); *Str. diacetilactis* DRC_1 (NCDO 1007).

Isolation and inoculation of reference flora organisms. Curd samples were taken after milling and salting at 5 different factories. The samples were homogenized (Naylor & Sharp, 1958) and plated on selective diagnostic media. Non-starter lactic acid bacteria were isolated on trypticase acetate agar (TCA); Gram-negative rods, including coli-aerogenes, on crystal violet agar; staphylococci and micrococci on salt–mannitol agar, and group D streptococci on thallous acetate–tetrazolium agar (Reiter *et al.* 1965; Fryer *et al.* 1966). The reference floras (RF3–RF7), consisting of the above groups or organisms isolated from the 5 commercial cheese curds, were inoculated into the cheesemilk by adding the appropriate numbers of organisms of each group, so that the experimental curds contained each group of organisms in numbers similar to those in the commercial curds from which they had been isolated originally.

Enumeration of the microflora during maturation. Organisms were enumerated on the various selective media at intervals during maturation and the total numbers of viable lactic acid bacteria determined by plating on yeast glucose agar.

Identification of non-starter lactic acid bacteria. The organisms isolated from the commercial cheese curds on TCA (Rogosa, Mitchell & Wiseman, 1951) were identified by the tests described by Franklin & Sharpe (1963) and Sharpe, Fryer & Smith (1966).

Chemical analyses

Determination of free fatty acids (FFA). The FFA were determined by chromatography on silicic acid columns, the eluted fractions being titrated with 0·01 N-KOH (Harper, Schwartz & El-Hagarawy, 1956). The acids determined were acetic acid (C_2), propionic acid (C_3), butyric acid (C_4) and higher FFA ($> C_4$).

Flavour assessment

The flavour of the cheeses was assessed by a panel of 6 experienced tasters. The descriptions of the flavours given in the tables represent a consensus of the opinions

of the members of the tasting panel. For the statistical analysis of the data from the group of 7 cheeses referred to in Table 10 the incomplete block method described by Elliott & Beckett (1959) was used.

RESULTS

Origins of the cheese flora in a factory

Before isolating the reference floras, the flora of a bulk milk from a creamery was examined before and after heat treatment at 155 °F for 15 s. Only heat-resistant corynebacteria and spore-formers survived. Group D streptococci which would have survived this heat-treatment were not present in the raw milk on this occasion. The

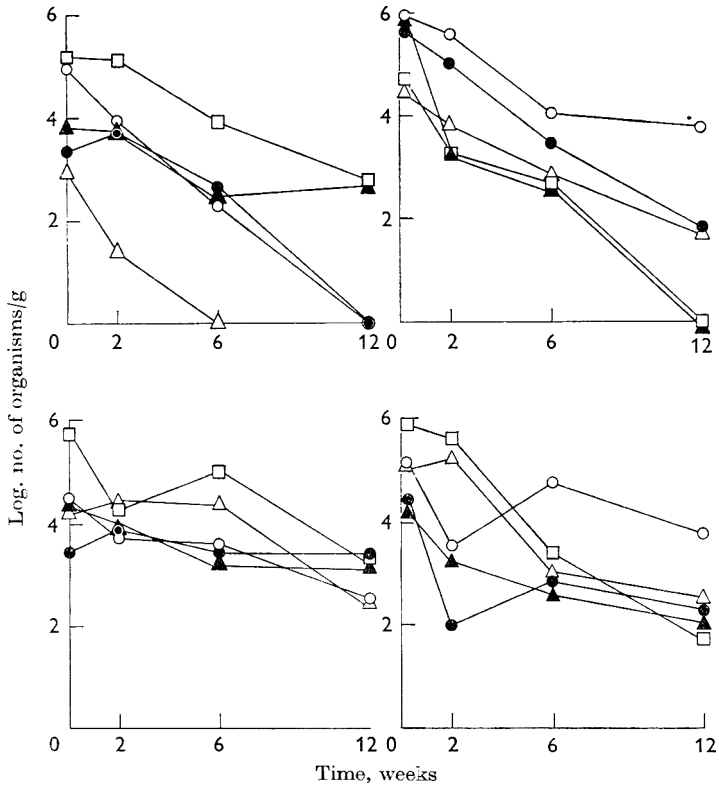

Fig. 1. Survival of micro-organisms in 5 commercial cheeses and the corresponding reference flora cheeses. Top row: Gram-negative rods in commercial (left) and reference flora cheeses (right). Bottom row: staphylococci and micrococci in commercial (left) and reference flora cheeses (right). ○——○, RF3, Co 3; ●——●, RF4, Co 4; △——△, RF5, Co 5; ▲——▲, RF6, Co 6; □——□, RF7, Co 7.

cheese curd derived from this heat-treated milk contained staphylococci, pediococci and lactobacilli in addition to corynebacteria and spore-formers. This indicates that an important part of the cheese microflora is derived from the air or from dairy equipment during cheese-making and therefore a more suitable reference flora may be isolated from cheese curd than from cheese milk.

Comparison of the bacterial flora of the commercial and reference flora cheeses

The numbers of organisms in the different bacterial groups consisting of non-starter lactic acid bacteria, group D streptococci, Gram-negative rods, and staphylococci and micrococci, present in 5 commercial cheeses and in the experimental cheeses containing the corresponding reference floras, are shown in Figs. 1, 2. As observed in an earlier study (Fryer, *et al.* 1966) only the non-starter lactic acid bacteria increased in numbers during maturation. All the other groups of organisms gradually died out,

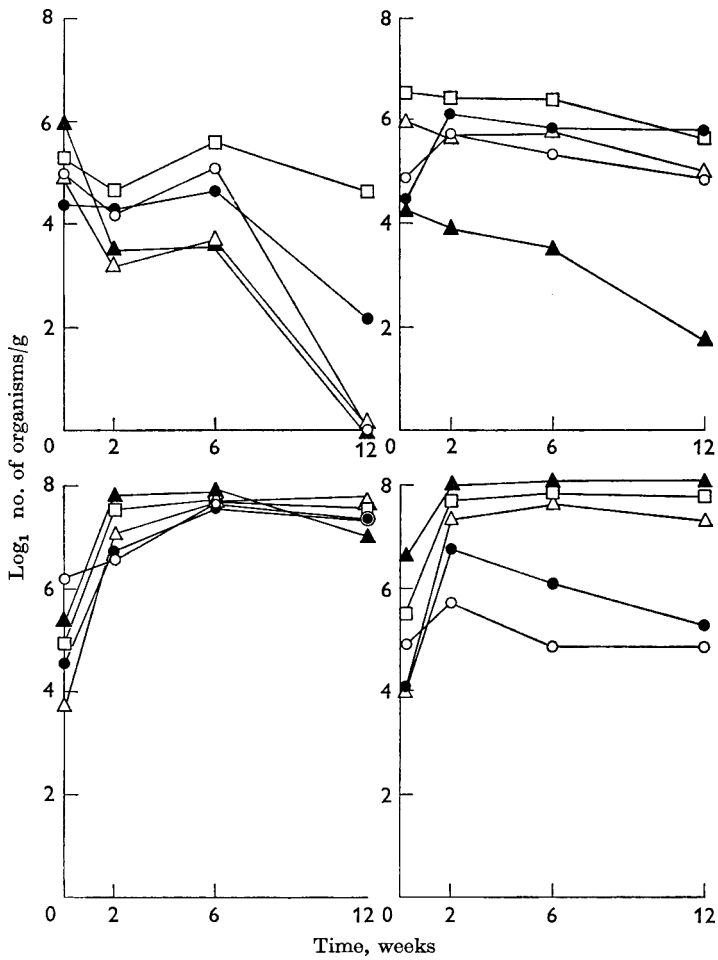

Fig. 2. Survival of micro-organisms in 5 commercial cheeses and the corresponding reference flora cheeses. Top row: group D streptococci in commercial (left) and reference flora cheeses (right). Bottom row: non-starter lactic acid bacteria in commercial (left) and reference flora cheeses (right). ○——○, RF 3, Co3; ●——●, RF 4, Co4; △——△, RF 5, Co5; ▲——▲, RF 6, Co6; □——□, RF 7, Co7.

although at different rates in the different commercial cheeses, and the rate of decrease in numbers of particular groups of organisms was not always the same in the reference flora cheeses as in the corresponding commercial cheeses.

Non-starter lactic acid bacteria. Except in RF 3 and RF 4 (Fig. 2) this group of

organisms was the one best replicated in the Co and RF cheeses. In both, similar rates of increase occurred, and to the same levels, and was followed by a very slow decline in numbers.

