Vol. 32, No. 2 June, 1965

THE JOURNAL OF DAIRY RESEARCH

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W.1
American Branch: 32 East 57th Street, New York, N.Y. 10022

Price:—THIRTY SHILLINGS *Net*
(U.S.A. $5.00)

The Journal of Dairy Research

was established in 1929 as a medium for the publication of the results of original research in dairy science and cognate subjects. It is published by the Cambridge University Press.

The subscription to the *Journal* is 80*s*. per volume, for orders placed in advance of publication, and covers three issues. Single copies may be purchased at 30*s*. each. Postage extra. Subscriptions may be sent to any bookseller or to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. The subscription price in the U.S.A. is $13.50. Enquiries should be sent to the Cambridge University Press, American Branch, 32 East 57th Street, New York, N.Y. 10022.

Back numbers of the *Journal* may be obtained from the publishers. Volumes 1 to 4, each part 25*s*. net. Volumes 5 to 29, each single part 25*s*., double parts 50*s*. net. Postage extra. (With the exception of Volume 6, No. 2, Volume 9, No. 1, Volume 10, Volume 11, No. 1 Volume 12, No. 1 and Volumes 13 and 14 complete, which are out of print.)

The influence of liveweight at calving on milk production in Friesian heifers

By B. RIDLER, W. H. BROSTER and D. R. WESTGARTH
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 26 *October* 1964)

Summary. Records of 81 heifers, daughters of 6 bulls, were examined to determine the regressions of milk yield, corrected for lactation length, on initial liveweight and age at calving.

Age was found to have no significant effect and was discarded in the final analysis.

The regression equations were similar for the different groups of half-sibs, except for one group whose members had not been fed according to milk yield. After removal of this group, a highly significant positive relationship between lactation yield and liveweight at calving was found 'within bull groups'. The mean values of groups of half-sibs did not show this relationship, and significant differences in milk yield still persisted between groups after adjustment for liveweight differences.

It was concluded that herd improvement by choosing heifers solely on the basis of liveweight shortly after calving appears unlikely to be effective unless the sire's potential is known and the feeding during lactation takes into account the greater maintenance requirements of heavier animals.

While the existence of a positive relationship between liveweight shortly after calving and total milk yield in subsequent lactation, within a population, has frequently been demonstrated (see Clark, 1960; Erb, 1962; Reid *et al.* 1964, for references) its practical value in terms of herd selection has yet to be established. Thus Bailey & Broster (1954), dealing with the Shorthorn section of the Institute herd, showed that while the relationship existed for the data as a whole and for 'within groups' of paternal half-sibs the mean milk yields of the progeny groups were independent of their mean liveweights.

This finding has been partially confirmed by Mason, Robertson & Gjelstad (1957) who, in a study of Danish progeny station data, analysed initial liveweight and lactation milk yield data and found a positive but non-significant correlation between these factors for both 'between' and 'within' progeny groups.

In view of the paucity of the data on the milk yield and liveweight relationship when applied to progeny groups, and its possible importance in relation to large-scale breeding programmes, and also the desirability of confirming the findings of Bailey & Broster (1954) with data from another breed, it was decided to examine the appropriate records of the Friesian section of the Institute herd.

The relationship of milk yield with liveweight immediately after calving has been expressed mathematically by Brody (1942) and Kleiber (1947) in the equation

$Y = cW^b$, where Y is 4% fat-corrected lactation yield in lb, W is the initial liveweight in lb, and b and c are unknowns to be determined. While the exponent, b, shows considerable variation between species when determined from the experimental data analysed by these authors, they have suggested an average value of $b = 0{\cdot}75$. A comparable figure, $b = 0{\cdot}82$, was obtained by Bailey & Broster (1954).

METHOD

The general management of the milking herd has been described by Bailey (1952) and by Bailey & Broster (1954). All the heifers calved in the autumn. They were milk-recorded twice daily, and their milk was sampled monthly for butterfat estimation by the Gerber method. The heifers were weighed on the 2nd and 3rd day after calving, and the mean of these weights used as the initial liveweight. All the heifers were included in feeding trials in early lactation (Bailey, Broster, Brown & Foot, 1954; Broster, Ridler & Foot, 1958; Broster, Tuck & Balch, 1964). Animals were allocated to blocks according to date of calving and liveweight. With the exception of one group of half-sibs noted below, the heifers were fed for milk production according to Woodman's (1957) standards, or fixed proportions of these standards, but the maintenance ration was constant for all the animals of an experiment. They were not used experimentally in the later stages of lactation, and were subjected to normal herd management until the next calving. They were served at the 1st heat after the 90th day in milk.

Records of 81 Friesian heifers sired by 6 different bulls and having their 1st lactation during the period 1952–61 were used for a study of the inter-relationship of the following factors: (1) age at calving in days; (2) initial post-calving liveweight in lb; (3) lactation length in days; (4) lactation yield of 4% fat-corrected milk in lb. The formula developed by Gaines (1947) was used to calculate lactation yield of 4% fat-corrected milk. Mean values of liveweight, age, and milk production for the bull groups are given in Table 1.

The relationships between yield and the other factors were expressed as simple regressions, and as a multiple regression fitted by the method of least squares (e.g. Snedecor, 1956).

RESULTS

Simple regression equations of the logarithm of fat-corrected lactation yield (lb) on the logarithms of age at calving (days), initial liveweight (lb) after calving, and lactation length (days) were calculated initially for all the data. The regression coefficients were: $0{\cdot}2502\,\mathrm{NS} \pm 0{\cdot}2367$; $1{\cdot}1577^{**} \pm 0{\cdot}2604$; and $1{\cdot}2781^{**} \pm 0{\cdot}1617$, for age at calving, initial liveweight after calving, and lactation length, respectively.

A multiple regression equation for the logarithms of these variables was then calculated for each group of half-sibs and a pooled estimate obtained. Similar multiple regressions were fitted to group means and to the total data. The partial regression coefficient of yield on age was non-significant for each individual bull group, and for the 'total' data and 'within group' analysis. It just reached significance at the 5% level of probability for the 'between group' analysis. The influence of age was judged to be unimportant for the range of values in the present data and it was decided to exclude this factor from the final analysis.

The partial regression coefficients of fat-corrected lactation milk yield on initial liveweight after calving, and lactation length, with age at calving disregarded, are given in Table 2, for individual groups of half-sibs, for the total data, and for 'between' and 'within' bull groups.

Table 1. *Age (days), initial liveweight at calving (lb), lactation length (days) and 4% fat-corrected milk yield (lb) for the daughters of 6 bulls*

Bull	No. of daughters	Mean liveweight at calving, lb	Mean age at calving, days	Mean lactation length, days	Mean lactation yield of 4% fat-corrected milk, lb
A	11	1113	1053	347	8931
B	9	1083	950	335	9039
C	23	1097	1010	329	7651
D	21	1162	1026	343	10198
E	9	1096	936	324	8116
F	8	1186	1082	337	8606
Total	81	1124	1012	336	8786

Table 2. *Partial regression coefficients showing effect of initial liveweight after calving (W, lb), lactation length (L, days), on fat-corrected lactation yield (Y, lb), all expressed as logarithms to the base* 10

Bull	$b_{\log Y \log W . \log L}$	$b_{\log Y \log L . \log W}$
A	0·9038** ± 0·1824	1·2938*** ± 0·1498
B	1·4883 ± 1·5737	2·9399* ± 1·0768
C	0·6515* ± 0·3018	0·8353*** ± 0·1772
D	1·0680* ± 0·4170	0·8091 ± 0·4144
E	−1·5984 ± 1·1329	1·2311 ± 0·6300
F	0·7206 ± 1·1972	−0·1229 ± 1·1871
Total	0·7519*** ± 0·2107	1·1288*** ± 0·1566
Between	0·8201 NS ± 1·1563	3·2713 NS ± 1·5939
Within	0·6047** ± 0·2059	1·0633*** ± 0·1452

*** Significant at $P < 0·001$. ** Significant at $P < 0·01$. * Significant at $P < 0·05$. NS Not significant at $P = 0·05$.

Significant differences at the 5% level of probability were established between individual group regressions. Of the 6 bull groups, the progeny of bull E had been used in an experiment by Broster *et al.* (1964) in which a fixed ration was given to each heifer regardless of its milk yield, in contrast with other heifers in the present study whose rations varied according to the amount of milk produced. All the heifers on the fixed ration experiment, including the daughters of bull E, showed a negative correlation between initial liveweight and milk yield (Broster, 1962); this was considered a sound reason for regarding them as distinct from the other 5 groups of half-sibs and they were accordingly withdrawn from the analysis.

Recalculated values of the partial regression coefficients, with bull group E discarded are given in Table 3 for total data 'between groups' and 'within groups'. Differences between the regressions of the 5 remaining groups were no longer significant (Table 4), but there were significant differences in yield level between the groups. The partial regression coefficients corresponding to 'between groups' were both non-significant, those for 'within groups' and 'total' were highly significant. Hence the

individual group regressions may reasonably be regarded as parallel but not coincident. The pooled regression equation was

$$\log Y = -0{\cdot}8979 + 0{\cdot}7537 \log W + 1{\cdot}0035 \log L,$$

where Y is lactation yield of fat-corrected milk (lb), W is initial liveweight after calving (lb) and L is the number of days in milk. Taking antilogs,

$$Y = 0{\cdot}1265 W^{0{\cdot}7537} L^{1{\cdot}0035}.$$

The partial regression coefficients 0·7537 and 1·0035 were pooled values representative of the individual groups; the constant term 0·1265 does not apply however to the individual groups, which have been shown to have different levels of yield.

Table 3. *Partial regression coefficients from Table 2 recalculated after excluding bull E*

	$b_{\log Y \log W . \log L}$	$b_{\log Y \log L . \log W}$
Total	0·8834*** ± 0·2079	1·0690*** ± 0·1577
Between	0·7175 NS ± 1·2725	3·9102 NS ± 1·9586
Within	0·7537*** ± 0·1974	1·0035*** ± 0·1416

*** Significant at $P < 0{\cdot}001$. NS Not significant at $P = 0{\cdot}05$.

Table 4. *Analysis of variance of regressions of log lactation yield of fat-corrected milk (lb) on log liveweight at calving (lb) and log lactation length (days)*

Source	D.F.	S.S.	M.S.
Total	71	0·7660	—
Due to overall regression	2	0·4292	0·2146***
Residual	69	0·3368	0·0049
Between bulls	4	0·1860	—
Due to regression	2	0·1534	0·0767 NS
Residual	2	0·0326	0·0163
Within bulls	67	0·5800	—
Due to pooled regression	2	0·3296	0·1648***
Residual	65	0·2504	0·0039
Differences between group regressions	8	0·0464	0·0058 NS
Differences between group levels	4	0·0864	0·0216***
Combined group residuals	57	0·2040	0·0036

*** Significant at $P < 0{\cdot}001$. NS Not significant at $P = 0{\cdot}05$.

The variance of log lactation yields among individuals of a bull group was reduced 46 %, on average, by adjusting for difference in log lactation length, 22 % for difference in log liveweight after calving, and 55 % by adjusting for both covariates jointly. Similarly, the variance of means of bull groups was reduced by 36, 37 and 53 %, by adjustment for log lactation length, log liveweight after calving, and for these covariates jointly. This reduction in variance following adjustment for the 2 variables was appreciable. Nevertheless, the differences between bull group means, adjusted for both covariates, remained highly significant. The effect of adjustment on bull group means may be further illustrated as follows:

	Log lactation yield					
Bull group	A	B	C	D	F	Weighted means
Original means	3·934	3·945	3·874	4·004	3·926	3·936
Means after adjustment by multiple regression	3·931	3·959	3·897	3·982	3·907	3·936

DISCUSSION

The present study confirms the findings of earlier workers that milk yield is directly related to initial liveweight after calving. Of particular interest is the close agreement of the present result with that of Bailey & Broster (1954), with animals of another breed, though it must be remembered that both analyses were conducted on data from 1 herd. Here the overall partial regression coefficient of log lactation yield of fat-corrected milk on log initial liveweight was +0·88 (Table 3); the comparable

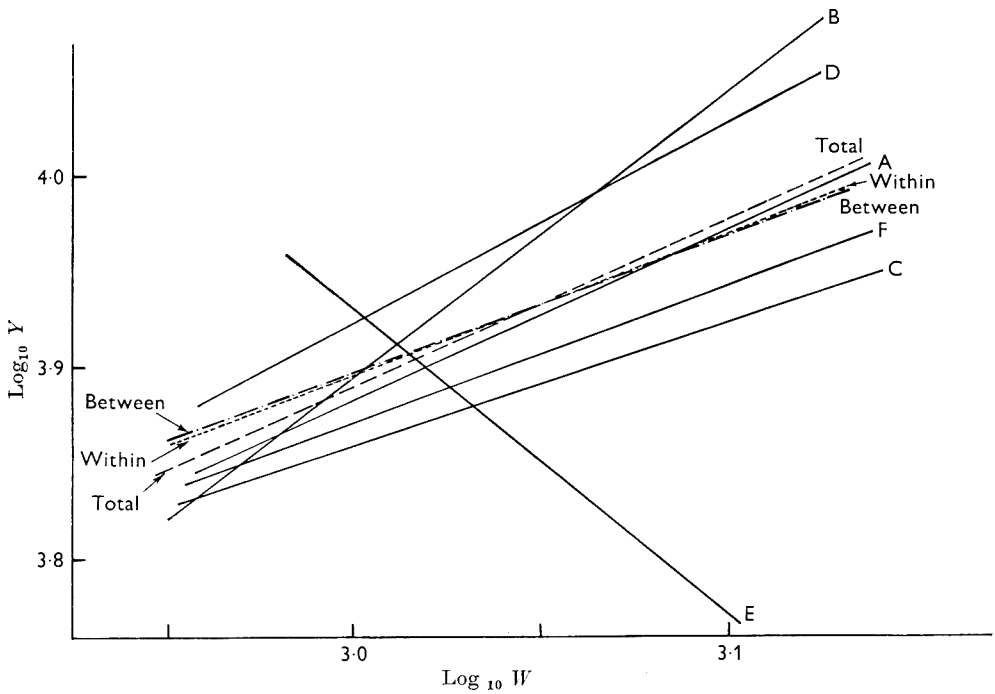


Fig. 1. Graphical representation of the partial regression equations of log fat-corrected milk yield (Y), adjusted to constant lactation length (equal to the grand mean), on log initial liveweight (W) after calving. Individual bull group (A–F) and, total, 'between' and 'within' group partial regressions are shown.

figure obtained by Bailey & Broster (1954) for 305-day fat-corrected milk yield was +0·82. Whereas Bailey & Broster (1954) used a linear multiple regression equation, here a multiple regression equation on the logarithms of the variates appeared to be more justified. The present mathematical model is a convenient device for adjusting for unequal periods of lactation. It is appropriate in the present range of lactation lengths but would be inappropriate, for example, in estimating yields at early stages of lactation. It should be noted that in the pooled multiple regression equation quoted above the partial regression coefficient of log lactation length is very nearly unity (1·0035). Hence the equation corresponds closely to a simple linear relation between log mean yield and log liveweight at calving.

The equation for the pooled data shows that at initial liveweights of 800, 1000, 1200 and 1400 lb, the 305-day fat-corrected milk yields are estimated as 6070, 7182,

8240 and 9255 lb; the increase per 100 lb liveweight approximated to 530 lb in all or 1·7 lb/day of the 305-day lactation.

Fig. 1 shows graphically the partial regression equations of log yield on log liveweight corrected to a mean lactation length of 336 days, and indicates that while the regressions are reasonably parallel (apart from bull E), they are not coincident. That is, because of significant differences in group levels of milk yield due to factors other than liveweight, the regression fitted 'within groups' does not apply 'between groups'. As in the earlier study by Bailey & Broster (1954), there was no significant partial regression of milk yield on liveweight among group means, and an effective study of such relationship would require more bull groups than the present data provide.

Attempts to improve yields by choosing heifers by weight alone are likely to be inefficient, as seen in Fig. 1, because heavier animals may be from sire groups of low milk-yield potential, a conclusion also drawn by Bailey & Broster (1954). Choosing the heavier heifers of proven sires of good yield potential could lead to a further improvement in yield. However, the question not broached here, but necessarily considered in assessing the value of liveweight as a criterion for selecting animals for milk production, is the efficiency of food conversion in lactation. A heavy animal requires a greater supply of nutrients for maintenance than a light one. The effect of this requirement on the efficiency of food conversion is seen in the negative partial regression of milk yield on liveweight in the animals in bull group E, fed a fixed amount of food daily. The other groups, showing a positive partial regression, were given increasing amounts of food as their yield increased. The greater maintenance requirement of the heavier animal is at least partially masked by a greater food intake consequent upon its greater milk yield.

A further explanation for the positive relationship of milk yield and liveweight may lie in the fact that liveweight represents a combination of physique and body condition. Animals in good condition at calving have a greater reserve upon which to draw in lactation, particularly in the event of small rations after calving (Broster *et al.* 1958). This is supported by the negative relationships observed between milk yield and change of liveweight after calving (Johannson, 1954; Mason *et al.* 1957; Broster, 1962).

It is possible that the small effect of age on milk yield observed here arises from the limited range of the data. Other authors in Scandinavia and the U.S.A., dealing with very much larger numbers of animals and herds, have shown a positive and highly significant relationship between age at calving and milk yield (Johannson, 1954; Clark & Touchberry, 1962; Erb, 1962).

It is obvious that further large-scale work on this subject is desirable, such as that recently initiated by the Milk Marketing Board of England (1960) with the object of studying the physiological efficiency at a given liveweight of the daughters of bulls of proven production potential.

We are grateful for the interest shown by Dr C. C. Balch and Mr A. S. Foot in this investigation.

REFERENCES

BAILEY, G. L. (1952). *J. Dairy Res.* **19**, 89.
BAILEY, G. L. & BROSTER, W. H. (1954). *J. Dairy Res.* **21**, 5.
BAILEY, G. L., BROSTER, W. H., BROWN, B. B. & FOOT, A. S. (1954). *J. Dairy Res.* **21**, 1.
BRODY, S. (1942). *Science*, **95**, 485.
BROSTER, W. H. (1962). *Rep. natn. Inst. Res. Dairy*, p. 45.
BROSTER, W. H., RIDLER, B. & FOOT, A. S. (1958). *J. Dairy Res.* **25**, 3.
BROSTER, W. H., TUCK, V. J. & BALCH, C. C. (1964). *J. agric. Sci., Camb.*, **63**, 51.
CLARK, R. D. (1960). *Diss. Abstr.* **21**, 1310.
CLARK, R. D. & TOUCHBERRY, R. W. (1962). *J. Dairy Sci.* **45**, 1500.
ERB, R. E. (1962). *J. Dairy Sci.* **45**, 699.
GAINES, W. L. (1928). *Bull. Ill. agric. Exp. Stn*, no. 308.
JOHANNSON, I. (1954). *Z. Tierzücht. ZüchtBiol.* **63**, 105.
KLEIBER, M. (1947). *Physiol. Rev.* **27**, 4.
MASON, I. L., ROBERTSON, A. & GJELSTAD, B. (1957). *J. Dairy Res.* **24**, 135.
MILK MARKETING BOARD (1960). *Milk Prodr.* **7**, 12.
REID, J. T., LOOSLI, J. K., TRIMBERGER, G. W., TURK, K. L., ASDELL, S. A. & SMITH, S. E. (1964). *Bull. Cornell Univ. agric. Exp. Stn*, no. 987.
SNEDECOR, G. W. (1956). *Statistical Methods*. Iowa State University Press.
WOODMAN, H. E. (1957). *Bull. Minist. Agric. Fish Fd., Lond.* no. 48.

Printed in Great Britain

J. Dairy Res. (1965), **32**, 143

The effect of antioxidants on the keeping quality of whole milk powder

II. Tocopherols

By J. ABBOT and R. WAITE
The Hannah Dairy Research Institute, Ayr

(*Received* 24 *November* 1964)

Summary. Spray-dried whole milk powders were prepared containing 0·01 % of γ-tocopherol and of a mixture of α-, γ- and δ-tocopherols. Control powders and powders containing 0·01 % of dodecyl gallate, an antioxidant of known effectiveness, were also made. The powders were stored at 37 °C and examined at intervals for taste, peroxide value and absorbed oxygen. The protection against oxidation afforded by both the tocopherol additions was small, although γ-tocopherol was more effective than the mixture of tocopherols. Dodecyl gallate was again found to be a good antioxidant for whole milk powder.

The widespread occurrence of tocopherols in plants and in the lipids of herbivores makes them attractive substances for use as antioxidants in foods intended for human consumption. In dairy research, tocopherols have been added to fresh milk, cream, butter and whole milk powder in attempts to delay fat oxidation. Conflicting results on their effectiveness have been reported. A mixture of tocopherols prevented 'spontaneous' oxidation in susceptible milks for 24–48 h (Williams & Burgwald, 1941; Krukovski, Loosli & Whiting, 1949), but α-tocopherol was not only without beneficial effect in cream and butter but appeared to be slightly pro-oxidative and its presence led to the formation of a fishy flavour (Richardson, El-Rafey & Long, 1947; Swartling, 1949; Nielsen *et al.* 1953). Findlay, Smith & Lea (1945) found it difficult to decide whether mixed tocopherols in whole milk powder had any antioxidative effect because of the development of oily flavours. Most of these earlier tocopherol concentrates contained only 15–30 % of total tocopherols and the off-flavours observed during the storage trials may have been derived more from the other constituents of the preparation or their oxidation products than from the tocopherols themselves. With the recent availability of more concentrated preparations, particularly of individual tocopherols, it appeared worthwhile briefly to re-examine their effectiveness as antioxidants in spray-dried whole milk powder.

EXPERIMENTAL

The preparation of the powders followed the system previously described (Abbot & Waite, 1962). Milk was obtained from a 3000-gal batch at a creamery of the Scottish Milk Marketing Board. It was preheated at 190 °F (88 °C) for approximately 15 sec

and concentrated to 34 % total solids under vacuum (28 inHg). Two tocopherol concentrates were available, DL-γ-tocopherol of 80 % purity and a mixture of α-, γ- and δ-tocopherols (10:57:33) which had a total tocopherol content of 55 %. The tocopherol concentrate in an amount sufficient to give 0·01 % tocopherol in the powder was dissolved in 4 g of hydrogenated, deodorized coconut oil at 98 °F (37 °C) and added with continuous stirring to the concentrated milk at the same temperature during homogenization at 2500 lb/in^2. (The concentration of 0·01 % antioxidant was chosen to conform with British legislation for other foodstuffs and because several antioxidants were known to be effective at that concentration.) The homogenized, concentrated milk was dried on a centrifugal disk type of laboratory-scale machine operating at an inlet air temperature of 248 °F (120 °C), a mid-chamber temperature of 176 °F (80 °C) and a flow rate of evaporated milk of 20 ml/min.

In previous work (Abbot & Waite, 1962) it was found that dodecyl gallate was the most effective antioxidant of several tested. To allow comparison of the present results with those of that earlier series, powders containing dodecyl gallate were made in addition to control powders. Two powders for each antioxidant and 2 control powders were prepared, the duplicate powders being made on different days. Immediately after manufacture the moisture content, fat percentage, peroxide value and ferricyanide reducing power of the protein were determined as previously described (Abbot & Waite, 1962), and the taste of the powder reconstituted in water was recorded by 5 experienced observers. The powders were packed on the day following manufacture in tinplate cans in air and stored at 37 °C. Cans were withdrawn at intervals of 28 days, the composition of the gas within the can was analysed and the powder examined for the peroxide value of the fat and for taste.

RESULTS

The initial analyses showed that the powders were very similar in composition; moisture contents lay between 2·4 and 2·9 %, fat contents between 25 and 27 %, peroxide values between 0·5 and 0·8 m-equiv./kg fat and protein-reducing values between 2·2 and 2·9 mg ferricyanide/g protein. The taste of all the reconstituted powders was good and none of the antioxidant additions had imparted any off-flavour to the powders. The powders thus appeared normal and the manufacturing process unlikely to influence their subsequent performance during storage. This was borne out by the agreement between the results for pairs of duplicate powders, and the values in the tables are the means for each pair.

From the relationship between taste score and duration of storage, the times for the powders to reach a taste score of 1, the first discernible oxidized flavour, and a score of 2, the limit of acceptable oxidized flavour, were obtained. These times are given in Table 1 together with previous results for control powders and those containing 0·01 % of dodecyl gallate. In the present series, all the powders containing antioxidant had a better keeping quality than the control powder, although the advantage conferred by the mixed tocopherols was small; γ-tocopherol was more effective than the α-, γ-, δ-mixture, but considerably less so than dodecyl gallate. The results from the earlier experiment show that the milk from which the present series of powders were made was much more susceptible to fat oxidation.

The general pattern of peroxide development in the fat of whole milk powders during storage and the absorption of oxygen is well established (see, for example Lea, Moran & Smith, 1943; White, Smith & Lea, 1947). The changes in peroxide value and oxygen uptake in the present powders followed normal courses and need not be shown in detail. A measure of the relative effectiveness of the antioxidants in delaying these changes is given in Table 2 where the values after storage for 160 days at 37 °C are shown. This was the time which the best keeping powder, containing

Table 1. *Flavour deterioration in whole milk powders stored at* 37 °C

(Values are means of duplicate determinations.)

Antioxidant	Days at 37 °C to reach Score 1*	Score 2*
None (control)	15	48
Mixed tocopherols	23	60
γ-Tocopherol	40	95
Dodecyl gallate	65	160
From Abbot & Waite (1962)		
None (control)	43	100
Dodecyl gallate	120	250

* Score 1, oxidized flavour only just discernible. Score 2, very slight oxidized flavour, powder on borderline of acceptance. (Above score 2, definite oxidized flavour, powder not acceptable.)

Table 2. *The effect of antioxidants on the keeping quality of whole milk powder stored for* 160 *days at* 37 °C

(Values are means of duplicate determinations.)

Antioxidant	Peroxide value, m-equiv./kg fat	Oxygen absorption, mg/100 g powder
None (control)	4·0	19·5
Mixed tocopherols	4·2	19·0
γ-Tocopherol	3·1	17·0
Dodecyl gallate	0·5	9·3
From Abbot & Waite (1962)		
None (control)	2·0	8·0
Dodecyl gallate	0·3	4·0

dodecyl gallate, had taken to reach the limit of acceptable oxidized flavour. The difference between the 2 powders containing tocopherol additions was smaller by these tests than by taste, although, again, γ-tocopherol was more effective than the preparation of mixed isomers. Dodecyl gallate was much superior to either of the tocopherol preparations. The greater susceptibility to oxidation of the milk used for the present powders is again seen from the comparison of peroxide and oxygen absorption values.

DISCUSSION

Using a variety of substrates Lea & Ward (1959) and Lea (1960) found that the relative ability of 7 tocopherols to extend the induction period of peroxide formation depended to some extent upon the degree of unsaturation of the substrates and the temperature at which they were held, but in all systems γ-tocopherol gave a consis-

tently good performance. Of the other tocopherols, δ- was usually an effective antioxidant but α- was not, despite the fact that as vitamin E α-tocopherol is the most active. Under the conditions of this experiment neither of the tocopherol preparations was particularly effective as an antioxidant for spray-dried whole milk powder. Of the 2 preparations, that containing only γ-tocopherol retarded the development of an oxidized flavour in the powder appreciably longer than the mixture of the α-, γ- and δ-tocopherols but both preparations were much less successful in this than the same concentration of dodecyl gallate. Neither of the tocopherol preparations introduced any fishy or oily flavour into the powders as earlier concentrates had been reported to do.

