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CORRECTIONS

Journal of Dairy Research, **33**, 3, p. 288.

Partition of caesin between polymer phases

N. J. Berridge and D. L. Suett

Lines 20 and 23 *for* '74' *substitute* '67'

Journal of Dairy Research **33**, 3, p. 295

The induction of ketosis in the lactating dairy cow

K. G. Hibbitt

Table 4 *for* '2·0 ± 4·0 (4)'
substitute '26·0 ± 4·0 (4)'

Changes in the quantity and composition of mammary gland secretion in the dry period between lactations

I. The beginning of the dry period

By J. V. WHEELOCK, A. SMITH*, F. H. DODD and R. L. J. LYSTER
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 3 *June* 1966)

Summary. The changes in the yield and composition of the mammary gland secretion in the first 16 days of the dry period have been investigated in 6 cows. The quantity of secretion, lactose, potassium, casein, α-lactalbumin, β-lactoglobulin and fat present in the gland decreased rapidly. A decrease was also observed for sodium, chloride, residual albumin and proteose-peptone + globulin, but was much less marked. Changes in composition were qualitatively similar to those normally occurring at the end of lactation but they were much more pronounced. Within 8 days after drying-off the concentrations in the secretion of sodium, chloride and potassium were similar to those of blood. There were differences between animals in the rate at which the changes developed. As the secretory activity of the gland ceased, the constituents of the mammary gland secretion appeared to be resorbed.

Most dairy cows would probably lactate continuously through several pregnancies from the time of their first parturition, provided that the milk was removed twice daily, although the quantity of secretion obtained in the period before each subsequent parturition might be very small. In practice, milking is usually suspended for 1–3 months before each parturition giving the so-called dry period. The reason for this husbandry practice is that it is uneconomic to remove the small amount of milk secreted in late lactation.

There is evidence that the duration of the dry period is related to secretory activity of the mammary gland in the succeeding lactation. Several workers (Johansson & Hansson, 1940; Klein & Woodward, 1943; Sanders, 1928) have observed that a dry period of less than 8 weeks is associated with a decrease in milk yield of the following lactation. More recently, Swanson (1965) observed that cows which were milked throughout the whole of pregnancy yielded, in the following lactation, much less milk than their twins which had a dry period of 2 months. It is unlikely that this effect is due to either hormonal or nutritional factors since Smith, Wheelock & Dodd (1966) have obtained similar results using different quarters of one animal.

It has also been demonstrated that the dry period is possibly important in other respects. After infection of a mammary gland quarter by pathogenic bacteria, there is usually a decrease in the yield and change in the composition of milk. When

* On visit from Animal Husbandry and Dairy Research Institute, Irene, South Africa.

infection is eliminated, maximum recovery in milk yield and composition does not occur until after the subsequent dry period (Rowland, Neave, Dodd & Oliver, 1959). In addition, it appears that in cows the mammary gland becomes infected more frequently at the beginning of the dry period than at any other time during the reproductive cycle, and new infections are rare during the remainder of the dry period (Neave, Dodd & Henriques, 1950).

During the dry period considerable changes in the structure of the mammary gland take place; involution occurs and the gland becomes similar to that of a heifer before first pregnancy, although it is rare for the lobular structure to disappear completely (McFarlane, Rennie & Blackburn, 1949; Mossiman, 1949). There is little information about the rate of involution for cows, but for goats most of the lactating alveoli have disappeared at the end of a normal lactation, but when milking is discontinued at peak production about 48 days are required for the gland to change to the full resting phase (Turner & Reineke, 1936).

Woodman & Hammond (1923) examined the composition of secretions by heifers before parturition and found that during the first 5 months of pregnancy, small quantities of fluid, similar to milk, could be obtained from the mammary gland, but that after the 5th month there was a progressive change in composition. The characteristic milk constituents began to disappear and eventually the secretion contained about 40% total solids and 35% total globulins. During this phase it appeared that there was little or no synthesis of characteristic milk constituents.

Larson (1958) confirmed that the synthetic activity of the mammary gland cells was minimal for the greater part of the dry period and also demonstrated that large quantities of immune globulins were transferred from the blood into the mammary gland in the days immediately before parturition. He also noted that the transfer of these large quantities of immune globulins occurred even if the synthetic activity of the mammary gland cells was stimulated by milking twice daily in the usual way. This finding was confirmed by Wheelock, Rook & Dodd (1965*a*).

The large amount of synthesized milk constituents usually found in colostrum shows that there must be considerable synthetic activity in the days prior to parturition even if the animal is not milked. Wheelock & Rook (1966) showed that during the last week of pregnancy there was a progressive increase in the concentration of lactose in the urine which reached a peak at parturition and then decreased rapidly.

Rowland, Roy, Sears & Thompson (1953) commenced milking 16 days before the expected date of parturition and observed that there was a very low yield of secretion at the first milking, thereby demonstrating that there is little carry-over of secretion from the end of one lactation to the beginning of the next. In general, the concentration of total solids, solids-not-fat (SNF), total protein, albumin, globulin and chloride, which are characteristically high in colostrum, were even higher in the initial prepartum secretions, whereas the concentrations of fat, ash, calcium, phosphorus, casein, proteose-peptone and non-protein N were similar to the values normally observed for colostrum.

Despite the known significance of the dry period, the physiological changes in the mammary gland of the dry cow are imperfectly understood and there is little detailed information on the changes that take place in the mammary secretion during the period when the secretion is not removed. The investigation reported

below, which is the first of a series on the dry period, was designed to determine the quantitative changes that occur in the secretion during the first days of the dry period.

EXPERIMENTAL

Animals and management

The relevant details of the 6 lactating cows (170, Y 61, Y 65, Y 67, 186 and S 61) used for the experiment are given in Table 1. All the cows were free of udder infections throughout the period of the experiment although each of the cows 170, Y 61 and S 61 had been infected with pathogenic bacteria in one quarter of the udder during the preceding lactation. During the experiment the cows were kept in a cowhouse and had free access to water. Before drying-off they were given a diet of hay (12 lb/day) and a concentrate mixture balanced for milk production (4 lb/gal of milk produced). No concentrates were fed after drying-off. To reduce the risk of udder infection all the teats of the cows were dipped in a 4% hypochlorite solution immediately after the teats were handled during the dry period.

Table 1. *Details of experimental animals*

Cow	Lactation preceding dry period	Lactation length, days	Duration of dry period, days	Mean daily milk yield in the week before drying-off, g
170	2	257	Non-pregnant	8895
Y 61	3	191	143	8195
Y 65	2	256	Non-pregnant	7115
Y 67	2	227	Non-pregnant	4495
186	2	248	126	7090
S 61	3	278	128	3180

Experimental procedure

During the lactation the cows were machine-milked twice daily. One week before drying-off, the cows were milked at 4 consecutive milkings with a milking machine designed for the separate collection of milk from individual quarters. The milk yields of the quarters were recorded and samples taken. All the samples from a quarter were used to prepare one weighted composite sample for analysis.

Two days after drying-off all the available secretion was removed by hand from one quarter of each cow, the yield recorded and a sample taken. At 2-day intervals the procedure was repeated on the other 3 quarters in succession. The complete procedure of removing the secretion from the individual quarters was then repeated starting 2 days after the end of the first cycle.

Urine samples were collected from the cows at 12- or 24-h intervals for 7 days before and 14 days after drying-off.

Methods of analysis

Milk and mammary gland secretion samples were analysed for total solids, fat, lactose, total N, non-casein N, sodium, potassium and chloride by methods previously described (Wheelock, Rook & Dodd, 1965*b*). Residual albumin and proteose-peptone + globulin protein fractions were determined by the method of Aschaffenburg