In order that the Co and RF cheeses could be analysed and tasted together it was necessary to make the RF cheeses before the strains of non-starter lactic acid bacteria present in their respective reference floras had been identified. After these cheeses had been made it was found that the non-starter lactic acid reference flora used for RF 3 consisted only of leuconostocs, whilst that for RF 4 consisted of group D and N streptococci and 2 strains of *Lactobacillus fermenti* which were unable to grow

Table 1. *Lactic acid bacteria isolated from commercial cheese curds on trypticase acetate medium (TCA) and used in reference floras for making experimental cheeses*

	No. of strains isolated from reference flora:				
Organism	RF 3	RF 4	RF 5	RF 6	RF 7
Streptococci					
Str. bovis	—	33	—	—	5
Str. faecium	—	—	3	—	7
Str. faecalis	—	—	—	—	4
Str. lactis	—	10	—	—	—
Unclassified str.	—	—	—	—	1
Leuconostocs*	13	—	20	21	1
Lactobacilli					
L. casei	—	—	6	9	5
L. plantarum	—	—	3	2	1
Streptobacteria†	—	—	2	—	1
L. brevis	—	—	2	—	—
L. fermenti	—	2	1	9	7
L. buchneri	—	—	1	—	2
Betabacteria†	—	—	6	3	7
L. salivarius	—	—	—	1	—
Total no. of isolates	13	45	44	45	41

* *L. dextranicum* or *L. mesenteroides* or intermediates between these 2 species.
† Could not be further identified by the tests used.

at the curing temperature of the cheese. The low numbers of non-starter lactic acid bacteria obtained during maturation in cheeses RF 3 and RF 4 are explained by the absence from these reference floras of types of lactobacilli which can multiply rapidly in cheese. An unexpected finding in this work was the isolation of the group D streptococci, *Str. bovis*, *Str. faecium* (and also of *Str. lactis*) on TCA, a medium previously found to inhibit these groups of organisms (Table 1). The young commercial cheeses contained high numbers of group D streptococci, which are known to survive pasteurization temperatures. They died out rapidly after 6 weeks in all the commercial cheeses except in Co 7 cheese. In the RF cheeses, however, except for RF 6, the numbers remained almost constant during the 12 weeks ripening period.

Staphylococci and micrococci. The decline in the numbers of these organisms during maturation in the RF cheeses was generally comparable with that in the commercial cheeses.

Gram-negative rods. Except in cheese RF 3 these organisms tended to die out rapidly in both the commercial and experimental cheeses although their rate of

decline was variable. The numbers of organisms present in the original inoculum in 3 of the RF cheeses) RF 3, RF 4 and RF 6) were high compared with those in the Co cheeses.

Starter streptococci. Counts were made of the numbers of these organisms in the S, M and RF cheeses. Representative counts are given in Table 2. The number of starter organisms in the fresh curds was much the same in all these experimental cheeses and was therefore not apparently influenced by the presence of the non-starter flora. It was not possible to enumerate the starter organisms in the reference flora cheeses after 2 weeks because of the growth of the non-starter lactic acid bacteria. The single-strain starters died out more rapidly during maturation than did the multiple-strain starter, although the counts were similar in the curd.

Table 2. *Counts of starter organisms in aseptic cheeses made with and without reference floras*

			No. of starter organisms/g curd or cheese, $\times 10^{-6}$				
			Age of cheese, weeks				
Cheese*	Date made	Curd	2	4	8	12	24
S/924	6. vii. 65	1080	—	30	—	3	—
S/924	4. xi. 65	840	—	40	—	0·4	0·003
S/TR	18. xi. 65	330	—	8	—	0·007	—
S/HP	9. xii. 65	960	—	15	—	0·4	0·0006
S/Ml_3	7. xii. 65	2400	—	120	—	0·2	0·003
M/CH	21. vi. 65	340	170	290	—	76	—
M/CH	2. xi. 65	740	160	—	100	14	0·3
M/CH	16. xi. 65	2100	410	—	37	32	0·01
S/924+RF3	8. vi. 65	—	78	†	—	—	—
S/924+RF4	9. vi. 65	1070	75	†	—	—	—
S/924+RF5	23. vi. 65	1380	38	†	—	—	—
S/924+RF6	20. vi. 65	1060	120	†	—	—	—
S/924+RF7	22. vi. 65	960	62	†	—	—	—

* S, cheese made from single-strain starter; M, cheese made from multiple-strain starter.

— No count made.

† No estimations could be made of numbers of starter streptococci in the RF cheeses after 2 weeks of maturation as non-starter lactic acid bacteria present in cheese sampled after this time also grew on the YGA plates.

FFA composition of commercial and experimental cheeses

Acetic acid. In the first series of cheeses (June–July 1965) at 9 months maturation all the Co cheeses, with the exception of Co 6, contained higher levels of C_2 acid than did the corresponding RF cheeses (Table 3). This appears to have been partly due to the use of multiple-strain starters for the Co cheeses, and of homofermentative single-strain starters for the RF cheeses; the cheese made with multiple-strain starter M/CH contained 97 μmoles C_2 acid/5 g cheese, whereas the cheese made with the single-strain starter S/924 contained only 28 μmoles (Table 3). This effect of starter was again found in the series of cheeses cited in Table 4, using as starters *Str. lactis* Ml_3 and C_{10}, and *Str. cremoris* HP, TR and K. From this finding it was expected that an aseptic cheese made with a multiple-strain starter and a reference flora containing lactobacilli would contain more C_2 than a similar cheese with the reference flora omitted. Table 3 (see values for RF 7, made 11. xi. 65) shows that this was not the case, and suggests that other factors might contribute to these results. The homo-

fermentative starters produced appreciable amounts of C_2 acid in the whey during cheese-making (Table 5).

The addition of *Str. diacetilactis* DRC1 to a homofermentative starter 924 considerably increased the C_2 acid content of the cheese (Table 4), but the addition of a strain of leuconostoc had no such effect probably because of the smaller numbers

Table 3. *FFA in commercial cheeses and in corresponding experimental cheeses*

				FFA, expressed in μmoles/5 g cheese			
Starter	Cheese	Date made	Age, months	C_2	C^4	$> C^4$	$\frac{C_4}{C_4 \times 7C_4}$
MM	Co3	15. vi. 65	9	138	14	55	0·2
S/924	RF3	8. vii. 65	9	18	7	37	0·16
S/924	RF3	23. xi. 65	9	30	6	36	0·14
M/CH	RF3	9. xi. 65	9	85	6	29	0·17
MM	Co4	15. vi. 65	9	118	8	40	0·17
S/924	RF4	9. vii. 65	9	34	7	41	0·15
MM	Co5	16. vi. 65	8·5	177	19	60	0·24
S/924	RF5	23. vii. 65	9	79	13	31	0·29
MM	Co6	16. vi. 65	9	150	16	53	0·23
S/294	RF6	20. vii. 65	9	146	10	31	0·24
MM	Co7	16. vi. 65	8·5	150	13	48	0·21
S/924	RF7	22. vii. 65	9	77	11	37	0·23
S/924	RF7	25. xi. 65	9	105	4	23	0·15
M/CH	RF7	11. xi. 65	9	89	6	23	0·21
S/924	S	6. vii. 65	9	28	10	41	0·20
M/CH	M	21. vi. 65	9	97	14	89	0·14

S = single-strain starter.
MM = multiple-strain starter different for each cheese factory.
M/CH = commercial starter used at N.I.R.D.

present—10^6/g as compared with 10^8/g for DRC1. The addition to the cheesemilk of heterofermentative lactobacilli, derived from the RF 7 flora also increased the C_2 acid content of the cheese. Unexpectedly, an even greater increase was observed when homofermentative lactobacilli from the same RF 7 flora were added (Table 4). As the RF 3 and RF 4 cheeses were devoid of lactobacilli it is not surprising that the C_2 acid levels of these cheeses when made with a single-strain starter were as low as those in cheeses made with S-starter only (Table 3).

The RF 3 cheeses made with the multiple-strain starter CH had the same C_2 acid content as the CH cheese made without reference flora (Table 3) presumably because of the absence of lactobacilli in RF 3, but it cannot as yet be explained why the C_2 acid content of the RF cheese made with CH starter was not higher than that in the RF cheese made with the single-strain starter. Similar experiments in an earlier series, using the same reference flora and starters, also showed that larger quantities of C_2 acid occurred in cheese made with a multiple-strain starter.

The GAL cheeses and cheese curds were found to have an even lower level of C_2 acid than the S cheeses (Tables 4, 5, 7). The C_2 acid was derived entirely from the milk and part of it was lost in the whey (Table 5). The increase in the level of C_2 acid observed in the 2 GAL cheeses (Table 4) before the use of antibiotics was possibly due to contamination of these cheeses with heat-resistant lactobacilli, which were not detected until after 3 months maturation but were undoubtedly present before that.

Propionic acid. None of the cheeses contained more than trace amounts of C_3 acid although small amounts could be detected in the milk.

Butyric acid and higher free fatty acids (FFA > C_4). With the exception of Co 4, the Co cheeses contained more FFA > C_4 than did the experimental cheeses (Table 3).