Although tocopherols appear to function best in low concentrations, 0·01–0·05 %, and 0·01 % was sufficient to delay oxidation in some unsaturated methyl esters (Lea & Ward, 1959), it may be that in whole milk powder, where the fat accounts for only one-quarter of the material, a concentration of 0·01 % is insufficient. Tocopherols, with a side chain of 13 carbon atoms, would be expected to be adsorbed preferentially at the surface of the fat globules, but homogenization, which was necessary to disperse the antioxidants through the concentrated milk, may at the same time have increased too much the surface area of fat. Against this possibility, however, must be set the much better results obtained with dodecyl gallate at the same concentration by weight, although it provided a slightly greater molar concentration ($3{\cdot}1 \times 10^{-5}$ moles dodecyl gallate/100 g powder compared with an average of $2{\cdot}4 \times 10^{-5}$ moles tocopherol/100 g powder).

Mention has been made of the greater susceptibility to oxidation of all the present powders compared with those made a year earlier. We have no certain explanation to offer for this, as the farms supplying the milk and the creamery receiving and bulking it were the same as before. Whatever the cause, it imposed a more stringent test on the effectiveness of the antioxidants.

Using the powders containing dodecyl gallate as a basis for comparison with earlier results (Abbot & Waite, 1962) it would appear that γ-tocopherol was slightly less effective than butyl hydroxyanisole, which, in turn, was less effective than propyl gallate, nordihydroguairetic acid and dodecyl gallate, in that order.

The authors thank Dr J. Green and Messrs Vitamins Ltd for the gift of the tocopherol concentrates.

REFERENCES

ABBOT, J. & WAITE, R. (1962). *J. Dairy Res.* **29**, 55.
FINDLAY, J. D., SMITH, J. A. B. & LEA, C. H. (1945). *J. Dairy Res.* **14**, 165.
KRUKOVSKY, V. N., LOOSLI, J. K. & WHITING, F. (1949). *J. Dairy Sci.* **32**, 196.
LEA, C. H. (1960). *J. Sci. Fd Agric.* **11**, 212.
LEA, C. H., MORAN, T. & SMITH, J. A. B. (1943). *J. Dairy Res.* **13**, 162.
LEA, C. H. & WARD, R. J. (1959). *J. Sci. Fd Agric.* **10**, 537.
NIELSEN, J., FISKER, A. N., PEDERSEN, A. H., PRANGE, I., SONDERGAARD, E. & DAM, H. (1953). *J. Dairy Res.* **20**, 333.
RICHARDSON, E. A., EL-RAFEY, M. S. & LONG, M. L. (1947). *J. Dairy Sci.* **30**, 397.
SWARTLING, P. (1949). *12th Int. Dairy Congr.* **2**, 375.
WILLIAMS, E. B. & BURGWALD, L. H. (1941). *J. Dairy Sci.* **24**, 539.
WHITE, J. C. D., SMITH, J. A. B. & LEA, C. H. (1947). *J. Dairy Res.* **15**, 127.

Printed in Great Britain

Steam distillation of taints from cream

X. Vapour liquid equilibrium relationships for benzyl mercaptan

By F. H. McDOWALL

The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

(*Received* 30 *November* 1964)

SUMMARY. The vapour/liquid equilibrium coefficient for benzyl mercaptan, which is the substance causing 'landcress' taint in butter from the milk of cows grazing pastures infested with the cruciferous weed *Coronopus didymus*, was found by use of a continuous vaporization equilibrium still to be 105 from solution in water and 1·30 from solution in cream of 30 % fat content. It was concluded that complete removal of heat-developed landcress taint from cream would not be commercially practicable.

The 'landcress' taint caused in milk and cream by the consumption of the cruciferous weed *Coronopus didymus* by cows is accentuated by the heat treatment applied in pasteurization of cream for the manufacture of butter. The taint appears to be only slowly produced under the action of heat from a mother substance in the cream (McDowall, McDowell, Morton, Singleton & O'Dea, 1951). The main constituent of the taint has been shown to be benzyl mercaptan (Forss, 1951). The developed taint can be destroyed by addition of hypochlorite. Experimental work on the treatment of heated cress-tainted cream with hypochlorite (McDowall, unpublished results) has indicated that heating to boiling point for 30 min is necessary to induce complete conversion of the mother substance. Even if this long period of heat treatment could be applied conveniently to cress-tainted cream, the need to remove the developed taint would still remain.

The normal method of removing tainting substances from cream for butter-making is by steam distillation in a Vacreator vacuum-pasteurizer or other suitable cream-treatment equipment. The rate of removal varies with the vapour/liquid equilibrium coefficient of the tainting substance being removed; and if the tainting substance is more soluble in fat than in water the coefficient may be very much lower for distillation from cream than for distillation from water (McDowall, 1959).

During the treatment of cress-tainted cream in a Vacreator the condensed water has a strong odour typical of cress taint, showing that the tainting substance is to some extent volatile in steam. In the present paper results are given of an investigation of the vapour/liquid equilibrium relationships for benzyl mercaptan when steam-distilled from water and from cream.

Since strong landcress taint in butter from a large vat of cream can result from the inclusion of cream from the milk of a few cows that have consumed landcress foliage, the concentration of benzyl mercaptan in landcress-tainted cream must be very low.

It is known that benzyl mercaptan is detectable in butter at a concentration of 1 pt in 2×10^9 pts. At such low concentrations normal chemical methods of estimation cannot be used and it was, therefore, necessary to use concentrations of 1–20 ppm. Although benzyl mercaptan is only slightly soluble in water, it is soluble over this range of concentrations.

EXPERIMENTAL

The vapour/liquid equilibrium relationships were measured in the continuous vaporization equilibrium still already described (McDowall, 1955). In the investigation of the vapour/liquid equilibrium coefficient from aqueous solution, the concentrations of mercaptan in the liquid and vapour at time of partition, i.e. in both the condensate and the residue, were estimated by measurement of the absorption at 218 mμm in a Beckman spectrophotometer. In the investigation of the coefficient from cream the concentration in the condensate was measured in this way, and the concentration in the residual cream was calculated by difference (McDowall, 1959).

As a check on the findings from estimations of the vapour/liquid equilibrium coefficient from cream, a quantity of cream containing 1 pt of added benzyl mercaptan in 10^9 pts cream was subjected to intense steam distillation treatment; 6 lb of steam per gal of cream, in a tandem Vacreator vacuum-pasteurizer, 3 times in succession. After each treatment a portion of the cream was set aside for conversion to butter, and the butter was graded for intensity of landcress taint.

RESULTS

Distillation from water. The results are illustrated in Fig. 1. Over the range of concentrations 1·0–2·0 ppm. in the liquid the relationship was linear, $C_V/C_L = 105$. At lower concentrations the indications are that the vapour/liquid equilibrium coefficient decreased. It is possible, however, that the curvilinear nature of the lower portion of the curve is to be attributed to errors in the method of estimation used. This aspect was not further investigated, since the results were adequate to show that benzyl mercaptan is readily steam volatile and that it should easily be removable from an aqueous solution by steam distillation.

Distillation from cream. The results in Fig. 2, over the range of concentrations 3–30 ppm. at time of partition in a cream containing 30 % fat, show that the relationship was linear, $C_V/C_L = 1{\cdot}30$. The effect of the two-phase nature of cream is thus to reduce the vapour/liquid equilibrium coefficient from 105 to 1·30. It is clear, therefore, that even partial removal of developed landcress taint from cream could only be effected with expenditure of large quantities of steam, and that complete removal would not be commercially feasible.

Repeated vacreator treatment of cream. Cress taint was still evident in the butter from cream containing 1 pt of benzyl mercaptan per 10^6 pts cream treated 3 times in succession in the Vacreator using greater quantities of steam for each treatment than would be economically possible commercially. These results thus confirm the above conclusions from the estimation of the vapour/liquid equilibrium coefficient for benzyl mercaptan in cream, that complete removal of this tainting substance from cream by steam distillation would not be commercially practicable.

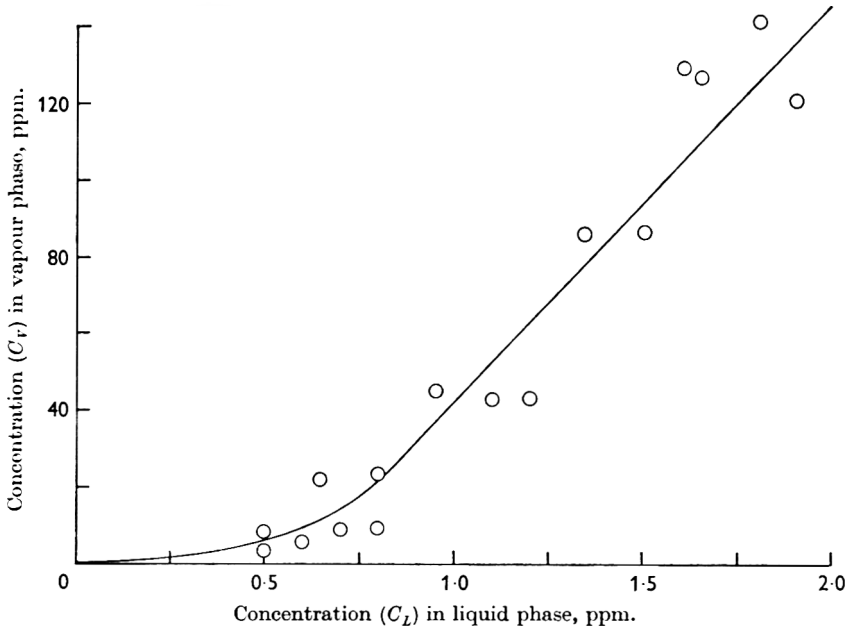


Fig. 1. Vapour/liquid equilibrium relationship for benzyl mercaptan in water.

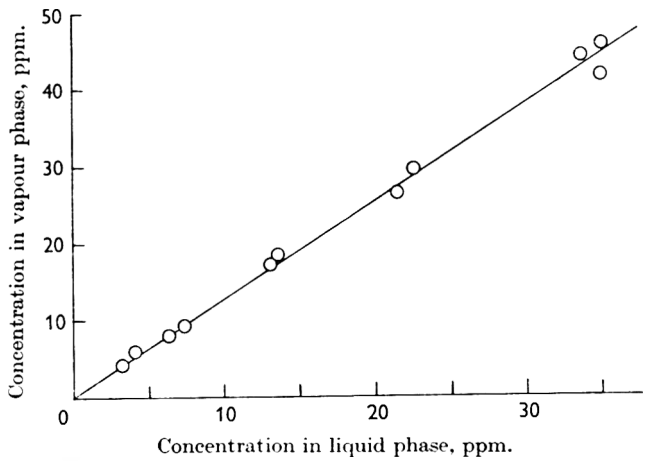


Fig. 2. Vapour/liquid equilibrium relationship for benzyl mercaptan in cream containing 30% fat.

The author is indebted to Mr A. K. R. McDowell of this Institute for assistance with the analytical work.

REFERENCES

McDowall, F. H., McDowell, A. K. R., Morton, I. D., Singleton, J. A. & O'Dea, J. J. (1951). *N.Z. Jl Sci. Technol.* **33**, 35.
Forss, D. A. (1951). *Aust. J. appl. Sci.* **2**, 396.
McDowall, F. H. (1955). *N.Z. Jl Sci. Technol.* **37**B, 1.
McDowall, F. H. (1959). *J. Dairy Res.* **26**, 46.

Printed in Great Britain

Survival of strains of lactic streptococci during frozen storage*

By C. A. GIBSON, G. B. LANDERKIN and PAMELA M. MORSE
Research Branch, Canada Department of Agriculture, Ottawa, Canada

(*Received* 11 *December* 1964)

Summary. Sixteen single-strain cultures, 10 of *Streptococcus cremoris*, 3 of *Str. lactis* and 3 of *Str. diacetilactis* were examined for their ability to withstand freezing and storage at −17·8 and −23·3 °C. The numbers of surviving bacteria decreased as storage time increased. For the majority of strains, the survival rate was greater at the lower temperature. At both storage temperatures there were differences between the percentages of surviving bacteria for different strains.

The results suggest that the relative proportion of strains in mixed cultures would not remain constant under frozen storage.

Extensive studies have been made on the freezing and storage of micro-organisms, including lactic streptococci used in the dairy industry. Heinemann (1958) found that the addition of glycerine improved subsequent activity of ripened lactic cultures after storage at 35, 5 and −20 °F. Richardson & Calbert (1959) found that the activities for freeze-dried and frozen (dry ice-alcohol) cultures of *Str. lactis* were 0·34 and 0·25 %, respectively, after storage at −18 °F for 6 months.

Johns (1956) observed that viability of commercial cultures was greater after frozen storage if they were frozen at the normal pH of milk; he neutralized ripened cultures before freezing. Other investigators have successfully frozen cultures at the normal pH of milk either by adding washed cells to skim-milk or by normal sub-culture into skim-milk followed by freezing without incubation. Simmons & Graham (1959) found satisfactory activity in a culture inoculated at the 1 % level in skim-milk after storage at −20 °F for 6 months. Moss & Speck (1962) reported that injury and death after 28 days at −20 °C was greater in cells frozen in distilled water than in cells frozen in skim-milk. Subsequent to the initiation of the present study, Cowman & Speck (1963) stored cells of *Str. lactis* suspended in skim-milk and found that viability was greater after 30 days of storage at 3 or −196 °C than at −20 °C.

Investigations on frozen storage of lactic streptococci have been confined mainly to single strains of the *Str. lactis* group and to commercial starter cultures of unknown composition. Strains of *Str. cremoris* and *Str. diacetilactis* used extensively in commercial starters have not received the same attention. The object of this investigation was to compare the viability of representative strains of lactic streptococci after frozen storage, as a preliminary step in investigating the extent to which the equilibrium of mixed-strain starters might be altered when similarly treated.

* Contribution no. 24 from the Food Research Institute, Canada Department of Agriculture.

METHODS

Sixteen single-strain cultures of New Zealand and Australian origin were used (see Table 1). Routinely all strains were subcultured twice weekly as 1% inocula in sterile skim-milk containing calcium carbonate with an incubation period of 16 h at 21 °C. Three propagations were made on successive days immediately prior to freezing.

Each culture was prepared for freezing by inoculating 1 ml of ripened culture into 99 ml of sterile skim-milk (10% T.S.; 10 lb/in² for 15 min) in each of 9 screw-cap Pyrex dilution bottles. Immediately after inoculation, 1 bottle was sampled for bacterial count and incubated for the activity test, 4 bottles were placed in storage at −17·8 °C and the remaining 4 bottles at −23·3 °C. The storage rooms were equipped with air circulating fans.

After 3 weeks, one bottle from each storage temperature was removed and thawed for 12 min in a water bath at 37·5 °C. Samples were taken for bacterial counts, then the bottles were incubated for 16 h at 21 °C before making activity tests. The same procedure was followed after 6, 12 and 24 weeks of storage, taking one bottle from each storage temperature each time.

The whole procedure was repeated for each strain, starting with different cultures and milks. There were thus 18 bottles for each strain, 9 in each complete replication.

Numbers of viable organisms were estimated, using the lactic agar of Elliker, Anderson & Hannesson (1956): 2% tryptone; 0·5% yeast extract; 0·25% gelatin; 0·5% of each of dextrose, lactose and sucrose; 0·4% sodium chloride; 0·15% sodium acetate; 0·05% ascorbic acid; 1·5% (Noble) agar; pH 6·8. The minimal agar medium of Moss & Speck (1962) was used in an attempt to estimate cell injury; its composition was the same as the lactic agar except that the tryptone and yeast extract were reduced to 0·5 and 0·1%, respectively. By definition, injured cells were those that grew on lactic agar but not on the minimal agar medium. Duplicate plates were poured at 3 dilutions and the plates were incubated at 21 °C for 72 h.

The activity tests were made on duplicate samples, using the method of Horrall & Elliker (1947). Starter was added at the rate of 0·3 ml per 10 ml of sterile skim-milk and titratable acidities determined after $3\frac{1}{2}$ h of incubation at 37·8 °C.

The bacterial counts were converted to logarithms for statistical analysis, in order to stabilize the variance. Significance tests were made by reference to the appropriate estimated variance between replicates.

RESULTS

The mean bacterial counts for the different strains and storage temperatures, are shown in Fig. 1, plotted against storage time. Although there were some overall differences between replicates, the relative disposition of the results remained fairly consistent from one replicate to another.

The numbers of viable cells decreased with increasing time, and the form of the relationship varied considerably among the different strains. In most cases, the log count decreased approximately linearly with increasing time. For some organisms (e.g. C_2, R_6, K), the higher temperature gave a more rapid early decrease in viable cells, the log count bearing a linear relation to log time, while a linear relation with

time held for the lower temperature. Strains ML_1 and KH gave a linear log count/log time relationship for both temperatures, while strain E_8 at the higher temperature gave an even sharper initial decrease in viable cells.

The higher storage temperature led to an appreciably lower bacterial count for many of the cultures. Comparisons are summarized in Table 1. For the cultures with a linear regression on time at both temperatures, the overall temperature effect was

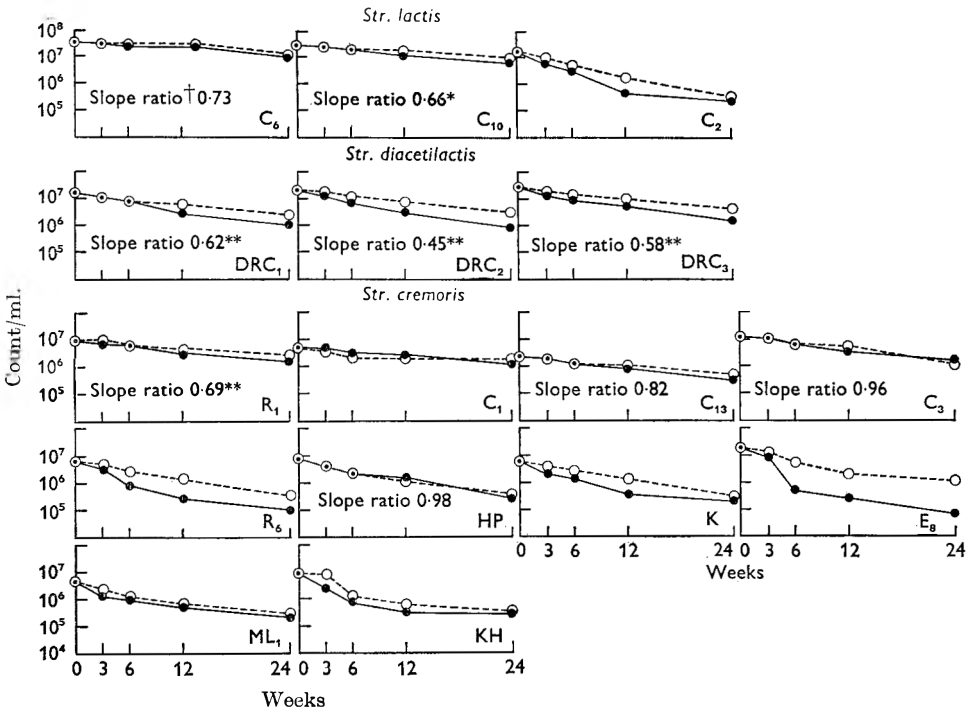


Fig. 1. Numbers of *Str. lactis*, *Str. diacetilactis* and *Str. cremoris* after various periods of frozen storage. ○----○, storage at −23·3 °C; ●——●, storage at −17·8 °C; *, $0{\cdot}05 \geqslant P > 0{\cdot}01$; **, $0{\cdot}01 \geqslant P > 0{\cdot}001$. †, see text for method of determining slope ratio.

assessed by slope-ratio techniques, fitting a pair of straight lines intersecting at zero time. The slope ratios are given in Fig. 1; in 5 of the 9 cases, the difference from unity was significant, reaching a probability level of 2 %, or less. Parallel regressions on log time for both temperatures could be fitted to the results for ML_1 and KH; in both cases the mean vertical distance between the lines (i.e. mean difference in log count) was significant, $0{\cdot}12 \pm 0{\cdot}030$ for ML_1 and $0{\cdot}26 \pm 0{\cdot}100$ for KH. Strains C_2, R_6 and E_8 gave obvious differences with temperature. This left only strains C_6, C_1, C_{13}, C_3 and HP giving no clear evidence of a consistent effect of storage temperature.

As seen from Fig. 1, the strains varied in their percentages of survivors, and in the rate of decrease with time. Even after only 3 weeks of storage at −17·8 °C, they ranged from 27 % survivors for KH to 90 % for C_1, while at −23·3 °C, they ranged from 51 % for ML_1 to 92 % for R_1. For simplicity, the percentage values presented in Table 2, with their standard errors, are restricted to those resulting from 24 weeks of storage. Differences among strains were highly significant ($P < 0{\cdot}001$) at both

temperatures, ranging from almost 0 to 29 % survivors at the higher temperature, and from 2 to 38 % survivors at the lower. The 3 strains of *Str. diacetilactis* gave similar results throughout, but large differences occurred among the 3 strains of *Str. lactis* and among the 10 strains of *Str. cremoris*.

Table 1. *Mean differences in log counts of cultures of lactic streptococci stored at* −17·8 *and* −23·3 °C.

(Mean at lower temperature minus mean at higher temperature.)

Culture	Initial population before freezing, millions/ml	Weeks of storage 3	6	12	24	S.E. (8 d.f.)
Str. lactis						
C_6	35	0·01	0·04	0·03	0·12**	±0·030
C_{10}	28	0·00	0·02	0·12	0·21*	±0·071
C_2	15	0·25*	0·72***	0·62***	0·11	±0·087
Str. diacetilactis						
DRC_1	16	0·04	0·00	0·36***	0·42***	±0·058
DRC_2	20	0·14	0·27	0·40	0·63*	±0·219
DRC_3	27	0·16	0·19	0·22	0·43**	±0·105
Str. cremoris						
R_1	10	0·04	0·01	0·14	0·27**	±0·065
C_1	5	−0·04	−0·12*	−0·06	0·14**	±0·041
C_{13}	2	0·00	0·01	0·05	0·16	±0·084
C_3	12	0·02	−0·01	0·17*	−0·03	±0·048
R_6	6	0·09	0·48***	0·70***	0·56***	±0·079
HP	8	−0·01	0·01	−0·10	0·08	±0·140
K	6	0·32*	0·33*	0·51**	0·19	0·103
E_8	17	0·14	1·08***	0·92***	1·11***	±0·078
ML_1	4	0·26**	0·04	0·13	0·08	±0·061
KH	9	0·53*	0·25	0·26	0·01	±0·200

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

The results of activity tests agreed well with bacterial survival estimates. When the number of surviving organisms became less than 5 % of the initial population, activities under the standardized conditions of these experiments were usually less than 0·40 % titratable acidity. Such cultures, however, regained satisfactory activity after serial transfer.

Preliminary work showed that most strains did not grow well on the minimal agar medium at 32 °C when incubated for 48 h. However, when incubated at 21 °C for 72 h counts were comparable to those obtained with lactic agar. Cell damage, as indicated by failure of the organisms to grow on minimal agar medium, could be clearly demonstrated with only 2 cultures, HP and KH. The percentage of injured cells during the 24 weeks of storage (see Table 3) was from 3 to 17 times higher with cultures HP and KH than with other cultures under the conditions used for incubation in this study.

Table 2. *Percentage of survivors in lactic cultures after 24 weeks of storage at −17·8 or −23·3 °C.*

Culture	Storage temp.		Storage temp.	
	−17·8 °C	−23·3 °C	−17·8 °C	−23·3 °C
	Survivors, mean log %		*Survivors, %	
Str. lactis				
C_6	1·46	1·58	29	38
C_{10}	1·34	1·56	22	36
C_2	0·23	0·34	2	2
Str. diacetilactis				
DRC_1	0·78	1·20	6	16
DRC_2	0·62	1·25	4	18
DRC_3	0·76	1·20	6	16
Str. cremoris				
R_1	1·19	1·46	15	29
C_1	1·42	1·56	26	36
C_{13}	1·14	1·32	14	21
C_3	1·06	1·04	12	11
R_6	0·19	0·76	2	6
HP	0·64	0·72	4	5
K	0·54	0·73	3	5
E_8	−0·37	0·82	0	7
ML_1	0·71	0·78	5	6
KH	0·55	0·56	4	4
†S.E. (16 d.f.)	±0·145	±0·144		

* Derived from mean log values.

† The standard errors given are for comparisons between strains. For comparisons between temperatures, see Table 1.

Table 3. *Percentage of viable cells showing injury as indicated by difference in counts on lactic agar (LA) and minimal agar medium (MAM)*

Culture	Weeks of storage				
	0	3	6	12	24
	% injured cells*				
HP	2	21	30	29	11
KH	2	60	26	35	36
All others	1·8	3·4	3·9	4·0	3·6

* Mean of both trials at the two temperatures of storage.
Percentage of injured cells = $[(LA-MAM)/LA] \times 100$.

DISCUSSION

While all strains tested singly showed a decrease in surviving cells during frozen storage in skim-milk, the differences among the strains in this respect indicate that certain strains may be more suitable than others for frozen storage.

The findings suggest that the proportions of strains in mixed cultures would not remain constant under frozen storage. The results cannot, of course, be used to predict the survival rates of the separate strains in mixed cultures but they do give some indication of the differences to be expected, and of factors which may influence the

results. The difference between −17·8 and −23·3 °C seems to be important for some strains, but not for others.

When lower counts were obtained on minimal agar medium than on lactic agar, Moss & Speck (1962) concluded that some cells of a strain of *Str. lactis* were injured during frozen storage. Similar results were obtained in this study only with strains HP and KH of *Str. cremoris*. With the other strains it would appear that different media, such as the restricted lactic agar described in a later paper by Moss & Speck (1963), or other methods may be necessary to demonstrate cell injury after freezing.

The lower incubation temperature of 21 °C employed in our studies as compared to 32 °C by Moss & Speck (1962) was selected because *Str. diacetilactis* and *Str. cremoris* have optimum growth temperatures below 30 °C (Schulz, 1963). This difference in temperature may explain in part our failure to demonstrate cell injury with the *Str. lactis* strains.

The authors wish to express their thanks to Dr D. B. Emmons and Dr J. A. Elliott for their advice and interest during these investigations; and to Mr M. D. Johnson for computational assistance.

REFERENCES

COWMAN, R. A. & SPECK, M. L. (1963). *J. Dairy Sci.* **46**, 609.
ELLIKER, P. R., ANDERSON, A. W. & HANNESSON, G. (1956). *J. Dairy Sci.* **39**, 1611.
HEINEMANN, B. (1958). *J. Dairy Sci.* **41**, 705.
HORRALL, B. E. & ELLIKER, P. R. (1947). *J. Dairy Sci.* **30**, 523.
JOHNS, C. K. (1956). *Can. Dairy Ice Cream J.* **35** (1), 32.
MOSS, C. W. & SPECK, M. L. (1962). *J. Dairy Sci.* **45**, 645.
MOSS, C. W. & SPECK, M. L. (1963). *Appl. Microbiol.* **11**, 326.
RICHARDSON, G. H. & CALBERT, H. E. (1959). *J. Dairy Sci.* **42**, 907.
SCHULZ, M. E. (1963). *Milchwissenschaft*, **18**, 443.
SIMMONS, J. C. & GRAHAM, D. M. (1959). *J. Dairy Sci.* **42**, 363.

Printed in Great Britain

The recovery of small numbers of *Staphylococcus aureus* infused into the bovine teat cistern

By F. H. S. NEWBOULD* and F. K. NEAVE
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 18 *December* 1964)

Summary. Small volumes of milk each containing an average of less than 10 colony-forming units of *Staphylococcus aureus* were injected into the teat cisterns of 39 quarters of 10 cows.

Typical staphylococci were recovered from only 14 quarters following overnight incubation of milk stripped manually from each quarter 10 min after injection. There was an inverse relationship between the total leucocyte count of the milk taken from the quarter before infusion and recovery of staphylococci.

From 4 of 8 quarters from which *Staph. aureus* could not be recovered by enrichment it was possible to recover the organism by centrifuging the milk and culturing the deposit of leucocytes and bacteria.