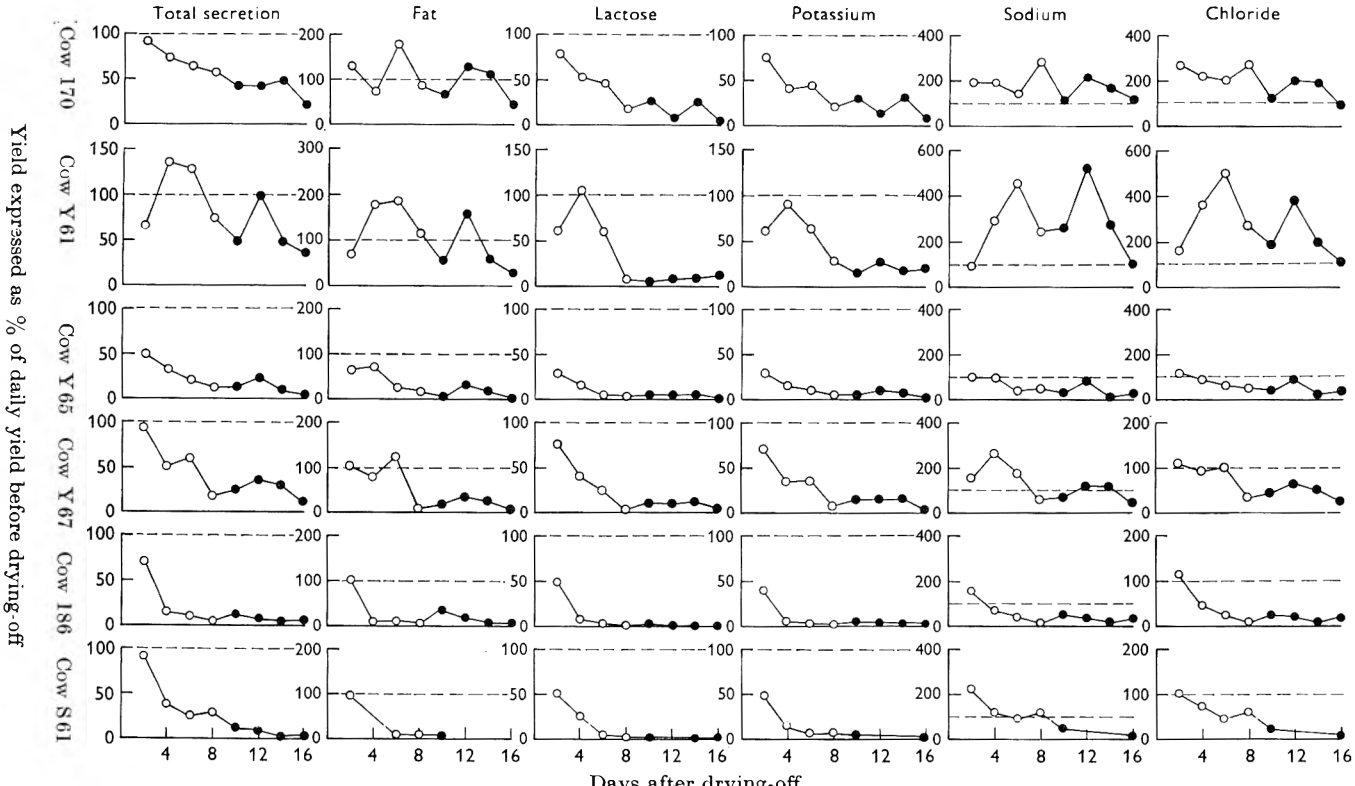

Fig. 1. The yield of total secretion, fat, lactose, potassium, sodium and chloride obtained from quarters of the mammary gland after drying-off. ○, values for the 1st sampling cycle; ●, values for the 2nd sampling cycle.

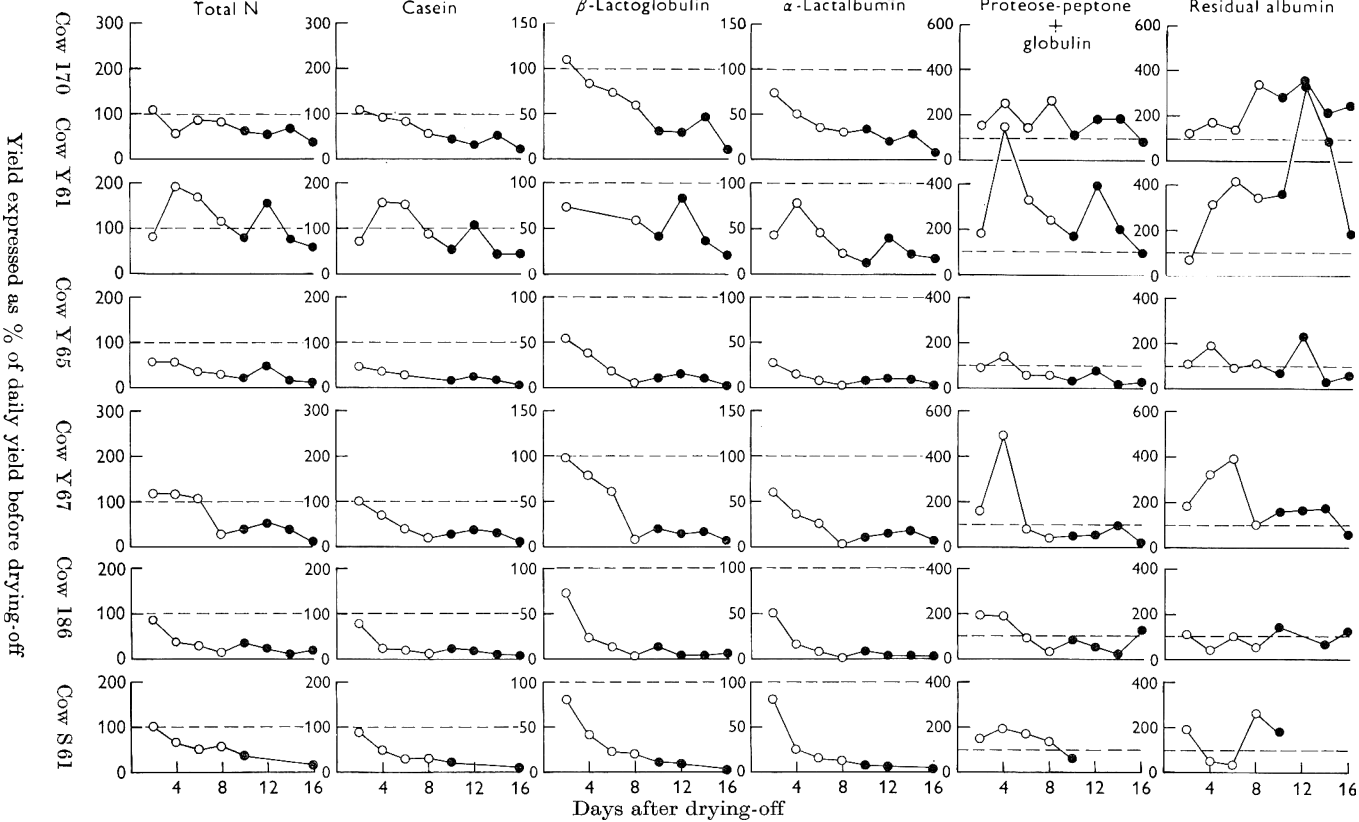

Fig. 2. The yield of total N, casein, β-lactoglobulin, α-lactalbumin, proteose-peptone + globulin and residual albumin obtained from quarters of the mammary gland after drying-off. ○, values for the 1st sampling cycle; ● values for the 2nd sampling cycle.

& Drewry (1959). Lactose in urine was determined as glucose by glucose oxidase after acid hydrolysis (Wheelock & Rook, 1966). α-Lactalbumin and β-lactoglobulin in milk, mammary gland secretions and urine were determined immunoelectrophoretically (Larson & Hageman, 1963; Larson & Twarog, 1961). Because of difficulty in pipetting the thick mammary gland secretions, the analyses were performed on samples which had first been suitably diluted with water.

RESULTS

Changes in the yield of mammary gland secretion and its individual constituents after drying-off (Figs. 1, 2)

In spite of the cessation of regular milking the yields of secretion after 2 days were, with the exception of cow Y 61, less than the milk yield in the 24 h before drying-off. The yield continued to decline so that after 8 days the average amount obtained was about 25% of the milk yield in 24 h. During the 2nd sampling cycle substantial yields of secretion were obtained only from cows Y 61 and 170. Milk dripped from the teats of both these cows in the period immediately after drying-off, and these were also the only cows in which milk ejection was observed 8 days after drying-off, when they were being prepared for sampling.

The reduction in yield of secretion was associated with similar changes in the yields of potassium, lactose, total N, casein, β-lactoglobulin and α-lactalbumin. The yield of fat also decreased but showed more variation, which probably reflected the degree of efficiency of fat evacuation when the milk was removed.

The yields of sodium and chloride were relatively much higher than the corresponding values for the synthesized constituents. In cows 170 and Y 61, these high values were maintained throughout the period of the experiment, but in the other cows the yields decreased progressively.

Proteose-peptone + globulin and residual albumin yields showed changes similar to those of sodium and chloride but in some of the cows the yields increased to a maximum 4 or 6 days after drying-off and then decreased.

Changes in the composition of the mammary gland secretion after drying-off (Figs. 3, 4)

The main changes in the concentrations of the individual constituents of the secretion occurred during the first 8 days of the dry period.

The SNF concentration of the secretion was steady throughout the period of the experiment in all the cows except cow 186, in which the concentration increased progressively from 9 to 14% during the first 8 days of the dry period. There was no consistent pattern in the changes of fat concentration. The concentrations of lactose and potassium decreased rapidly in all cases, so that within 8 days lactose was about 1% and potassium about 40 mg/100 g. The concentrations of sodium and chloride increased and remained constant once the maximum values were achieved, which was usually 6 or 8 days after drying-off; with cows 186 and S 61 the maximum value was observed on the 4th day of the dry period.

The concentrations of total N increased progressively during the first days of the dry period and then became stabilized, the most pronounced changes being