Table 4. *A comparison of the levels of FFA in cheeses made aseptically with or without starter*

Cheese made with	Date made	Age, months	FFA expressed in μmoles/5 g cheese		
			C_2	C_4	> C_4
GAL	23. vi. 66	0	0	5	50
		3	7	1	50
GAL	28. vii. 66	0	0	tr	41
		3	10	tr	42
S/924	17. v. 66	0	6	3	43
		3	15	3	42
		6	20	6	54
		9	30	8	67
S/ML_3	19. v. 66	0	21	5	50
		3	17	4	53
		6	20	5	56
		9	38	10	66
S/TR	24. v. 66	0	7	tr	59
		3	17	3	66
		6	24	5	72
		9	23	4·5	74
M/CH	7. vi. 66	0	55	7	53
		3	73	6	56
		6	ND	8	62
		9	82	9	62
S/924 + DRC1 (*Str. diacetilactis*)	9. vi. 66	0	48	2	48
		3	68	5	59
		6	68	7	71
		9	contaminated		
S/924 + leuconostocs (derived from CH)	14. vi. 66	0	12	5	73
		3	23	5	69
		6	20	6	80
		9	38	9	86
S/924 + DRC1 + leuconostoc	16. vi. 66	0	55	7	52
		3	79	5	64
		6	69	7	72
		9	85	14	73
S/924 + homofermentative lactobacilli derived from RF 7	16. viii. 66	0	12	2	57
		3	114	8	64
		6	115	9	70
		9	130	11	78
S/924 + heterofermentative lactobacilli derived from RF 7	18. viii. 66	0	6	tr	35
		3	91	6	46
		6	102	7·5	52
		9	107	11	62

GAL = δ-Gluconic acid lactone.
0 month = ex-press.
tr = trace.
ND = not determined.
The cheeses were also tested at 1 and 6 months but these figures have been omitted for the majority of examples.

The most obvious difference between these cheeses was that the Co cheeses were made with various multiple-strain starters, and the experimental cheeses with one single-strain starter. Of the 2 experimental cheeses containing starter only (Table 3), S/924 (made 6. vii. 65) and M/CH (made 21. vi. 65), the M cheese contained a higher level of $> C_4$ acid. However, later findings for cheeses made with this same multiple starter did not confirm this result and it became increasingly evident that the initial levels of C_4 and $> C_4$ acids varied considerably according to the source of the milk and the season of the year. This suggested that the initial level in the milk might vary

Table 5. *The distribution of FFA in whey and cheese curd: made without starter, with homofermentative single-strain starter, or with heterofermentative multiple-strain starter*

		FFA expressed in μmoles/5 g cheese		
Starter	Substrate	C_2	C_4	$> C_4$
	Milk	6	4·0	24
GAL*	Curd	6	4	19
S/924*	Curd	22	3	23
S/ML_3*	Curd	14	2	24
M/CH*	Curd	82	1	21
GAL†	Milk‡	14	1·5	14
	Whey‡	8	1	4·5
	Curd‡	5	1·5	13
S/924§	Milk‡	13	3	12
	Whey‡	37	2	9
	Curd‡	12	1·0	15

* The same bulk milk was used for all 4 cheeses.

† average of 2 experiments.

‡ The volumes of milk and whey and the weight of the curd were analysed in the proportions of the actual cheese-making of the 40 lb-cheeses, i.e. milk, 50 ml; whey 45 ml; curd 5·4 g.

§ average of 3 experiments.

GAL = cheese made without starter.

S = cheese made with single-strain starter.

M = cheese made with multiple-strain starter.

Table 6. *Concentrations of FFA in the cheese curd made from summer or from winter milk*

	FFA* expressed in μmole/5 g cheese			
	C_4 acid		$> C_4$ acid	
	Average	Range	Average	Range
May–June 1966	4·7	2–7	49	43–59
Dec.–Jan. 1966–7	0·2	0·0–0·3	14	10–16

* Each figure is an average for 12 cheeses.

in a similar way and might also be affected by the source of the milk and the season. To further illustrate the effect of season, the levels of C_4 acid and $> C_4$ acid were determined in the fresh curds of 12 aseptic cheeses made in May–June 1966, and of 12 aseptic cheeses made in December 1966–January 1967. The averages for each of these 2 groups are compared in Table 6. Both C_4 and $> C_4$ acids were considerably higher in concentration in the summer cheese than in the winter cheese.

During maturation of the cheese, a slight increase in amount of C_4 acid and of

> C_4 acids occurred in cheeses made with starter (Table 4). These results are consistent with the finding (Fryer, Reiter & Lawrence, 1967) that lactic acid bacteria are capable of weakly hydrolysing cheese fat. It was apparent that not only the single-strain starters but also the component bacteria of multiple-strain starters were capable of such hydrolysis. When the significant effect of the initial FFA C_4 acid and > C_4 acid content of the milk on the level of these acids in the cheese was realized the cheeses were analysed at the curd stage as well as at intervals during maturation. The FFA > C_4 and C_2 of 2 GAL cheeses remained unaltered throughout the 3 months maturation period (Table 6), whereas there was an appreciable increase in some cheeses containing starter and other lactic acid bacteria. Some starter streptococci appeared to be more lipolytic than others but none was as active as the leuconostocs and *Str. diacetilactis*. This was consistent with earlier results, which indicated that commercial starters were more lipolytic than single-strain ones.

Unfortunately the GAL cheeses for which results are presented in Table 4, and which were the key cheeses for the proof of the lipolytic activity of the cheese microflora, became contaminated after 3 months; the single-strain starter S/924, which appeared to be the least lipolytic (Table 6), consistently produced some lipolysis (Table 7).

Table 7. *A comparison of the levels of FFA present in 3-month-old cheeses made with and without starter*

	FFA* expressed in μmoles/5 g cheese		
Cheese	C_2	C_4	> C_4
GAL†	8	tr	16
S/924‡	15	3	22

* Average of 3 cheeses made on different occasions during Dec.–Jan. 1966–7.
† Made without starter, using δ-gluconic acid lactone.
‡ Made with single-strain starter.
tr = trace.

Assessment of flavour

Cheeses made with reference floras

It was hoped that the Co cheeses would prove to be of good Cheddar flavour and that this would be reproduced in the RF cheeses through the use of the individual reference flora. However, each of the Co cheeses (Table 8) had some off-flavour which was usually described as 'fruity' or 'yeasty', and this was reproduced to some extent in the experimental cheeses. The 2 cheeses, RF 3 and RF 4, which contained no lactobacilli, were the most acceptable cheeses and had a mild Cheddar flavour. Experimental cheeses which had strong off-flavours at 6 months lost the objectionable flavour and at 11 months were considered to be extremely strong and good flavoured cheeses. Compared with cheeses made with starter only, flavour developed more rapidly and intensely in the reference flora cheeses, suggesting that the non-starter flora makes some positive contribution to flavour.

Cheeses made with starter only

Table 9 shows the flavour assessment at 5 months of the cheeses which were made in the aseptic vat with starter only, and of the control cheese, containing the usual

Table 8. *A comparison of the flavours of experimental and commercial cheeses at 5 months maturation*

Commercial cheese		Experimental cheese with reference flora added*	
No.	Flavour	No.	Flavour
Co 3	Strong cheese flavour, sour, fruity off-flavour	RF 3†	Very mild Cheddar flavour, slightly sour
Co 4	Mild Cheddar flavour, sweet, fruity off-flavour	RF 4	Mild Cheddar flavour, sour, burnt off-flavour
Co 5	Off-flavour, fruity, rancid, faecal, a little Cheddar flavour	RF 5	Strong flavour, either mature Cheddar or nasty off-flavour
Co 6	Very salty, masks anything else. Probably rancid	RF 6	Very unpleasant, fruity, yeasty off-flavour
Co 7	Strong cheese with peppery fruity off-flavour	RF 7	Unpleasant fruity, yeasty off-flavour

* Control cheeses (non-aseptic) to RF 3, RF 5 and RF 7 without added reference flora, also tasted at 5 months and found to be mild Cheddar, with no pronounced off-flavours.
† Tasted at 4 instead of 5 months.