It is suggested that release of ingested staphylococci from defunct phagocytes may be important in initiation of infection, failure of antibiotic therapy and recurrence of infection.

It is difficult to determine whether or not micro-organisms which colonize the bovine teat duct gain entrance to the teat cistern unless they produce recognizable reactions in the cistern. It is also difficult to determine if and when micro-organisms from either the teat skin or milking equipment are forced through the duct into the cistern during milking as has been suggested by Plastridge (1958). A syringe collection of milk by the teat wall puncture has been described by Murphy & Stuart (1954), and McEwen & Samuel (1946) have given details of the aseptic examination of milk obtained from the cisterns after the cows were killed. In the latter work it was reported that no organisms could be isolated *post mortem* in the milk from 7 teat cisterns into which from 300 to 30 000 *Escherichia coli* had been injected *ante mortem*, and that organisms were isolated from only one of 3 cisterns infused with 300 000 organisms.

In an investigation of the mode of entry of staphylococci into the teat cistern through the teat canal it was necessary to determine whether they could be recovered regularly shortly after they had been introduced into the teat cistern.

This paper describes experiments in which *Staph. aureus* was recovered from milk samples stripped out manually 10 min after infusion of very small numbers of the bacteria into the teat cistern via the teat canal.

* Present address: Department of Veterinary Bacteriology, University of Guelph, Ontario, Canada.

MATERIALS AND METHODS

Cows. The number of lactations and the lactation stage of the 10 cows used is shown in Table 1. They were not secreting visibly abnormal milk and were not shedding any organisms except non-haemolytic corynebacteria.

Management and milking routine. The cows were housed in yards and milked as a group in a milking parlour. The interval between morning and afternoon milking was 10 h. Milking units were operated at 15 inHg vacuum, a pulsation rate of 60 c/min, and a pulsation ratio of 3:1. During the experimental period the udders were washed before each twice-daily milking with a solution of a commercial iodophor detergent* containing 100 ppm. available iodine and immediately after milking each teat was dipped in a commercial iodophor disinfectant* containing 5000 ppm. available iodine (Newbould & Barnum, 1960). After preliminary examinations of several aseptically taken foremilk samples to establish that the animals were not secreting *Staph. aureus*, milk samples and orifice swabs were taken daily for 5 days and *Staph. aureus* was infused immediately after milking on the 5th day.

Sampling procedure. Each morning, before milking, the udders were washed and the teats and teat apices thoroughly scrubbed with a pledget of cotton-wool soaked in 70 % (w/v) alcohol. Orifice swabs (Neave & Oliver, 1962) were then taken followed by a 1-oz sample of foremilk. Foremilk and orifice swabs were plated on nutrient blood agar (8 % calf blood). Five ml of each foremilk sample was transferred to a sterile bottle, enriched by incubation for 18–24 h at 37 °C and 0·025 ml spread on salt (8 % NaCl) nutrient blood agar. Post-infusion samples (see infusion procedure) were all enriched. Plates were incubated at 37 °C for 48 h. Total leucocyte counts were made on the fresh foremilks by the method of Dodd, Oliver & Neave (1957).

Preparation of the culture for injection. Staph. aureus strain *f* (Sharpe, Neave & Reiter, 1962) was used to infect a cow. The organism was recovered from the milk of this infected donor animal and used as described below. When originally examined this strain produced uniform growth in Lemco broth and in 18 h at 37 °C over 90 % of the organisms were single or in pairs.

Initial trials had shown that the infusion of 0·25 ml of sterile milk caused no obvious cellular reaction whereas 0·02 ml of Lemco broth could cause a marked cellular response. When grown in milk *Staph. aureus* produced little or no α-lysin.

In order to produce consistently a culture with about the same number of organisms at a given time the technique was standardized. Cultures and suspensions of the organism were in Lemco broth or antibiotic-free sterile whole milk in screw-cap bottles containing 24 glass beads. The milk for sterilization was collected aseptically and heated in boiling water for 30 min on 2 successive days.

The following procedure for growing the culture was adopted. Milk from the infected donor cow was plated on blood agar which was then incubated at 37 °C for 24 h. Twenty-two hours before infusion, 3 colonies on the blood plate were taken up in a loop and well triturated on the inner surface of a 2-oz bottle containing sterile milk. The contents were shaken thoroughly, incubated 16 h at 37 °C, shaken for 2 min and dilutions made in warm (37 °C) half-strength Lemco broth. After shaking 30 sec, 0·25 ml of the 10^{-2} dilution was transferred to 50 ml of sterile whole milk and the

* Iosan and Wescodyne: supplied by the West Chemical Company.

resulting suspension was incubated 5 h at 37°C. At the end of this time (1 h before infusion) the bottle was thoroughly wrapped in pre-warmed cotton wool and taken to the adjoining farm laboratory. Ten minutes before infusion the culture was shaken for 1 min and tenfold dilutions were made in warm sterile whole milk. Dilution bottles were shaken 30 sec and 0·2 ml of a 10^{-6} dilution was infused into each teat cistern. Simultaneously 0·2 ml of the 10^{-6} dilution was spread over the surface of each of 12 blood agar plates for estimation of the numbers of bacteria.

Infusion procedure: In outline the following operations were followed:

1. Orifice cleaned with alcohol swab.
2. Milk sample taken.
3. Orifice cleaned with alcohol swab.
4. Orifice swab taken.
5. Orifice cleaned with alcohol swab.
6. 0·2 ml milk containing staphylococci infused.
7. Orifice cleaned with alcohol swab.
8. After 10 min milk sample taken.
9. One tube of antibiotic infused.

Infusions were made by means of sterilized 1-ml glass syringes graduated to 0·01 ml, to each of which was attached a short (15 mm) cannula having an external diam. of 1·5 mm and completely free of sharp edges. After filling, the cannula was carefully wiped with a pledget of cotton wool soaked in 70% alcohol to remove any milk residue and bacteria, and thus prevent their deposition in the teat duct.

Number of colony-forming units (CFU). The number of *Staph. aureus* infused is based on the numbers of colonies which developed on 9–12 blood plates. The 1st group of 5 cows received $7{\cdot}55 \pm 3{\cdot}16$ CFU in each cistern, the 2nd group of 5 received $6{\cdot}25 \pm 1{\cdot}94$ units.

Table 1. *The recovery of* Staph. aureus 10 *min after infusion into the teat cistern*

Cow	Lactation no.	Period in milk, months	No. of quarters: Infused	No. of quarters: *Staph. aureus* recovered
M 78	6	7	3	1
N 19	5	4	4	1
N 20	5	7	4	0
O 73	3	7	4	2
O 74	3	10	4	2
O 81	3	7	4	1
S 3	1	13	4	4
S 6	1	11	4	2
Y 16	8	7	4	1
Y 48	7	< 1	4	0
Totals			39	14

RESULTS

All pre-infusion milk samples and swabs were negative for *Staph. aureus*. Table 1 shows that *Staph. aureus* was recovered from 14 of 39 quarters (35·9%) 10 min after infusion into the teat cistern. Non-haemolytic corynebacteria were shed from 29 of

the quarters and recovery of *Staph. aureus* was made from 7 of these compared with 7 of the 10 quarters not shedding these organisms. This difference is significant ($P < 0{\cdot}02$).

The 39 quarters were classified into 4 groups according to the leucocyte count of the milk immediately before injection. From Table 2 it will be seen that the milk from 10 quarters contained less than 1×10^6 leucocytes/ml and *Staph. aureus* was recovered from 9 of them, whereas only 1 quarter of 15 containing over 3×10^6 leucocytes/ml yielded the organism. The difference in recovery rate between groups is highly significant ($P < 0{\cdot}01$).

Table 2. *The effect of the number of leucocytes in the milk on the recovery of* Staph. aureus *from the teat cistern*

Leucocytes in milk before infusion, no./ml	No. of quarters	
	Total	*Staph. aureus* recovered
Less than 1×10^6	10	9
$1 \times 10^6 - 2 \times 10^6$	6	2
$2 \times 10^6 - 3 \times 10^6$	8	2
More than 3×10^6	15	1

$\chi^2 = 12{\cdot}495$. $P < 0{\cdot}01$.

Table 3. *Recovery of* Staph. aureus *from leucocytes*

Source (cow and quarter)	Cell count/ml of milk before infusion		*Staph. aureus* recovered
	Leucocytes $\times 10^{-6}$	Neutrophils $\times 10^{-6}$	
M78LF	> 3	2	+
N20RH	1–2	1·2	–*
N20LH	2–3	1·2	+
O74LH	> 3	2	+
O81LH	2–3	1·5	–
S6LH	< 1	0·007	+
Y16LH	> 3	> 3	–
Y48LH	> 3	> 3	–

* Non-haemolytic micrococci recovered.

Of the total of 14 quarters from which staphylococci were recovered, 7 were shedding corynebacteria and 7 were not. The leucocyte counts of the former were, on the average, twice as high as those of the latter. Thus it seems that the number of leucocytes in the milk had more effect on recovery of staphylococci than did the presence of corynebacteria. On the assumption that in the negative samples the organisms had been phagocytized, attempts were made to recover them from the leucocytes. Eight of these samples which, after the initial 18-h incubation at 37 °C had been stored in the refrigerator for 2–3 days, were centrifuged to throw down the cells, and after removal of the supernatant milk the sediment was resuspended in salt (8 % NaCl) nutrient broth, and incubated for 18 h at 37 °C. Where there was evidence of growth, 0·025 ml of the broth culture was spread on salt blood agar. The results are shown in

Table 3. It will be seen that recovery of typical *Staph. aureus* strain *f* was achieved from 4 of the samples, a pure culture of non-haemolytic micrococci from a 5th, and no organisms from the remaining 3 samples. Recovery by these means appeared to be unaffected by the numbers of leucocytes present.

DISCUSSION

Data from these experiments indicate that recovery of *Staph. aureus* shortly after infusion into teat cisterns was possible in the majority of cases when milk, withdrawn immediately before the introduction of the organisms, contained less than 1 million leucocytes/ml. The recovery rate rapidly diminished as the leucocyte count increased, indicating phagocytosis of the organisms. The fact that organisms could be recovered from centrifuged deposits suspended in salt nutrient broth, which lyses the leucocytes (Retnasabapathy, 1962), further substantiates the importance of leucocytes in ingesting staphylococci from milk. Because of the small numbers of organisms involved, it was necessary to pre-incubate the milk before isolation on plates could be attempted, and thus it is not known to what extent phagocytosis took place in the udder before incubation of the milk. These results are markedly different from those obtained with *E. coli* by McEwen & Samuel (1946) who had to infuse from 30 000 to 300 000 organisms in order to recover any from milk of the teat cistern shortly afterwards. The only information about the cows used in their experiments was that they were 'cows at the abattoir', which makes it reasonable to assume that they were probably shedding large numbers of leucocytes. If this were so it might explain the low rate of recovery which they experienced.

It is interesting to note the large numbers of leucocytes required to inactivate the small numbers of organisms injected during the present experiments. This would indicate that either there were few phagocytes among the leucocytes present or that those present were grossly inefficient. The latter would appear to be the case as the proportion of neutrophils in the total leucocyte count was high.

Murphy & Stuart (1952) concluded that the milk from normal glands does not prevent the multiplication of *Streptococcus agalactiae*. The data reported here strongly suggest that this is also true for *Staph. aureus* and Schalm, Lasmanis & Carroll (1964) have shown that 100 *Aerobacter aerogenes* will readily infect glands containing few, but not glands secreting many, leucocytes.

The fact that the organisms were able to multiply after release from the phagocytes even several days after ingestion, may be important in the genesis of infection. Recurrent infections might be explained by the release of ingested organisms from defunct leucocytes entrapped in small occluded ducts in the milk secreting tissue. Also, it might account in part for failures of antibiotic therapy, since intracellular staphylococci are known to be unaffected by large concentrations of antibiotic (Mackaness, 1960) in the suspending medium *in vitro*.

We wish to thank Mr D. L. Simpkin for his technical assistance, Mr W. A. Cuthbert and Mr A. S. Foot for the provision of facilities for the work and Dr F. H. Dodd and Mr N. Jackson for generous help and advice.

REFERENCES

DODD, F. H., OLIVER, J. & NEAVE, F. K. (1957). *J. Dairy Res.* **24**, 20.
MACKANESS, G. B. (1960). *J. exp. Med.* **112**, 35.
MCEWEN, A. D. & SAMUEL, J. MCA. (1946). *Vet. Rec.* **58**, 485.
MURPHY, J. M. & STUART, O. M. (1952). *Cornell Vet.* **42**, 133.
MURPHY, J. M. & STUART, O. M. (1954). *Cornell Vet.* **44**, 501.
NEAVE, F. K. & OLIVER, J. (1962). *J. Dairy Res.* **29**, 79.
NEWBOULD, F. H. S. & BARNUM, D. A. (1960). *J. Milk Fd Technol.* **23**, 374.
PLASTRIDGE, W. N. (1958). *J. Dairy Sci.* **41**, 1141.
RETNASABAPATHY, A. (1962). Thesis, University of Toronto.
SCHALM, O. W., LASMANIS, J. & CARROLL, E. J. (1964). *Am. J. vet. Res.* **25**, 83.
SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). *J. appl. Bact.* **25**, 403.

Printed in Great Britain

The response of the bovine mammary gland to an infusion of staphylococci

By F. H. S. NEWBOULD* and F. K. NEAVE
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 18 *December* 1964)

Summary. Small numbers of bovine staphylococci, usually less than 30, were infused into the teat cistern of 17 cows secreting less than 100 000 leucocytes/ml milk. Infection was assumed to have occurred if the organisms multiplied sufficiently to be recovered in the foremilk and inflammation was regarded as demonstrated if there was an increase in leucocytes. If the staphylococci were recovered at the 1st post-infusion milking they were consistently recovered thereafter and inflammation always followed but was never evident at the 1st post-infusion milking. Inflammation was usually evident by the 3rd but in one instance was delayed to the 11th post-infusion milking. There was never inflammation in the absence of infection. When there was a high probability that the infusion contained one organism or more, infection occurred in 2 of 10 quarters infused with coagulase-negative staphylococci and 23 of 25 quarters infused with coagulase-positive staphylococci.

A significant positive relationship was found between the number of staphylococci found at the 1st post-infusion milking and the number of leucocytes at the 3rd post-infusion milking.

It is accepted that the main portal of entry of bacteria to the bovine mammary gland is the teat duct (Dalling & Stableforth, 1948; Plastridge, 1958). It has been suggested (Davis, 1935; McEwen & Samuel, 1946; Pier, Schalm & Hage, 1956) that suction during milking either by hand or machine, results in the pathogens being carried through the duct to the teat cistern. Other evidence (Murphy & Stuart, 1953; Neave, Oliver & Dodd, 1957) indicates that multiplication within the teat duct between milkings enables pathogens to penetrate into the teat cistern. Assuming that a natural staphylococcal infection results from penetration to the teat cistern of small numbers of organisms, it seems probable from the work of Klastrup (1960) that after implantation in the teat cistern there is usually a subclinical infection followed by a leucocytosis and increase in the chloride-lactose ratio. Newbould & Neave (1965*a*) have shown that the level of leucocytes in the milk is important in determining the survival of small numbers of staphylococci implanted in the teat cistern. Diagnosis of infection would be much easier if we knew whether, in the presence of only small numbers of leucocytes in the milk, organisms gaining entrance to the teat cistern always multiply and produce a leucocytosis.

* Present address: Department of Veterinary Bacteriology, University of Guelph, Ontario, Canada.

It is probable that when only small numbers of bacteria gain entrance to a quarter significant bacterial multiplication would have to take place within the gland before demonstrable changes in the milk occurred. Changes in milk composition in response to infection (i.e. bacterial multiplication), may be measured chemically (e.g. by increases in blood constituents, reduction in lactose and changes in pH and conductivity) or microscopically by increases in leucocytes. The latter is likely to be the most sensitive and reliable single test in the early stages of infection. Some workers (Pattison & Holman, 1951; Howell, Pattison, Holman & Smith, 1954; Derbyshire, 1960) have counted neutrophils instead of total leucocytes, but this would seem unnecessary as a clear positive correlation ($r = 0{\cdot}651$) has been shown between total and polymorphonuclear leucocyte counts (Waite & Blackburn, 1957; Waite & Blackburn, 1963).

We have investigated the effects of the infusion of small numbers of staphylococci into the teat cisterns of a number of quarters as determined by the multiplication of the staphylococci and the leucocyte response of the glands. Further work in which small numbers of staphylococci were deposited in the teat duct is reported separately (Newbould & Neave, 1965*b*).

EXPERIMENTAL METHODS

Cows, management, milking routine and leucocyte count. Seventeen cows were used in these experiments, including one set of monozygous triplets. One animal was in her 3rd lactation, one set of twins in their 2nd and all others were 1st-lactation heifers. Thirteen animals were uninfected on calving. One heifer was infected in 2 quarters with *Streptococcus dysgalactiae* and the triplets were infected in a total of 4 quarters with *Str. uberis*. Except for one quarter which recovered spontaneously these 6 infections were eliminated by treatment. The 3rd lactation cow and the 2nd lactation twins each carried a *Micrococcus* (coagulase-negative staphylococci) in one quarter during their 1st lactation, 7 quarters, however, were shedding non-haemolytic corynebacteria at the time the cows were assigned to the experiment.

No quarter was secreting over 100 000 leucocytes/ml when challenged with staphylococci and in most cases there were less than 50 000 leucocytes/ml.

In a few early experiments the cows were kept in yards and milked in a milking parlour. In later experiments they were kept and milked in a cow house but exercised outside for several hours each day. The interval between morning and afternoon milking was 9½ h. Milking units were operated at a vacuum of 15 inHg, a pulsation rate of 60 c/min, and a pulsation ratio of 1:1. Udders were washed before milking with an iodophor detergent and the teats were dipped immediately after milking in an iodophor disinfectant (Newbould & Neave, 1965*a*). Before each cow was milked the teat cup clusters were pasteurized by drawing hot water (85–90 °C) through them for 6–8 sec (Neave, Dodd & Kingwill, 1961).

Examination of samples. Foremilk samples were taken aseptically from all quarters at regular intervals from the day of calving and, commencing at least 4 days before infusion, 1-oz foremilk samples were taken each morning before milking, as described by Newbould & Neave (1965*a*). Where samples could not be examined within 4 h of taking they were stored at 4 °C overnight. Total leucocyte counts were made using the strip method described by Dodd, Oliver & Neave (1957). A volume of 0·05 ml of

the samples was spread on nutrient blood agar (8 % calf blood), but in addition post-infusion samples were enriched by incubating at 37 °C for 18–24 h and 0·025 ml was then streaked on salt (8 % NaCl) nutrient blood agar. Plates were incubated at 37 °C for 48 h and examined for staphylococci.

Infusion of staphylococci. The preparation of the cultures, the infusion procedures and the estimation of the number of colony forming units (CFU) infused were as described by Newbould & Neave (1965*a*).

Strains of Staphylococcus aureus. Three bovine strains were used in these experiments. Strains *d* and *f* were typical *Staph. aureus* and have been described by Sharpe, Neave & Reiter (1962). Strain *ah*, from a mild udder infection, was coagulase-positive in bovine plasma but coagulase-negative in human plasma (from several donors) and fermented mannitol only slowly (1 week). It was considered to be representative of the weakly pathogenic staphylococci that commonly infect the udder and was chosen for this reason and because it was not found associated with any of the cows used in these experiments. All 3 strains were easily distinguished on blood agar plates.

Plan of experiment. Ten quarters of 5 cows were infused with strain *ah*, one quarter of each cow being used at a time. Infected quarters were treated and the organism eliminated before another quarter in the same cow was infused. Forty-three quarters of 15 cows were inoculated with strain *f*, all individually as above, except in the case of cow P 92 in which 2 quarters were infused simultaneously. To determine the within-cow variation in response to 2 different strains, while eliminating the effects of genetic and time differences and of previous infection, strain *f* and strain *d* were infused simultaneously into separate quarters of each of the triplets.

RESULTS

Table 1 shows the number of organisms infused into the teats and the number of infections caused by each strain. Two of 10 infusions with strain *ah*, 23 of 46 infusions with strain *f*, and 3 of 3 infusions with strain *d* resulted in infection, i.e. multiplication and recovery of the organisms followed by inflammation as indicated by an increase in numbers of leucocytes in the milk. In no case did inflammation develop without infection or infection without inflammation and whenever organisms were recovered inflammation developed.

Examination of the results of individual quarters infused with strain *f* revealed that the organism was recovered from the foremilk at the 1st post-infusion milking, either on direct plating (13) or by enrichment only (10), or not at all (23).

In Table 1 an association can be seen between the number of CFU of strain *f* infused and the number of quarters infected. Where the lower limit of the range on 12 plates was 0, i.e. when there was a low degree of probability that the infusion contained organisms, infection developed in only 3 of 24 quarters (13·4 %), whereas when the lower limit was 2 or more colonies, there was a high probability of some organisms being infused and infection developed in 20 of 22 quarters (91 %). This difference is highly significant ($P < 0{\cdot}001$). Also, from Table 1 it is evident that in one quarter an infection was established with probably only 1–3 CFU.

Table 2 gives for strain *f* the mean leucocyte count obtained from individual cows at the 2nd and 4th pre-infusion and the 1st, 2nd, 3rd and 7th post-infusion milkings.

In no case was an increased leucocyte count obtained at the 1st post-infusion milking. In the 23 infections which developed, inflammation, as evidenced by an increase in leucocytes, was seen in 17 quarters at the 3rd post-infusion milking, in 4 quarters at

Table 1. *Effect of numbers of staphylococci infused into the udder on the incidence of infection*

No. of CFU infused Mean ±S.D.	Range	Total	Number of quarters: Developed: Infection	Developed: Inflammation	No infection nor inflammation
Strain *ah*					
8·84±3·58	3–14	3	1	1	2
11·25±4·43	6–20	2	0	0	2
15·59±4·22	8–23	5	1	1	4
		10	2	2	8
Strain *f*					
0·33±0·78	0–2	5	0	0	5
1·0 ±0·85	0–3	5	1	1	4
1·0 ±0·95	0–3	5	0	0	5
1·5 ±1·60	0–5	4	0	0	4
2·25±1·55	0–5	4	1	1	3
2·92±1·51	0–5	1	1	1	0
5·0 ±1·76	2–8	4	4	4	0
9·25±3·90	4–16	4	4	4	0
12·82±3·07	8–22	5	3	3	2
19·1 ±8·01	11–37	2	2	2	0
22·8 ±3·92	17–30	4	4	4	0
52·5 ±9·69	40–67	3	3	3	0
		46	23	23	23
Strain *d*					
23·75±5·70	15·34	3	3	3	0

Table 2. *Inflammatory response of individual cows to infusion of small numbers of* Staph. aureus *strain* f

Cow	No. of infections	Leucocyte counts*: Pre-infusion milkings: 4th	2nd	1st	Post-infusion milkings: 3rd	5th	7th
29	2	6	19	5	19	87	172
S59	2	4	5	4	156	53	23
S60	2	1	3	7	7	482	358
S61	2	11	6	3	7	5	25
S62	2	7	6	3	10	71	123
S7	2	7	6	2	123	269	78
S8	2	6	9	2	2144	2710	870
P92	2	18	13	18	1615	1660	402
P77	1	22	15	12	115	146	107
P95	1	4	6	10	238	58	32
P96	1	2	0	6	2300	1450	480
S43	1	2	10	4	1500	2760	—
S44	1	4	0	4	4900	3580	—
S44T	1	6	6	8	4960	4080	—
O74	1	9	12	15	40	42	17

* Sum of cells in 1 strip across each of 2 Breed films. Multiply by 3850 to convert to cells/ml. Where 2 infections occurred, counts were averaged.

the 5th and in one quarter at the 7th. In one quarter of cow S61 no increase in leucocyte count was found until the 11th milking. There were marked differences in inflammatory response to infection among the cows, the minimum and maximum occurring in cows S61 and S44T.

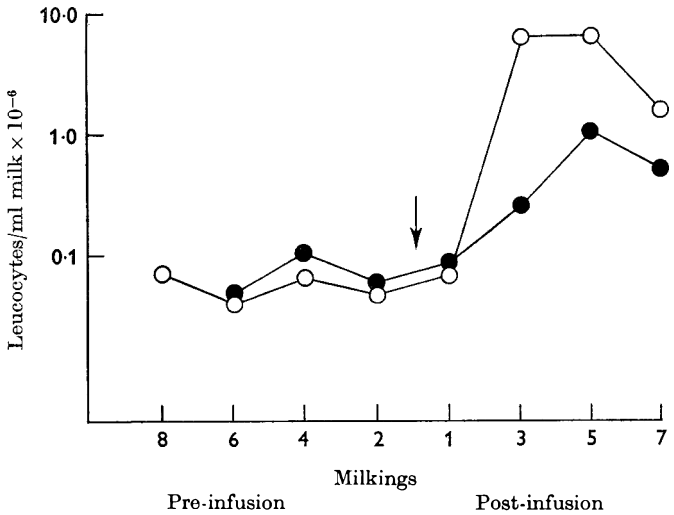


Fig. 1. Leucocyte response in a single cow to infusion into the teat cistern of 2 strains of *Staph. aureus* at different times. Each curve the mean of 2 quarters. ○, strain *f*; ●, strain *ah*.

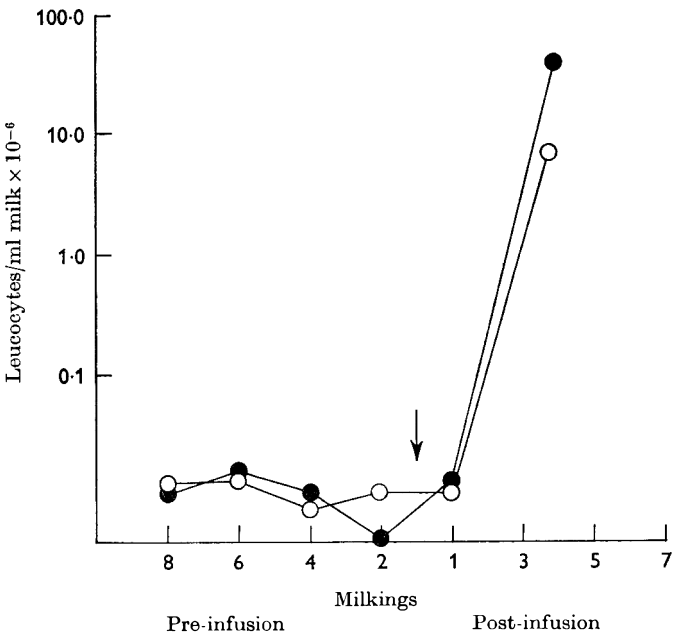


Fig. 2. Leucocyte response in monozygous triplet heifers infused simultaneously in 2 quarters each with equivalent small numbers of 2 strains of *Staph. aureus*. Each curve the mean of 3 quarters. ○, strain *f*; ●, strain *d*. Antibiotic treatment was started after the 3rd post-infusion milking.

There was also a marked difference in the response of individual cows to infection by different strains. This is shown in Figs. 1 and 2. Fig. 1 shows the mean leucocyte response curves of cow P92 to 2 infections with strain *ah* and 2 infections with strain *f*, the 4 quarters having been infused at different times with similar numbers of organisms. Fig. 2 shows the mean response curves of the monozygous triplets S43, S44 and S44T to simultaneous infusion of similar numbers of strains *f* and *d* in different quarters. Quarters of the same cow infused with any one strain responded similarly and the within-cow variation in response to different strains was apparent whether the infections were produced at the same or different times.

The response of the triplets was nearly identical. In each of the animals the quarter infused with strain *f* produced no visually abnormal milk but the 3 quarters infected with strain *d* became extremely hard and swollen and the secretion very abnormal in appearance. In spite of prompt and frequent antibiotic treatment these 3 quarters did not return to normal function.