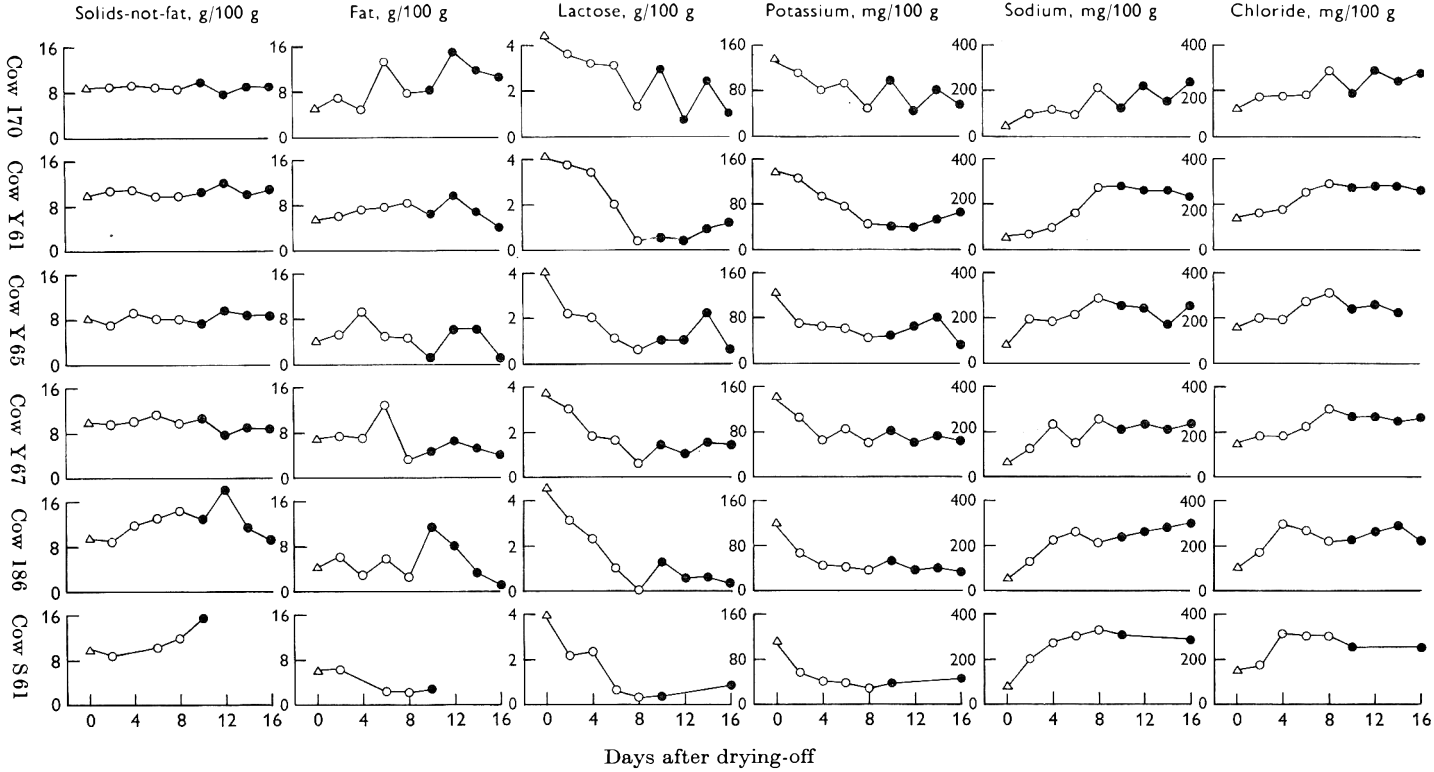

Fig. 3. The changes in concentration of SNF, fat, lactose, potassium, sodium and chloride in mammary gland secretion after drying-off. △, in milk before drying-off; ○ in mammary gland secretion during the 1st sampling cycle; ●, in mammary gland secretion during the 2nd sampling cycle.

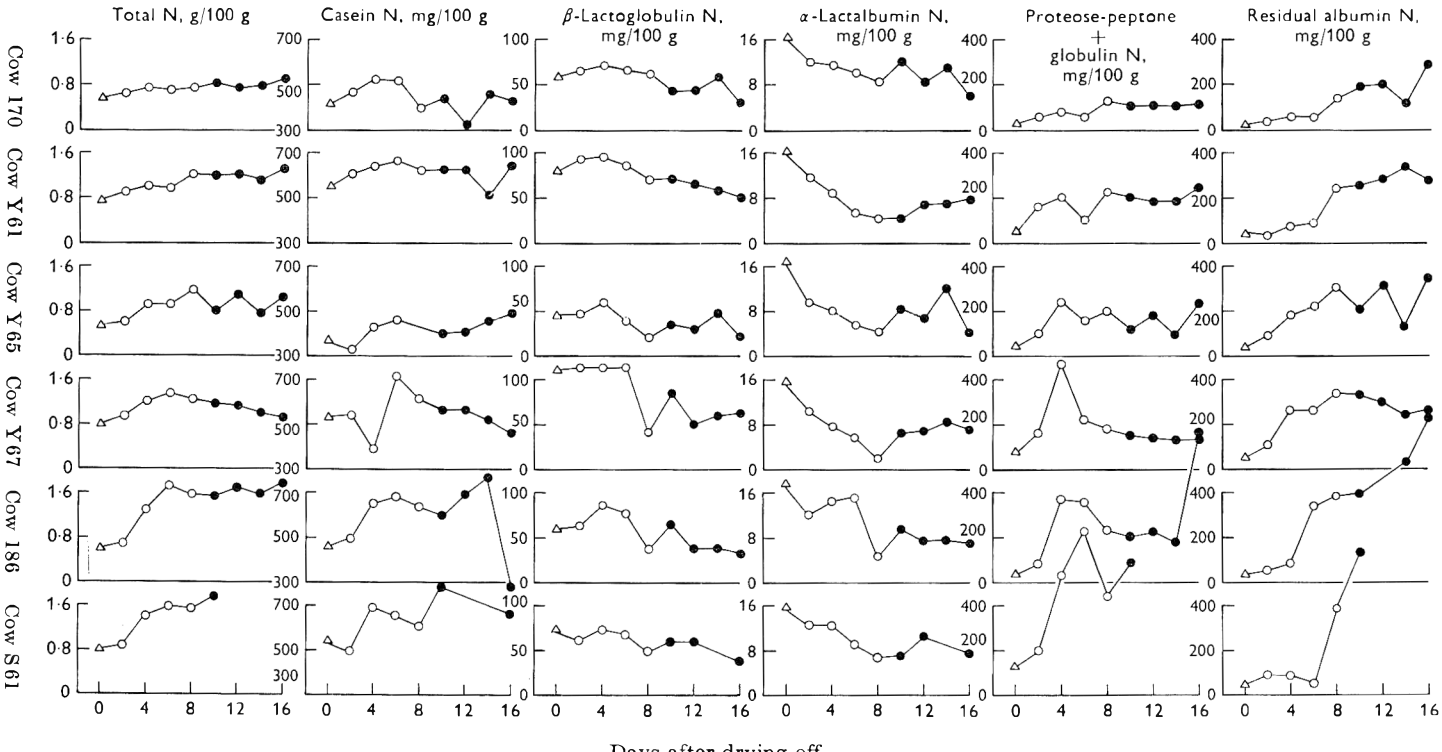

Fig. 4. The changes in concentration of total N, casein N, β-lactoglobulin N, α-lactalbumin N, proteose-peptone + globulin N and residual albumin N in mammary gland secretion after drying-off. △, in milk before drying-off; ○, in mammary gland secretion during the 1st sampling cycle; ●, in mammary gland secretion during the 2nd sampling cycle.

shown by cows 186 and S61. The casein and β-lactoglobulin concentrations tended to increase during the first days of the dry period and then the casein value fluctuated but β-lactoglobulin decreased. The other synthesized protein, α-lactalbumin, decreased progressively, reaching a minimum within 8 days. These changes for α-lactalbumin are remarkably similar to those for lactose. During the first days of the dry period, the concentration of proteose-peptone + globulin increased to a maximum and then remained constant or decreased again. Fivefold increases in concentration were observed for cows Y67, 186 and S61.

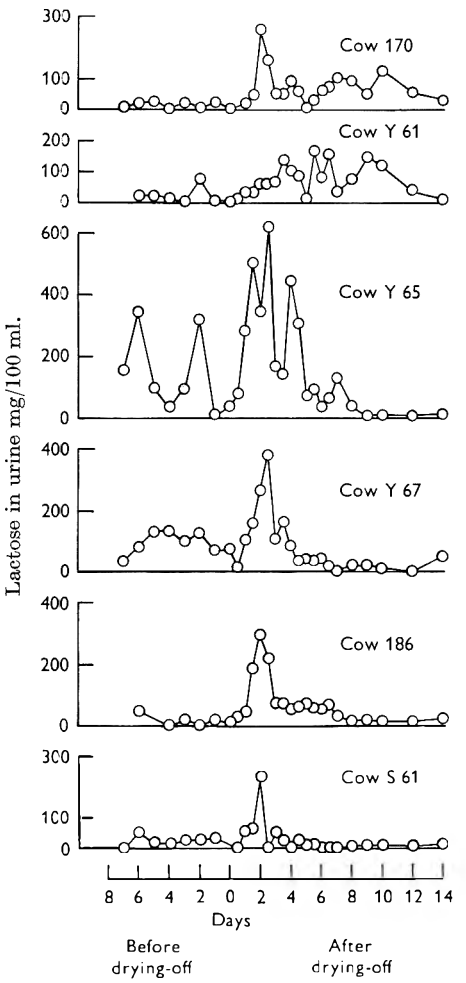

Fig. 5. The concentration of lactose in urine before and after drying-off.

Excretion of lactose in the urine (Fig. 5)

With all the cows the concentration of lactose in the urine increased markedly after drying-off and then decreased again to very low levels. In cows Y65, Y67, 186 and S61 these low values were reached within 8 days of drying-off but in cows 170 and Y61 the high concentrations persisted for a further 6 days. During the

period before drying-off, the lactose concentrations were much higher for cows Y65, Y67 and S61 than those observed by Wheelock & Rook (1966) for cows in mid-lactation.

DISCUSSION

The decrease in yield of secretion and synthesized constituents during the first 8 days after drying-off demonstrates that water and the individual constituents are resorbed from the mammary gland and that this resorption must take place after the contents of the secretory cells have been ejected into the alveoli. The changes in the lactose concentration in the urine show that resorption of lactose begins about 24 h after the last milking, builds up rapidly and may be completed within 4 days in certain animals.

The further observation of lactose in the urine in the period immediately before drying-off when the cows were still being milked twice daily indicates that there is a loss of lactose from the mammary gland at this time. It has previously been shown (Wheelock & Rook, 1966) that the excretion of lactose in the urine is negligible for cows in mid-lactation with udders free of pathogenic infections, but that there is a marked increase in the excretion of lactose when milk accumulates within the mammary gland during an extended milking interval. It is unlikely that there is any accumulation of milk in the gland in late lactation as the production of milk is much lower than in the early part of the lactation, and in the present investigation the highest concentrations of lactose in the urine before drying-off were observed in those cows which were giving the lowest yield of milk. A possible explanation is that the increased degree of involution of mammary gland tissue in late lactation (Turner & Reineke, 1936) facilitates the resorption of lactose from the gland.