Table 9. *A comparison of the flavours of 12 aseptic cheeses, made with starter only, and their controls made in the open vat*

Starter	Date of make	Age, months	Flavour	
			Aseptic cheese	Control cheese (non-aseptic)
M/CH	21. vi. 65	5	Cheddar flavour, some fruity off-flavour	Mild Cheddar, some fruitiness
S/294	24. vi. 65	5	Mild Cheddar	—
S/924	28. vi. 65	5	Very mild Cheddar, bitter	Mild Cheddar, slightly bitter
S/924	6. vii. 65	5	Mild Cheddar, some off-flavour	Mild Cheddar, a little more flavour than aseptic cheese
M/CH	2. xi. 65	6	Mild Cheddar, fruity off-flavour	Mild Cheddar, slight off-flavour
M/CH	16. xi. 65	6	Mild Cheddar, sharp	Mild Cheddar, sharp
*M/CH	30. xi. 65	6	Mild Cheddar, typical sour flavour	Mild Cheddar, typical sour flavour
S/924	4. xi. 65	6	Mild Cheddar, slightly sharp	Mild Cheddar, slightly sharp
*S/TR	18. xi. 65	6	Fairly strong Cheddar not full flavour	Strong, sharp Cheddar
S/924	2. xii. 65	9	Mild mature Cheddar, lacking in fulness	Strong mature Cheddar
S/HP	9. xii. 65	9	Mature Cheddar, lacking fulness, some bitterness	Strong mature Cheddar, some bitterness
S/ML_3	7. xii. 65	9	Mature Cheddar, unpleasant fruity flavour	Unpleasant fruity flavoured cheese

— Not tasted.
* At 6 months, but not at 3 months maturation, small numbers of colonies (10^2–10^4/g cheese) found on TCA and identified as lactobacilli.

cheese flora, and made at the same time in the open vat. The aseptically made cheeses had a distinct Cheddar flavour, although they did not possess the fulness of flavour of a mature Cheddar. The starter itself must therefore be partly responsible for the development of cheese flavour. Different starters produced characteristic flavours, the fruity flavour of cheese made with *Str. lactis* ML_3 being very characteristic.

Whilst these starter-only cheeses possessed a mild flavour up to about 6 months' maturation, after further ripening up to 12 months extremely strong Cheddar flavour developed in all of these cheeses, indistinguishable from that of mature Cheddar. This was confirmed in several later experiments.

Table 10. *Ranking of 4 cheeses according to the intensity of cheese flavour, and disregarding any off-flavours*

(The figures represent rank totals. Method of scoring is that used by Elliott & Beckett (1959).)

	Cheese made with:			
	δ-gluconic acid lactone	Starter S/ML_3	Starter S/924	Starter S/924 + RF 7
Average ranking				
Ottawa	16·8	13·4	9·8	8·0
N.I.R.D.	16·4	12·0	10·8	8·5
Calculated*	18	14	10	6

* Average ranking: when there is perfect agreement between tasters and consistency within tasters.
S = single-strain starter.

Cheeses made with δ-gluconic acid lactone

Of the 10 GAL cheeses made without starter and tasted at intervals during maturation, none developed any Cheddar cheese flavour, nor any cheesy flavour whatsoever. All that could be detected was slight bitterness and acidity.

Intensity of cheese flavour

Seven tasters at the N.I.R.D. and 5 tasters at the Food Research Institute, Central Experimental Farm, Ottawa, were asked to rank the intensity of cheese flavour, disregarding any off-flavours, in 4 cheeses made as follows: 1 cheese made with GAL, 2 S-cheeses, one made with S/924 and the other with ML_3, and 1 RF cheese (S/924 + RF7). All were made at approximately the same time. Table 10 shows the statistical analysis of the ranking trial and the ratings assigned by the 2 tasting panels.

The ratings of the 2 panels agreed well: starter by itself was found to produce Cheddar cheese flavour and the reference flora intensified the flavour; cheeses made without starter (GAL) possessed no Cheddar or other cheese flavour. Subsequent experiments have confirmed these results.

DISCUSSION

The cheeses made with starter only were produced regularly without other bacteria being detected at any stage but some of the early GAL cheeses, made without the addition of antibiotics became contaminated with heat-resistant lactobacilli. Repro-

duction of the reference floras from commercial cheese was difficult, in that it was not easy to duplicate exactly the numbers and type of organisms; thus in RF 3 and RF 4 virtually no lactobacilli were included. The nearest replicate was obtained with RF 6. Nevertheless, the results established that by strict control of the bacterial flora and of the milk supply, cheeses were obtained having a predictable flavour intensity and free fatty acid composition.

It has been generally assumed that the free fatty acids are associated in some way with flavour intensity, although this had not yet been directly proven. Patton (1963), for instance, was of the opinion that the volatile fatty acids, of which acetic acid is the predominant one, constitutes the *sine qua non* of Cheddar cheese flavour. In the present work, multiple-strain starters produced considerably more C_2 acid than single-strain starters, because of the presence in them of *Str. diacetilactis*. Although their relative merits have been discussed for many years, this is to our knowledge the first comparison of FFA in cheese made with single- and multiple-strain starters. At a later stage in cheese maturation C_2 acid is produced by the lactobacilli present. It was therefore possible to control the C_2 acid production in aseptically made cheeses by means of the addition of starter with or without lactobacilli. However, as mature cheeses of widely different C_2 acid content possessed strong Cheddar flavour, it seems unlikely that C_2 acid is an essential flavour component. The role of C_2 acid in flavour development has been discussed previously (Mabbitt, 1961; Marth, 1963; Forss & Patton, 1966; Kristoffersen, 1967).

The production of C_4 and $> C_4$ FFA also proved to be predictable. The determination of C_4 acid is likely to be inaccurate as the quantities were small, but the average ratio of $C_4:(C_4 + > C_4)$ in Table 2 for the Co cheeses was 0·22, and for the RF cheeses 0·21, which agrees well with the figure of 0·2 quoted by Bills & Day (1964) for Cheddar cheese. Although Wolf (1941) reported that *L. casei* produced FFA from milk during prolonged incubation it has been assumed generally that only 'actively' lipolytic organisms such as micrococci and Gram-negative organisms contribute to fat hydrolysis. Using very sensitive methods for the detection of lipolysis it has now been shown that many lactic acid bacteria show weak lipolytic activity (Lawrence, Fryer & Reiter, 1967; Fryer, Lawrence & Reiter, 1967). This suggests that starter streptococci, although only weakly lipolytic, contribute towards fat hydrolysis because of their great numbers, and because they remain in contact with the substrate for a long period. During the present experiments it became evident that the initial content of higher FFA varied considerably between milks of different herds, and with season and type of feed. The data in Table 6 show the effect of season on the initial level of FFA in cheese made from winter and summer milk. Types of feeding, and treatment of the milk, are known to affect the fatty acid composition of milk (Tarassuk, Laben & Yaguchi, 1962). Recent work (Sorokin & Reiter, unpublished) has shown that the type of feed and the mode of milking also have considerable effects on the initial levels of C_4 and $> C_4$ FFA in the cheese.

When the flavour of the experimental cheeses was assessed there was no doubt that the GAL cheeses, devoid of bacterial flora, were also devoid of cheese flavour. A slight bitterness which appeared after some months maturation was probably due to peptides produced by the rennet and not further broken down in the absence of starter streptococci. In this total lack of cheese flavour the GAL cheese differed markedly

from the starter-only cheese, which developed a characteristic Cheddar flavour, sometimes with an off-flavour, depending on the strain of starter used.

Observations by previous workers on the influence of starters on cheese flavour have shown that in cheese made with *Str. lactis* distinct off-flavours occurred (Perry, 1961; Perry & McGillivray, 1964) and that freshly isolated strains of *Str. cremoris* and *Str. lactis* with good activities for Cheddar cheese-making had to be abandoned because they consistently led to abnormal flavours in the mature cheese (Robertson, 1966). Whereas in the past the starter was considered to exert an all-important influence on the quality of the cheese produced, it was supposed that its influence on flavour was indirect and resulted from the importance of acidification in controlling maturation. Our results show that the starter organisms themselves make a direct contribution to cheese flavour.

It is generally accepted that different starters die out at different rates during maturation of cheese (Dawson & Feagan, 1957; Perry, 1961). We found that cheese flavour continued to develop in the 'starter-only' cheese after the death of even the most long-lasting strain of starter, up to at least 12 months maturation. It appears, therefore, from our work that the flavour products must have been produced by the enzymes of the dead organisms. It is immaterial whether the starter or other organisms present actually lyse after death, as even without lysis the permeability of the dead cell would increase, so permitting enzymes to diffuse into the surroundings, or substrates to diffuse into the cell.

Franklin & Sharpe (1963) showed that different pairs of single-strain starters gave cheeses having fairly large differences in average flavour score; they also found that the development of cheese flavour correlated with the numbers and types of bacteria present. A clear separation of the role of starter was not possible because these cheeses were not made under controlled (aseptic) conditions. The use of the aseptic vat has enabled us to confirm that differences in flavour are conferred by the use of different starters. The technique using the reference flora needs further development and study because the RF cheeses contained too many off-flavours, although these disappeared on prolonged ripening. However, this appears to be a most useful procedure for investigating bacterial association, and the study of the metabolic products in relation to cheese maturation.

The findings of this work show unequivocally that basic Cheddar flavour is produced by starter. Further work should be concentrated on the enzymic activities of the organisms; in this way the problems of identification of cheese-flavour components will be greatly simplified.