It appeared from the results obtained that there was some relationship between the number of organisms recovered at the 1st post-infusion milking and the total leucocyte count at the 3rd post-infusion milking. The number of quarters at the 1st post-infusion milking yielding less than and more than 100 CFU, respectively, were 13 and 10, while the corresponding numbers for 250 000 leucocytes/ml at the 3rd post-infusion milking were 4 and 9 ($\chi^2 = 5{\cdot}839$, $P < 0{\cdot}02$).

The results of infusion were not obviously related to quarters that had been previously infected and treated, nor to the number of CFU infused within the range used, i.e. 1–70.

With one exception no infections occurred in uninfused quarters. This exception involved a quarter, with a severely eroded teat canal, of a cow that had been artificially infected in one other quarter. Both infections were with strain *f*.

DISCUSSION

While only 3 strains of staphylococci have been used in this study it is evident that they differed greatly in pathogenicity for the bovine udder. Strain *d* was more pathogenic than strain *f* (this is in agreement with the report of Sharpe *et al.* 1962) and both these typical *Staph. aureus* strains were more pathogenic than the atypical *Staph. aureus* strain *ah*.

When small numbers of typical *Staph. aureus* were infused into the teat cistern the organisms were recovered at the 1st post-infusion milking or not at all. In those quarters in which infection did not follow infusion there was no inflammation. In this case either no organisms were in the inoculum or they were prevented from multiplying presumably by some additional antibacterial factor present in the quarter, although their destruction by leucocytes cannot be ruled out completely. However, when there was a high probability that the inoculum contained organisms, infection occurred in all but 2 quarters. This supports our previous conclusion (Newbould & Neave, 1965*a*) that milk in normal uninfected glands would be unable to prevent multiplication of *Staph. aureus*. In contrast to this the atypical *Staph. aureus* strain *ah* caused infection in only 2 of 10 quarters so infused.

Infection was always followed by inflammation; in one quarter inflammation was

delayed as long as the 11th post-infusion milking, although generally it was present by the 5th and usually by the 3rd post-infusion milking. No inflammation was recorded at the 1st post-infusion milking and this disagrees with the results published by Klastrup (1960) in which 3 quarters showed large increases in leucocyte count at the 1st milking after infusion. It is possible that differences between strains could account for this, but even in the 3 cows infected with strain *d*, despite very severe general toxaemia and nephritis developing later, no increase in leucocyte count was found at the 1st post-infusion milking.

Wide differences were shown in the response of individual cows to infection. It is not clear whether this is due to the more rapid and perhaps more extensive multiplication of the organisms in some cows as is suggested by the relationship between the number of bacteria at the 1st, and the number of leucocytes at the 3rd post-infusion milking. An alternative explanation could be the differences in efficiency of phagocytosis in leucocyte from different cows as suggested by the observations of Ward (1938), Lush (1950) and Young, Legates & Leece (1960).

Initial multiplication could be affected either by inhibitory or stimulatory substances. Since in quarters showing delayed inflammation a marked increase in bacterial numbers was noticed 24 h before an increased leucocyte count was observed, there is a possibility that the initial delay in this increase in bacteria resulted from lack of some essential metabolite, which was overcome either by adaptation or mutation.

The fact that no bacteria were recovered from 8 of the quarters infused with strain *ah* indicates that small numbers of some types of bacteria may gain entrance to the teat cistern and be eliminated without being detected, as claimed by McEwen & Samuel (1946).

In general, the data support the conclusion of Spencer & McNutt (1950) that differentiation between infection and inflammation is difficult, since the latter always follows the former. Our data show that any inflammation may be delayed by as little as 10 h or as much as 130 h after challenge with small numbers of *Staph. aureus*. This observation that bacteria can be present for at least 5 days without detectable inflammation is of considerable interest. However, it is known that in natural infections with streptococci or staphylococci often there are periods when the number of leucocytes decreases from millions/ml to less than 50 000/ml and remains low for several days or weeks before rising again (Neave, unpublished).

The data indicate that in addition to strain of organism, the individuality of an animal is a major determining factor in the establishment of an infection and inflammation; of 5 cows challenged each in 2 quarters with about 12 CFU of strain *ah*, one cow only became infected and this in both quarters. Neither the leucocyte count nor the udder history of the animals differed significantly prior to infusion. It is thus most important in studies of the pathogenicity of different strains to make within-cow comparisons.

We wish to thank Mr D. L. Simpkin for his technical assistance, Mr W. A. Cuthbert and Mr A. S. Foot for the provision of facilities for the work, Dr F. H. Dodd and Mr N. Jackson for generous help, and Mr B. Reiter and Dr M. Elisabeth Sharpe for criticisms.

REFERENCES

DALLING, T. & STABLEFORTH, A. W. (1948). *J. Frms' Club*, Pt 4, 39.
DAVIS, D. J. (1935). *J. Bact.* **29**, 43.
DERBYSHIRE, J. B. (1960). *Res. vet. Sci.* **1**, 350.
DODD, F. H., OLIVER, J. & NEAVE, F. K. (1957). *J. Dairy Res.* **24**, 20.
HOWELL, D. G., PATTISON, I. H., HOLMAN, H. H. & SMITH, I. M. (1954). *J. comp. Path. Ther.* **64**, 335.
KLASTRUP, D. (1960). *Nord. VetMed.* **8**, 193.
LUSH, J. L. (1950). *J. Dairy Sci.* **33**, 121.
MCEWEN, A. D. & SAMUEL, J. MCA. (1946). *Vet. Rec.* **58**, 485.
MURPHY, J. M. & STUART, O. M. (1953). *Cornell Vet.* **43**, 465.
NEAVE, F. K., OLIVER, J. & DODD, F. H. (1957). *Rep. natn. Inst. Res. Dairy.*
NEAVE, F. K., DODD, F. H. & KINGWILL, R. G. (1961). *Rep. natn. Inst. Res. Dairy.*
NEWBOULD, F. H. S. & NEAVE, F. K. (1965*a*). *J. Dairy Res.* **32**, 157.
NEWBOULD, F. H. S. & NEAVE, F. K. (1965*b*). *J. Dairy Res.* **32**, 171.
PATTISON, I. H. & HOLMAN, H. H. (1951). *J. comp. Path. Ther.* **61**, 26.
PIER, A. C., SCHALM, O. W. & HAGE, T. J. (1956). *J. Am. vet. med. Ass.* **129**, 347.
PLASTRIDGE, W. N. (1958). *J. Dairy Sci.* **41**, 1141.
SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). *J. appl. Bact.* **25**, 403.
SPENCER, G. R. & MCNUTT, S. H. (1950). *Am. J. vet. Res.* **11**, 188.
WAITE, R. & BLACKBURN, P. S. (1957). *J. Dairy Res.* **24**, 328.
WAITE, R. & BLACKBURN, P. S. (1963). *J. Dairy Res.* **30**, 23.
WARD, A. H. (1938). *N.Z. Jl Sci. Technol.* **20**, 109A.
YOUNG, C. W., LEGATES, J. E. & LEECE, J. G. (1960). *J. Dairy Sci.* **43**, 54.

Printed in Great Britain

The effect of inoculating the bovine teat duct with small numbers of *Staphylococcus aureus*

By F. H. S. NEWBOULD* and F. K. NEAVE
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 18 *December* 1964)

Summary. Fifty-seven inoculations of a strain of *Staphylococcus aureus* were made into the outer 4 mm of the teat ducts of 8 cows. The inocula ranged from 10 to 600 colony-forming units (CFU) and were made with a stainless steel instrument designed to overcome the disadvantages of glass rods and cotton swabs hitherto used.

A single inoculation resulted in either no colonization of the duct, in temporary colonization for up to 7 days, or in colonization followed by intramammary infection.

The recovery of the organisms depended on the size of the inoculum. When 70–100 CFU were placed in the teat duct no organisms were recovered from 12 of 24 quarters after the 1st post-inoculation milking. When 500–600 CFU were used, organisms were recovered from all of 31 quarters for at least 3 milkings, and from 61 % for 6 milkings or more, in spite of dipping the teats in a strong disinfectant twice daily.

Intramammary infection developed in 1 of 12 quarters (8 %) inoculated in the teat duct with about 600 CFU when the animals were milked twice daily, and in 5 of 19 (23 %) quarters if the 1st post-inoculation milking was omitted.

There was no evidence of sensitization resulting from previous infection.

Inoculation of small numbers of *Staph. aureus* into the teat cisterns of a number of lactating cows (Newbould & Neave, 1965*b*) nearly always resulted in infection, i.e. multiplication of the organisms as shown by their recovery from foremilk drawn at the 1st post-infusion milking. Infection always produced inflammation, i.e. leucocytosis, but the time between inoculation and inflammation and the severity of inflammation varied with the cow and with the strain of organism within cows.

Inoculation of the teat duct with *Streptococcus agalactiae* on glass rods (Jones & Little, 1934) and on cotton swabs (Hadley, Frost, Gumm & Welsh, 1930; Hopkirk, 1934; Murphy & Stuart, 1953) has been reported to cause intramammary infection, but we are not aware of any experiments with *Staph. aureus*. The technique of using glass rods gives some control over the number of organisms introduced into the duct, but cotton swabs do not permit even approximate estimation of the number of organisms. Also, organisms may be forced into the teat duct further than intended by both methods, particularly if there is milk in the duct.

A stainless steel instrument designed to overcome some of the disadvantages noted above is described here together with experiments in which the first 4 mm of the teat duct were inoculated with relatively small numbers of staphylococci.

* Present address: Department of Veterinary Bacteriology, University of Guelph, Ontario, Canada.

METHODS

Management of cows and milking routine. Eight cows were used, all of which had been previously infected with staphylococci in 2 quarters by infusion into the teat cisterns. Their housing, management and milking hygiene routine have been described previously (Newbould & Neave, 1965*b*).

Instrument for teat duct inoculation. One end of a stainless steel rod approximately 7 in. long and 4 mm in diam. was reduced to 2 mm diam. for a distance of 5 mm and a transverse channel 1 mm wide and 0·5 mm deep was made in the reduced section, as shown in Fig. 1. The shoulder prevented the instrument being inserted into the teat

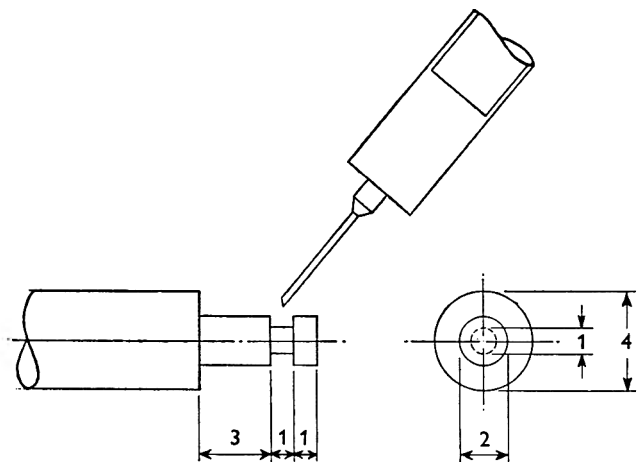


Fig. 1. Details of the inoculator, with syringe in position for filling the channel (all measurements in mm).

duct more than 5 mm, and the 1 mm head in front of the channel reduced the likelihood of organisms from the inoculum being introduced into the duct further than 4 mm. The inoculum was held in the channel, and by inserting and withdrawing the instrument a film of inoculum was left on the epithelium of the outer 4 mm of the teat duct and orifice.

Organisms and preparation of culture for inoculum. Staph. aureus strain *f* (Sharpe, Neave & Reiter, 1962) was grown in milk as previously described (Newbould & Neave, 1965*a*) and diluted as required in sterile milk.

Filling and callibration of inoculator. The channel of the inoculator was filled carefully using a syringe and fine needle so that the inoculum did not protrude beyond the level of the shoulder (Fig. 1). The number of CFU was estimated by washing thoroughly the end of the inoculator in 0·5 ml of half-strength nutrient broth in a Petri dish into which was then poured 10 ml of nutrient blood agar (8 % calf blood). The agar plate was incubated at 37 °C for 24 h. The charging and estimation of the number of CFU was repeated 5 times. Between chargings, the inoculator was washed well in sterile water and placed in 70 % ethanol for at least 30 sec after which the excess alcohol was shaken off and the inoculator allowed to dry.

Use of inoculator. Immediately after an evening milking the teats were dipped in

disinfectant (Newbould & Neave, 1965*b*) and, about 30 min later, the ends of the teats to be inoculated were rubbed with cotton wool soaked in 70 % ethanol and allowed to dry. The filled inoculator was then gently inserted into a teat duct, rotated 10–12 times and withdrawn while being rotated. Teats were not dipped in disinfectant immediately after inoculation but were dipped after each subsequent milking.

Some teat ducts were inoculated more than once but only after the leucocyte count on the foremilk had fallen to pre-inoculation level and the milk was consistently free of *Staph. aureus* and other pathogens.

Estimation of numbers of organisms left in teat duct. After withdrawal of the inoculator from the teat duct it was washed in 0·5 ml of half-strength nutrient broth and the number of bacteria estimated. The difference between this count and the mean calibration count was taken as the approximate number of CFU of staphylococci left in the duct. Preliminary trials in which each of 2 inoculators was filled 15 times from each dilution showed mean counts of $12{\cdot}14 \pm 3{\cdot}72$ and $10{\cdot}53 \pm 3{\cdot}9$ when filled from a 10^{-4} dilution of the culture, and $186{\cdot}5 \pm 44{\cdot}19$ and $178 \pm 43{\cdot}67$ when filled from a 10^{-3} dilution.

Milk samples and swabs. Foremilk samples (first 1 oz) were taken aseptically as described by Newbould & Neave (1965*a*) immediately before every morning milking and also before the evening milking on the day after each inoculation. At the end of each experiment, i.e. 12 days after inoculation, 1 oz of foremilk was taken by cannula and 1 oz manually, from those quarters with an intramammary infection or which had been shown to contain *Staph. aureus* in their foremilk.

Orifice swabs (Neave & Oliver, 1962) were taken immediately before the teat ducts were inoculated but not afterwards so as not to interfere with the growth of the organisms.

Erosion round the opening of the teat canals was in all cases either very slight or absent.

Examination of samples and swabs. Each morning sample was examined for the total leucocyte count (Newbould & Neave, 1965*a*) and 0·05 ml streaked on nutrient blood agar (8 % calf blood). The samples were then enriched by incubation at 37° C for 24 h and again examined by plating. Cannula samples and afternoon foremilks were examined by plating only. Orifice swabs were streaked on the surface of nutrient blood agar.

Peak milk flow. This was determined by the method of Griffin & Dodd (1962) but because the milk yields were low the determination was made after a 24-h interval between milkings.

Intramammary pressure. A relatively high intramammary pressure was obtained in 5 cows by leaving the animals unmilked for 24 h. The teat ducts were inoculated before the 24-h milking interval but foremilk samples were taken at the same intervals as stated above.

RESULTS

No staphylococci or corynebacteria were recovered from pre-inoculation orifice swabs, i.e. the external 2–3 mm of all teat canals were free of bacterial infection.

In 6 experiments in which a total of 56 inoculations were made (Table 1), only 8 intramammary infections resulted as determined by a leucocyte count. Confirmation that only 8 infections existed was obtained from the 2 successive foremilk samples

taken on the 12th day. The infected quarters yielded similar numbers of staphylococci in both samples whereas the non-infected quarters, with a teat duct infection, yielded staphylococci only in the sample drawn by hand.

Table 1. *The persistence of* Staph. aureus *when placed in the teat duct and the number of quarters becoming infected*

Expt. no.	Staph. placed in duct*	No. of cows	No. of ducts inoculated	No. of quarters that became infected†	No. of quarters that did not become infected but from which *Staph. aureus* was recovered before the following number of milkings						
					1	3	5	7	9	11	13
1	11	1	2	0	.	.	.	.	.	.	.
2	71	1	2	0	.	1	.	.	.	.	1
3	108	5	10	1	3	1	2	.	.	.	1
4	72	3	12	1	4	3	.	.	.	.	.
5	580	5	12	1	.	2	3	3	3	.	.
6	520	5‡	19	5	.	1	6	4	3	.	.
Total			57	8	7	8	11	7	6	.	2

* Average number of colony-forming units for all ducts inoculated in each experiment.
† *Staph. aureus* recovered at all milkings. A leucocytosis was observed in the milk of these quarters only.
‡ Not milked for 24 h after inoculation.

Table 2. *The failure of* 90–130 *colony-forming units of* Staph. aureus *to cause intramammary infection after implantation in the teat duct*

(All teat ducts inoculated after p.m. milking on 5th day. Normal twice-daily milking.)

Cow and quarter ...		S59RF		S60RH		87RH		88RF	
Day	a.m. or p.m.	Staphylococci count*	Leucocyte count†	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count
1	a.m.	0	3	0	2	0	3	0	5
2	a.m.	0	1	0	7	0	3	0	1
3	a.m.	0	1	0	3	0	7	0	2
4	a.m.	0	3	0	2	0	1	0	2
5	a.m.	0	3	0	1	0	4	0	4
6	a.m.	0	2	‡	2	4	0	2	2
	p.m.	0	—	0	—	14	—	5	—
7	a.m.	0	0	0	7	30	0	14	2
8	a.m.	0	3	0	2	1	5	250	2
9	a.m.	0	3	0	3	0	8	500	6
10	a.m.	0	2	0	3	0	7	> 500	1
11	a.m.	0	3	0	1	0	7	200	3
12	a.m.	0	3	0	2	0	8	350	3

* Number in 0·05 ml foremilk.
† Multiply by 7700 to convert to cells/ml.
‡ Recovered from incubated milk only.

In 41 of the remaining 49 teat duct inoculations staphylococci were recovered in the foremilk taken 12 h after inoculation. In 26 of these the organisms were detected in foremilk for 5 or more milkings and in 2 cases for at least 13 milkings.

There was a relationship between the number of organisms placed in the duct and the length of time for which they were recovered from the foremilk (Table 1). In

those experiments in which about 70–100 CFU were used no organisms were recovered from 50 % of the quarters after the 1st post-inoculation milking, whereas in the 31 quarters where 500–600 CFU were used, organisms were recovered from all quarters for at least 3 milkings and from 61 % for 6 milkings or more.

With normal twice daily milking, the size of the inoculum had no marked effect on the number of intramammary infections developing.

Table 2 shows the leucocyte counts and staphylococcus counts of 4 representative quarters in which under normal milking conditions no intramammary infection resulted from inoculation of the ducts. No staphylococci were recovered from the right fore quarter of S59, whereas the other 3 cows yielded staphylococci in the foremilk at 1–8 successive milkings.

Table 3. *The development of intramammary infections following inoculation of the teat duct with small number of* Staph. aureus

(All teat ducts inoculated after p.m. milking on 5th day. Normal milking.)

Cow and quarter ...		S59RH		87RF		88LF	
Day	a.m. or p.m.	Staphylococci count*	Leucocyte count†	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count
1	a.m.	0	3	0	3	0	2
2	a.m.	0	1	0	2	0	1
3	a.m.	0	2	0	2	0	3
4	a.m.	0	7	0	2	0	1
5	a.m.	0	0	0	5	0	1
6	a.m.	‡	1	9	4	8	1
	p.m.	7	—	62	—	23	—
7	a.m.	> 500	2	300	6	160	1
8	a.m.	300	177	235	1330	> 500	5
9	a.m.	350	87	100	501	75	3050
10	a.m.	> 500	72	250	246	18	2458
11	a.m.	> 500	51	81	149	51	990
12	a.m.	> 500	21	64	57	4	271

* Number in 0·05 ml of foremilk.
† Multiply by 7700 to convert to cells/ml.
‡ Recovered from incubated milk only.

The staphylococci and leucocyte counts for the 3 quarters in which intramammary infections developed under normal milking conditions, following teat ducts inoculation, are shown in Table 3, and those for the 5 quarters which became infected when the first post-inoculation milking was omitted are shown in Table 4. It is evident that while *Staph. aureus* was recovered from all post-inoculation milk samples, a leucocytosis was observed in 36 h in 2 of the 5 quarters shown in Table 4, but in none of the quarters shown in Table 3 was an increase in leucocytes detected until 60 h.

Although there was no clear relationship between peak milking rates and intramammary infections resulting from teat-duct inoculations, it should be pointed out that all but one animal were in a high peak rate group, i.e. over 6·1 lb/min. However, in cow 29 with the highest peak flow (11·0 lb/min) only one intramammary infection occurred from 19 inoculations. It was noted that the 2 cows with the lowest peak flow rates were the only animals not leaking milk by 12.30 p.m. following the omission of the morning milking, and neither of them became infected in any teat duct inocula-

tion experiment. In cows S59 and S60 which had the next 2 lowest peak flow rates (7·1 and 6·7 lb/min, respectively) only one infection resulted from 10 teat-duct inoculations in each.

DISCUSSION

The teat duct was considered to be colonized when organisms were detected in successive foremilk samples without enrichment, but no increase in leucocytes could be detected. On this basis it has been shown that a single introduction of about 10–600 CFU of *Staph. aureus*, strain *f*, into the outer 4 mm of the teat duct may or may not result in colonization. When colonization does occur it is only very rarely followed by intramammary infection.

Table 4. *The development of intramammary infections following inoculation of the teat duct with small numbers of* Staph aureus.

(Cows not milked for 24 h after inoculation. Teat ducts inoculated after p.m. milking on 5th day.)

Cow and quarter ...		29LH		S61RF		S61RH		87RF		87RH	
Day	a.m. or p.m.	Staphylococci count*	Leucocyte count†	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count	Staphylococci count	Leucocyte count
1	a.m.	0	4	0	2	0	1	0	8	0	4
2	a.m.	0	2	0	2	0	1	0	6	0	3
3	a.m.	0	6	0	6	0	0	0	4	0	3
4	a.m.	0	9	0	2	0	3	0	2	0	4
5	a.m.	0	7	0	1	0	2	0	2	0	10
6	a.m.	‡	11	41	4	13	1	> 500	3	28	2
	p.m.	13	—	189	—	450	—	> 500	—	134	—
7	a.m.	300	20	350	4	> 500	77	250	4200	85	18
8	a.m.	> 500	29	300	5	> 500	104	44	3190	13	31
9	a.m.	> 500	2580	200	93	120	10	200	880	250	81
10	a.m.	> 500	709	160	238	> 500	79	> 500	131	68	87
11	a.m.	> 500	523	> 500	73	500	71	> 500	84	> 500	313
12	a.m.	400	2820	350	52	50	62	> 500	52	> 500	175

* Number in 0·05 ml of foremilk.
† Multiply by 7700 to convert to cells/ml.
‡ Recovered from enriched milk only.

These results confirm our unpublished findings that the presence of organisms in aseptically taken foremilk is not necessarily an indication of intramammary infection unless the milk contains significantly higher numbers of leucocytes than the milk of the normal quarters of the cow.

The colonization of the duct for at least 7 days (Table 1), even when the teats were dipped twice daily in a strong disinfectant, demonstrates the importance of preventing infectious organisms from contaminating the teat apex. Our other unpublished studies showed that without teat dipping the teat ducts can often remain naturally infected with staphylococci for many weeks before causing an intramammary infection or the duct infection can spontaneously disappear. If, however, hygiene including teat dipping is introduced most of these teat canals are freed of *Staph. aureus* within a week.

It has been shown (Newbould & Neave, 1965*b*) that at the start of an artificial infection of the teat cistern, multiplication of the organism is clearly evident within

12 h but an increase in leucocytes is not observed at this time and may be delayed for more than 24 h. If we assume that this delay in cellular response is typical under normal twice-daily milking then there was a delay of at least 24 h between inoculation of the teat duct and penetration to the teat cistern, for as shown in Table 3 there was no increase in leucocytes until after 36 h. Table 3 shows also a marked increase in *Staph. aureus* before inflammation occurred, which is similar to the findings of Murphy & Stuart (1953) with *Str. agalactiae.*

When, however, the 1st post-inoculation milking was omitted inflammation was observed in 2 quarters 36 h after inoculating the teat duct (Table 4). It would appear that penetration into the teat cistern of these 2 quarters took place in much less than 24 h. A high bacterial count was evident by the 1st post-inoculation milking in one of them and by the 2nd milking in the other. Although the 5 cows involved were not milked at the 1st normal milking time, let-down was stimulated by sampling and other factors normally associated with milking; thus milk commenced leaking from some quarters and this became more copious as time elapsed. If, as has been suggested, the filling of the duct with milk under intramammary pressure facilitates the entry of micro-organisms to the teat cistern, it seems highly probable that organisms growing in the inoculated teat duct would, under these conditions, be transferred into the cistern by the normal movement of the udder and teats. Further support for this hypothesis comes from the findings of Oliver, Dodd & Neave (1956) that higher internal udder pressure, built up after the last milking in lactation, increases the incidence of new early dry-period infection.

While most teat ducts are probably 7–12 mm in length (Murphy & Stuart, 1956) some have been reported to be only 5 mm (Johnston, 1938). Therefore, once colonization of the duct has occurred, rapid penetration into the teat cistern could be expected even without movement of the duct caused by the act of milking, or by an increase in internal udder pressure (Espe & Cannon, 1942). Furthermore, the studies of Neave, Dodd & Henriques (1950) demonstrate that intramammary infection can be more frequent when the animal is not milked. The explanation of why colonization of the duct does not regularly lead to infection of the udder awaits further detailed experimentation. It has not been established whether this colonization is confined to the first few mm only nor has it been shown that udder infection is necessarily preceded by colonization of the teat duct.

Whatever factors operated in establishing the organisms in the teat duct they seemed to be variable, particularly in the case of cows 87 and 88 (Tables 2–4). Murphy & Stuart (1953), working with *Streptococcus agalactiae*, recovered organisms from the foremilk of all quarters for at least 2 milkings, but they used very much larger numbers of bacteria which makes comparison with our results difficult. In our experiments recovery of the organism from the fresh unincubated milk indicated that some multiplication had occurred.

When no organisms were recovered, even from incubated samples, at the 1st post-inoculation milking, one must assume that either the staphylococci were killed by some bactericidal factor in the duct as postulated by Adams & Rickard (1963), or they died out for lack of some essential metabolite. This, however, cannot apply to those instances where multiplication took place at first and the organisms died out after a few days, unless a growth factor was depleted or some delayed defence mechanism

was involved. Such effects could result from a combination of the two hypotheses, i.e. some factor or factors enable initial multiplication of the organisms in the duct, but if this factor becomes depleted, or modified in some way, multiplication is slowed down and bactericidal factors, which might themselves be variable, are able to act effectively on the reduced numbers. In those teat ducts in which the organism became established for relatively long periods, but did not always cause intramammary infection, sufficient essential metabolites or stimulatory factors, must have been present in the duct to enable the organisms completely to overgrow any antibacterial factors present. These stimulatory factors could conceivably be associated with erosion of the epithelium of the teat duct (Kennedy, 1943), a condition which, when visible externally, was found to be related to the incidence of natural infection of the duct (Report, 1959).

The data presented here do not support the hypothesis of Hopkirk (1934) that leucocytes pour into the milk as the result of infection in the teat duct, but they confirm other findings (Report, 1959) that the leucocyte count does not increase in the foremilk unless intramammary infection has taken place.

If previous intramammary infection had sensitized the quarters to further infection, one would have expected more intramammary infections in the left side quarters than the right side ones, as all quarters on the left side of all but cow S 43 had been infected during the infusion experiments about 3 months earlier (Newbould & Neave, 1965*b*). Of the 8 intramammary infections produced, 6 were in quarters on the right side and 2 on the left. In cow S 61 when all 4 teat ducts were inoculated, both intramammary infections occurred in the right side quarters. In cow 87 three occurred in right side quarters and none in the left. In cow S 59 the RH quarter was infected in trial 4, but in trial 6, in which this duct was again inoculated, no intramammary infection resulted. While the number of quarters involved is small, these results indicate that previous infection by *Staph. aureus*, strain *f*, produced no sensitization but rather, increased resistance.