The changes in composition of the mammary gland secretion which occur after drying-off are similar to those observed in late lactation (Barry & Rowland, 1953), with extended milking intervals or incomplete removal of milk from the mammary gland (Wheelock, Rook & Dodd, 1965*c*) and with bacterial infections of the mammary gland (Wheelock, Rook, Neave & Dodd, 1966) but much more pronounced. Although careful bacteriological tests showed that the cows were free of udder infections, in 4 of the 6 cows, clots similar in appearance to those usually obtained in milk from quarters with clinical mastitis were observed in one or more samples during the period of the experiment. The changes in composition of milk after intervals of 24, 36 and 120 h have been studied by Petersen & Rigor (1932–33) and although their experiments were performed at an earlier stage of lactation, trends in composition similar to those in the present work were observed.

The occurrence of high concentrations of lactose in the urine shows that some of the lactose is eventually excreted by the kidneys, and Wheelock & Rook (1966) have shown that during a 39-h milking interval almost all of the lactose lost from the mammary gland is accounted for in the urine. Traces of casein, β-lactoglobulin and α-lactalbumin were also found in the urine before and after drying-off, but the actual amount after drying-off, was much less than that lost from the mammary gland as determined by the decrease in yield.

In several of the cows the excretion of lactose had decreased to very low levels within 4 days of drying-off and only very small quantities of synthesized constituents

could be obtained from the mammary gland. These results demonstrate that the synthesis of milk constituents by the alveolar cells must have been depressed considerably or possibly had ceased completely within 2 or 3 days of drying-off. However, in cows 170 and Y61, high concentrations of lactose in the urine were maintained and significant yields of synthesized constituents were obtained from the mammary gland for much longer periods. This evidence suggests that in these cows the synthetic activity of the alveolar cells continued for a longer period than in the other cows on the experiment. Differences in the rate at which the primary secretion of the alveolar cells was depressed would therefore explain the differences between animals in the rate at which the changes in composition developed.

There was a much more rapid decrease in the yields of the synthesized constituents than in the yields of sodium, chloride, proteose-peptone + globulin and residual albumin, which contains serum albumin. The secretion of the non-synthesized constituents, therefore, is probably regulated by a process which differs from that which controls the synthetic activity of the mammary gland. It has previously been suggested (see Barry & Rowland, 1953) that the changes in composition of milk in late lactation or due to bacterial infections of the udder are caused by a relative increase in the rate at which a transudate of blood serum enters the mammary gland. It is interesting, therefore, that as the concentration of lactose, the main osmotically active synthesized constituent, approached zero the concentrations of sodium, potassium and chloride approached those normally found for these constituents in blood.

However, by the end of the first 8 days after drying-off there were also marked decreases in the yields of sodium, chloride, residual albumin and proteose-peptone + globulin. Previously it has been shown (Turner, 1955) that the capacity of the mammary gland decreases with stage of lactation and it is likely that after drying-off the capacity continues to decrease, but at an increased rate. This would reduce the available volume for secretion and as constituents can apparently move between blood and the mammary gland more easily at that time than during lactation, as shown by the transfer of lactose, there would be a tendency for the amount of all the constituents in the gland to be reduced.

Information on the mechanism of resorption of constituents has been provided by Lascelles (1961). He found that in sheep the lymph draining the mammary gland became opalescent in character when the gland was acutely distended with milk. The opalescent lymph was found to contain high concentrations of calcium, inorganic phosphorus and esterified fatty acids. Specific milk proteins were also identified. In a later paper, Lascelles (1962) introduced human serum albumin and casein labelled with radio-active iodine into the mammary gland and found that when milk accumulated in the gland, both appeared in the interstitial fluid of the gland, but the casein was transferred directly to the blood while the serum albumin was removed by the lymphatics.

These experiments indicate that the changes in the mammary gland develop very quickly after the last milking of the lactation but the reasons for them remain obscure. Since there was no difference in response between the pregnant and non-pregnant cows, it may be concluded that the approach of parturition was not an important factor in bringing about the changes, although it probably affected the

rate of decline in the milk yield before drying-off (Gaines & Davidson, 1926). It is more likely that the depression in activity after drying-off is mainly due to the failure to remove the products of secretion rather than the absence of the milking stimulus since in a previous experiment (Smith *et al.* 1966) it was observed that 2 quarters of a cow will apparently dry off normally even if the other 2 quarters are milked twice daily.

We are grateful to Miss M. Weston, Miss S. Futcher, Miss P. Cowley and Mr P. Higgs for skilled technical assistance.

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Changes in the quantity and composition of mammary gland secretion in the dry period between lactations

II. The complete dry period

By A. SMITH*, J. V. WHEELOCK and F. H. DODD
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 27 *July* 1966)

Summary. The changes in yield and composition of mammary gland secretion throughout the whole of the dry period have been investigated in 6 cows. The yields of lactose, potassium, fat and casein decreased to very low values for the greater part of the dry period but then increased slightly just before parturition. Similar trends were observed for sodium and chloride but they were much less marked. During the middle of the dry period the concentrations of sodium, potassium and chloride tended towards those of blood serum while the concentrations of fat and lactose decreased to $< 1\%$.

In the previous paper Wheelock, Smith, Dodd & Lyster, (1967), described the changes in the secretion of the mammary gland at the beginning of the dry period between consecutive lactations. In this paper the results of a similar type of study for the complete dry period are presented.

EXPERIMENTAL

Animals and management

The yield of milk produced by the individual quarters of the mammary gland of each cow was determined in the period before drying-off by milking with a milking machine designed to collect the milk from each quarter separately. During the dry period at the first sampling date all the available secretion was removed by hand from one quarter of the mammary gland and the yield recorded. This procedure was repeated on the other quarters at regular intervals throughout the dry period. A total of 25 cows was used, but frequently the quantity of secretion obtained during the middle of the dry period was insufficient for chemical analysis. Samples of colostrum were obtained as soon as possible after parturition. All the cows were free of bacterial infections of the udder throughout the period of the experiment and were managed in the same way as described previously (Wheelock *et al.* 1967).

Methods of analysis

Milk and mammary gland secretion samples were analysed as described in the previous paper (Wheelock *et al.* 1966).

* On visit from Animal Husbandry and Dairy Research Institute, Irene, South Africa.

RESULTS

Changes in the yield of mammary gland secretion and of individual constituents throughout the dry period (Table 1; Figs. 1, 2)

Table 1, which summarizes the available data for all the 25 cows, shows that the yield of mammary gland secretion decreased after drying-off. In the 3rd and 4th weeks of the dry period yields as low as 1 g of secretion were obtained from some quarters, with mean yields of 78 g and 43 g, respectively. From about 3 weeks before parturition the yield increased slowly. Because several of the cows yielded such a small quantity of secretion for much of the dry period, changes in the secretion of individual constituents could be determined only for those cows which gave sufficient secretion for analysis. The relevant details for these 6 cows are shown in Table 2 and the changes based on their results are described below.

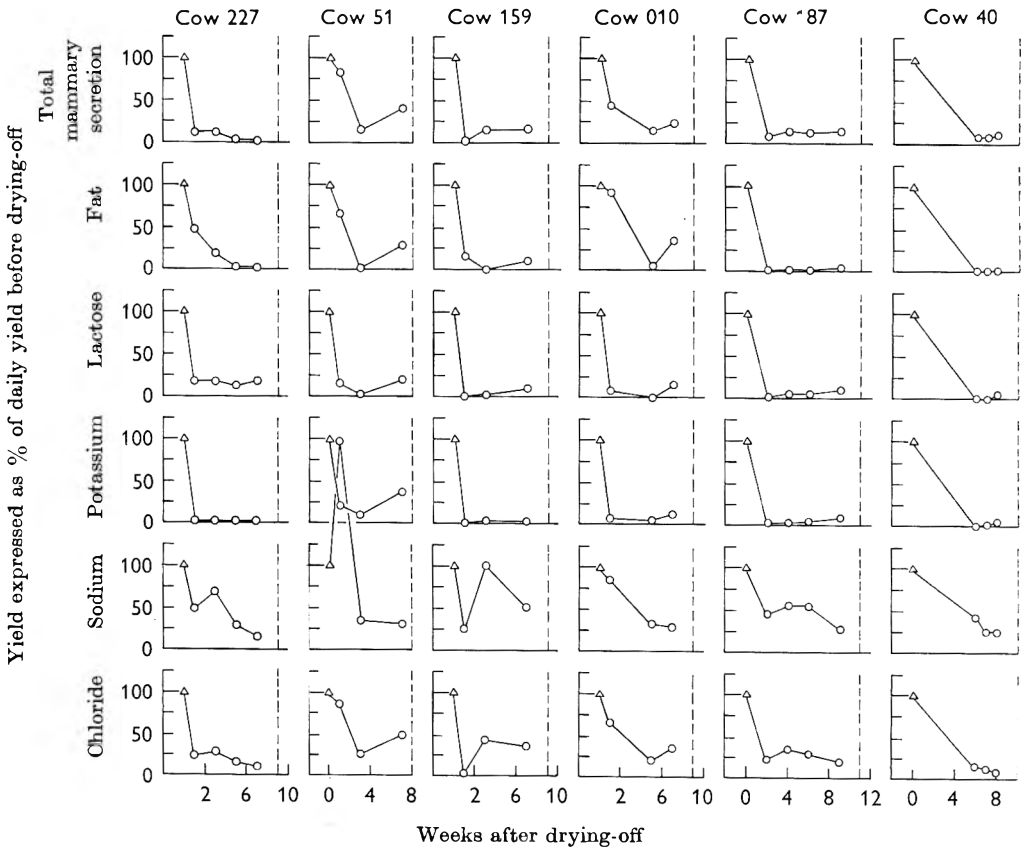

Fig. 1. The yield of total secretion, fat, lactose, potassium, sodium and chloride in the dry period. △, last day of lactation; ○, dry period (value for 1 quarter only). The dotted line indicates the time of parturition.