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Reviews of the progress of dairy science

Section C. Dairy chemistry. The secretion of water and of water-soluble constituents in milk

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CONTENTS

INTRODUCTION

Folley (1947) defined milk secretion as the synthesis of milk by the cells of the alveolar epithelium and the passage of this milk from the cytoplasm of the cells into the alveolar lumen. The passive withdrawal of milk from cisterns or sinuses and the ejection of milk from the alveolar lumen was referred to as milk removal; milk secretion and milk removal together were held to comprise lactation. There is, however, circumstantial evidence that in addition to the primary secretion, a 'tissue exudate' or a 'blood plasma transudate' contributes to the fluid which accumulates within the ducts and cisterns of the gland (Davies, 1933; Peskett & Folley, 1933; Barry & Rowland, 1953). Also, resorption of ions from the gland has been demonstrated experimentally, and Azimov, Orlov & Belugina (1962) have expressed the view that diffusion and resorption across the mammary epithelium are normal and integral parts of milk secretion. For the purposes of the present article, the term milk secretion will be used in the broadest sense, to include resorption and diffusion, but without implying that these processes necessarily make a constant or a significant contribution under normal physiological conditions.

Fat globules and protein granules have been shown to be transferred from the secretory cell into the alveolar lumen without rupture of the apical membrane, but

mechanisms for the excretion of lactose and other water-soluble substances have still to be described. There is, moreover, no information on the means by which the ionic composition of the fluid of the secretory cell may be regulated, or the composition of the excreted fluid altered during its passage through the ducts and cisterns of the gland. This situation undoubtedly reflects a lack of suitable direct methods for the study of the processes that contribute to milk secretion. Considerable information exists, nevertheless, on variations in the milk content of water-soluble constituents and on the rates of their secretion and that of water, which permits certain broad conclusions to be drawn about the physiology of milk secretion. The object of this review is to summarize this information (a complete bibliography will not be attempted) and to examine the explanations given for the observed variations in milk composition.

Most of the published work relates to the cow and only where reference is made to the milk of other species will the species be identified.

VARIATIONS IN THE CONCENTRATIONS IN MILK AND IN THE RATES OF SECRETION OF WATER-SOLUBLE CONSTITUENTS

Factors that affect milk composition may be conveniently divided into 2 main groups. In one group are factors such as stage of lactation, and nutrition, that affect the whole animal and exert their effect on the milk of all 4 quarters. In the other group are factors such as bacterial infection and incomplete removal of milk, that affect the individual quarters separately. Emmerson (1946) demonstrated conclusively, by X-ray studies of whole udders after the injection of barium sulphate into a single quarter, that there is no connexion between individual quarters; when the yield of a single quarter is depressed there may, however, be an increase in the yield of the other quarters which partly compensates for the decreased yield of the affected quarter (Wheelock, Rook & Dodd, 1965*b*). The physical separateness of individual mammary units has not always been taken into account in studies of factors known to affect the quarters separately: results have been reported for the milk of the whole udder, when there would be dilution of the milk from the affected quarter with that of unaffected quarters or perhaps of quarters affected in a different way. Also, as Waite, Abbott & Blackburn (1963) have pointed out, for many studies of factors affecting the whole animal the results may be inaccurate because of undetected udder damage. Such inaccuracy could be largely eliminated if analyses were made on the milk of the separate quarters rather than on the bulked milk of the whole udder.

Whole animal effects

Breed and individuality. There are only small differences between the milk of different breeds in the average contents of lactose, potassium, sodium and chloride. Values for 4 main British dairy breeds reported by Rowland & Rook (1949) are given in Table 1; as lactose content increased sodium and chloride contents decreased. There was no consistent trend in potassium content. Within a single breed, however, the milk content of water-soluble constituents recorded for individual animals varies widely. Much of this variation may be attributed to environmental and physiological effects (*vide infra*), but estimates of heritability for lactose content, which range from

0·36 to 0·70 (Hansson, 1949; Politiek, 1957; Robertson, Waite & White, 1956), and the similarity of lactose, sodium and potassium values for monozygous pairs (Hansson, 1949; Comberg, Andreae & Meyer, 1962) indicate that there is an important genetic component.

Table 1. *Average values for the lactose, potassium, sodium and chloride contents of the milk of 4 main British dairy breeds (Rowland & Rook, 1949)*

Constituent, %	Ayrshire	Friesian	Guernsey	Shorthorn
Lactose (anhydrous)	4·57	4·46	4·62	4·51
Potassium	0·151	0·158	0·154	0·152
Sodium	0·054	0·058	0·048	0·059
Chloride	0·099	0·113	0·096	0·102

Rook & Wood (1959) have shown that for heifers maintained free of infection of the udder the lactose, potassium and sodium contents of milk are constant from about the 2nd week of lactation to the 3rd or 4th month, and appear to be characteristic for the individual animal. Within a single breed, there was a close inverse relationship between lactose content and the molar sum of sodium plus potassium contents of milk water and, with the exception of results for one or two animals, only a slightly less good inverse relationship between lactose and potassium contents (Walsh & Rook, 1964). The reported values for lactose varied from 4·7 to 5·8 g/100 g, for potassium from 150 to 210 mg/100 g and for sodium from 29 to 64 mg/100 g of milk water. It was further shown that though lactose, potassium and sodium values were altered by a number of environmental and physiological factors, the lactose : potassium ratio was little affected. The heritability of this ratio calculated from values for monozygous pairs was 0·95, and from the within-herd regression of daughter group means on the records of their dams $0{\cdot}95 \pm 0{\cdot}270$.

Stage of lactation. Some of the first recorded studies of milk composition established that the lactose content of milk is at a maximum early in lactation and tends to decrease as the lactation progresses. The content in colostrum is exceptionally low. There are converse changes in the concentrations of sodium and chloride (see Richmond, 1899). Numerous investigations have since confirmed these general trends and have shown that changes in potassium content broadly follow those in lactose (cf Rook & Campling, 1965). In few of these investigations, however, has it been possible to separate effects which are directly dependent on the stage of lactation of an animal from the effects of other factors which may tend to vary with stage of lactation. Where an attempt was made to exclude variations in the level of feeding and infection of the udder, the concentrations of lactose, potassium and sodium were reasonably constant throughout the greater part of the lactation, from about the 2nd day to the 7th month (Rook & Campling, 1965). Towards the end of lactation there is a point, at about the 5th month of pregnancy (Gaines & Davidson, 1925–6), at which the decline in milk yield is accelerated, and Rook & Campling (1965) found that from this point there was a progressive decrease in the concentrations of lactose and potassium and an increase in that of sodium.

A detailed study of the changes in milk composition immediately after parturition was made by Garrett & Overman (1940). At parturition, the concentrations of lactose

and potassium were low and of sodium and chloride high, but within 2 days values typical of the mid-lactation period were obtained. Those findings were confirmed in more recent investigations (White & Davies, 1958; Rook & Campling, 1965), except that values for the potassium content of colostrum were variable and on average similar to those for mid-lactation. The changes are not entirely a direct result of parturition but arise, in part, because milk secreted before parturition is normally allowed to accumulate within the udder and a selective resorption of secreted materials takes place. Lactosuria has been recorded in pregnant cows from about 20 days before parturition and in the days immediately before calving there was a temporary, marked increase in the excretion of lactose in urine (Campus, 1920; Wheelock & Rook, 1966*a*). Similar observations have been made in the human (Watkins, 1928; Hubbard & Brock, 1935; Date, 1964).

Milking pre-partum gives initially a small volume of a secretion which may have a lower lactose content and a higher content of sodium and chloride than is typical for colostrum. Some days before parturition there is a rapid rise in milk yield which continues for about 6 weeks of lactation, and until shortly after parturition there is an associated increase in lactose and potassium and a decrease in sodium and chloride concentrations (Rowland, Roy, Sears & Thompson, 1953; Wheelock, Smith & Dodd, unpublished). If milking is continued throughout the whole of pregnancy, the gland continues to produce a secretion, though the yield may become very small. As pregnancy advances the changes in composition which occur normally in late lactation continue and their degree corresponds roughly to the decrease in milk yield. Some days before the minimum in yield, however, there is a sharp decrease in potassium content and corresponding increases in sodium and chloride contents of milk water without a distinctive change in lactose content. As the minimum in yield is reached, lactose content also begins to fall, the contents of sodium and chloride then steady and the trend in potassium content is immediately reversed. The potassium content subsequently reaches maximum at or about parturition and thenceforward normal lactational trends are observed. This sequence of changes is thought to relate to a decreased metabolic activity of the secretory cells, which could result in the partial replacement of cellular potassium by sodium (Wheelock *et al.* 1965*c*).

Fluid removed from the udder during the dry period is virtually free of lactose and has contents of sodium, potassium and chloride similar to those of extracellular fluids (Mackenzie & Lascelles, 1965*b*; Smith, Wheelock & Dodd, 1967).