Neave, Oliver & Dodd (1957) inferred that infections occurred more frequently during the interval between milkings than during milking. If it is supposed that the organisms grow through the duct in the period between one milking and the next, this seems unlikely, in view of the results reported in this paper, unless we presuppose that the strains were more vigorous or the initial numbers were much greater.

We wish to thank Mr D. L. Simpkin for his technical assistance, Mr W. A. Cuthbert and Mr A. S. Foot for the provision of facilities for the work, Dr F. H. Dodd and Mr N. Jackson for generous help and Mr B. Reiter for criticisms.

REFERENCES

ADAMS, E. W. & RICKARD, C. G. (1963). *Am. J. vet. Res.* **24**, 122.
ESPE, D. & CANNON, C. V. (1942). *J. Dairy Sci.* **25**, 155.
GRIFFIN, T. K. & DODD, F. H. (1962). *J. Dairy Res.* **29**, 207.
HADLEY, F. B., FROST, W. D., GUMM, M. & WELSH, W. E. (1930). *J. Am. vet. med. Ass.* **30**, 328.
HOPKIRK, C. S. M. (1934). *Aust. vet. J.* **10**, 143.
JOHNSTON, T. (1938). *J. comp. Path. Ther.* **51**, 69.
JONES, F. S. & LITTLE, R. B. (1934). *12th Int. vet. Congr.* **2**, 563.
KENNEDY, A. J. (1943). *Vet. Rec.* **55**, 45.
MURPHY, J. M. & STUART, O. M. (1953). *Cornell Vet.* **43**, 465.

MURPHY, J. M. & STUART, O. M. (1956). *Cornell Vet.* **45**, 112.
NEAVE, F. K., DODD, F. H. & HENRIQUES, E. (1950). *J. Dairy Res.* **17**, 37.
NEAVE, F. K., OLIVER, J. & DODD, F. H. (1957). *Rep. natn. Inst. Res. Dairy.*
NEAVE, F. K. & OLIVER, J. (1962). *J. Dairy Res.* **29**, 79.
NEWBOULD, F. H. S. & NEAVE, F. K. (1965*a*). *J. Dairy Res.* **32**, 157.
NEWBOULD, F. H. S. & NEAVE, F. K. (1965*b*). *J. Dairy Res.* **32**, 163.
OLIVER, J., DODD, F. H. & NEAVE, F. K. (1956). *J. Dairy Res.* **23**, 204.
REPORT (1959). *Bull. Minist. Agric. Fish Fd., Lond.*, no. 177, p. 116. London: H.M.S.O.
SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). *J. appl. Bact.* **25**, 403.

Printed in Great Britain

J. Dairy Res. (1965), **32**, 181
With 2 plates

The labelling of certain milk proteins with iodine^{131}

By H. A. VERINGA and M. F. KERKHOF MOGOT
Netherlands Institute for Dairy Research, Ede, The Netherlands

(*Received* 1 *January* 1965)

Summary. Whole casein, β-casein, β-lactoglobulin and euglobulin were labelled with ^{131}I. The conditions under which iodination was carried out were chosen so as to avoid any modification of the original characteristics of the proteins. This was checked by starch-gel electrophoresis and determination of sedimentation constants, apparent molecular weights and, for euglobulin, the clustering effect on fat globules.

As was shown by autoradiograms of the starch-gel plates, the radioactivity was incorporated in all zones of the electrophoresis pattern.

To enable experiments to be carried out on the creaming of raw milk, protein complex formation and coagulation of serum proteins on heating, it was found necessary to have labelled milk proteins available. In the present investigation ^{131}I was used as the labelling agent.

^{131}I is a beta- and gamma-emitter, with a half-life of 8·04 days. Its radioactivity can be detected by scintillation counting.

A number of methods for the labelling of proteins with iodine has already been published, but in only a few cases has it been proved that they have no effect on the properties of these proteins. The method described in this paper is based on the procedure of De Zoeten (1959) for the iodination of insulin.

EXPERIMENTAL

Materials and methods

All reagents used were of 'Analar' grade.

Whole casein was precipitated from fresh skim-milk by slowly adjusting the pH to the isoelectric point (4·6) with HCl. The protein was washed with distilled water and subsequently freeze-dried.

β-Casein was prepared by the urea method of Hipp, Groves, Custer & McMeekin (1952) (with some slight modifications of the alcohol method) and by Warner's isoelectric precipitation, as described by Payens & van Markwijk (1963).

Crystalline β-lactoglobulin was isolated according to Larson & Jenness (1955) and euglobulin was prepared by the method of Smith (1946).

Carrier-free Na^{131}I without reducing agent was obtained from Philips Duphar, Amsterdam, Holland.

Starch-gel electrophoresis was performed by the method of Smithies (1959), and starch-gel electrophoresis in presence of urea by the procedure of Wake & Baldwin

(1961). After wrapping the starch-gels in non-porous transparent film, as proposed by Smithies (1959), autoradiograms were made by exposing them to Gevaert X-ray film Structurix D7-DW.

Sedimentation was studied with a Phywe air-driven ultracentrifuge. Apparent molecular weights were determined by the Archibald method as described by Schachman (1959). All measurements were made at 2 °C.

Clustering times of the fat globules of 'agglutinin-depleted' milk were estimated according to Dunkley & Sommer (1944) by visual observation of a thin, 0·5 mm layer of the milk.

Iodine was prepared by oxidation of KI and $Na^{131}I$ with KIO_3 using a small excess of the KI.

Reagents:

A. Dissolve 327 mg KI in distilled water and make up to 100 ml.
B. Dissolve 85 mg KIO_3 in distilled water and make up to 100 ml.
C. Hydrochloric acid sp.gr. 1·18.
D. Na ^{131}I with an activity of 1 mC.

To obtain a solution of 30 mg iodine, mix 11 ml of solution A and 10 ml of solution B in a 50-ml volumetric flask. Inject 2 ml of this mixture into the rubber-stoppered capsule which contains the radioactive iodide, and transfer the liquid to the volumetric flask. To rinse the capsule, the operation is repeated 4 times with 5 ml distilled water. Acidify the solution with 0·5 ml of solution C, adjust the volume with distilled water to 50 ml and cool to 4 °C.

General remarks

To prevent oxidation during iodination, the procedure should preferably be carried out at pH values higher than 6 and at a temperature of 4 °C. The optimum concentration of iodine is different for the various proteins. For instance, iodination of β-casein can be carried out with amounts of iodine up to about 6 mg/100 mg of protein. When, however, only 1 mg of iodine per 100 mg of β-casein is used, the specific activity of the iodinated protein becomes too low to ensure reliable results after the dilution involved in the intended experiments. In contrast the addition of iodine to euglobulin in a quantity corresponding to 6 mg/100 g proteins leads to destruction of the clustering effect on the fat globules in milk. Fortunately, however, 1 mg of iodine per 100 mg of euglobulin gives a specific activity high enough to permit counting at a considerable dilution but does not destroy the clustering properties.

Iodination of the various milk proteins

Whole casein

One gram of casein was dissolved in 50 ml of 0·01 M sodium tetraborate buffer at pH 9·2, and the solution was cooled to 4 °C. An iodine solution containing 30 mg of iodine was added to the protein solution. Care was taken to keep the pH at a constant value of 9·2. The solution was stirred for 15 min at 4 °C, after which the pH was adjusted to 4·5 by means of N-HCl. The precipitated casein was centrifuged and redissolved in distilled water with N-NaOH. This acidification procedure was repeated twice, after which the iodinated protein was dialysed against water until all free iodine was completely removed. This was checked by comparing the decrease in radioactivity of the

protein solution with that of a standard. If the decrease in radioactivity is due only to the decay of ^{131}I, the protein solution contains no free iodine. The iodinated protein was then freeze-dried and stored in a desiccator over P_2O_5. The yield was about 750 mg, and the specific activity amounted to 40 nC/mg casein.

Starch-gel electrophoresis revealed no difference between the iodinated and non-iodinated casein, as is shown in Plate 1 (1). An autoradiogram made from the starch plate showed radioactivity for all zones of the iodinated casein (Plate 1 (2)).

Average molecular weight determinations showed no significant difference between the iodinated and the original preparation of whole casein. The values of the molecular weight found for both preparations were 239 000 ± 5000 and 241 000 ± 5000, respectively.

β-Casein

One gram of β-casein was dissolved in 50 ml of 0·01 M-sodium tetraborate buffer at pH 9·2. The amount of iodine used was 30 mg/g β-casein. The iodine-containing protein solution was stirred for 1 h at 4 °C before the pH was adjusted to 4·6. The precipitated β-casein was centrifuged and redissolved in distilled water by adding N-NaOH. This purification procedure was repeated 4 times, after which the protein was dialysed and freeze-dried. The yield was about 750 mg, and the specific activity was found to be 90 nC/mg β-casein. The starch-gel electrophoresis patterns of the labelled and unlabelled β-casein (Plate 2 (3)) were very similar.

An autoradiogram of the starch plate after electrophoresis showed one single zone for the iodinated β-casein (Plate 2 (4)).

β-Lactoglobulin

One gram of β-lactoglobulin was dissolved in 50 ml of sodium tetraborate-HCl buffer at pH 8·0. The iodination was carried out with 60 mg of iodine. The solution was stirred for 30 min at 4 °C. The iodinated lactoglobulin was purified by precipitation with 3·3 M-$(NH_4)_2SO_4$ solution at pH 8·0. This procedure was repeated twice.

The yield of iodinated β-lactoglobulin was about 750 mg, with a specific activity of 80 nC/mg.

The sedimentation constant of the labelled β-lactoglobulin was 3·00 *S*, whereas that of the original preparation was 3·12 *S*. From these data and from the starch-gel electrophoresis patterns (Plate 3) the treatment with iodine appeared to be harmless.

Euglobulin

One gram of euglobulin was dissolved in 50 ml of 0·01 M-NaCl solution and cooled to 4 °C. This quantity of protein was iodinated with 10 mg of iodine. Care must be taken to maintain the pH value of the iodine-protein mixture between 6·5 and 7·5. The solution was stirred for 6 min at 4 °C, after which the pH was adjusted to 6 by means of N-HCl. Ammonium sulphate was added until 0·4 saturation was reached, and the precipitated protein was centrifuged, redissolved in distilled water and dialysed. The water-insoluble euglobulin was collected and dissolved in distilled water with the aid of ammonia, and subsequently freeze-dried.

The yield of iodinated euglobulin was about 650 mg, with a specific activity of 100 nC/mg.

The values of the sedimentation constants of ^{131}I-labelled euglobulin and of normal, inactive euglobulin are given in Table 1. In our opinion the observed differences are due only to experimental error.

Table 1. *Sedimentation constants of non-iodinated and iodinated euglobulin*

	Slow moving component	Fast moving component
	($S^0_{20,w}$)	($S^0_{20,w}$)
Non-iodinated euglobulin	7·20	21·75
Iodinated euglobulin	8·17	22·92

The clustering properties of the iodinated and non-iodinated euglobulin towards the fat globules were compared in 'agglutinin-depleted' milk at 5 °C. The results are given in Fig. 1; no differences were observed.

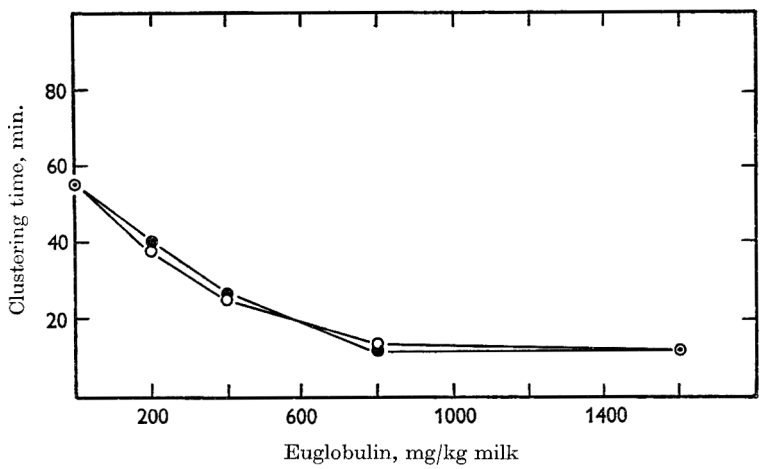
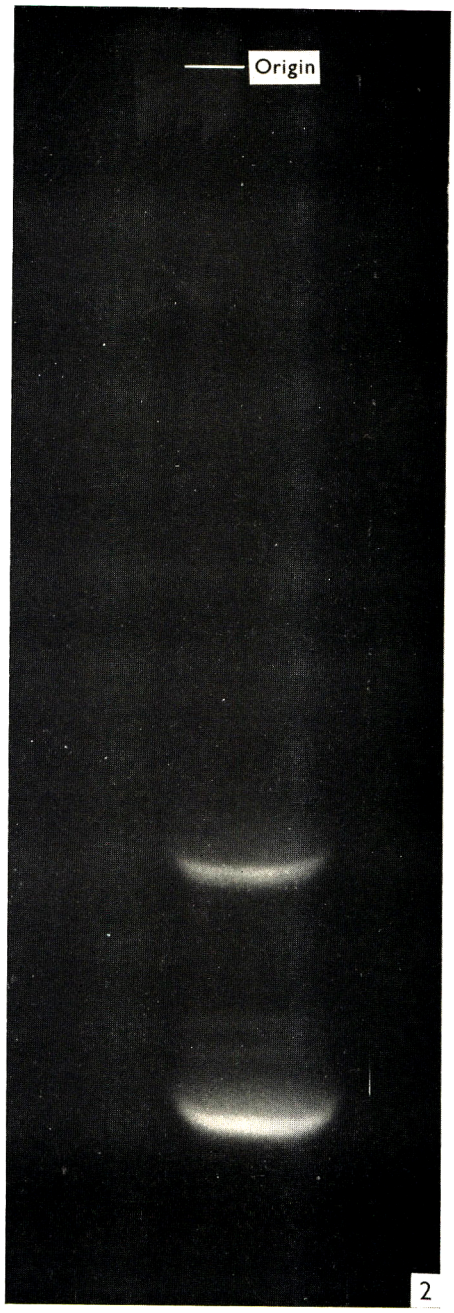


Fig. 1. Effect of added euglobulin on the clustering time of milk-fat globules of 'agglutinin-depleted' milk at 5 °C. ●, Non-iodinated euglobulin; ○, iodinated (^{131}I) euglobulin.

DISCUSSION

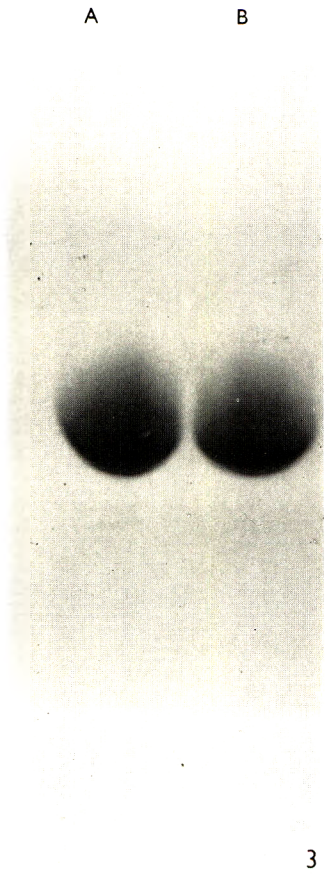
From the literature it is well known that treatment of proteins with iodine mainly results in substitution of iodine in the tyrosine rings of these proteins. Moreover, dependent on the conditions under which iodination is carried out, side reactions may occur, e.g. iodination of histidyl residues and oxidation of disulphide bridges, sulphydryl groups and other residues such as tryptophane. It is assumed that these oxidation reactions have a particularly detrimental effect on the electrophoretic and sedimentation characteristics of the proteins. Consequently, the results of the iodination of the milk proteins described in this paper indicate that the side reactions probably do not take place to any great extent, in agreement with the results of the iodination of insulin by De Zoeten (1959).

The possibility of labelling milk proteins without changing the characteristic properties enables investigations in dairy research to be carried out which would otherwise be impossible.

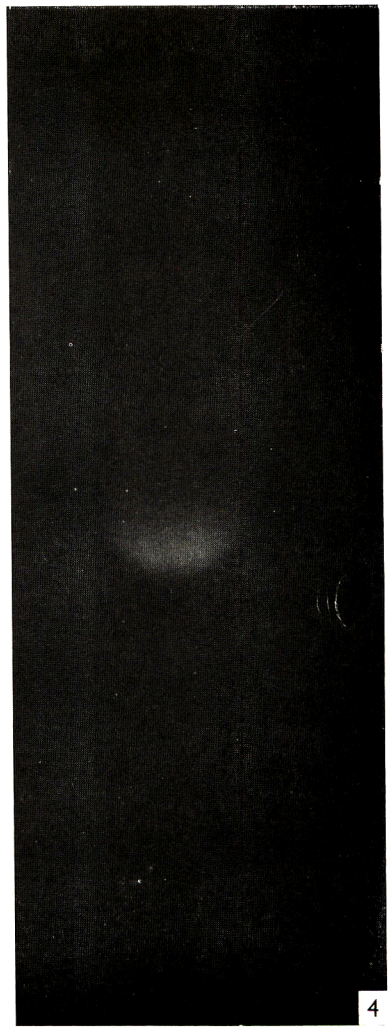


H. A. VERINGA AND M. F. KERKHOF MOGOT

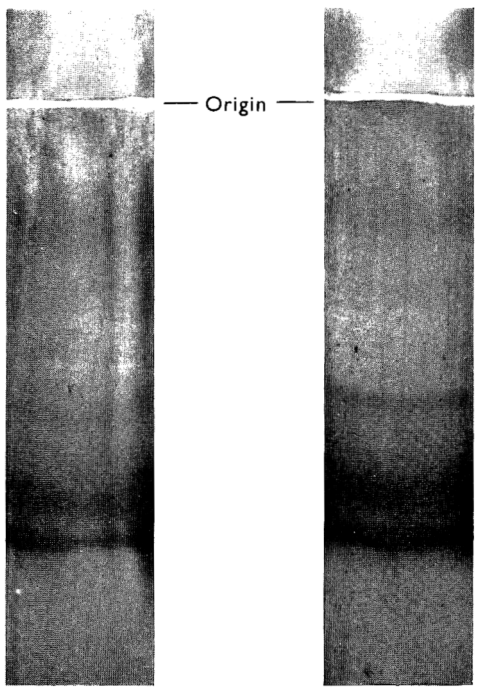
(*Facing p.* 184)



3



4


Origin
A
B

The authors are indebted to Mr B. W. van Markwijk of this Institute for the determination of molecular weights and sedimentation constants.

REFERENCES

DUNKLEY, W. L. & SOMMER, H. H. (1944). *Res. Bull. agric. Exp. Stn Univ. Wis.* no. 151.
HIPP, N. J., GROVES, M. L., CUSTER, J. H. & MCMEEKIN, T. L. (1952). *J. Dairy Sci.* **35**, 272.
LARSON, B. L. & JENNESS, R. (1955). *Biochemical Preparations*, **4**, 23. London: Chapman & Hall, Ltd.
PAYENS, T. A. J. & MARKWIJK, B. W. VAN (1963). *Biochim. biophys. Acta*, **71**, 517.
SCHACHMAN, H. K. (1959). *Ultracentrifugation in Biochemistry*. New York: Academic Press.
SMITH, E. L. (1946). *J. biol. Chem.* **165**, 665.
SMITHIES, O. (1959). *Adv. Protein Chem.* XIV, 65.
WAKE, R. G. & BALDWIN, R. L. (1961). *Biochim. biophys. Acta*, **47**, 225.
ZOETEN, L. W. DE (1959). Thesis, Leiden.

EXPLANATION OF PLATES

PLATE 1

(1) Patterns of starch-gel electrophoresis in the presence of 7M-urea of non-iodinated (A) and iodinated (B) whole casein.

(2) Autoradiogram of a starch plate, after electrophoresis in the presence of 7M-urea, of iodinated whole casein.

PLATE 2

(3) Patterns of starch-gel electrophoresis in the presence of 7M-urea of iodinated (B) and non-iodinated (A) β-casein.

(4) Autoradiogram of a starch-gel plate, after electrophoresis in the presence of 7M-urea, of iodinated β-casein.

PLATE 3

Starch-gel electrophoresis patterns of non-iodinated (A) and iodinated (B) β-lactoglobulin.

Printed in Great Britain

A survey of the incidence of coagulase-positive staphylococci in market milk and cheese in England and Wales

By M. ELISABETH SHARPE, B. GILLIAN FEWINS, B. REITER and W. A. CUTHBERT

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 6 *January* 1965)

Summary. The occurrence of coagulase-positive staphylococci in 954 samples of raw or heat-treated market milk and in 910 samples of cheese of different varieties, taken at 40 cheese factories or farms in England and Wales was determined. Only 9% of the cheeses contained more than 500 000 staphylococci/g, and these were all made from raw milk. All but the mildest of the heat treatments used were effective in greatly reducing the numbers of staphylococci present in milk and usually resulted in cheese virtually free from staphylococci. The effect of sublethal heat treatment on multiplication of these organisms in cheese curd is discussed.

The increased incidence of staphylococcal mastitis and the resultant increase in coagulase-positive staphylococci in raw milk over the last 10–15 years is well recognized (Munch-Petersen, 1960). The occurrence of a number of outbreaks of staphylococcal food poisoning attributed to cheese has led to studies of the conditions under which staphylococci are likely to multiply during cheese-making and of methods of preventing such multiplication (Takahashi & Johns, 1959; Thatcher & Ross, 1960; Jezeski, Morris, Zottola, George & Busta, 1961; Roughley & McLeod, 1961; Reiter, Fewins, Fryer & Sharpe, 1964). To investigate whether these organisms occur in large numbers in commercial cheese sufficiently frequently to constitute a serious health hazard, a survey was made of their incidence in cheese made in England and Wales.

At the same time milk used for cheese-making was examined and the pH of the cheeses was measured, to determine whether there was the same correlation between very large numbers of staphylococci and sweet, slow cheeses (high pH) as was observed in experimental Cheddar cheeses (Sharpe, Neave & Reiter, 1962; Reiter *et al.* 1964). The work was carried out in collaboration with the Advisory Bacteriologists of the National Agricultural Advisory Service (N.A.A.S.) in cheese-making areas of England and Wales. One dairy laboratory also took part.

METHODS

During 1962 samples were taken over 3–12 months at 17 farms and 33 cheese factories in 10 different N.A.A.S. regions. One farm and 26 factories used heat-treated milk. Sampling was carried out at weekly intervals at each farm or factory, apart

from 8 farms where sampling was only done once. Where coagulase-positive staphylococci were consistently absent over a period of 3 months, sampling was usually discontinued.

Milk samples

Heated milk samples were obtained either directly from the pasteurizer or from the vat. Thermograph charts were checked at each sampling. Raw milk samples (667) and heat-treated milk samples (287) were examined.

Cheese samples

Cheeses (910) were tested approximately 14 days after making. For each cheese two samples bored from closely adjacent positions were minced together: one portion was homogenized in warm 2% citrate for bacteriological examination, the other portion was homogenized with distilled water and the pH determined electrometrically. The varieties of cheese and the number of farms and factories at which they were sampled is shown in Table 1. Most of the creameries were primarily making Cheddar and Cheshire cheese, but other varieties were sampled at 14 centres. At some places more than one type of cheese was manufactured so that comparisons within factories could be made; as there was no evidence of any tendency for higher numbers of staphylococci to occur in any one variety, results on all cheeses were combined.

Table 1. *Number of creameries at which different types of cheese were examined*

	Variety of cheese							
	Cheddar	Cheshire	Wensley-dale	Lancs.	Leics.	Derby-shire	Caer-philly	Double Gloucester
Factory	12	10	4	2	3	1	3	1
Farm	5	8	—	—	—	—	—	—

Isolation and enumeration of staphylococci

Coagulase-positive staphylococci were isolated and enumerated on the selective and diagnostic egg-yolk glycerine pyruvate tellurite medium of Baird-Parker (1962), previously shown by Sharpe *et al.* (1962) to be suitable for this purpose. For milk samples, duplicate 0·1 ml amounts of milk and of serial dilutions were spread on the surface of well-dried agar plates. For cheese samples, the portion homogenized in citrate was plated in serial dilutions using the Miles & Misra (1938) technique but spreading each drop out on a quarter of the plate. All plated samples were incubated at 37 °C for 40–45 h before counting. Representative diagnostically positive colonies were picked into nutrient broth and after incubation at 37 °C for 18 h their identity confirmed by tube coagulase test. Stocks of basal medium, additives to it, and of human plasma for coagulase test were all issued to the different laboratories from this Institute and standard techniques were adopted throughout.

RESULTS

Numbers of staphylococci in raw and heat-treated milks

Table 2 shows the numbers of staphylococci in the raw and heat-treated milks. Of the heat-treated samples, approximately 90 % contained less than 10^3 staphylococci/ml whilst only 30 % of the raw milks contained less than this number.

Table 2. *Numbers of coagulase-positive staphylococci in raw and heat-treated milk*

	% of milk samples having staphylococci/ml within the range of						No. of samples
	< 10	10–10^2	10^2–10^3	10^3–10^4	10^4–10^5	10^5–10^6	
Raw milk							
Factory	5·4	2·3	23·1	40·8	19·1	9·6	550
Farm	3·4	5·9	23·2	35·9	23·1	8·5	117
Heat-treated milk							
Factory	73·9	4·4	12·0	5·9	2·9	0·37	272
Farm	93·3	0	0	6·7	0	0	15
						Total	954

Table 3. *Numbers of coagulase-positive staphylococci found in milks at a single creamery, after different heat treatments*

	No. of samples having staphylococci/ml within the range of					
Heat treatment	< 10	10–10^2	10^2–10^3	10^3–10^4	10^4–10^5	Total
150 °F, flash	1	0	6	5	1	13
150 °F, 17–19 sec	11	3	4	2	1	21
155 °F, flash	11	3	4	2	1	21
161 °F, flash	4	0	0	0	0	4
161 °F, 17 sec	3	0	0	1	0	4
	28	7	12	11	3	63

Heat treatment varied considerably, not only from one creamery to another but also sometimes from day to day. The range recorded in the whole survey was from 145 °F flash to 165 °F for 17 sec, so that some of the heat treatments were milder and others more severe than required for normal pasteurization. The few samples of heated milks which contained more than 10^3 staphylococci/ml were isolated samples from centres where the heat treatment was only flash-heating to 145–155 °F. At one creamery, where sometimes flash treatment and sometimes holding treatments at different temperatures were used, the effect of the different heat treatments on the numbers of staphylococci was studied in some detail (Table 3). Previous work (Reiter *et al.* 1964) indicated that staphylococci were destroyed by a heat treatment well below 161 °F for 17 sec, so that in the one sample where $> 10^3$/ml survivors were found after this treatment, it is likely that post-pasteurization contamination occurred.

Numbers of staphylococci in cheeses made from raw and heat-treated milks

The effect of heat treatment of the milk on the numbers of staphylococci in the cheese is shown in Table 4. 92 % of the cheeses made from heated milk contained less than 10^4/g. There was a difference between the numbers of staphylococci found in the raw milk cheeses made at factories and those made at farms; a higher proportion

containing less than 10^4/g occurred in factory (46%) than in farmhouse cheeses (10%). As the numbers of staphylococci in farm raw milks were almost identical with those in factory raw milks, more rapid multiplication or further contamination must have occurred at the farms. This suggests less active starters or poorer hygiene.