The changes in yield of secretion for the 6 individual animals (Fig. 1) after drying-off show trends similar to those observed for all the 25 cows (Table 1). In the period before parturition, however, the yield of secretion increased only with cows 51, 159 and 010.

With all 6 cows, the yields of lactose and potassium were very low throughout the greater part of the dry period but they increased slightly just before parturition. Sodium and chloride yields tended to decrease throughout the dry period, although in some of the cows an increase was observed just before parturition.

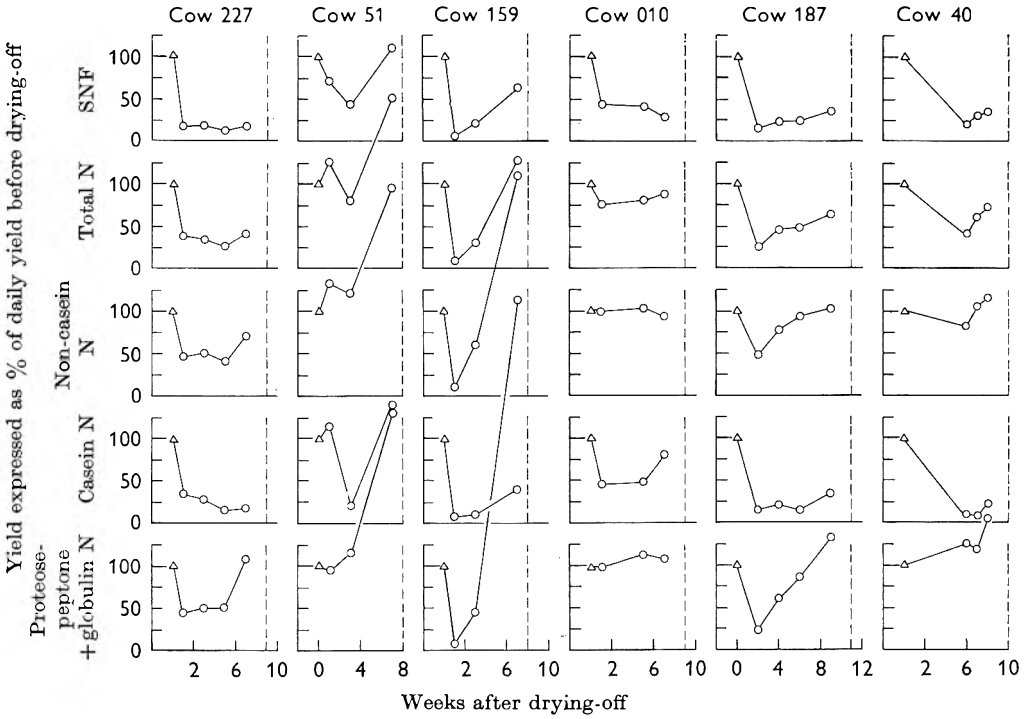

Fig. 2. The yield of SNF, total N, non-casein N, casein N, and proteose-peptone + globulin N in the dry period. △, last day of lactation; ○, dry period (value for 1 quarter only). The dotted line indicates the time of parturition.

Table 1. *Yield of secretion obtained from the individual quarters of the mammary gland at drying-off and during the dry period*

Time of sampling	Number of quarters sampled	Average quarter yield, g	Range of yields, g
At drying-off	100	997	80–2420
1 week after drying-off	14	215	40–1080
2 weeks after drying-off	11	83	3–210
3 weeks after drying-off	14	78	1–263
4 weeks after drying-off	12	43	1–210
3 weeks before parturition	19	82	5–285
2 weeks before parturition	11	116	1–325
1 week before parturition	18	258	25–1020

After drying-off the yield of SNF decreased and then in all but cows 227 and 010 it increased again before parturition. The yields of total N, non-casein N and proteose-peptone + globulin N decreased to a minimum value and then increased again at the end of the dry period. Very high yields of proteose-peptone + globulin N were observed in cows 51 and 159, 2 weeks before parturition. For the greater part

of the dry period the yield of casein was low compared with that normally obtained in lactation, but 5 of the 6 cows showed an increased yield some weeks before parturition.

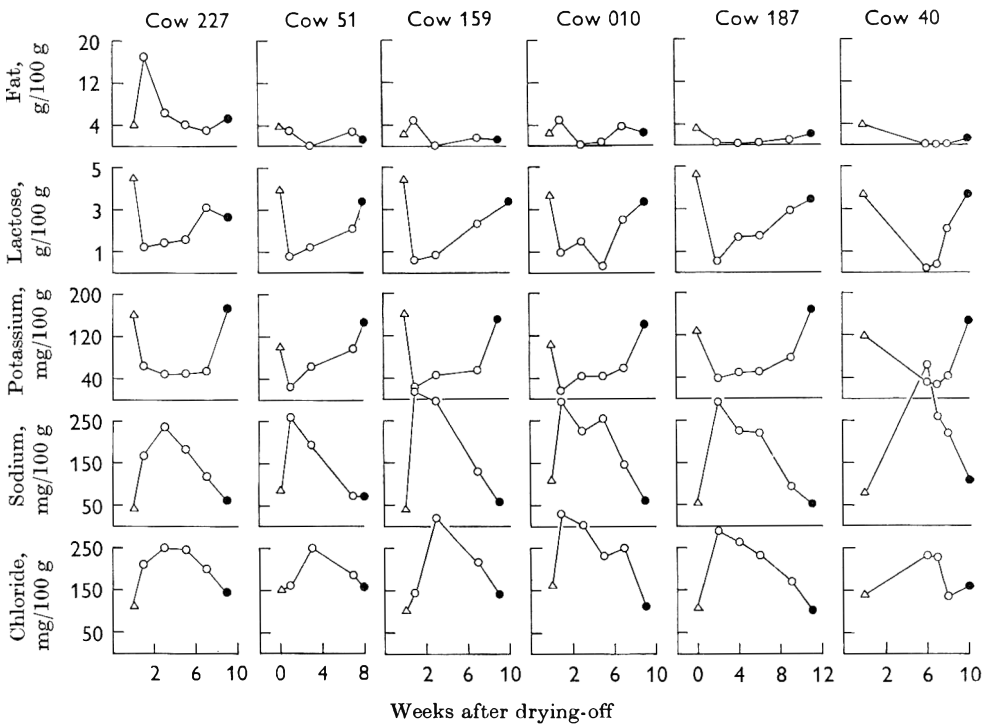

Fig. 3. The concentration of fat, lactose, potassium, sodium and chloride in mammary gland secretions. △, last day of lactation (average value for 4 quarters); ○, dry period (value for 1 quarter only); ●, parturition, (average value for 4 quarters).

Changes in the composition of the mammary gland secretion throughout the dry period (Figs. 3, 4)

In cows 227, 159 and 010 the concentration of fat in the mammary gland secretion 1 week after drying-off was higher than in the milk obtained during the lactation, but in 5 of the 6 cows it then decreased to < 1% for the greater part of the dry period. Some of the variation in the period immediately after drying-off may be due to incomplete removal of secretion but the very low values in the middle of the dry period are consistent. There was a small increase in the concentration of fat before parturition.

The lactose concentration decreased to a minimum value of < 1% soon after drying-off and then increased gradually, although at parturition it was usually still lower than at drying-off. With 3 of the 4 cows, from which samples were obtained 1 week after drying-off, the minimum value was observed at this time. The potassium concentration changed in very much the same way as that of lactose. Corresponding to the decreases in lactose concentration there were increases in those of sodium and chloride, with the exception that in cows 51 and 159, the minimum lactose concentrations were observed 1 week after drying-off, whereas the maximum value

for chloride was observed 2 weeks later. The maximum values for the concentrations of sodium and chloride and the minimum value for potassium are similar to the concentrations of these constituents normally found in blood.

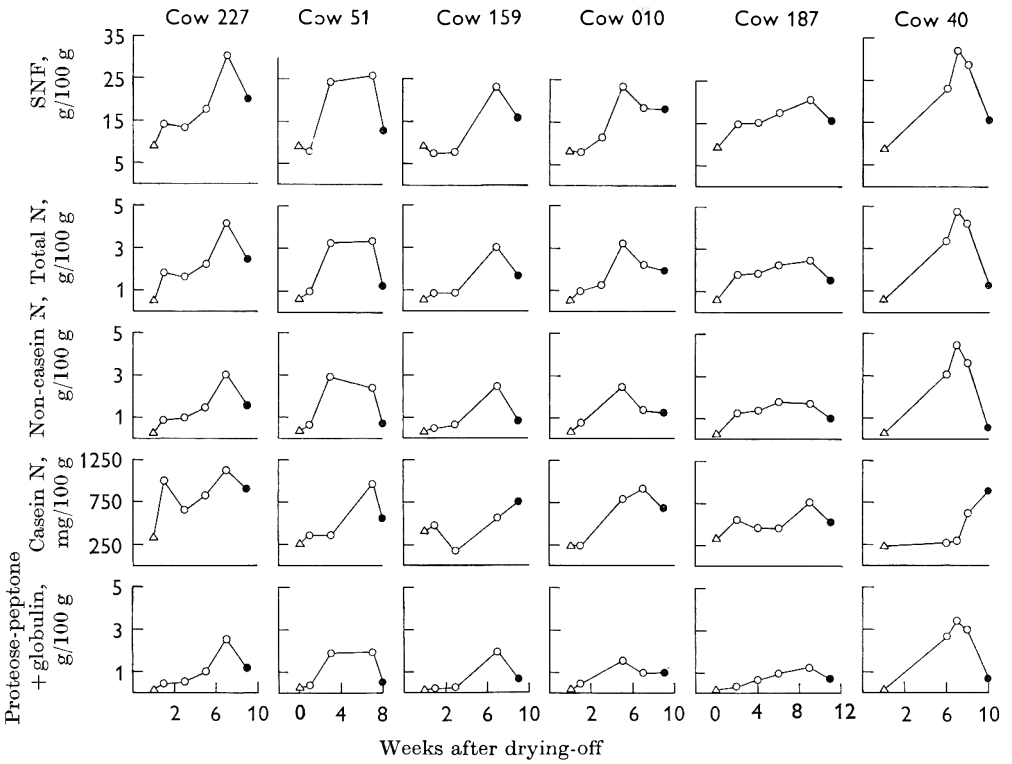

Fig. 4. The concentration of SNF, total N, non-casein N, casein N, and proteose-peptone + globulin N in mammary gland secretions. △, last day of lactation (average value for 4 quarters); ○, dry period (value for 1 quarter only); ●, parturition (average value for 4 quarters).