Age. Under commercial conditions there is, on average, a progressive decrease from lactation to lactation in the concentrations in milk of lactose and potassium and an increase in the concentration of sodium (Rensburg, 1947; Waite, White & Robertson, 1956; Politiek, 1957; Vanschoubroek, 1963; Vanschoubroek, Willems & Lampo, 1964). The extent to which the changes are directly attributable to the age of the cow is uncertain, as bacterial infections of the udder, the incidence of which tends to increase with age, would be expected to contribute to the observed changes. Where an attempt was made to exclude udder infection, the changes in the lactation average for lactose content from the 1st to the 3rd lactation in the 3 animals studied were $-0{\cdot}09$, $\pm 0{\cdot}00$ and $-0{\cdot}08$ percentage units (Rook & Campling, 1965).

Feeding. Intake of energy in excess of the standards of Woodman (1957) was without effect on milk lactose content (Rook, 1953; Rook & Line, 1961), but under-

feeding caused a small depression (Rowland, 1946; Rook & Line, 1961). Variations in dietary protein content have little influence on milk lactose content (Rowland, 1946; Frens & Dijkstra, 1959; Rook & Line, 1961). Milk yield and the yield of lactose, and presumably the yields of other water-soluble constituents are, however, sensitive to changes in the dietary supply of energy and protein.

During starvation, milk yield falls rapidly in the first few days to less than a third of the original value and then continues to fall more slowly (see, for example, Smith, Howat & Ray, 1938). There is an associated decrease in lactose content and an increase in chloride content which, according to Smith *et al.* (1938), is insufficient to maintain isotonicity. They suggested that a substance not usually present in milk in such large amounts is secreted in quantities sufficient to maintain the usual osmotic pressure. The results of Robertson, Paver, Barden & Marr (1960) with cows and of Linzell (1967*a*) with goats show that this substance is citric acid.

Hormones. The hormonal mechanisms responsible for the initiation and maintenance of lactation and for milk ejection are reasonably well established, but the effects of administered hormones on the concentrations and yield of individual milk constituents have been studied to only a limited extent.

The action of insulin has been most widely investigated. In both the cow and the goat, dose rates sufficient to depress the concentration of glucose in blood plasma to less than about 40 mg/100 ml caused a depression in milk yield and to a lesser extent in milk lactose content (Gowen & Tobey, 1931–2; Brown, Petersen & Gortner, 1936; Kronfeld, Mayer, Robertson & Raggi, 1963; Rook, Storry & Wheelock, 1965; Schmidt, 1966; Linzell, 1967*b*) but in goats there was no effect on potassium or sodium content (Rook & Hopwood, unpublished). Administration of insulin over periods of 5–8 days has been reported to cause a complete inhibition of milk secretion (Bucciardi, 1928).

Subcutaneous injection of DL-thyroxine (10 mg/day) increased milk yield and milk lactose content and decreased chloride content; converse changes were observed with thiouracil (20 mg/day) (Chanda, McNaught & Owen, 1952). Injection of pilocarpine (1 mg) was reported to increase milk lactose content (Bucciardi, 1928). Implantation of adrenaline tablets beneath the skin of 4 lactating goats (*ca.* 50 mg adrenalin/goat) and 1 lactating cow (*ca.* 600 mg adrenalin) gave a rise in blood glucose and milk lactose content but milk yield was not increased (Bottomley, Folley, Walker & Watson, 1939).

Injection of oxytocin (20 i.u. Syntocinon), to permit the removal of residual milk after the completion of the usual milking procedure, was found to affect the composition of the milk obtained at subsequent milkings: the contents of sodium and chloride were increased and those of lactose and potassium decreased (Wheelock *et al.* 1965*d*). The effects were more marked when injections were given over several successive milkings. On discontinuing the injections there was a progressive return to the original composition. Mackenzie & Lascelles (1965*a*) showed that when, at a single milking, the residual milk was removed in portions after successive injections of oxytocin (5 i.u. Syntocinon), the later portions showed changes in composition similar to the above, except that potassium content was little affected. In the goat, milked 1–4 times/h with the aid of oxytocin (0·05–0·4 i.u.) removal of the milk by cannula, or by gentle hand milking caused a slight fall in potassium content only. When

milking was more vigorous, there were more marked changes in potassium content, accompanied by a decrease in the lactose content and increases in the contents of sodium and chloride (Linzell, 1967*a*). These changes could not be accounted for entirely by assuming that the act of milking causes exudation of tissue fluid into milk and it was considered possible that the doses of oxytocin used caused some changes in the composition of the milk obtained from the gland.

Gatschew (1963) found that with rabbits in late lactation, the administration of prolactin and reserpine was associated with a pronounced and immediate increase in the lactose content of the milk. A similar but less rapid effect was observed with hydrocortisone acetate, but no effect was observed with adrenocorticotropin, thyrotropin, serum gonadotropin, oestrogen or progesterone. In later work (Gatschew, 1965) it was demonstrated that the administration of prolactin (10–15 i.u./kg live weight, intramuscularly) in late lactation restored the concentrations of potassium sodium and lactose to values similar to those observed during the period of maximum milk production.

After adrenalectomy of lactating goats, Cowie & Tindal (1958) observed in some of the animals a decrease in milk yield, with an associated decrease in the milk content of potassium and a compensatory increase in sodium: invariably the sodium concentration in blood plasma decreased and that of potassium increased. Milk lactose values were not reported.

Inter-quarter effects

Accumulation of milk within the udder. The accumulation of milk within the udder, which occurs when there is incomplete removal of milk at successive milkings or failure to remove milk over an extended period, causes a decrease in net milk yield, and the effect persists after the accumulation is relieved (Elliott, Dodd & Brumby 1960; Dodd & Clough, 1962). There is a concomitant decrease in lactose and potassium contents and an increase in sodium and chloride, and the extent of these changes varies with the degree of accumulation (Petersen & Rigor, 1932–3; Hansson, Dassat & Claesson, 1954; Wheelock *et al.* 1965*b*; Wheelock, Rook, Dodd & Griffin, 1966). The rates of secretion of water and of water-soluble constituents measured over milking intervals varying from 6 to 36 h decreased curvilinearly with the length of interval, the degree of curvilinearity varying with the constituent in the following increasing order: sodium, chloride, water, lactose, potassium (Wheelock, Rook, Dodd & Griffin, 1966). The reduced rates of secretion with the longer intervals persisted for a period after resumption of normal milking but virtually the original rates of secretion were recovered within a few days. When the original yield of milk had been recovered, sodium and chloride were, in some animals, temporarily secreted at a rate slightly above the pre-treatment values (Wheelock *et al.* 1965*b*; Wheelock, Rook, Dodd & Griffin, 1966). Suspension of milking for periods of 2 weeks caused similar but more marked effects on the concentrations of lactose, potassium, sodium and chloride. When milking was resumed the original concentrations were practically recovered, although the yield of milk remained depressed until the end of the lactation (Wheelock, Smith & Dodd, 1967).

Petersen & Rigor (1932–3) suggested that resorption of lactose occurs when milk is allowed to accumulate within the udder, an idea repeated by Johannson & Claesson

(1957) and for which there is now direct experimental evidence: lactosaemia and lactosuria have been demonstrated in cows towards the end of an extended milking interval (Wheelock & Rook, 1966*a*). Garrison & Turner (1936), however, attempted to explain the effects of an extended milking interval on milk secretion in terms of an impairment of the activity of the secretory cells due to an increase in pressure within the mammary gland resulting from the accumulation of milk. The initial reduced rate of secretion of lactose observed when normal milking is resumed after an extended milking interval indicates that secretion rate must be reduced at the end of the extended interval. The effect is demonstrated most dramatically at the end of a lactation when milking is suspended. Initially lactose is resorbed, but only for a matter of days. Eventually there is only a very small quantity of lactose left in the gland and secretion must then have virtually ceased (Wheelock, Smith, Dodd & Lyster, 1967). It appears that the observed reduction in lactose yield results both from impairment of lactose synthesis and from partial resorption.

Short-interval milking. Zaks (Zaks, 1964; Zaks, Natochin, Sokolova, Tanasiĭchuk & Tverskoĭ, 1965) has claimed that the milking of goats at 15-min intervals produces a milk with increased contents of sodium and decreased contents of lactose and potassium, and that this composition is characteristic of alveolar milk. Linzell (1967*a*) however, has shown that such changes probably arise from excessively large doses of oxytocin used to induce milk ejection prior to the milking out of the udder.

Variations in the composition of milk throughout a milking. For quarters milked at regular 12-h, intervals no change in the concentrations of water-soluble constituents (expressed as a percentage of milk water) is observed throughout a milking, and the residual milk removed after the intravenous injection of oxytocin is of similar composition to that removed by the normal milking process. With an extended milking interval, however, the concentrations of lactose and potassium decrease and those of sodium and chloride increase throughout the normal milking, but the residual milk is similar in composition to that of the first portions of milk removed (Wheelock *et al.* 1965*b*).