Table 4. *Numbers of coagulase-positive staphylococci in cheese made from raw and heat-treated milks*

	% of cheese samples having staphylococci/g within the range of							No. of samples
	< 500	500–10^3	10^3–10^4	10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^8	
Cheeses made from raw milk								
Factory	23·0	5·0	17·8	26·5	20·9	6·6	0	286
Farm	4·27	0·6	5·7	33·3	39·5	15·3	1·8	162
Cheese made from heat-treated milk								
Factory	82·3	2·3	8·8	5·1	1·4	0	0	430
Farm	65·6	0	25·0	9·4	0	0	0	32
							Total	910

Table 5. *The effect of heat treatment of milks on the numbers of coagulase-positive staphylococci found in cheeses made from these milks*

	% of samples having numbers* of staphylococci within the range of							No. of samples†
Cheese made from	< 500	10^2–10^3	10^3–10^4	10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^9	
Raw milk								
Milk	2·2	19·2	40·0	27·5	9·2	1·7	0	116
Cheese	4·1	0	0·85	33·2	42·6	17·7	1·6	120
Heat-treated milk								
Milk	0	33·3	22·2	33·3	11·1	0	0	9
Cheese	44·4	44·4	0	0	11·1	0	0	9

* Numbers are expressed as /ml milk or /g cheese.

† Numbers of samples of milk do not exactly correspond to numbers of cheeses as occasionally more than one cheese from a single vat was sampled.

Cheeses made from milks previously sampled

It was only possible at 14 factories and 9 farms to follow the milk samples to the cheeses made from them, so that the numbers of staphylococci in the milks could be related to those in the cheese. At 8 of these 14 factories where the milk was heated no staphylococci were found in the milk or in the cheeses made from them. At 2 others, although no staphylococci were found in the heated milk, staphylococci were found in numbers of 10^3–10^4/g in the cheese, indicating that post-pasteurization contamination had occurred. At the 4 other factories where heat treatment was used, the 9 samples of milk examined contained 10^2–10^6 staphylococci/ml after heat treatment, but in all but one of these the numbers were considerably lower in the corresponding cheeses (Table 5), particularly allowing for the fact that organisms are concentrated 8–10 times in the cheese curd. In cheeses made from raw milk, however, there was an increase in numbers of staphylococci during cheese-making of 60–100-fold, indicating that multiplication had occurred. This suggests that the heat-shocked cells were not able to multiply under the unfavourable conditions in the curd, whilst the unheated organisms were not retarded in the same way.

Cheeses containing large numbers of staphylococci

Of the 910 cheeses examined only 82 (9·0 %) contained more than 500 000 staphylococci/g. These cheeses were all made from raw milk and came from 4 farmhouses and 5 factories. The numbers of staphylococci varied from 5×10^5 to 6×10^8/g. Five of them contained more than 10^7/g and all came from one farm where conditions of hygiene were poor.

pH *of cheeses*

There was no correlation between the pH of the cheeses and the numbers of staphylococci occurring in them. Only 3 of the high count cheeses had a pH as high as 5·5–5·7. In the others the pH varied between 4·7 and 5·5. In some of the cheeses containing only small numbers of staphylococci the pH was as high as 5·7, whilst in others it was only 4·8. However, as far as could be ascertained, no really slow cheeses were included in the survey. Although a particular lookout was kept for them, any reported slowness entailed a delay of only an hour or two.

Table 6. *Numbers of coagulase-positive staphylococci in cheeses of different ages*

Age of cheese, days	% of cheeses having staphylococci/g within the range of								No. of samples
	< 500	500–10^3	10^3–10^4	10^4–10^5	10^5–10^6	10^6–10^7	10^7–10^8	10^8–10^9	
6–11	36	7·1	17·9	7·1	21·4	7·1	3·5	0	28
12–18	50·4	2·6	11·0	16·4	14·0	4·6	12	12	815
19–27	34·2	2·9	8·5	22·8	17·1	8·5	2·9	2·9	35
28–42	50	6·3	15·6	6·3	15·6	6·3	0	0	32
								Total	910

Variation in numbers of staphylococci with age of cheese

Although it was intended that cheeses should be sampled when approximately 2 weeks old, this was not always practicable and the age of the cheeses sampled varied from 6 to 42 days. Whilst most of the cheeses were sampled when 12–18 days old, sampling when the ages were outside these limits did not appear to affect the numbers of staphylococci; there was no tendency for the older cheeses to contain lower numbers of staphylococci (Table 6). However, when a few (10) commercial cheeses with high counts were sampled after 14 days and again after 4–5 weeks maturation, half of them showed no reduction in numbers over this period, whilst the others showed a considerable decrease in numbers. In our own work with experimental Cheddar cheese (Reiter *et al.* 1964) an approximate fivefold decline in numbers occurred over this period.

DISCUSSION

The numbers of staphylococci found in raw milks are likely to vary under different conditions. McPhillips & Cunningham (1962) found the maximum number of coagulase-positive staphylococci in grade B raw tanker milk to be 10^4/ml. Murray (1963) found no staphylococci in 30 % of raw milks used for processing, and only 1·6 % contained 10^3/ml or more. Seeleman, Obiger, Rachov & Welz (1963) found no staphylococci in 68 % of raw milks examined and $> 10^3$/ml in only 11 %. These are lower numbers than reported in our survey where only 5 % of the samples contained $< 10^2$/ml and 30 % contained $> 10^4$/ml; however, Thatcher, Comtois, Ross & Erdman (1959) found 68 % of the cheese milks at one factory to contain $> 10^4$/ml.

In young Cheddar and Cheshire cheeses Murray (1963) found a low incidence of staphylococci, 94% containing $< 10^4$/g. Thatcher *et al.* (1959) observed that high count raw milk cheeses might contain $> 10^6$/g, and Takahashi & Johns (1959) found that over 6% of 2–3 weeks old commercial raw milk cheeses contained > 500 000/g. These last authors also found a marked seasonal variation, much higher numbers occurring in June–August than in other months. No such variation was observed in our survey.

Our findings show that raw milk may contain large numbers of staphyococci which by multiplication and concentration in the curd during cheese-making may reach a level of 10^5–10^6/g in the curd and in the young cheese. Large numbers were only found in raw milk cheeses. Under normal conditions of cheese-making an initial infection in the milk of 10^3/ml would result in a cheese containing about 5×10^5/g. Only 9% of heated milks contained more than this number of staphylococci, occurring only sporadically and usually when flash heating at low temperatures was used. Other workers have also found that heat treatment of the milk greatly reduced the number of staphylococci present (Zottola & Jezeski, 1963) and that heat treatment which is not severe (Takahashi & Johns, 1959; Murray, 1963; Reiter *et al.* 1964) usually results in milk virtually free from staphylococci. The low incidence of staphylococci in cheeses made from milk exposed to sublethal heat treatment (only 8% contained more than 10^4/g) confirms our previous finding with experimental Cheddar cheese that in the unfavourable conditions of low pH, high salt concentration and starter competition in cheese curd, the surviving heat-shocked cells of staphylococci fail to multiply and that even a heat treatment of 150 °F for 15 sec is adequate (Reiter *et al.* 1964). The suggested use of a higher heat treatment of 154 °F for 15 sec (Milk and Milk Products Technical Advisory Committee, 1964) which destroys them in the milk would give an ample margin of safety.

The authors wish to thank the Advisory Bacteriologists of the N.A.A.S. for their valuable contributions to this work; the many dairies who made facilities available and members of the Dairy Department, Reading University for helpful discussions. This survey and the previous studies were supported in part by a grant from the National Association of Creamery Proprietors.

REFERENCES

BAIRD PARKER, A. C. (1962). *J. appl. Bact.* **25**, 12.
JEZESKI, J. J., MORRIS, H. A., ZOTTOLA, E. A., GEORGE, E. & BUSTA, F. F. (1961). *J. Dairy Sci.* **44**, 1160.
MCPHILLIPS, J. & CUNNINGEAM, E. A. (1962). *Aust. J. Dairy Technol.* **17**, 95.
MILES, A. A. & MISRA, S. S. (1938). *J. Hyg., Camb.*, **38**, 732.
MILK AND MILK PRODUCTS TECHNICAL ADVISORY COMMITTEE 1962 (1964). *J. Soc. Dairy Technol.* **17**, 114.
MUNCH-PETERSEN, E. (1960). *Aust. J. Dairy Technol.* **15**, 25.
MURRAY, J. G. (1963). *J. Soc. Dairy Technol.* **16**, 201.
REITER, B., FEWINS, B. G., FRYER, T. F. & SHARPE, M. E. (1964). *J. Dairy Res.* **31**, 261.
ROUGHLEY, R. J. & MCLEOD, R. W. (1961). *Aust. J. Dairy Technol.* **16**, 110.
SEELEMAN, M., OBIGER, G., RACHOV, H. G. & WELZ, W. (1963). *Dairy Sci. Abstr.* **25**, 3270.
SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). *J. appl. Bact.* **25**, 403.
TAKAHASHI, I. & JOHNS, C. K. (1959). *J. Dairy Sci.* **42**, 1032.
THATCHER, F. S., COMTOIS, R. D., ROSS, D. & ERDMAN, I. E. (1959). *Can. J. publ. Hlth*, **50**, 497.
THATCHER, F. S. & ROSS, D. (1960). *Can. J. publ. Hlth*, **51**, 226.
ZOTTOLA, E. A. & JEZESKI, J. J. (1963). *J. Dairy Sci.* **46**, 600.

Printed in Great Britain

J. Dairy Res. (1965), **32**, 193

The action of rennin on casein: the effect of modifying functional groups on the casein

By R. D. HILL and RAIONE R. LAING
Division of Dairy Research, C.S.I.R.O., Melbourne, Australia

(*Received* 18 *January* 1965)

SUMMARY. κ-Casein and whole casein when photo-oxidized in the presence of methylene blue lose the ability to clot when treated with rennin. Two effects are involved—first the photo-oxidation alters the κ-casein fraction so that the rennin is unable to split off the glycopeptide fragment, and secondly, in whole casein the photo-oxidation interferes with the aggregation in the presence of Ca^{++} that normally follows rennin action. As a result of amino acid analysis and specific treatments which affect other photo-oxidizable side chains, it is concluded that both of these effects are caused by the alteration of histidines.

The action of rennin on casein has both an industrial and a theoretical interest, and this no doubt accounts for the numerous studies of the reaction. These studies have established that the substrate for the reaction is the κ-casein fraction (Waugh & von Hippel, 1956; Wake, 1959) and that this action releases a glycopeptide having a molecular weight of about 8000 (Jollés, Alais & Jollés, 1961); the composition of this glycopeptide has also been determined (Nitschmann & Beeby, 1960; Alais & Jollés, 1961). After the release of the glycopeptide, rennin-treated milks gel because functional groups exposed by the removal of the glycopeptide can take part in a polymerization reaction. The information regarding the nature of these groups is, however, not certain. Because casein is a phospho-protein and Ca^{++} is required for gelation to occur, it has been suggested that Ca^{++} forms intermolecular links between phosphate groups (McFarlane, 1938). However, Hsu *et al.* (1958) showed that, although dephosphorylation resulted in marked increases in clotting time at a Ca^{++} concentration of 0·01 M, enzymically dephosphorylated casein would clot following rennin treatment if the Ca^{++} content were sufficient (0·02 M). This suggests that, in addition to the postulated links of Ca^{++} to phosphate groups, other types of intermolecular bonds are involved in the clotting reaction. In this connexion Bargoni (1963) has reported that casein in which the carboxyl or the amine groups are blocked is not acted on by rennin; these treatments however cause such extensive alteration of the protein that the results are difficult to interpret. To obtain a less drastic alteration of the protein, we have used the technique of photo-oxidation with methylene blue as sensitizer (Weil & Seibles, 1955). Our initial tests showed that κ-casein and whole casein treated in this way would no longer clot on treatment with rennin. Since this work was commenced Zittle has reported that κ-casein photo-oxidized in the presence

of methylene blue lost its ability to stabilize α_s casein, and did not precipitate on treatment with rennin (Zittle, 1964): the photo-oxidation resulted in the destruction of mainly histidine and tryptophan, and to a lesser extent tyrosine, side chains. However, the fact that this treatment simultaneously affects several different types of side chain causes uncertainty in interpreting the experimental results. We have, therefore, combined this technique with that of photo-oxidation in the presence of proflavine (Sluyterman, 1962), which allows a greater control of the effects of the treatment. At pH 3 tryptophan and methionine are oxidized while histidine and tyrosine are not affected below pH 4 and pH 7, respectively. We have also used treatments that specifically affect a given side chain such as the modification of tryptophan with 2-hydroxy-5-nitro-benzyl bromide (Koshland, Karkhanis & Latham, 1964), the oxidation of the methionine with peroxide at pH 3 (Schachter & Dixon, 1964), and the oxidation of tyrosine with tyrosinase (polyphenoloxidase) according to the method of Lissitzky & Rolland (1962).

The purpose of the study was to determine which of the photo-oxidizable side chains of the casein was instrumental in the failure of the casein to clot, and whether the effect was exerted on the reaction between the rennin and the casein, or on the subsequent aggregation of the casein itself. To this end, κ-casein, whole casein and para-casein were used as substrates and the effects of the treatments were followed by analysis of the modified proteins; their clotting behaviour and NPN release on treatment with rennin were also compared with those of controls.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade, and deionized or glass-distilled water was used throughout. Tyrosinase was obtained from Worthington Biochemical Corporation, New Jersey, and pronase (proteolytic enzyme from *Streptomyces griseus*) was supplied by the Kaken Chemical Co., Tokyo. Sephadex was the product of Pharmacia, Uppsala. Rennin was prepared from Benger's powdered rennet using at least threefold precipitation with NaCl (cf. Berridge & Woodward, 1953). Assayed amino acids were supplied by Mann Research Laboratories, New York. Whole casein was prepared from fresh raw milk by double precipitation at pH 4·5 with HCl and re-solution at pH 7 with NaOH. κ-Casein was prepared as described in Hill & Hansen (1963) and also by the method of alcohol fractionation of McKenzie & Wake (1961). Micellar casein was prepared by gel filtration on G 100 Sephadex (Hill & Hansen, 1964). These materials were preserved by freeze-drying.

Photo-oxidation with methylene blue

Solutions containing 50–100 mg of protein in 20 ml of phosphate buffer (I = 0·1, pH 7·4) were equilibrated at 30 °C in the Warburg apparatus (Braun, type V 185). At equilibrium, 0·08 ml of methylene blue solution (2·5 mg/ml) was allowed to flow into the reaction vessel, the taps were closed and the lights switched on. One vessel contained the control solution without methylene blue, and another contained buffer. All vessels had 1 ml 1 N-NaOH in the central well. Uptake of oxygen was followed manometrically. In these conditions the rate of photo-oxidation was independent of the

concentration of methylene blue. When the reaction had proceeded sufficiently the vessels were removed from the bath and the methylene blue removed by adsorption on a short column of glass micro-beads (100–150 mesh). The methylene blue adsorbs firmly on the glass but it can be removed by boiling in 1 N-HCl. When necessary phosphate buffer was removed by dialysis against sodium acetate buffer (0·03 N, pH 6·25) and the samples either used immediately for analysis and tests or preserved by freeze-drying.

Photo-oxidation with proflavine

The protein was dissolved in distilled water and the solution made 0·05 N in acetic acid, pH 3·2. The temperature of the Warburg bath was 30 or 37 °C. When the contents of the reaction vessels had reached the temperature of the bath, 0·5 ml of proflavine solution (2·5 mg/ml) was injected into the vessel through a rubber (Suba) seal by means of a hypodermic syringe. A few minutes were then allowed for the contents to reach temperature equilibrium before switching on the high pressure mercury lamp (Phillips 57131 G/92, 400 W). After completion of the photo-oxidation, the proflavine was separated from the protein by adjusting the solution to pH 6·5 and passing it through a Sephadex G25 column, in 0·05 N-sodium acetate buffer also at pH 6·25.

Modification with tyrosinase

Twenty ml of phosphate buffer (I = 0·1, pH 7·4) containing about 50 mg protein was heated to 37 °C in the Warburg bath. The central wells of the reaction vessels contained 1 ml 1 N-NaOH. Tyrosinase solution (0·5 ml, 0·5 mg/ml) prewarmed to 37 °C was injected by hypodermic syringe into the reaction vessel, which was shaken for a short time, the taps closed, and the uptake of oxygen followed manometrically. The samples were freeze-dried for analysis.

Modification with 2-hydroxy-5-nitro-benzyl bromide

The reagent was synthesized as described by Koshland *et al.* (1964). Its m.p. was 145 °C (lit. 145 °C). The solid reagent (6–20 mg) was stirred at room temperature with 10 ml of solution (pH 6·25 in sodium acetate buffer or pH 3 in 0·001 HCl) containing 0·5–1 % protein. In some cases the solution also contained 20 % formamide in which the reagent was rather more soluble. Stirring was continued for periods of up to 5 h as the reaction was rather slow. The separation of the uncombined reagent and the formamide from the modified protein was accomplished by passing the solution through a column of G25 Sephadex previously equilibrated with a solution of the same pH. The eluting solutions were monitored with a LKB Uvicord which recorded transmission at 253 mμm. The eluted protein was used in tests with rennin and analysed to determine the extent of modification of tryptophan.

Oxidation with H_2O_2 in perchloric acid

Protein solutions (0·5 %) at pH 3·2 in $HClO_4$ (approx. 1 mM), EDTA (0·5 mM) and H_2O_2 (0·38 M, added last) were incubated for 2 h at 30 °C. Control solutions contained no H_2O_2. This treatment is reported to result in the specific oxidation of methionine to methionine sulfoxide (Schachter & Dixon, 1964). After incubation the solutions were freeze-dried to remove the H_2O_2, and the freeze-dried samples were used for tests with rennin.

Analysis

The contents of tryptophan, tyrosine and histidine were estimated by methods applicable to the intact protein. However, it was found that more reproducible results could be obtained if the protein were given a preliminary digestion with pronase, presumably because the side chains became more available for reaction. A solution (3–4 ml) containing about 15 mg of protein was adjusted to pH 9 with 1 N-NaOH and 0·5–1 ml of pronase solution (0·25 mg/ml) added. The digestion was continued for at least 4 h at 37 °C. Samples of the digest were used for determining nitrogen by a semimicro-Kjeldahl method, and for the amino acid estimations. Histidine was determined by a modified Pauly method (Greenstein & Winitz, 1961), tyrosine by the method of Cobbett, Kenchington & Ward (1962) and tryptophan by that of Saifer, Gerstenfeld & Vecsler (1961).

Infra-red spectra were obtained on a Beckman IR 7 spectrophotometer to test for the presence of oxidized methionine. Solutions containing 2·5 mg of protein and 500 mg KBr were freeze-dried, and 400 mg of the dried powder used to make KBr disks at 1400 kg/cm^2 pressure. Disks containing photo-oxidized methionine alone, and protein plus photo-oxidized methionine were similarly prepared.

RESULTS

κ-Casein

The relation between O_2 uptake and residual amino acid content for the κ-caseins photo-oxidized with methylene blue are presented in Fig. 1. The different preparations of κ-casein showed similar behaviour in that in all of them the histidine was photo-oxidized faster than the tryptophan, while the tyrosine reacted more slowly again than tryptophan. In some cases the tyrosine was scarcely affected after 20 min of photo-oxidation, when half of the histidine had been destroyed. It is also apparent from Fig. 1 that some of the histidine reacts much more rapidly than the rest. The average histidine content was 2·4 % or about 18 moles/10^5 g protein; from the graph the rapidly reacting histidine may be estimated at 20–25 % of this or 3–4 moles/10^5 g.

Photo-oxidized κ-casein samples were treated for ½ h at 37 °C with 1 pt of rennin to 8–10 000 pts by weight of κ-casein, and the release of NPN determined by deducting the amount of material soluble in 12 % TCA at the start of the rennin treatment from that at the finish. The amount released from the photo-oxidized κ-caseins expressed as a percentage of the amount released from the respective controls, is plotted against residual histidine, tyrosine and tryptophan content in Fig. 2. The proportion of NPN released decreases approximately linearly with residual histidine content, while the relation between NPN release and tryptophan content is rather less regular. In a number of cases, the tyrosine content is scarcely changed by the photo-oxidation. (In these respects no difference was found between the κ-casein prepared by alcohol fractionation and that prepared by chromatography on DEAE cellulose.) The decline in NPN release and the observed failure of the κ-casein to clot with increasing photo-oxidation is therefore most likely to be associated with destruction of the histidine.

To clarify this point κ-casein was photo-oxidized at pH 3 with proflavine as sensitizer; in these conditions tryptophan and possibly methionine are oxidized

(Sluyterman, 1962). In 2 treatments, tryptophan was destroyed to extents of 25 and 46 %. When the samples were treated with rennin there was no visible difference in the clotting behaviour and the NPN releases of the controls and treated samples were

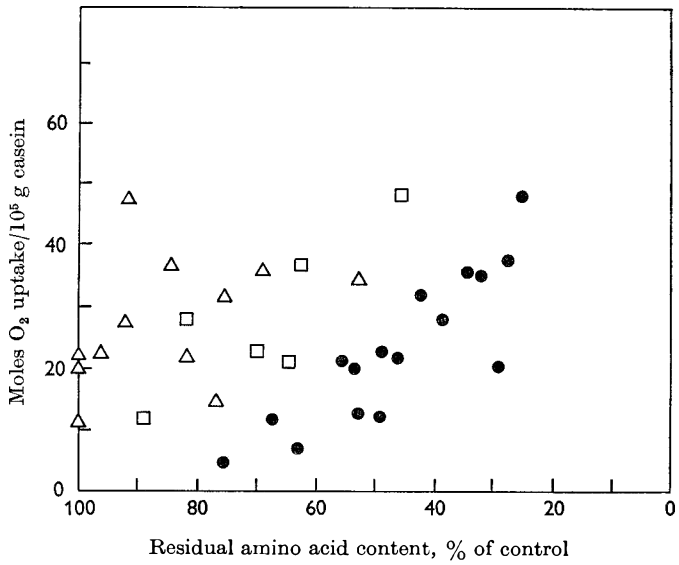


Fig. 1. Effect of photo-oxidation on the tyrosine, tryptophan and histidine content of κ-casein. △, Tyrosine; □, Tryptophan; ●, Histidine. Several preparations of κ-casein were used in these experiments.

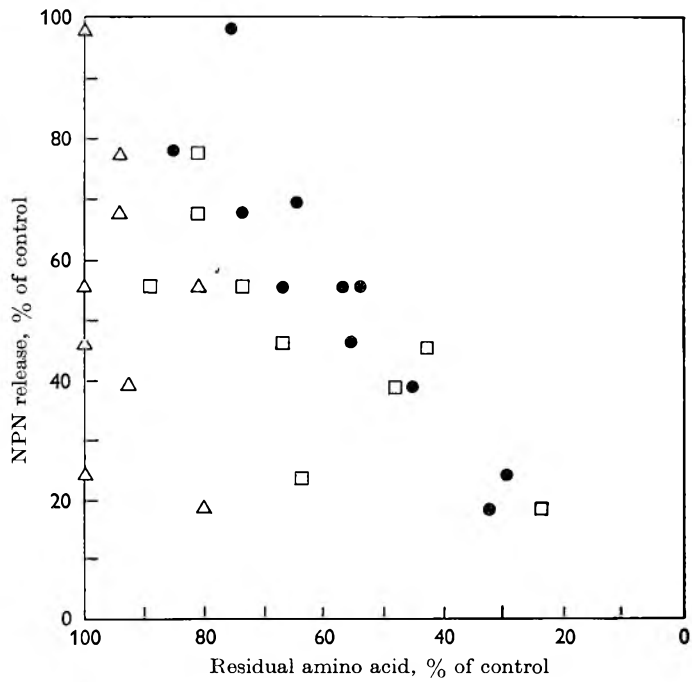


Fig. 2. Effect of photo-oxidation of κ-casein on the NPN release by rennin. △, Tyrosine; □, Tryptophan; ●, Histidine. Several preparations of κ-casein were used; they were not identical with those used to obtain the results shown in Fig. 1.

closely similar. From this it can be inferred that the reduced NPN release of the κ-caseins photo-oxidized with methylene blue is not associated with destruction of the tryptophan.

κ-Casein was also incubated with tyrosinase in an attempt to determine the effect of oxidizing the tyrosine side chains only. When more than 50 % of the tyrosine was destroyed, the treated κ-casein still clotted when treated with rennin but quantitative comparison of the NPN releases in the control and treated samples was rendered meaningless because the tyrosinase treatment also resulted in considerable degradation of the casein, with consequent high zero time NPN values. This effect may have been caused by proteolytic impurity in the tyrosinase or the tyrosinase may itself possess such activity.

Whole casein

Sodium caseinate and micellar casein (prepared as in Hill & Hansen, 1964) when photo-oxidized with methylene blue, show behaviour similar to that of κ-casein, in that the tyrosine content is relatively unaffected while both histidine and tryptophan are destroyed. After photo-oxidation, these samples were dialysed against sodium acetate at pH 6·25 to remove phosphate buffer, and made 0·025 M in $CaCl_2$. The photo-oxidized samples in which more than 65–70 % of the histidine had been destroyed either failed to clot, or clotted very feebly on treatment with rennin. At this stage 0–10 % of the tyrosine was destroyed and 30 % of the tryptophan. This failure to clot is to be expected, because the photo-oxidation of the κ-casein fraction results in reduced NPN release by the rennin (Fig. 2), but another effect may also be present, viz. that the photo-oxidation may destroy side chains required for the aggregation of the casein which follows the rennin action. This possibility was investigated by treating sodium caseinate with rennin at pH 6·5 and 30 °C for $\frac{1}{2}$ h prior to photo-oxidation. Following the rennin action, the samples were adjusted to pH 8–8·5 with NaOH to inactivate the rennin, and photo-oxidized with methylene blue. After photo-oxidation, the methylene blue was removed, and control and treated samples adjusted to the same volumes at pH 6·5. To a portion of each sample $CaCl_2$ was added with shaking to 0·025 M. These portions were then warmed to 37 °C for 5 min, centrifuged, and the nitrogen content of the clear supernatant determined. The proportion of para-casein precipitated by the Ca^{++} was then calculated and is plotted in Fig. 3 against the residual histidine, tyrosine and tryptophan content, determined on the other portions of the solutions and expressed as a percentage of the content in the control.

The amount of casein precipitated decreases with increasing destruction by photo-oxidation of tryptophan and histidine.

Treatment of casein with 2-hydroxy-5-nitro-benzyl bromide after rennin action

In order to distinguish between the roles of the tryptophan and histidine side chains, para-casein was prepared as described above and after inactivation of the rennin was treated with the tryptophan reagent 2-hydroxy-5-nitro-benzyl bromide at pH 3 in 20 % formamide. By this means 82 % of the tryptophan on the para-casein was destroyed without any effect on the precipitation of the para-casein by 0·025 M-$CaCl_2$ at pH 6·5, and 37 °C, after the removal of the benzyl bromide reagent and the formamide. The experiment was repeated, and a more extensive destruction (87 %) of

tryptophan achieved. Again, there was no difference between the amount of casein precipitated by Ca^{++} in control and treated samples. From this, it can be concluded that the tryptophan side chains are not essential for the aggregation of para-casein in the presence of Ca^{++}.

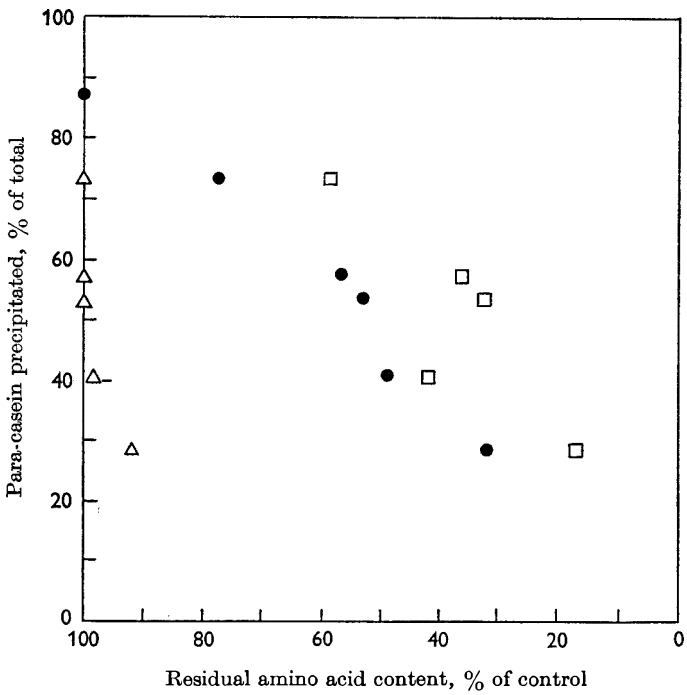


Fig. 3. Effect of photo-oxidation of para-casein on its precipitation by Ca^{++}. △, Tyrosine; □, Tryptophan; ●, Histidine.