Table 2. *Details of the* 6 *cows for which full results were obtained*

Cow	Number of lactations preceding the dry period	Length of dry period, days	Daily milk yield at drying-off, g
187	2	71	6080
227	1	55	7155
51	3	52	3120
159	2	66	3465
010	7	60	2125
40	3	65	6610

SNF concentration increased progressively throughout the dry period reaching a maximum between 1 and 4 weeks before parturition and then it decreased again, but the value at parturition was always much higher than the value usually observed during lactation. Similar trends were observed for the concentrations of total N, non-casein N and proteose-peptone + globulin N. There was no consistent pattern for casein N, except that the concentration was much higher just before and at parturition than at the end of lactation. In comparison with normal milk, the

concentration of casein in mammary gland secretions during the dry period was higher, whereas concentrations of the other synthesized constituents, lactose and fat, were much lower.

DISCUSSION

The results of this experiment confirm the changes observed in the mammary gland secretion during the early part of the dry period described previously (Wheelock *et al.* 1966) and provide additional data on the quantity and composition of the secretion during the remainder of the dry period. The activity of the gland during the period when secretion is not removed appears to have a definite pattern. Within a very short period of drying-off the synthetic activity ceases and constituents present in the gland are resorbed leaving a small quantity of secretion, in which the concentrations of sodium, chloride and potassium are similar to those of blood. During the middle of the dry period the synthetic activity of the gland is negligible and with most animals the yield of secretion that can be obtained is < 100 g/quarter and with many animals is < 10 g/quarter. Several weeks before parturition the direction of change in concentration of sodium, chloride and potassium is reversed and then the values tend towards those normally observed for these constituents in milk. The increase in the yields of lactose, fat and casein shows that synthesis has commenced again. These observations suggest that the marked difference in concentration of sodium, chloride and potassium between blood and milk may be related to the synthetic activity of the alveolar cells.

Histological studies with small laboratory animals and heifers have shown that the most rapid growth of the mammary gland occurs during the first two-thirds of pregnancy and that in the period immediately before parturition there is little development of the gland (see Hammond, 1927; Elliott & Turner, 1953, for references). However, there appears to be little information on the changes in the mammary gland cellular structure of cows lactating during the greater part of pregnancy. Altman (1945) observed that, in lactating cows, mitosis is rare and concluded that a dry period is important to allow for the rapid regeneration of the secretory epithelium before the next lactation. This conclusion was confirmed by Swanson (1965) and Smith, Wheelock & Dodd (1966) who found that milk yields after parturition were reduced by maintaining lactation throughout the whole of the previous pregnancy. It is probable, therefore, that in cows which lactate for the greater part of pregnancy the development of the mammary gland occurs in the dry period when there is little or no synthetic activity.

The presence of colostrum in the mammary gland at parturition confirms that secretory activity of the alveolar cells occurs in the period before parturition in the absence of any milking stimulus. The present results show that this activity begins several weeks before parturition.

Our results also confirm the findings of Larson (1958) that large quantities of globulins are transferred into and accumulate in the mammary gland in the weeks before parturition. Invariably the concentration of proteose-peptone + globulin in the mammary gland secretion 1–3 weeks before parturition was higher than in the colostrum. This suggests that in the days immediately before parturition there was a rapid increase in the secretory activity of the alveolar cells coupled with a movement of water into the gland which diluted the globulins already present.

We are grateful to Miss M. Weston, Miss S. Futcher and Miss E. Jenkins for skilled technical assistance.

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Estimation by gas chromatography of 5β-pregnane-3α, 20α-diol in the urine of normal lactating and ketosed cows

By R. J. HEITZMAN

*Department of Anatomy, University of Birmingham**

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Summary. The measurement by gas chromatography of pregnanediol in the urine of normal and ketosed cows in the first 3 months of lactation is described. Mean pregnanediol levels, corrected for recovery losses, are reported for 3 groups of cows, namely: normal lactating cows 30·7 μg/l urine; experimentally induced ketosed cows 22·3 μg/l urine; and primary spontaneously ketosed cows, 31·4 μg/l urine. No significant differences between the 3 groups of animals were found.

Balfour, Comline & Short (1957) stated that the major source of circulating progesterone in the cow was the adrenal gland. Thus, the assay of blood progesterone or one of its major catabolites, e.g. urinary 5β-pregnane-3α,20α-diol (pregnanediol) would give some indication of adrenal function with respect to progesterone secretion. At Compton, interest has been centred on the disease bovine ketosis, and one hypothesis being studied is the concept of bovine ketosis as a pituitary adrenal syndrome as proposed by Hatziolos & Shaw (1950) and reviewed by Shaw (1961). If adrenal function in ketosis is impaired, then the levels of adrenal steroids secreted and thus the rate of gluconeogenesis would be decreased. This present investigation deals with the assay of urinary pregnanediol in an attempt to correlate ketosis with adrenal function.

The application of gas chromatography to the estimation of pregnanediol has been reported by several workers (Cox, 1963; Wotiz, 1963; Lau & Jones, 1964). Heitzman & Thomas (1965*a*) reported the estimation of pregnanediol in the urine of pregnant cows by a suitable gas chromatographic modification of the assay procedure of Klopper, Michie & Brown (1955). This method has now been extended to the assay of pregnanediol in the urine of normal and ketosed cows in the first 3 months of lactation.

The availability of neutral non-ketone fractions prepared in the course of other experimental work (Heitzman & Thomas, 1965*a*, *b*; Heitzman & Hibbitt, unpublished) made it possible to measure the levels of pregnanediol in the urine of ketosed and normal animals.

* Present address: Institute for Research on Animal Diseases, Compton, Berks.

METHODS AND RESULTS

Details concerning the animals used in this study are described in Table 1. Ketosis was induced in the 4 cows by the method of Hibbitt, (1964, 1966). Cows with primary spontaneous ketosis were purchased from farms in the vicinity of Compton. Blood samples (20 ml) were taken on the day of urine sampling and blood 'ketone body' levels were measured by the method of Dumm & Shipley (1946). The ketone body levels, which are shown in Table 1, were an indication of the degree of ketosis in the cows.

Collection and fractionation of urine. The urines of the normal lactating cows were collected for 24 h as described by Heitzman & Thomas (1965*a*). Because time was not sufficient to accustom the ketosed animals to wearing a harness, the samples were taken during micturition. The samples were collected at 2–5 p.m. from successive micturitions until the volume exceeded 2 l and stored at −15 °C in the presence of toluene (5 ml), until required.

Table 1. *Levels of urinary pregnanediol and blood ketone bodies in lactating and ketosed cows*

Group of cows	Days since parturition	Blood ketone bodies, mg %	Pregnanediol, μg/l urine	Mean value pregnanediol, μg/l urine	Breed
Normal lactating	4*	< 10 for all cows	4·3	7·55† 30·7‡	A
	11*		5·3		A
	18*		8·9		A
	24*		7·2		A
	32*		14·7		A
	46*		11·9		A
	94*		5·3		A
	16		6·4		A
	27		3·9		A
Experimentally induced ketosis	21	30	7·3	5·45† 22·3‡	A
	26*	125	7·7		A
	27*	125	3·5		A
	31	75	3·4		A
Primary spontaneous ketosis	16	50	11·3	7·64† 31·4‡	F
	24	60	5·2		G
	38	45	6·4		F

* The figures refer to samples taken from a single animal at different stages of lactation.
† Uncorrected for recovery losses.
‡ Corrected for recovery losses.
A = Ayreshire; F = Friesian; G = Guernsey.

Samples (1·6 l) of the urine were extracted and fractionated into water-soluble, phenolic, and neutral fractions as described by Cooke, Rogers & Thomas (1963). The neutral fraction was divided into a ketone and non-ketone fraction with Girard T reagent. The non-ketone fraction contained the pregnanediol.

Klopper assay procedure. 5% of the non-ketone fraction was dissolved in 100 ml toluene and shaken for 10 min with 50 ml freshly prepared solution of 4% (w/v) potassium permanganate in N sodium hydroxide. The procedure from this point was as described by Klopper *et al.* (1955), but instead of measuring the pregnanediol

diacetate obtained from the alumina column by their method a gas chromatographic analysis was used.

Gas chromatographic analysis. The fraction containing pregnanediol diacetate was evaporated to dryness under nitrogen and an internal standard, androst-4-en-3,17-dione (10 μg) added in 0·1 ml acetone. About one-sixth of the sample was applied to the gas chromatography column by the method of solid injection described by Heitzman & Thomas (1965*a*). A typical chromatogram is shown in Fig. 1. The apparatus was a Pye Panchromatograph with a strontium-90 detector. The column was glass (150 cm × 4 mm) and was packed with 3% QF–1 on siliconized celite (100–120 mesh). The operating temperatures were 232 and 250 °C, respectively, for the column and detector ovens with a flow rate of the argon carrier gas of 45 ml/min. Under these conditions, retention times of about 4 min were observed for 5α-cholestane and 15–17 min for pregnanediol diacetate.