Mackenzie & Lascelles (1965*a*) have reported that successive portions of residual milk removed after the completion of normal milking, and following successive injections of oxytocin, show progressive increases in sodium and chloride contents and decreases in lactose and potassium contents in the whey. On the basis of their results, they postulated that the fluid secreted by the alveolar cells is modified during its passage through the ducts by the absorption of water, sodium, chloride and possibly potassium. In the light of Linzell's (1967*a*) observations, it might equally be assumed that the observed effects arose from the action of the oxytocin.

Infections of the udder and udder damage. The literature on the effects of infections of the udder on the yield of milk and milk constituents including the water-soluble constituents, is extensive (see Munch-Petersen, 1938, for literature up to 1935; McDowall, 1945) and reference will be made only to selected papers. Much published work is open to the criticism that the detection of infection was based on indirect tests, the unreliability of which has been demonstrated by Johns & Hastings (1938); they pointed out that even with bacteriological methods it is essential to test on a number of occasions, as bacteria are not necessarily shed at each milking when an infection is present. The interpretation of some of the results is further confounded by the

chemical analyses having been made on the bulk milk of the 4 quarters of the udder or on the foremilk of individual quarters, instead of the bulk milk of a single affected quarter.

The effects of udder infections on the yield of milk and the yield and content of water-soluble constituents are, nevertheless, well established. There is invariably a fall in the milk yield, a decrease in the contents of lactose and potassium in the milk and an increase in those of sodium and chloride. With infections induced by the infusion of bacteria into an udder quarter (Wheelock, Rook, Neave & Dodd, 1966), these changes were particularly marked in the first few days after the infusion, when clinical signs were most pronounced. There was a partial recovery in composition as the lactation progressed, with a further recovery in the succeeding lactation; recovery was enhanced by the elimination of the infection. The magnitude of the changes in concentration during the development of an infection were closely related to the extent of the depression in milk yield. During the period of infection, the depression in the yield of lactose was more marked than that of protein or fat, but similar to that of potassium. With sodium, and to a lesser extent chloride, the increase in concentration tended to compensate for the decrease in yield, and in some instances an increase in the yield of sodium was obtained after infection.

INTERRELATIONSHIPS OF WATER, LACTOSE, POTASSIUM, SODIUM AND CHLORIDE

Direct, rectilinear relationships between sodium/chloride and lactose/potassium and inverse rectilinear relationships between lactose/sodium, lactose/chloride, potassium/sodium and chloride/potassium values for milk have been established in numerous investigations. Barry & Rowland (1953) showed that these relationships were precise for inter-quarter comparisons within a single cow, and that the observed variation could be explained in terms of a mixing of a 'true milk' with a diluent which, for the constituents in question, differed only slightly from blood plasma. A similar interpretation of other evidence was made both by Davies (1933) and Peskett & Folley (1933).

For comparisons between cows at varying stages of lactation, Barry & Rowland found similar but less good relationships between sodium/chloride, chloride/potassium and potassium/sodium values for the milk of the whole udder, but the relationships with lactose were not reported. Rook & Wood (1958), in a similar analysis with values expressed on a water basis, demonstrated close inverse relationships between sodium and both lactose and potassium, but found variations in lactose and potassium to be largely independent. From their results they concluded that milk sodium could be considered to be derived from 2 sources, a part associated with lactose and the remainder with potassium; the water of milk could be represented as 2 phases in one of which sodium and lactose, and in the other sodium and potassium, varied inversely.

In later papers (Rook & Wood, 1959; Walsh & Rook, 1964) it was shown that when variations due to stage of lactation, age and udder damage (including infection) were excluded, lactose and potassium values were constant for an individual animal and there was, for the majority of animals studied, a close inverse relationship between

these values. In previous studies, this inverse relationship was confounded with the direct relationship due to variations with age, stage of lactation and udder infection, and the poor correlations reported may be thus accounted for. To explain these observations, it is necessary to represent milk water as a 3-phase system. One phase would be associated with milk lactose, and a second phase with milk potassium though containing small amounts of sodium and chloride. These 2 phases together would constitute the 'true milk' of Barry & Rowland (1953): the proportions would be constant within an individual animal but would vary between animals. The third phase, rich in sodium and chloride, would correspond to the diluent of Barry & Rowland (1953).

OSMOTIC REGULATION

The osmotic pressure of milk is close to that of blood and other body fluids (Altman & Dittmer, 1961) and, in common with the osmotic pressure of these fluids, normally varies only within close limits. The ingestion of a massive dose of sodium sulphate (Van der Laan, 1915) or the restriction of water intake to a short period each day (Aschaffenburg & Rowland, 1950; Hillman, Provan & Steane, 1950; Aschaffenburg, 1955) has been shown in the cow, however, to cause marked variation throughout the day in the freezing-point depression (Δ) of the blood and unusually large morning–evening differences in the Δ of milk. Wheelock *et al.* (1965*a*), by experimentally restricting access of cows to drinking water to 1 short period each day and by using a short-interval milking technique, have studied more fully the relationship between the osmotic pressure of milk and that of blood. They showed that the values for milk agreed more closely with those for mammary-venous blood than those for jugular-venous blood, and that changes in the concentrations of individual milk constituents were proportional to the changes in Δ and were consistent with a movement of water into or out of the gland in response to any change in osmotic pressure of the blood. They concluded that milk present in the mammary gland is in continuous equilibrium with the blood flowing through the gland and not only during its formation.

THE RELATIONSHIP BETWEEN LACTOSE YIELD AND MILK YIELD

Taylor & Husband (1922) observed that when the yield of milk is depressed by underfeeding or by starvation, there is a decrease in milk lactose content and an increase in fat and protein contents and that when animals are again given normal amounts of food, the original composition of the milk is gradually regained. In the light of these observations, they suggested that the rate of lactose synthesis regulated the secretion of milk water. Gowen & Tobey (1931–2) and Brown *et al.* (1936) reported experiments with the goat in which both milk yield and milk lactose content were decreased by the administration of insulin or phloridzin. Numerous reports have since appeared confirming these observations (Kronfeld *et al.* 1963; Rook *et al.* 1965; Schmidt, 1966) and demonstrating that there is no associated reduction in the yield of protein or fat. There is strong circumstantial evidence that the reduced lactose secretion is due to hypoglycaemia, as when insulin was infused together with glucose to maintain the glucose level in the blood plasma, the effects of insulin on lactose yield were not observed (Kronfeld *et al.* 1963; Schmidt, 1966). The relationship between

lactose yield and plasma glucose concentration has been shown to be curvilinear, with negligible changes in the yield of lactose with variations in glucose concentrations above about 40 mg/100 ml (Rook & Hopwood, unpublished). Storry & Rook (1962) observed a similar specific depression in milk lactose synthesis, with an associated reduction in milk yield and a compensatory increase in fat and protein contents, in response to massive intraruminal infusions of butyric acid which caused a marked depression in the glucose content of the blood plasma.

THE PERMEABILITY OF THE MAMMARY EPITHELIUM

The permeability of the mammary epithelium to ions present in milk was first demonstrated for labelled phosphorus and calcium. Such a transfer could be accounted for simply in terms of an exchange diffusion process, but Azimov *et al.* (1962), on the basis of observations mainly with labelled phosphorus, proposed that resorption from the alveoli and exchange across the epithelial border is an integral part of milk secretion.

Knutsson (1964*a*) has studied in detail in the goat the transfer of ^{42}K, ^{36}Cl and ^{24}Na. Exchange across the epithelium was demonstrated both after intravenous injection and introduction into the gland through the teat. The half-time (the time needed for the amount of tracer in the udder to decrease to 50 % of the dose value, or for the specific activity of milk constituents to rise to half that of the constituents in the plasma) for ^{42}K and ^{36}Cl was of the order of 2–4 h. ^{42}K was found to leave the milk slightly faster than ^{36}Cl, but to appear in milk more slowly, differences which Knutsson ascribed to 'equilibration with the intracellular pools which are of different size for potassium and chloride'. For ^{24}Na, the half time after infusion through the teat was about 30 min or less, and even shorter after intravenous injection. This difference was thought to be due to exchange being mainly within the alveolar region (after intravenous administration of ^{24}Na, ^{42}K and ^{36}Cl, the concentrations in milk removed from the udder were highest in the later portions) when the rate of equilibration within the udder would be a factor limiting the rate of resorption. In a later paper Knutsson (1964*b*) calculated from measurements of fluid volume determined by a polyethylene glycol dilution method, that a net resorption of both sodium and chloride took place when isotonic saline was infused into the gland. Resorption of the sodium was significantly greater than that of chloride, and part of the sodium appeared to have exchanged with potassium. Fluid removed from the udder 1 h after the introduction of hypertonic solutions of lactose and of sucrose, showed depressed concentrations of potassium, calcium, chloride and phosphate, as compared with those of milk removed immediately before the infusion, but the concentration of sodium was only slightly decreased and of bicarbonate unchanged (Knutsson, 1966). It was concluded that water, sodium and bicarbonate had entered the gland.