Oxidation of casein with H_2O_2

Photo-oxidation with either methylene blue or proflavine may cause the oxidation of methionine to the sulphoxide which remains undetected by amino acid analysis of acid hydrolysates of the protein (Schachter & Dixon, 1964). We endeavoured to detect the presence of methionine sulphoxide by means of the strong infra-red absorption band at 1025–1050 cm^{-1} of the S=O stretching vibration (Bellamy, 1958). This band was strongly present in photo-oxidized methionine, but could not be detected in photo-oxidized caseins. However, 1·6 % by weight (expressed as residue weight) of photo-oxidized methionine added to casein could readily be detected by this means. As casein contains about 2·8 % of methionine (Tristram, 1949) this would suggest that the methionine in casein was not greatly affected by the photo-oxidation.

The effect of oxidation of the methionine was investigated by treating casein with H_2O_2 according to Schachter & Dixon (1964) (see Methods). Solutions of approximately 9·4 % of the treated and control caseins were made 0·025 M in $CaCl_2$ at pH 6·5 and 37 °C, and incubated with rennin. The clotting times of control and treated samples were identical (5 min), although more of the control sample (84 %) sedimented on centrifuging (5 min at 2000 *g*) than did the treated sample (76 %). This result also

leads to the conclusion that the methionine side chains are not essential for either the rennin action nor for the subsequent aggregation of the casein in the presence of Ca^{++}.

DISCUSSION

The results of the experiments with κ-casein and whole casein may usefully be summarized.

(*a*) Photo-oxidation of κ-casein with methylene blue affected mainly the histidine and tryptophan, and caused a reduction in the amount of NPN that can be released by rennin.

(*b*) Photo-oxidation of κ-casein with proflavine affected mainly the tryptophan, and had little effect on the amount of NPN released by rennin.

(*c*) When whole casein was photo-oxidized with methylene blue, histidine and tryptophan were again the residues mainly affected, and the ability to clot in the presence of Ca^{++} following rennin action was impaired. As well as effect (*a*) above, photo-oxidation of whole casein also interferes with the casein-casein aggregation which follows rennin action.

(*d*) Extensive modification of the tryptophan in whole casein by 2-hydroxy-5-nitro-benzyl bromide was without effect on the aggregation of the rennin-treated casein in the presence of Ca^{++}.

(*e*) Treatment of whole casein with H_2O_2 in conditions in which methionine is oxidized had relatively little effect on the clotting behaviour.

These results suggest strongly that the reduced NPN release in (*a*) above was caused by the alteration of histidine, and this is supported by the approximately linear relation between the NPN released and the residual histidine content (Fig. 2). The effect may be a specific one such as would occur if a particular histidine residue were part of the site on to which the rennin absorbs during its action, or may merely be due to the change of charge on the casein as a result of the photo-oxidation. The latter is less likely, as the change of charge which accompanied the destruction of the tryptophan alone was without these effects.

The evidence also shows that effect (*c*)—the interference with the clotting of rennin-treated caseins—is to be attributed to the destruction of histidine. The histidine involved here need not be on the κ-casein, and in this connexion Zittle (1963) has shown that α_s-casein photo-oxidized with methylene blue is no longer sensitive to Ca^{++}. The particular side chain responsible for this effect was not determined. The inference that histidine side chains play a part in the gelling of rennin-treated casein is supported by unpublished observations made in this laboratory of the marked change in the physical nature of the rennet curd between pH 5 and 6, at about the pK of the histidine imidazole group. Higgins & Fraser (1954), on the basis of quite different evidence, viz. titration data and differences between the spectra of azo-derivatives of casein and para-casein, also suggested the likely participation of histidine in the gelling of the rennet curd.

REFERENCES

ALAIS, C. & JOLLÉS, P. (1961). *Biochim. biophys. Acta*, **51**, 315.
BARGONI, NORA (1963). *Enzymologia*, **26**, 108.
BELLAMY, L. J. (1958). *The infra-red spectra of complex Molecules*, 2nd ed., p. 358. London: Methuen.
BERRIDGE, N. J. & WOODWARD, C. (1953). *J. Dairy Res.* **20**, 255.
COBBETT, W. G., KENCHINGTON, A. W. & WARD, A. G. (1962). *Biochem. J.* **84**, 468.
GREENSTEIN, J. P. & WINITZ, M. (1961). *Chemistry of the Amino Acids*, p. 1979. New York: Wiley.
HIGGINS, H. D. & FRASER, D. (1954). *Aust. J. biol. Sci.* **7**, 85.
HILL, R. D. & HANSEN, RAIONE R. (1963). *J. Dairy Res.* **30**, 375.
HILL, R. D. & HANSEN, RAIONE R. (1964). *J. Dairy Res.* **31**, 291.
HSU, R. Y. H., ANDERSON, L., BALDWIN, R. L., ERNSTROM, C. A. & SWANSON, A. M. (1958). *Nature, Lond.*, **182**, 798.
JOLLÉS, P., ALAIS, C. & JOLLÉS, J. (1961). *Biochim. biophys. Acta*, **51**, 309.
KOSHLAND, D. E., KARKHANIS, Y. D. & LATHAM, H. G. (1964). *J. Am. chem. Soc.* **86**, 1448.
LISSITZKY, S. & ROLLAND, M. (1962). *Biochim. biophys. Acta*, **56**, 95.
MCFARLANE, A. S. (1938). *Nature, Lond.*, **142**, 1023.
MCKENZIE, H. A. & WAKE, R. G. (1961). *Biochim. biophys. Acta*, **47**, 240.
NITSCHMANN, H. & BEEBY, R. (1960). *Chimia*, **14**, 318.
SAIFER, A., GERSTENFELD, S. & VECSLER, F. (1961). *Clin. Chem.* **7**, 626.
SCHACHTER, H. & DIXON, G. H. (1964). *J. biol. Chem.* **239**, 813.
SLUYTERMAN, L. A. AE. (1962). *Biochim. biophys. Acta*, **60**, 557.
TRISTRAM, G. R. (1949). *Advances in Protein Chemistry* (ed., M. L. Anson and J. T. Edsall), vol. 5, p.103. New York: Academic Press.
WAKE, R. G. (1959). *Aust. J. biol. Sci.* **12**, 479.
WAUGH, D. F. & HIPPEL, P. H. VON (1956). *J. Am. chem. Soc.* **78**, 4576.
WEIL, L. & SEIBLES, T. S. (1955). *Archs. Biochem. Biophys.* **54**, 368.
ZITTLE, C. A. (1963). *J. Dairy Sci.* **46**, 607.
ZITTLE, C. A. (1964). *J. Dairy Sci.* **47**, 672.

Printed in Great Britain

J. Dairy Res. (1965), **32**, 203
With 2 plates

The composition of milk deposits in an ultra-high-temperature plant

By R. L. J. LYSTER
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 22 *February* 1965)

Summary. Milk deposits from an ultra-high-temperature (UHT) plant were analysed for protein, fat and minerals. The physical appearance and composition of the deposits was found to vary with modifications of the plant. For each particular arrangement of the plant, a characteristic pattern of deposits occurred. Possible mechanisms involved in the formation of the deposits are discussed.

The deposits formed when milk is exposed to heated surfaces can give rise to serious problems in plant used for the processing of milk. The mechanism of formation of these deposits is not known and there is only a small amount of published information on their composition. Samples of 'milk stone' from plant used for holder pasteurization were analysed by Parker & Johnson (1930), but since the samples included accretions from the alkaline solutions used for cleaning, the results were rather variable. However, protein, calcium and phosphate were recognized as major components. A similar conclusion was reached by Ito, Sato & Suzuki (1962*a*, *b*) who analysed deposits from a UHT plant. In addition, they observed the presence of fat and found considerable differences in the composition of the deposits from different parts of the plant.

An opportunity arose recently to examine the deposits formed in a commercial UHT plate heater arranged in 3 different ways, but heating the milk to the same maximum temperature of 280 °F in each case. To assist in studies on the factors affecting deposit formation, the deposits were analysed for protein, ash and fat, and the ash was analysed for calcium and phosphate.

The results of the analyses, together with a description of the physical appearance of the deposits, are discussed briefly in relation to possible factors involved in deposit formation.

METHODS

The UHT plant, manufactured by the A.P.V. Co. Ltd and operated by Express Dairies Co. Ltd, was used to process milk at the rate of 474 gal/h. Details of the arrangements of the plant are given, for convenience, with the results. After the milk had been heat treated, the plant was rinsed briefly with cold water to remove residual milk and then opened. Individual plates were removed as required for examination and collection of samples of the deposits. The samples were dried at 100 °C and ground to a fine powder. Ash was determined as the residue after heating for 16 h at 530–

550 °C. Protein content was taken to be 6·38 times the nitrogen content, as determined by Kjeldahl analysis. Fat was determined by a solvent extraction procedure based on the British Standard (1963) method for the analysis of casein for fat. Calcium in the ash was determined by precipitation as the oxalate, followed by permanganate titration (1st and 2nd arrangement of the plant), or by the direct titration method of Ntailianas & Whitney (1964), using the sodium salt of ethylenediamine tetraacetic acid as titrant and calcein as indicator (3rd arrangement of the plant). Phosphate in the ash was determined by a colorimetric method using a vanadate-molybdate reagent (Simonsen, Wertman, Westover & Mehl, 1946). Determinations were done in duplicate, except occasionally when insufficient material was available; the results have been expressed as % of the dry weight of the deposit or % of the ash.

RESULTS

In the first arrangement of the plant the milk was heated to 185 °F in a regenerative heating section, then held in a tank for 6 min, homogenized at 3000 lb/in.^{2} and rapidly heated to 280 °F in a steam-heating section. After the steam section the milk entered a water-cooled section where the temperature fell rapidly to 215 °F, and finally passed to the hot side of the regenerative section. On both the occasions when deposits from the plant in this arrangement were studied only two kinds of deposit were found. One kind was present in the last 2 passes of the cool side of the regenerative section and in the pipe from there to the holding tank, and consisted of white tufts of material attached to an almost clear membranous film. This type of deposit is shown in Plate 1, in which the appearance of the tufts can be seen. The other type of deposit occurred in the steam section and the pipe leading to the cooling section, and was quite different in appearance, being compact, gritty to touch, and yellow to pale brown in colour; these deposits were thickest on the last pass of the steam section.

Table 1 shows representative values of the composition of both these types of deposit, and since the deposits from the regenerator clearly consisted of two quite different components, values are given for the composition of the membranous material separately. As can be seen from Table 1, the composition of deposits from the steam section varied slightly from one pass to another. The results are very similar to, and may be compared with, the figures given by Ito *et al.* (1962*a*, *b*). Some of the fat extracted from deposits in both the regenerative and steam sections was analysed for fatty acid composition by gas chromatography, and found to resemble normal milk fat in this respect, indicating that no preferential inclusion of the phospholipid fraction of milk fat into the deposits had occurred. Since so much of the deposits from the steam section was mineral in nature, 2 samples were submitted to the Warren Spring Laboratory of D.S.I.R. for examination by X-ray diffraction. Both samples were found to contain β-$Ca_3(PO_4)_2$ as very small crystallites ($10–14 \times 10^{-6}$ mm) with a distorted lattice. This mineral has a Ca/P atom ratio of 1·5; however, it is noticeable that the samples of ash from the steam section have a consistently lower Ca/P atom ratio.

The 2nd arrangement of the plant differed from the 1st only in the omission of the holding tank, so that milk passed directly from the regenerative heating section to the homogenizer and then to the steam section. Since extensive denaturation of the whey proteins occurs when milk is held at 185 °F for 6 min, the main effect of this change

was to increase the amount of native whey protein in the milk entering the steam section from about 15 % to about 84 % of the whey proteins in unheated milk. Consequently, the change in arrangement of the plant had a considerable effect on the character and composition of the deposits in the steam section but left the deposits on the cool side of the regenerative section unchanged. The appearance of plates from the steam section is shown in Plate 2. The 1st pass carried a deposit of moderate thickness, but the most noticeable feature is the deposit on the lower half of the 2nd pass. This deposit, which in many places bridged the gap between the 2 plates forming the pass and is, therefore, referred to as the blocking deposit, was composed of a brown brittle material in contact with the plates and supporting a soft curd-like mass. The later passes carried a brittle gritty deposit showing a progressive decrease in thickness, the 5th pass carrying virtually no deposit at all.

Table 1. *Composition of deposits from a UHT plant working in the 1st arrangement described in the text*

Source	% of dry weight				% of ash			Ca/P atom ratio
	Protein	Fat	Ash	Other	Ca	PO_4	Other	
Regenerative section								
Last pass	60	12	25	3	36	57	7	1·50
Last pass, membranous film	46	—	53	—	—	54	—	—
Steam section								
1st pass	19	3	71	7	33	59	8	1·32
3rd pass	15	9	72	4	34	60	6	1·33
5th (last) pass	12	8	71	9	33	60	7	1·31

Table 2. *Composition of deposits from a UHT plant working in the 2nd arrangement described in the text*

Source	% of dry weight				% of ash			Ca/P atom ratio
	Protein	Fat	Ash	Other	Ca	PO_4	Other	
Steam section								
1st pass	56	4	34	6	35	54	11	1·55
2nd pass	51	8	34	7	35	56	9	1·46
3rd pass	27	6	59	8	35	61	4	1·36
4th pass	19	—	71	—	35	—	—	—
Water cooler								
Upper part	95	—	2	—	—	—	—	—
Lower part	90	—	6	—	—	—	—	—
Regenerator, hot side								
1st pass	87	—	5	—	—	—	—	—
2nd pass	91	—	4	—	—	—	—	—
3rd pass	82	—	4	—	—	—	—	—

Unlike the 1st arrangement, the 2nd arrangement gave rise to deposits in the cooling sections. The upper part of the water-cooled section carried a clear deposit, rather slimy to the touch, while the lower half and the first 3 passes of the hot side of the regenerative section contained a white deposit with long strings developing from its surface. The compositions of the deposits are given in Table 2. The blocking deposit was rather variable in composition. The deposits from the cooling passes contained so little ash that reliable analyses could not be obtained.

In the 3rd arrangement of the plant, the holding tank was again omitted and the steam section was partially replaced by a hot-water section of 6 passes which heated the milk to 250 °F. The milk was then brought to 280 °F in the steam section which was shortened from 5 passes to 3. The other parts of the plant were left unchanged. All plates of the hot water and steam sections carried deposits, and the composition of some of these deposits is given in Table 3. The slower rate of heating in this arrangement gave deposits with no sign of the browning seen in the other arrangements. The first 4 passes of the hot water section carried firm white deposits of increasing grittiness and ash content. Part of the 6th and last pass carried a soft curd-like deposit, the remainder of which had obviously become detached from the plate; this deposit contained more protein than the similar blocking deposit in the second arrangement. In the steam section, deposits were gritty and high in ash content; as in the 2nd arrangement, they became progressively thinner, with so little deposit on the last pass that no sample for analysis could be taken. As in the 2nd arrangement, deposits also occurred in the cooling sections, but they were so similar in appearance that they were not examined further.

Table 3. *Composition of deposits from a UHT plant working in the 3rd arrangement described in the text*

	% of dry weight				% of ash			Ca/P atom ratio
Source	Protein	Fat	Ash	Other	Ca	PO_4	Other	
Water-heater								
1st pass	81	4	8	7	—	56	—	—
3rd pass	61	—	30	—	38	56	6	1·61
6th pass	77	4	11	7	32	49	19	1·55
Steam section								
1st pass	—	—	72	—	35	57	8	1·43
2nd pass	—	—	68	—	35	59	6	1·41

DISCUSSION

The formation of deposits always lowers the efficiency of processing and eventually limits the length of time that a plant can be operated without cleaning. It is to be expected that the presence of deposits will lead to an increase in the resistance to the flow of heat from the heating medium to the milk, and to an increase in the resistance to the flow of milk through the plant. Although both these effects must occur simultaneously whenever deposits are formed, it is found in practice that one effect or the other predominates. Thus, in the 1st arrangement, the length of run was limited by the rising pressure of steam necessary to maintain a constant rate of heating. In the 2nd arrangement, however, the length of run was limited by the rising pressure needed to maintain a constant flow of milk through the steam section.

The appearance and composition of the deposits indicated clearly that they did not consist simply of dried milk solids but that different parts of the plant had characteristically different deposits in them. Since the changes in arrangement had such a marked effect, it is concluded that the nature of the deposits at any particular point in the plant depended both on the temperature of the milk at that point and on the

heat treatment received by the milk before reaching that point. Deposit formation is clearly a complex process involving several of the many components of milk.

The mineral part of the deposits has been found in one case to contain β-$Ca_3(PO_4)_2$, and since the ash from all types of deposit is remarkably constant in composition, this mineral is probably present in all deposits, except those from the cooling sections. If the milk becomes supersaturated with respect to β-$Ca_3(PO_4)_2$ as the temperature rises towards 280 °F, this would account not only for the high-ash deposits on the plates of the steam section but also for the similar deposits present on the walls of the pipe leading from the steam section to the cooling section in the 1st arrangement. Unfortunately, the solubility of β-$Ca_3(PO_4)_2$ in hot milk at various temperatures is unknown.

Protein was present in all the samples analysed and was the major component of most of the deposits. The principal origin of the protein in the deposits is believed to have been the whey protein fraction of milk, because deposits start to form at temperatures at which these proteins begin to denature. The whey protein fraction contains a number of components, including β-lactoglobulin and α-lactalbumin, with different heat stabilities and it is likely that different proportions of these proteins provide the protein part of deposits in different parts of the plant. The deposits with a high protein content formed at relatively low temperatures, i.e. the deposits found in the regenerative heating section and in the cooling sections, tend to occur as tufts or strings, suggesting that their rate of formation depends more on velocity gradients and turbulence in the milk than on the temperature gradient at the walls. It is known that the whey proteins tend to aggregate on heat-denaturation and it is probable that the processes involved in this aggregation are involved in the formation of at least some of the deposits; however, little is known of the kinetics of such aggregation. There appears to be considerable interaction between mineral and protein in deposit formation; this is suggested by the fact that although a deposit of high ash content was present in the steam section in all 3 arrangements, it became progressively thicker only in the 1st arrangement; in the other 2 arrangements, this type of deposit became progressively thinner once the milk had passed through the blocking deposits. Two possible mechanisms for such an interaction can be envisaged; either denatured protein becomes absorbed on the surface of the mineral crystallites, or denatured protein might act as nuclei for crystal growth; however, these possibilities remain purely speculative.

It appears that the fat is present in the deposits simply from being entrained by other material. Since deposits are known to occur in plants working with skim-milk, no direct role of fat in deposit formation seems likely.

From the results, materials other than protein, fat and mineral appear to be present to the extent of 5–10 %. Nothing is known of their nature or possible significance.

It is a pleasure to thank the Express Dairies Co. Ltd, the A.P.V. Co. Ltd, and members of their staff for their helpful co-operation; also to thank Miss P. Cowley for technical assistance, Mr N. G. J. Gruber for help in photographing the deposits, Mr A. Wagstaff for nitrogen determinations, Mr A. J. Hall for fat analyses by gas chromatography, the Warren Spring Laboratory of D.S.I.R. for analyses by X-ray diffraction and Mr H. Burton for helpful discussions.

REFERENCES

BRITISH STANDARDS INSTITUTION (1963). *Br. Stand.* no. 1417, 9.
ITO, R., SATO, M. & SUZUKI, M. (1962*a*). *Jap. J. Dairy Sci.* **11**, 330.
ITO, R., SATO, M. & SUZUKI, M. (1962*b*). *Jap. J. Dairy Sci.* **11**, 380.
NTAILIANAS, H. A. & WHITNEY, R. McL. (1964). *J. Dairy Sci.* **47**, 19.
PARKER, M. E. & JOHNSON, A. H. (1930). *Proc. int. Ass. Milk Dlrs.* 23*rd Ann. Conv.* (*Pl. Sect.*), p. 5.
SIMONSEN, D. G., WERTMAN, N., WESTOVER, L. M. & MEHL, J. W. (1946). *J. biol. Chem.* **166**, 747.

EXPLANATION OF PLATES

PLATE 1

Part of a plate from the last pass of the cool side of the regenerative heating section, with the plant operating in the 1st arrangement. The milk flowed from right to left.

PLATE 2

Plates from passes 1–5 of the steam section with the plant operating in the 2nd arrangement. Milk flowed np pass 1, down pass 2, up pass 3, etc. Note the heavy deposit on the lower half of pass 2.

Printed in Great Britain

R. L. J. LYSTER

(*Facing p.* 208)


5
4
3
2
1

β-Lactoglobulin variation in milk from individual cows

By C. A. KIDDY, R. E. TOWNEND, W. W. THATCHER and S. N. TIMASHEFF

Animal Husbandry Research Division, Beltsville, Maryland (C.A.K. and W.W.T.) and Eastern Utilization Research and Development Division, Philadelphia, Pennsylvania (R.E.T. and S.N.T.) United States Department of Agriculture

(*Received* 2 *February* 1965)

Summary. The β-lactoglobulin types of 812 individual cows of various breeds were determined by paper and polyacrylamide gel electrophoresis. The results support the hypothesis that β-lactoglobulins A and B are controlled genetically by codominant autosomal alleles (Lg^A and Lg^B).

Milks from 5 heterozygous cows (Lg^A/Lg^B) were examined by free electrophoresis and column chromatography to estimate the relative quantities of β-A and β-B produced by these animals. The results indicate that β-A and β-B are produced in approximately equal amounts, with β-A being slightly in excess of β-B.

Lacteal secretions were obtained from 2 castrated males and typed for β-lactoglobulin by means of paper electrophoresis. These animals were found to produce β-lactoglobulins (B and AB) consistent with their expected genotypes as deduced from pedigree analysis.

In 1955 Aschaffenburg & Drewry discovered the existence of 2 electrophoretically distinguishable forms of β-lactoglobulin, β-A and β-B, in cow's milk. After studying the β-lactoglobulins produced by individual animals, they reported that the observed variation was genetically controlled by 2 autosomal alleles with no dominance. This genetic hypothesis has been confirmed by workers in our group (Plowman, Townend, Kiddy & Timasheff, 1959), by Moustgaard, Møller & Havskov Sørensen (1960), and by Bell (1962) who discovered a 3rd allele producing β-lactoglobulin C which moves even slower than β-B in electrophoresis.

This paper presents further extensive data confirming the genetic hypothesis. It also presents the results of studies on the relative production of β-A and β-B by heterozygous animals, and on the occurrence of β-lactoglobulins in lacteal secretions from hormone-treated castrated males.

EXPERIMENTAL

The sample for electrophoresis was prepared from the portion of the whey soluble in 20% (w/v) Na_2SO_4; it was dialysed free of sulphate and concentrated tenfold by pervaporation. The typing work was initially done by paper electrophoresis using a modified technique of Aschaffenburg & Drewry (1955).

Later, vertical polyacrylamide gel electrophoresis was used for typing and is now used routinely (Peterson, 1963); it may be done directly on skim-milk (Aschaffenburg, 1964). Buffer was pH 8·6 veronal, ionic strength 0·025 and the polyacrylamide gel 50 g/l (Cyanogum 41†). One ml of skim-milk was diluted with 1 ml of the buffer and 0·3 ml of amido black (0·2 % solution in 60 % ethanol) was added as an indicator dye. Sucrose (about 150 mg) was added to increase the density and 20 μl of the sample mixture was applied to the gel. Electrophoresis was for 4 h at 60–65 mA (about 200 V) with circulation of cooling water at 10–15 °C.

Tiselius electrophoresis experiments were performed at 1 °C in a Spinco Model H† apparatus. Column chromatography was performed at room temperature using carboxymethylcellulose (CMC) and diethylaminoethylcellulose (DEAE) resins.

RESULTS AND DISCUSSION

Plate 1 (*a*) shows the results of electrophoresis in polyacrylamide gel of crystalline preparations of β-A, B, and C and their combinations in pairs. Plate 1 (*b*) is a photograph of a pattern obtained with skim-milk in routine typing. The β-lactoglobulin types of 812 cows of various breeds are shown in Table 1; data for 145 cows, from a

Table 1. *Distribution of β-lactoglobulin types by breeds*

Breed		Number of cows				χ^2	P	Gene frequency	
		Total	A	AB	B			A	B
Ayrshire	Observed	27	0	7	20	0·60	0·43	0·13	0·87
	Expected		0·5	6·1	20·5				
Brown Swiss	Observed	24	2	9	13	0·07	0·83	0·27	0·73
	Expected		1·8	9·5	12·7				
Guernsey	Observed	200	20	79	101	0·61	0·43	0·30	0·70
	Expected		18·0	84·0	98·0				
Holstein	Observed	406	86	205	115	0·09	0·77	0·46	0·54
	Expected		87·3	201·8	116·6				
Jersey	Observed	39	7	18	14	0·08	0·80	0·41	0·59
	Expected		6·6	18·9	13·6				
Cross-breds	Observed	116	2	58	56	8·82	0·003	0·27	0·73
	Expected		8·2	45·4	62·3				
Over-all	Observed	812	117	376	319	0·13	0·72	0·38	0·62
	Expected		114·5	380·8	316·7				

previous preliminary report (Plowman *et al.* 1959), have been included. For each of the pure breed groups, as well as the over-all group of 812, the distribution of the genotypes agrees well with that expected, assuming control by codominant autosomal alleles and random mating (P ranges from 0·35 to 0·83). In the cross-bred group, however, the difference between the observed and expected numbers of the various genotypes is highly significant ($P \simeq 0{\cdot}003$). The reason for this is that the random mating assumption is not valid for the cross-bred group. Of the 48 sires whose daughters were represented in the group of 116 crossbreds, 4 had 57 of the daughters in the group and these sires (2 Ayrshire and 2 Brown Swiss) were of the genotype

† It is not implied that the U.S. Department of Agriculture recommends the above companies or their products to the possible exclusion of others in the same business.

Lg^B/Lg^B. The average size of the other 44 sire groups was 1·3 daughters. Chi-square values for goodness-of-fit with the Hardy–Weinberg expectations were 7·9 ($P \simeq 0{\cdot}005$ for the 57 daughters of the 4 Lg^B/Lg^B sires and 1·9 ($P \simeq 0{\cdot}16$) for the remaining 59 cows in the cross-bred group.

The segregation data in Table 2 provide further evidence to support the genetic hypothesis for the control of the β-lactoglobulin variants. The agreement with the expected results is very good as it has been in other studies (Aschaffenburg & Drewry, 1957*b*; Moustgaard *et al.* 1960).

Table 2. *Segregation of β-lactoglobulin alleles in female offspring from certain mating combinations*

Mating types		Offspring by β-lactoglobulin genotype A/A*	A/B	B/B
A/A × A/A	Observed	10	0	0
	Expected	10	0	0
A/A × A/B	Observed	26	25	0
	Expected	25·5	25·5	0
A/A × B/B	Observed	0	45	0
	Expected	0	45	0
A/B × B/B	Observed	0	43	49
	Expected	0	46	46
A/B × A/B	Observed	14	38	13
	Expected	16·25	32·5	16·25
B/B × B/B	Observed	0	0	19
	Expected	0	0	19

* Shortened form for Lg^A/Lg^A.