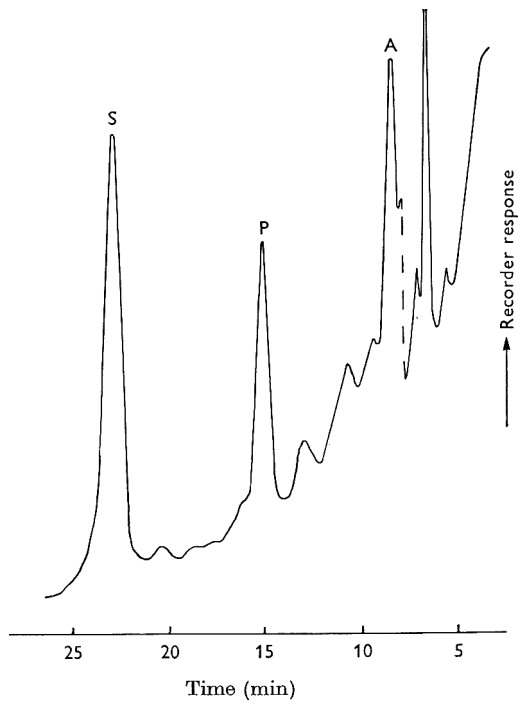

Fig. 1. Typical gas chromatogram of normal lactating cow 46 days after parturition. Key: P, 5β-pregnane-3α,20α-diol diacetate; S, androst-4-en-3,17-dione added as internal standard; A, 5β-androstane-3α,17α-diol diacetate which was present in the extracts from all the cows. The dotted line represents a change in the sensitivity of the detector. Column packing, 3% QF-I on siliconized celite (100–120 mesh).

The areas of the pregnanediol diacetate and androstenedione peaks were measured by the method of triangulation. In order to ascertain whether the standard and the pregnanediol diacetate gave a differing response in the detector, a number of peak area measurements were carried out. The standard and diacetate were made into 5 mixtures containing 4·0 and 1·25, 2·0 and 1·25, 2·0 and 2·0, 1·0 and 2·0, 2·0 and 2·5 μg, respectively. Each mixture was chromatographed and the area of each peak calculated. The area response per μg of the diacetate compared with the

standard was within the range 0·85–1·04 with a mean value of 0·93. Applying this correction the quantity of pregnanediol expressed as the diacetate in cow's urine was given by the expression:

$$\frac{1}{0{\cdot}93} \times \frac{Ap}{As} \times \frac{1000}{V} \times \mathrm{S}\ \mu\mathrm{g/l},$$

Where Ap and As are the areas of the peaks for pregnanediol diacetate and standard respectively, V is the volume in ml of urine assayed (80 ml) and S is the quantity in μg of the standard added (10 μg). The pregnanediol values calculated using this expression are shown in Table 1. The values are uncorrected for overall recovery losses.

The overall recovery of pregnanediol from the urine was measured by evaluating the recovery of pregnanediol (150 μg) added to urines (1·61). Blank determinations on samples of the same urine were carried out. The mean recovery for 5 evaluations was 31 % and the range 29–34 %. This value was unexpectedly low and could be attributed to the initial extraction process, since the recovery of pregnanediol diacetate (5 μg/assay) added to the neutral non-ketone fractions was 94·6–99·5 % with a mean of 97·5 %.

DISCUSSION

Methods of analysis of steroids in biological fluids need to be rapid, reproducible and capable of detecting very small quantities of steroid. The use of gas chromatography in conjunction with other separation techniques often provides the ideal answer to these problems.

In the present study the strontium-90 detector was capable of the accurate determination of 0·1 μg of pregnanediol diacetate. Many such detectors vary considerably in sensitivity, and experience has shown that the normal limiting sensitivity is about 0·5 μg and higher sensitivity is but rarely achieved. Using a very sensitive detector no difficulty was experienced in estimating 5 μg/l (uncorrected for losses) urine.

5β-pregnane-3α,20α-diol has been detected in the urine of late pregnant cows by earlier workers (Marker, 1938: Klyne & Wright, 1959; Heitzman & Thomas, 1965*a*), but its presence in the urine of lactating cows has not been reported.

Heitzman & Thomas (1965*a*) reported levels of urinary pregnanediol of 200 μg/l in the urine of late pregnant cows, whereas using a similar method, the highest value obtained for the lactating cow in the present study was 14·7 μg/l and the mean of the normal samples was 7·55 μg/l. This was compatible with the fall in levels (about 10-fold) of progesterone in the peripheral plasma of the cow during the last month of pregnancy reported by Short (1958). Unfortunately, Short seems only to have recorded the levels in lactating cows, which were also pregnant.

The urines from the normal animals were collected over 24 h. However, it was not possible to collect 24-h urine samples for the ketosed animals. In order to obtain comparable results for the spontaneous and induced ketosed animals, each sample (2 l) was collected between 2 and 5 p.m. No assessment was made of the diurnal variation of pregnanediol excretion. This, however, did not detract from the value of the results for the ketosed cows since they quite clearly showed that

pregnanediol was excreted. The levels were similar to those measured in the 24-h samples of the normal animals. If in ketosis the pituitary adrenal system is severely impaired, then it would be expected that the levels would have been considerably lower. The prevention of ketosis in the cow, however, may demand higher than normal levels of secretion of steroids by the adrenal gland. That the gland does not attain these higher levels may arise from an insufficient secretion of ACTH by the pituitary gland.

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Occurrence of milk proteins in the urine of cows during an extended milking interval

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

(*Received* 12 *September* 1966)

Summary. Immunological methods have been used to test samples of urine from 5 cows for the presence of milk proteins. None could be detected when the cows were milked twice daily at the usual intervals, but during an extended milking interval α-lactalbumin was found in the urine of all 5 cows and β-lactoglobulin in the urine of 2 cows. The urine of one cow during and after a milking interval of 39 h contained 1·63 g α-lactalbumin, 1·12 g β-lactoglobulin and a small amount of casein. One of the factors affecting the transfer of these milk constituents from the udder to the urine appears to be their molecular weight.

Lascelles (1961, 1962) showed that, in the lactating ewe, milk proteins can pass to the lymph when the suspension of milking has caused distension of the udder. More recently Wheelock & Rook (1966) observed that the concentration of lactose in the blood of lactating cows rose sharply during an extended milking interval and that considerable amounts of lactose were excreted in the urine. Some of these samples of blood and urine have now been examined by immunological methods for the presence of milk proteins, and the results are reported here.

EXPERIMENTAL

The samples of urine were from cows 1–5 of Wheelock & Rook (1966). After milking at the usual 9-h day and 15-h night intervals, milking was suspended for 39 h except for cow 4 for which the period was 24 h; milking was then resumed at the usual intervals. The entire output of urine was collected during these periods; the blood samples were from cow 5.

Specific antisera to α_{s1}-casein, β-casein, and β-lactoglobulin were prepared from rabbits by the procedure recommended by Gell & Coombs (1963); anti-α-lactalbumin serum was obtained commercially. The ring test (Campbell, Garvey, Cremer & Sussdorf, 1964) was used as a qualitative method for the presence of milk proteins; for all 4 proteins, the lowest detectable concentrations were of the order of 1 mg/l. Quantitative methods for β-lactoglobulin and α-lactalbumin in urine samples were based on Oudin's method, using the procedures described by Larson & Twarog (1961) and Larson & Hageman (1963) except that the standard solutions were diluted with a sample of cow's urine known to be free of milk proteins, and that all tubes were incubated in an inverted position.

RESULTS

Milk proteins were not detected in any sample of urine collected during the control period of 24–48 h before milking was suspended. Urine from cows 1, 2 and 3 was collected at intervals of 12 h throughout the experiment, and α-lactalbumin was found in at least one sample of urine from each cow; β-lactoglobulin was found only in the urine of cow 3.

Milking of cow 4 was suspended for 24 h, and urine samples were collected every hour. The concentrations of α-lactalbumin in these samples were measured and the total amount excreted was 0·09 g; however, β-lactoglobulin could not be detected in the urine of this cow.

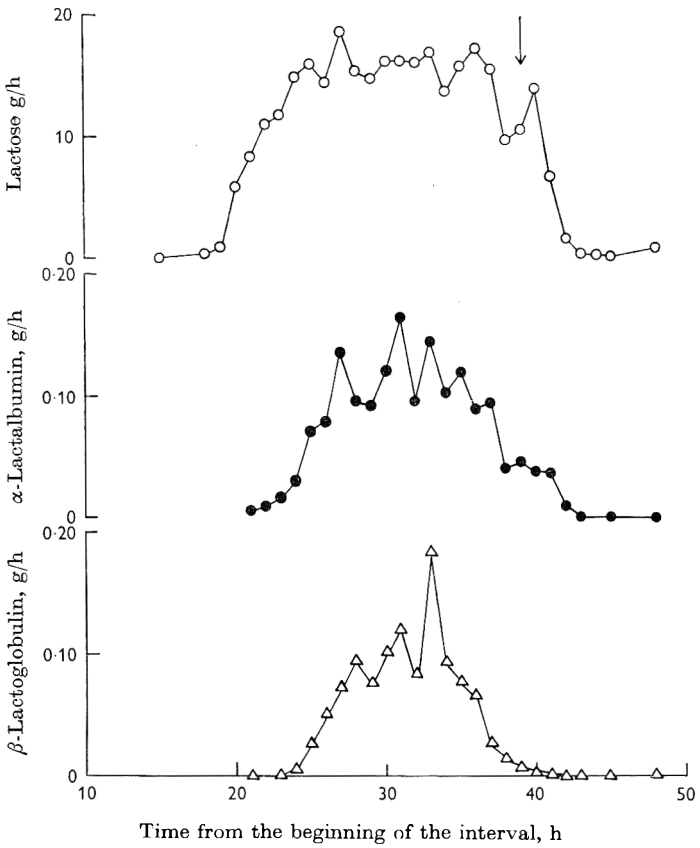

Fig. 1. Excretion of lactose and protein in g/h in the urine of cow 5 during an extended milking interval. The lactose results are those shown in Fig. 2. of Wheelock & Rook (1966). ○, lactose; ●, α-lactalbumin; △, β-lactoglobulin; ↓, cow milked.