Mackenzie & Lascelles (1965*b*) have confirmed with ewes that a net movement of ions can take place across the mammary epithelium. Solutions containing electrolytes and lactose, and differing markedly in composition from that of milk, when injected into the gland changed in composition towards that of milk, or in the dry gland towards that of involution secretion which is higher than milk in sodium and chloride and

lower in potassium and lactose. Thus, sodium, potassium and chloride were secreted into solutions in which the concentrations were lower than in milk and absorbed when the concentrations were higher. Lactose was absorbed from the dry gland but not from the lactating one. Water absorption occurred only from solutions containing sodium and chloride at a higher concentration than in milk. This is surprising since the administered solutions were hypotonic and a rapid and complete osmotic equilibrium between milk and blood has been demonstrated (Wheelock *et al.* 1965*a*). The use of hypotonic solutions may well seriously affect the extent, and even the nature, of any movement of ions across the mammary epithelium.

Water-soluble compounds that distribute throughout the whole of body water, urea and *N*-acetyl-4-aminoantipyrine, for example, diffuse freely into milk from blood and from milk back into blood, and equilibration appears to be rapid and complete (Whiting, Balch & Campling, 1960; Wheelock & Rook, 1966*b*). Rasmussen (1961) has shown that for weak electrolytes there is diffusion equilibrium between plasma and milk for the free (i.e. unbound to protein) unionized fractions. Compounds, such as thiocyanate and thiosulphate, which when administered intravenously show a more limited distribution which follows mainly extracellular water, enter milk only to a limited extent; the ratio of the concentration in milk to that in blood is usually between about 0·25 and 0·50, the value varying with stage of lactation and between quarters of the udder in association with variations in the chloride content of the milk. From the limited information available it appears also that once such materials have entered milk there is little movement back to the blood. These results have been interpreted as demonstrating a continuous secretion into the udder of a fluid of extracellular origin the volume of which, relative to the alveolar secretion, increases in advanced lactation and with other physiological changes associated with an increase in the chloride content of milk (Wheelock & Rook, 1966*b*).

THE CONTROL OF IONIC COMPOSITION

Although the concentrations of lactose, potassium, sodium and chloride in milk are affected by a wide variety of factors, the observed changes in composition are of 2 main types, one associated with variation between animals, the other with variation within animals. For 1st-lactation cows, managed normally and maintained free of udder infection, the milk concentrations of lactose, potassium, sodium and chloride are remarkably constant throughout the main part of the lactation. The values vary between animals but there is a close inverse rectilinear relationship between lactose content and the molar sum of sodium plus potassium contents, and for a majority of animals there is also a good inverse relationship between lactose and potassium alone. Within-animal effects—age, advanced lactation, accumulation of milk within the udder, udder infection and intravenous injection of oxytocin—all cause similar changes in milk composition, namely a decrease in the contents of lactose and potassium and an increase in those of sodium and chloride. The lactose to potassium ratio is usually maintained and there is a close rectilinear relationship between the increases in the concentrations of sodium and chloride. These changes in milk composition almost invariably occur in association with a reduction in milk volume, and though sodium and chloride are present in increased concentrations, they are usually

secreted in reduced amounts: the only recorded exceptions are for short periods after the development of a bacterial infection (Wheelock, Rook, Neave & Dodd, 1966), in the period following an extended milking interval (Wheelock, Rook, Dodd & Griffin, 1966) and after the administration of oxytocin (Wheelock *et al.* 1965*d*).

There can be little doubt that lactose originates within the cells of the alveolar epithelium. Since the ratio of potassium to sodium in the mid-lactation milk of healthy heifers is similar to that for intracellular fluid, it seems reasonable to assume that under those circumstances these constituents also are mainly cellular in origin. To account for the constancy of the lactose to potassium ratio within an animal and for the variation between animals, Rook & Wood (1959) postulated that milk fluid arises by the continuous synthesis within the cell of lactose, and other constituents and the movement of water into the cell to maintain osmotic equilibrium until a fairly constant ratio of secretory to intracellular fluid is obtained, when the cellular contents are expelled into the alveolar lumen. The ratio of secretory to intracellular fluid at the moment of expulsion (and, presumably, to some extent the composition of the intracellular fluid itself) will vary from animal to animal. Variation in the extent to which lactose contributes to the total osmotic activity of synthesized materials would result in some change in the lactose to potassium ratio, as for example is known to occur in colostral milk which is rich in immune globulins. The constancy of the lactose to potassium ratio in circumstances such as accumulation of milk within the udder and udder infection, which are known to result in lactose resorption, may also be accounted for if it is assumed that resorption occurs primarily from the secretory cell. During the drying-off period, lactose resorption must in part be from the ducts and cisterns of the gland but the associated changes in milk potassium content have not been recorded. The series of events described by Rook & Wood (1959) are thus consistent with many experimental observations, but any consideration of the physiological processes that might be involved must await more direct evidence on the type of excretion process involved in the discharge of water-soluble constituents from the cell.

Increases in the sodium and chloride contents of milk were explained by Turner (Garrison & Turner, 1936; Turner, 1946) in terms of an impairment of the activity of the alveolar cells, resulting in a decreased rate of excretion of milk from the cell, an increased permeability of the cell to certain constituents, especially chloride (and presumably sodium) and, to maintain osmotic equilibrium with blood, a selective depression of lactose synthesis. This view was based mainly on results of experiments in which there was serious disturbance of udder function (see Knutsson, 1964*a*), nevertheless the high concentration of potassium and low concentration of sodium within the secretory cell must presumably be maintained by a 'sodium pump' mechanism, which would be ATP dependent, and a restriction on the supply of metabolites to the gland could result in an alteration of the ionic composition of cell contents towards that of extracellular fluid. A lowered mammary blood flow has been found in animals in advanced lactation (Linzell, 1960) and during fasting (Linzell, 1967*a*) and in a few animals with distended or diseased udders also (J. L. Linzell, personal communication). The mechanism by which the lactose to potassium ratio would be maintained is not clear.

An impairment of the activity of the sodium pump of the secretory cell could

account for the temporary slight rise in sodium yield during the recovery phase after an extended milking interval. Additional mechanisms, however, would be required to account fully for the observed changes in milk secretion after a severe bacterial infection, when invariably there is a permanent decrease in the yield of milk due to involution of certain areas of secretory tissue. The increased yield of sodium and chloride under those circumstances can reasonably be accounted for only in terms of an entry of fluid of extracellular origin, as discussed by Barry & Rowland (1953). They, in common with several earlier workers (Davies, 1933; Peskett & Folley, 1933), have favoured the view that milk secreted by the alveolar cell is of constant composition, and is mixed after secretion with a diluent in which the concentrations of potassium, sodium and chloride are approximately the same as in blood serum. This diluent, they suggested would continually filter into the alveolar lumen without passing through the epithelial cells, and when the volume of milk secreted by the cells was depressed, in late lactation, after infection or with other treatments, the proportion of transudate in the milk withdrawn from the mammary gland would rise. Certain treatments, udder damage in particular, but possibly also accumulation of milk within the udder and administration of oxytocin, would be expected to alter permeability and allow an additional increased entry of transudate. The invariable presence of traces of blood plasma albumin and globulins in milk and the marked increase in the entry of plasma proteins that occurs in association with increases in the sodium and chloride contents gives support to this hypothesis. There is no direct, physiological evidence for the existence of such a secretory process but an interchange of ions between milk and blood, which appears to be most active in the region of the alveolus, has been clearly demonstrated and the evidence on the entry of intravenously administered thiocyanate into milk (Wheelock & Rook, 1966*b*) is strong indication of a net flow of extracellular fluid into the gland.

Azimov *et al.* (1962), Knutsson (1964*b*) and Mackenzie & Lascelles (1965*b*) favour an alternative explanation for the observed variations in milk sodium and chloride contents. They have suggested that the fluid secreted by the cell is richer in sodium and chloride than that obtained from the gland at milking and that there is a net resorption, primarily of sodium and chloride, as the fluid excreted from the cell passes through the ducts into the cistern of the gland. Such a transport across the duct membranes would be against the concentration gradient and presumably, therefore, ATP linked. The ducts are richly supplied with blood vessels (J. L. Linzell, personal communication) and a reduced blood flow would possibly interfere with this active process. These ideas arose mainly out of experiments in which a net resorption or entry of sodium and chloride was demonstrated following the introduction into the gland of solutions differing in ionic composition from that of milk. Such a movement of ions does not necessarily point to a resorption of sodium and chloride across the ducts under normal physiological conditions. It is generally accepted that sodium and potassium ions diffuse into the cell from extracellular fluid and that the high potassium and low sodium concentrations are maintained through the action of a sodium pump, which transfers sodium out of and potassium into the cell. There is no evidence that the sodium pump operates on that part of the cell membrane proximal to the lumen but it is possible that there is an interchange of ions across this membrane. Experimentally induced differences in ionic composition between the fluid of the secretory cell and

that within the alveolar lumen could result in a limited net transfer of ions that would not occur when the composition of the 2 fluids was identical.

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