With the paper electrophoresis technique the β-C variant cannot be detected as its mobility, under these conditions, is essentially that of β-B. Therefore, the earlier data (about 300 animals) may have an occasional β-C animal listed as β-B. The chances of this are small, however, since most of the animals involved were Holstein–Friesians and the occurrence of β-C has not been reported in this breed. Furthermore, the Lg^C allele appears to be rare in the 2 breeds in which it has been found (Bell, 1962). Bell reported the occurrence of C in 24 of 135 Jersey cows and one of 85 Australian Illawarra Shorthorns. No gene frequencies were given, but a minimum figure for Lg^C in Bell's Jersey sample would be 0·09 (if all 24 animals were heterozygotes and assuming no technical error such as the acknowledged one which led to the report that A, B and C could be found in milk from an individual cow). Since Bell's report, a few heterozygous Lg^C animals and 2 homozygotes have been found among Jerseys and Jersey–Brahman crosses in the United States (Kalan & Thompson, personal communication). It remains to be seen if Lg^C is present to any appreciable extent in breeds other than Jersey.

A summary of gene frequency data reported by various workers is shown in Table 3. It appears that Lg^B is definitely the most common allele in all but the Holstein–Friesian (0·55 Lg^B) and Jersey (0·47 Lg^B) breeds. There is a possibility, as stated above, that the figure for Lg^B in the Jersey breed may be slightly inflated as a result of failure to distinguish between B and C in the early stages of the present study.

The information summarized in Table 3 does not indicate much similarity between related breeds. It must be remembered, however, that the Red Dane samples were not random ones (Moustgaard *et al.* 1960); this is also probably true of most of the other breed samples. Because of this and the small number of animals sampled in some breeds and locations, many of these gene frequency figures should be considered as preliminary estimates.

Table 3. *Summary of β-lactoglobulin gene frequencies in various breeds of cattle*

Breed	No. of cows	Gene frequencies A	Gene frequencies B	Location and reference
Ayrshire	27	0·13	0·87	United States*
	54	0·31	0·69	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Brown Swiss	24	0·27	0·73	United States*
Campine	66	0·33	0·67	Belgium (Préaux & Lontie, 1961)
Guernsey	200	0·30	0·70	United States*
	27	0·22	0·78	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Holstein–Friesian	406	0·46	0·54	United States*
	161	0·61	0·39	Denmark (Moustgaard *et al.* 1960)
	87	0·40	0·60	Great Britain (Aschaffenburg & Drewry, 1957*b*)
	1056	0·43	0·57	Germany (Comberg, Meyer & Gröning, 1964)
Icelandic Cattle	52	0·34	0·66	Iceland (Blumberg & Tombs, 1958)
Jersey	39	0·41	0·59	United States*
	158	0·56	0·44	Denmark (Moustgaard *et al.* 1960)
Shorthorn	87	0·11	0·89	Great Britain (Aschaffenburg & Drewry, 1957*b*)
Red Dane	293	0·14	0·86	Denmark (Moustgaard *et al.* 1960)
White Fulani	58	0·21	0·79	Nigeria (Blumberg & Tombs, 1958)
Zebu	138	0·09	0·91	India (Bhattacharya, Roychoudhury, Sinha & Sen, 1963)

* Extracted from Table 1.

Lacteal secretions of castrated males

As a check on the presumption that males would express their β-lactoglobulin genotype in lacteal secretions, 2 castrated males were treated with oestrogen and progesterone. Treatment consisted of subcutaneous injections of the hormones in corn oil, 100 mg/day of progesterone for 4 days, alternating with 20 mg/day of diethylstilbesterol for 4 days. Milk was obtained from the first animal, 4120, after 6 weeks on this regimen. This male had been castrated at the age of 2 months and hormone injections were started at 1 year of age. Milk was not obtained from the 2nd male, 4104, until 3 months of treatment had elapsed, and then in smaller quantities than from 4120. This animal had been castrated at 18 months of age and hormone treatment began 2 months later. One to three ml of apparently normal milk was obtained each day from 4120 and was stored at -10 to -15 °C. The average composition of four 20 ml pooled samples was 12·8 % total solids, 3·2 % milk fat, and 3·9 % protein. Thus, the milk seemed to be essentially normal for these components.

The ultracentrifugal pattern given by the whey (pH 6·5, phosphate buffer) could not be distinguished from that given by normal cow's milk. Tiselius electrophoresis of

the whey in pH 8·63 veronal buffer, 0·05 ionic strength, also gave a pattern very similar to that of normal milk.

For the typing experiments 23 ml of the milk was worked up following the procedure of Aschaffenburg & Drewry (1957*a*). Paper electrophoresis on the pervaporated Na_2SO_4 whey (Plate 2(*b*)) gave a pattern essentially identical with that given by the milk of an A/B cow (Plate 2(*a*)) with a few extra bands running slower than α-lactalbumin, and an unmistakable A/B β-lactoglobulin. This result was as expected since the dam of 4120 was A/A and the sire's type had been deduced as B/B.

The milk production of 4104 was very poor, averaging about 1 ml/day. A sample of the milk was freed of casein and fat by centrifugation and acid precipitation at pH 4·4. After dialysis of the whey against water and pervaporation, a paper electrophoresis run was made using whey from a known A/B cow prepared in the same manner as a control. These strips are shown in Plate 2(*c*) and 2(*d*). The milk can be seen to be rich in components moving more slowly than α-lactalbumin and in the range of mobilities known to be possessed by the immune globulins of milk (Larson & Jenness, 1955). The β-lactoglobulin band is single, however, and occurs at the position of β-B. Again, this was as expected since 4104's sire and dam were both B/B.

From these experiments it is concluded that the milk produced by such artificially stimulated mammary tissue in bovine males does express the β-lactoglobulin phenotype characteristic of the genotype deduced by pedigree analysis.

Table 4. *Compositional changes during β-lactoglobulin preparation*

Sample	Concentration, g/l	% β-B*
$(NH_4)_2SO_4$ ppt.	2·2	0·51
First supernatant	13·1	0·49
Second supernatant	7·6	0·60
Second crystal crop	12·6	0·36

* Electrophoretic analysis at pH 5·6 (Timasheff & Townend, 1960).

Relative fractions of β-A and β-B in AB milk

Crystallization of the mixed β-lactoglobulin and subsequent electrophoretic determination of its composition (Timasheff & Townend, 1960) was not the method of choice for determination of the relative amounts of β-A and β-B synthesized by a heterozygous animal. Observation has shown that β-A by itself forms crystals much more readily than β-B. As a result of this, crystallization of the mixture might well cause fractionation, as has been observed by Ogston & Tombs (1957), and a non-uniformity of the crystals forming at the beginning and end of crystallization. This was checked in a simple experiment as follows. An 800-ml sample of whey from an A/B animal (X-617) was carried through the Aschaffenburg & Drewry (1957*a*) preparation procedure and samples were taken of the ammonium sulphate-β-lactoglobulin precipitate, the first supernatant after crystals had just begun to form (a single overnight dialysis), and the supernatant and a part of the crystal crop after 4 days dialysis with three changes of dialysate. The β-lactoglobulin B contents of these fractions are given in Table 4.

It can be seen that some fractionation does occur, and the effect of this on the final composition might well be a function of the volume of the last crystallizing

solution, and other factors generally not reported. Other workers (Johannsen, 1958; Yaguchi, Tarassuk & Hunziker, 1961) have reported separation of whey protein fractions using chromatographic methods, and an attempt was made to apply quantitatively the ion exchange cellulose resins of Sober & Peterson (1960) to this system. Preliminary experiments using gradients of ionic strength at fixed pH on DEAE or CMC were not satisfactory in separating the numerous components of milk whey (Brunner *et al.* 1960), although under other conditions (Yaguchi *et al.* 1961;

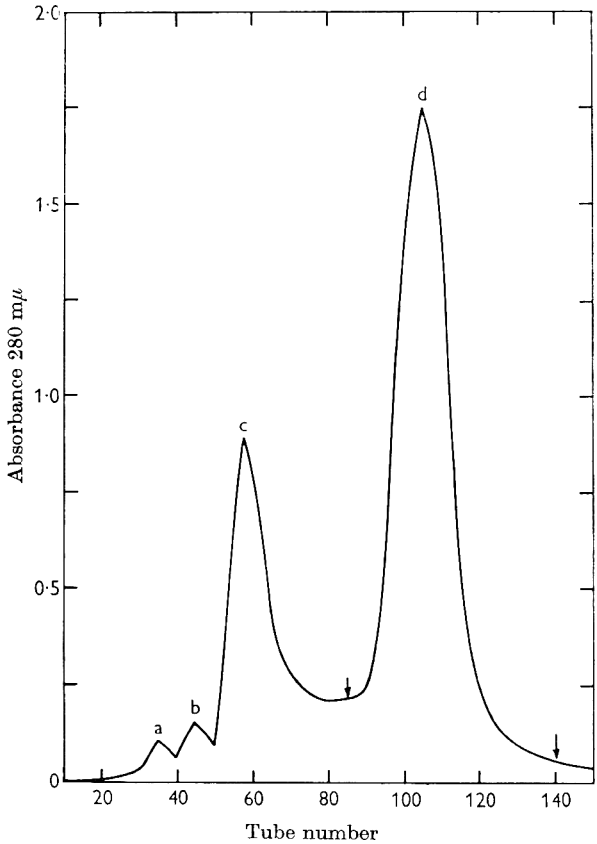


Fig. 1. Whey proteins, unprecipitated by 20% (w/v) Na_2SO_4, chromatographed on carboxymethylcellulose. Ascending pH gradient at 0·02 ionic strength; 11 ml fractions collected. Peak *d* is β-lactoglobulin and contains both the A and B variant.

Table 5. *Electrophoretic mobilities of the fractions obtained by chromatography of whey proteins on carboxymethylcellulose*

Peak	Tube number	Descending mobility, cm^2/volt-sec × 10^5	Identification
a	30–38	−4·64	Unknown
b	41–48	−5·9 (40%)	Probably blood serum albumin
		−3·9 (60%)	Unknown
c	52–67	−4·5 (90%)	α-Lactalbumin
		−8·3 (10%)	Unknown
d	85–140	−6·3	β-Lactoglobulins A and B

Kalan, Greenberg & Walter, 1964) this approach is successful. The technique finally arrived at was as follows: whole milk from a heterozygous animal (A/B) was warmed to 40 °C and 200 g/l of anhydrous Na_2SO_4 was added to precipitate the fat, casein, and the immune globulins, as described by Aschaffenburg & Drewry (1957*a*). About 200 ml of the clear greenish filtrate was dialysed against distilled water until free of colour and salt and then against an excess of 0·02 ionic strength NaAc buffer, pH 4·60. In some instances a portion of the α-lactalbumin precipitated at this stage and was removed by centrifugation. The clear solution was applied to a 1·9 × 13 cm column of CMC previously equilibrated with the buffer, and rinsed on with 50–100 ml buffer.

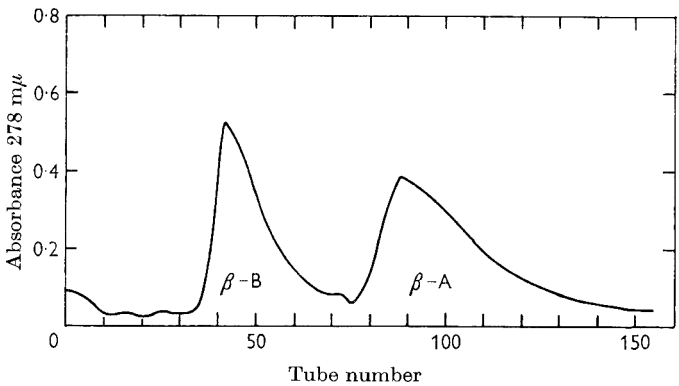


Fig. 2. Peaks of β-A and β-B lactoglobulin obtained by applying the β-lactoglobulin fraction from a carboxymethylcellulose column (Peak *d*, Fig. 1) to a diethylaminoethylcellulose column and eluting with acetate buffer at 0·02 ionic strength using a descending pH gradient. 11 ml fractions collected.

Table 6. *Proportion of β-A in β-lactoglobulin AB from individual Holstein cows*

Animal number	β-lactoglobulin A, % total β-lactoglobulin
3082	52, 51*
3407	54, 56*
3827	57
3806	54
3858	57

* Two samples of the milk of each of these cows were studied.

No u.v. absorbing material was eluted. A continuous pH gradient was made in a closed mixing vessel containing 250 ml of the pH 4·60 buffer, fed by a reservoir containing 1800 ml 0·02 M-NaAc, adjusted to pH 6–6·5 with a few drops of acetic acid. A flow rate of 150 ml/h was easily obtained and a typical elution pattern is shown in Fig. 1. The major peaks obtained were separated and their electrophoretic mobility determined at pH 8·7 in 0·05 ionic strength veronal buffer, as shown in Table 5.

Peak *d* was the β-lactoglobulin peak, containing both the A and B variants, which are not resolved upon Tiselius electrophoresis at pH's near 8·6 (Timasheff & Townend, 1960). The pH of the pooled material under this peak was between 5·2 and 5·4. This entire cut (shown by the arrows on Fig. 1) was then applied to a DEAE column

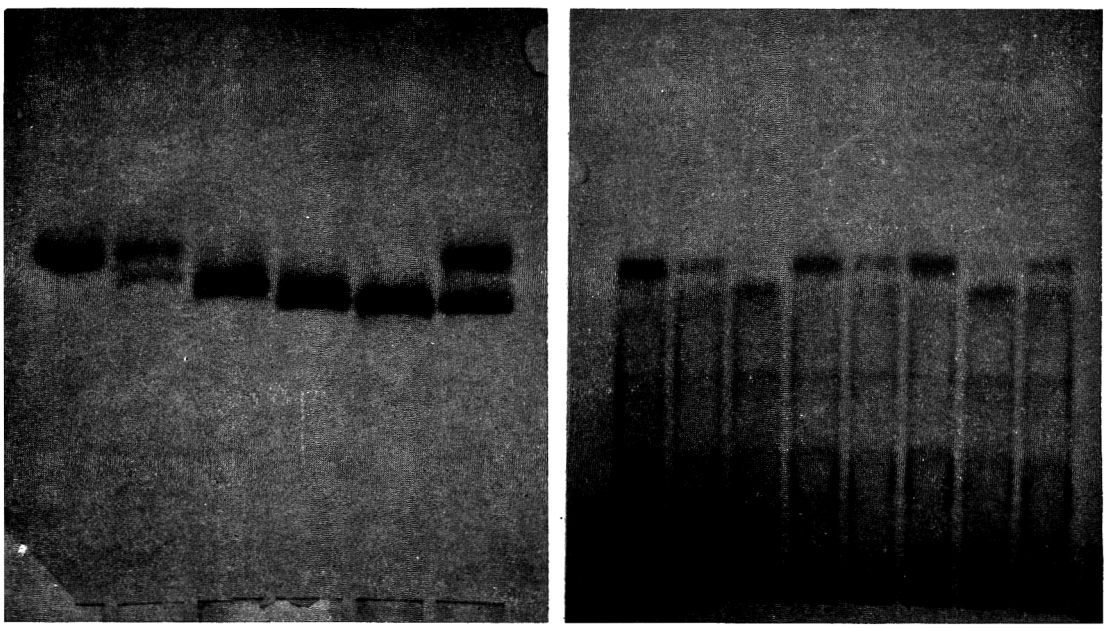
(1·9 × 13·0 cm), previously equilibrated with a pH 5·2, 0·02 ionic strength NaAc buffer. The β-lactoglobulins are quantitatively retained on this column. A downward pH gradient was applied. A constant volume mixing vessel containing 1100 ml pH 5·2 acetate buffer, 0·02 ionic strength was used with a reservoir containing pH 4·5 buffer of the same ionic strength. The β-lactoglobulins elute as shown in Fig. 2 with better than 95% recovery. As both β-A and β-B have identical absorbancies (Tanford & Nozaki, 1959) the integrated areas under the peaks may be used for quantitation. This was checked using synthetic mixtures of recrystallized β-A and β-B, and results were as follows—%A: 85, found 86; 50, found, 52; 40, found, 37; 15, found 18. Milks from 5 heterozygous animals were analysed by this technique, and the results are given in Table 6.

Within the significance of the analyses, it seems that β-A and β-B are produced in almost equal amounts by heterozygous animals. This result is in disagreement with Lontie, van Goethem, DeWeer & Préaux (1964) who found by agar electrophoresis at pH 8·6, followed by staining, that β-A is produced in much greater amount than β-B. This latter technique should be interpreted with caution, however, since β-A and β-B have almost identical free electrophoretic mobilities at this pH (Timasheff & Townend, 1960) and the effectiveness of the separations obtained on fixed media (Aschaffenburg & Drewry, 1955; Lontie *et al.* 1964) may be due to the differences in rate, and possibly extent, of denaturation during the electrophoresis. Gough & Jenness (1962) have shown that there is a difference in rate of denaturation of β-A and β-B in the range 67–75°C.

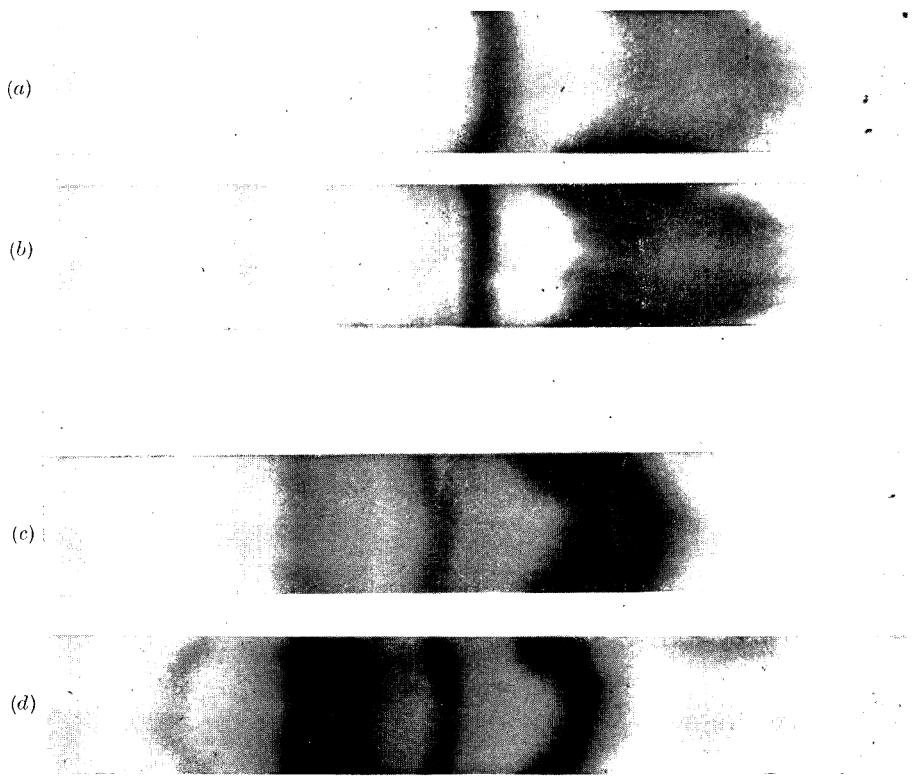
The authors wish to thank Mr Leon D. Cerankowski for his able performance of the column fractionation experiments, Mr A. Kreft for carrying out the Tiselius electrophoresis experiments, Mr John McGrath for the paper electrophoresis typing runs, and Dr Marvin P. Thompson for supplying the β-lactoglobulin C used in the preparation of Plate 1 (*a*).

REFERENCES

ASCHAFFENBURG, R. (1964). *Biochim. biophys. Acta*, **82**, 188.
ASCHAFFENBURG, R. & DREWRY, J. (1955). *Nature, Lond.*, **176**, 218.
ASCHAFFENBURG, R. & DREWRY, J. (1957*a*). *Biochem. J.* **65**, 273.
ASCHAFFENBURG, R. & DREWRY, J. (1957*b*). *Nature, Lond.*, **180**, 376.
BELL, K. (1962). *Nature, Lond.*, **195**, 705.
BHATTACHARYA, S. D., ROYCHOUDHURY, A. K., SINHA, N. K. & SEN, A. (1963). *Nature, Lond.*, **197**, 797.
BLUMBERG, B. S. & TOMBS, M. P. (1958). *Nature, Lond.*, **181**, 683.
BRUNNER, J. R., ERNSTROM, C. A., HOLLIS, R. A., LARSON, B. L., WHITNEY, R. M. & ZITTLE, C. A. (1960). *J. Dairy Sci.* **43**, 901.
COMBERG, G., MEYER, H. & GRÖNING, M. (1964). *Züchtungskunde*, **36**, 248.
GOUGH, P. & JENNESS, R. (1962). *J. Dairy Sci.* **45**, 1033.
JOHANNSEN, B. (1958). *Nature, Lond.*, **181**, 996.
KALAN, E. B., GREENBERG, R. & WALTER, M. (1964). *Biochem. biophys. Res. Commun.* **16**, 199.
LARSON, B. L. & JENNESS, R. (1955). *J. Dairy Sci.* **38**, 1.
LONTIE, R., GOETHEM, G. VAN, DEWEER, C. & PRÉAUX, G. (1964). *Protides of the Biological Fluids* (ed. H. Peeters). Amsterdam: Elsevier Publishing Company.
MOUSTGAARD, J., MØLLER, I. & HARSKOV SØRENSEN, P. (1960). *Ann. Rep. Royal Vet. and Agric. College Steril. Res. Inst., Copenhagen*, p. 111.
OGSTON, A. G. & TOMBS, M. P. (1957). *Biochem. J.* **66**, 3399.
PETERSEN, R. F. (1963). *J. Dairy Sci.* **46**, 1136.
PLOWMAN, R. D., TOWNEND, R. E., KIDDY, C. A. & TIMASHEFF, S. N. (1959). *J. Dairy Sci.* **42**, 922.



(*a*) (*b*)


(a)
(b)
(c)
(d)

PRÉAUX, G. & LONTIE, R. (1961). *Archs. int. Physiol.* **69**, 598.
SOBER, H. A. & PETERSON, E. A. (1960). *Amino Acids, Proteins, and Cancer Biochemistry* (ed. J. T. Edsall). New York: Academic Press.
TANFORD, C. & NOZAKI, Y. (1959). *J. biol. Chem.* **234**, 2874.
TIMASHEFF, S. N. & TOWNEND, R. (1960). *J. Am. chem. Soc.* **82**, 3157.
YAGUCHI, M., TARASSUK, N. P. & HUNZIKER, H. G. (1961). *J. Dairy Sci.* **44**, 589.

EXPLANATION OF PLATES

PLATE 1

Polyacrylamide gel electrophoresis of β-lactoglobulins. (*a*), Six possible combinations (types) obtained with recrystallized preparations of β-A, β-B and β-C; left to right A, AB, B, BC, C and AC; (*b*), routine typing run obtained with skim-milk from individual cows; left to right the β-lactoglobulin types are A, AB, B, A, AB, A, B, and AB.

PLATE 2

Paper electrophoresis patterns of milk proteins. (*a*), 20% (w/v) Na_2SO_4-soluble proteins from an AB cow; (*b*), 20% (w/v) Na_2SO_4-soluble proteins from castrated male 4120; (c), whole whey from an AB cow; (*d*), whole whey from castrated male 4104. Line of application at left edge of Plate.

Printed in Great Britain

DIRECTIONS TO CONTRIBUTORS

GENERAL. The onus of preparing a paper in a form suitable for publication in the *Journal of Dairy Research* lies in the first place with the author. In their own interests authors are strongly advised to follow these directions carefully and to consult a current issue for guidance on details of layout and use of headings.

Typescripts may be sent to the Editors at any time, and submission of a paper will be held to imply that it reports unpublished original work which is not under consideration for publication elsewhere.

FORM OF PAPERS. Papers should be typed with double spacing, and the title followed by the names and initials of the authors, women supplying one Christian name. The name and postal address of the laboratory must be stated.

Papers should be divided into the following parts in the order indicated: (*a*) Summary, brief and self-contained; (*b*) Introductory paragraphs, briefly explaining the object of the work but without giving an extensive account of the literature; (*c*) Experimental or Methods; (*d*) Results; (*e*) Discussion and Conclusions; (*f*) Acknowledgements without a heading; (*g*) References. Only with some exceptional types of material will headings different from (*c*), (*d*) and (*e*) be necessary.

The spelling adopted is that of the *Shorter Oxford English Dictionary*. Underlining should be used only to indicate italics. Every effort should be made to avoid the use of footnotes. Proper nouns, including trade names, should be given a capital initial letter.

TABLES. Each table should be numbered and should have a heading that enables its contents to be understood without reference to the text. Tables must be typed on separate sheets and their approximate positions indicated in the text.

ILLUSTRATIONS. Line drawings, which must be originals, should be numbered as Figures and photographs as Plates, in Arabic numerals Drawings should be in indian ink, on Bristol board or cartridge paper. However, a technique which may be more convenient to authors is to use a double-sized piece of tracing paper, or translucent graph paper faintly lined in *bule* or *grey*, folded down the centre with the drawing on one half and the other acting as a flyleaf.

Attached to every figure and plate there should be a translucent flyleaf cover on the outside of which should be written legibly: (*a*) title of paper and name of author; (*b*) figure or plate number and explanatory legend; (*c*) the figures and lettering, which is intended to appear on the finished block, in the correct position relative to the drawing underneath. For each paper there should also be a separate typed sheet listing figure and plate numbers with their legends, and the approximate position of illustrations should be indicated in the text.

As a rule the photographs and diagrams should be about twice the size of the finished block and not larger over-all than the sheets on which the paper itself is typed. For general guidance in preparing diagrams, it is suggested that for a figure measuring 9 in. × 6 in. all lines, axes and curves, should have a thickness of 0·4 mm, thus ————. Graph symbols in order of preference should be ○ ●, △ ▲, □ ■, × +, and for a 9 in. × 6 in. graph the open circles should be $\frac{1}{8}$ in. in diameter. The open triangles should be large enough to contain circles of $\frac{5}{32}$ in. diameter and the open squares circles of $\frac{1}{4}$ in. diameter. The crosses should have lines $\frac{1}{8}$ in. long. The block symbols should be slightly smaller than the corresponding open symbols. Scale marks on the axes should be on the inner side of each axis and should be $\frac{1}{8}$ in. long.

REFERENCES. In the text references should be quoted by whichever of the following ways is appropriate: Arnold & Barnard (1900); Arnold & Barnard (1900*a*); Arnold & Barnard (1900*a*, *b*); (Arnold & Barnard, 1900). Where there are more than two authors all the surnames should be quoted at the first mention, but in subsequent citations only the first surname should be given thus, Brown *et al.* (1901). If there are six or more names, use *et al.* in first instance. Also, if the combinations of names are similar, repeat names each time, e.g. Brown, Smith & Allen (1954); Brown, Allen & Smith (1954).

References should be listed alphabetically at the end of the paper, title of journals being abbreviated as in the *World List of Scientific Periodicals*. Author's initials should be included, and each reference should be punctuated in the typescript thus: Arnold, T. B., Barnard. R. N. & Compound, P. J. (1900). J. Dairy Res. 18, 158. References to books should include name of author, year of publication, title, town of publication and name of publisher in that order, thus, Arnold, T. B. (1900). Dairying. London: Brown and Chester.

It is the duty of the author *to check all references* and to ensure that the correct abbreviations are used.

SYMBOLS AND ABBREVIATIONS. The symbols and abbreviations used are those of British Standard 1991: Part 1: 1954, *Letter Symbols, Signs and Abbreviations*.

DESCRIPTIONS OF SOLUTIONS. Normality and molarity should be indicated thus: N-HCl, 0·1 M-NaH_2PO_4. The term '%' means g/100 g solution. For ml/100 ml solution write '% (v/v)' and for g/100 ml solution write '% (w/v)'.

REPRINTS. Order forms giving quotations for reprints are sent to authors with their proofs.

CONTENTS OF VOL. 32, No. 2

Printed in Great Britain at the University Printing House, Cambridge
(Brooke Crutchley, University Printer)