Urine samples from cow 5 were also collected hourly; the period of the extended milking interval was 39 h. Both α-lactalbumin and β-lactoglobulin were found in the urine of this cow; the hourly excretion is shown in Fig. 1, together with the corresponding values for lactose in the same samples found by Wheelock & Rook (1966). In general, excretion of the whey proteins followed a pattern very similar

to that shown by lactose, except that the proteins were slower to appear in the urine and disappeared earlier.

The urine samples from cow 5 were also examined by the ring-test for the presence of α_{s1}- and β-casein. Positive reactions for both proteins were observed in all samples collected 27–40 h after milking was suspended. This period coincides closely with that for maximum output in the urine of the other milk constituents; however, the reactions were weaker than those observed with ring-tests for the whey proteins. Samples of blood plasma from this cow were also tested with the antisera and some weak or doubtful reactions were observed, but no milk proteins could be detected with certainty in most of the samples taken while milk proteins were being excreted.

DISCUSSION

These results show that α-lactalbumin and occasionally other milk proteins appear in the urine of the lactating cow during an extended milking interval, and presumably more sensitive methods would lead to the detection of milk proteins in the blood during such an experiment. Comparison of our failure to detect milk proteins in blood samples, implying concentrations of not more than 1 mg/l, with the values for lactose of 320–360 mg/l found in the same samples by Wheelock & Rook (1966), shows that lactose is lost from the mammary gland much more readily than milk protein.

Table 1. *Total amounts of milk constituents excreted in the urine of cow 5 compared with average daily yields in the milk before milking was suspended*

Milk constituent	Average daily yield, g/24 h	Amount excreted in the urine, g	Molecular weight
Casein	360*	< 2	> 10^6†
β-Lactoglobulin	40*	1·12	36000
α-Lactalbumin	21*	1·63	16000
Lactose	708	304	342

* See text.
† Assuming casein to be in micellar form.

The amounts of milk protein in the urine are small relative to the amounts of lactose excreted, as may be seen from Table 1 where the total amounts found in the urine of cow 5 are compared with the average daily yield before milking was suspended. The values for the yield of proteins in milk are only a rough estimate, since they were calculated by assuming that the milk contained 25 g/l casein, 2·8 g/l β-lactoglobulin and 1·5 g/l α-lactalbumin. Table 1 also shows the molecular weights of the milk constituents and consideration of these, even if lactose is excluded, suggests that low molecular weight facilitates transfer from the mammary gland to the urine. This would be expected for the transfer of proteins from the blood stream to the urine, since it has been known for many years that foreign proteins of high molecular weight, when introduced into the blood stream, are less readily excreted by the kidney than those of low molecular weight (Bayliss, Kerridge & Russell, 1933).

We are grateful to Miss P. Cowley for skilled technical assistance and to Dr M. P. Thompson (Philadelphia) for the specially purified samples of α_{s1}-casein and β-casein used in preparing antisera.

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The production of L(+) and D(−) lactic acid in cultures of some lactic acid bacteria, with a special study of *Lactobacillus acidophilus* NCDO 2

By ELLEN I. GARVIE

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 16 *September* 1966)

SUMMARY. A study of the production of L(+) and D(−) lactic acid in cultures of DL forming lactic acid bacteria has shown that they can be divided into 2 groups. The first includes the thermobacteria and *Pediococcus cerevisiae* in which the percentage of L(+) lactic acid is high initially but decreases as the cultures grow. The second includes *Lactobacillus plantarum* and the heterofermentative lactobacilli and in cultures of these species the percentage of L(+) acid changes little during growth and is generally below 50% of the total.

In a strain of *Lactobacillus acidophilus* it has been found that if the pH is kept constant during growth the proportion of L(+) lactic acid decreased. However, when acid production stopped the proportion of total acid which is the L(+) isomer is greater than the proportion at the same stage of development in cultures where the pH was not adjusted. Cells harvested from young cultures formed more L(+) acid than cells harvested from older cultures.

There is some evidence that the composition of the medium can affect the ratio of L(+):D(−) lactic acid.

The isomeric type of lactic acid formed by lactic acid bacteria is of taxonomic importance but until the recent development of enzymic determinations of L(+) lactic acid and D(−) lactic acid (van den Hamer & Elias, 1958) the methods available were tedious and demanding in the quantity of culture required. The enzymic methods were tested on a number of known lactic acid bacteria, and the expected results were obtained. With leuconostocs (7 strains), group N streptococci (6 strains) and species of lactobacilli previously reported as forming only D(−) or L(+) lactic acid (12 strains) only one isomer was detected, and in those species of lactobacilli (37 strains) and pediococci (11 strains) which were reported to make DL acid, both isomers were found. In the DL forming bacteria the quantitative enzymic methods showed that equal amounts of both isomers were not present but indicated that there might be a relationship between the species and the proportion of L(+):D(−) lactic acid. Kitahara & Obayashi (1955) reported in a few strains of lactobacilli and pediococci, that one or other acid was predominant and suggested that this acid was formed direct from pyruvate and that the other isomer was formed by a racemization.

When trying the effect of different media on the production of lactic acids, it was observed that the proportion of L(+):D(−) acid was not constant for any one strain and also changed with the age of the culture and therefore a study was made of the production of both D(−) and L(+) lactic acid during the growth of a number of lactic acid bacteria.

METHODS

Source of cultures. The strains of lactic acid bacteria were from the National Collection of Dairy Organisms (NCDO).

The strain of *Escherichia coli* (NCDO 1246) used in the determination of D(−) lactic acid was received from Dr van den Hamer.

Growth of lactic acid bacteria. Cultures of lactobacilli were maintained in MRS broth (de Man, Rogosa & Sharpe, 1960). The thermobacteria and *Lactobacillus fermenti* were incubated at 37 °C and other strains at 30 °C. Pediococci were grown in a yeastrel-glucose-citrate broth (YGCB; E. I. Garvie, unpublished) and incubated at 30 °C. The addition of 0·5 ml of sterile 1·0% (w/v) cysteine hydrochloride to each 10-ml media was made prior to inoculation with strains of *Pediococcus parvulus* (Garvie, Gregory & Mabbitt, 1961).

For studying lactic acid production well-grown cultures were inoculated into EPB because neither MRS nor YGCB were suitable. The composition of EPB was (w/v) Evans peptone, 0·75%; glucose, 1·0%; Yeastrel, 0·25%; sodium acetate, 0·25%; KH_2PO_4, 0·25%; triammonium citrate, 0·1%; $MgSO_4 7H_2O$, 0·02%; $MnSO_4 4H_2O$, 0·005%; and (v/v) Tween 80, 0·1%; tomato juice, 10%. The final pH was 6·5 and the medium was sterilized by autoclaving at 15 lb for 15 min. EPB was inoculated at the rate of 1 ml of $\frac{1}{100}$th dilution of culture for every 250 ml of medium, and cultures were incubated at 30 or 37 °C according to species. A few strains were grown in litmus milk + 1% glucose and 0·3% Yeastrel and the whey examined for lactic acid.

Sampling cultures. Ten-ml samples of the cultures were taken at intervals which depended on the growth rate. The first sample was taken soon after growth became visible and the last sample about 24 h after the first. Generally, 4–5 samples were taken from each culture. The total time of incubation for the various cultures ranged from 35 to 48 h.

After measurement of pH the samples were placed in boiling water for 5 min, the cells removed by centrifuging and the supernatant stored at −20 °C until analysed.

Growth at constant pH. 120-ml quantities of EPB were inoculated with *L. acidophilus* NCDO 2 and incubated at 37 °C. The pH of the culture was then maintained by additions of N/1 KOH using an automatic titrator, (Pye Ltd., Cambridge). During incubation the culture was agitated by a magnetic stirrer. Air in contact with the surface of the culture inhibited growth but in an atmosphere of hydrogen—carbon dioxide, (90:10, v/v) good growth was obtained. An unstirred control culture in which the pH was not maintained constant was incubated in the same atmosphere.

Examination of cell suspensions. At least 100–120 ml of culture was used. In one experiment the samples were taken from 3000 ml EPB. On other occasions the cultures were grown in small volumes and the whole of the culture used at one time. The cells were removed by centrifuging, washed twice in $\frac{1}{4}$ strength Ringers solution and resuspended in a small volume of the same strength solution.

Tubes containing 2·5 ml buffer, 1 ml 50% glucose, 1 ml cell suspension and 0·5 ml water were prepared and incubated at 37 °C until a fall in pH indicated that acid had been formed. In experiments starting at pH 7·4, phosphate buffer (0·08 M) was used and in those starting at pH 5·0 sodium acetate/acetic acid buffer (0·2 M). After incubation the material was treated in the same way as culture samples.

Counts of L. acidophilus NCDO 2. Estimates of numbers were made on MRS agar plates incubated anaerobically at 37 °C for 3 days.

Optical density. The optical density of cultures was measured in $\frac{5}{8}$-in. test tubes in a Hilger Biochem absorptiometer using an orange (580 mμm) filter.

Estimation of lactic acid

Estimation of D(−) *lactic acid.* The method of van den Hamer & Elias (1958) modified by Garvie (1966) was used. Samples were diluted with water to contain between 0·6 and 1·6 mg D(−) lactic acid/ml and 0·5 ml used in the estimation.

Estimation of L(+) *lactic acid.* The final method used was developed from several published methods, it most closely resembled that given by Gercken (1960).

Solutions were prepared as follows:

(A) 0·4 ml of the solution prepared for analysis for D(−) lactic acid was diluted to 10 ml with water.

(B) As_2O_3 buffer (Gercken, 1960). 19·8 g As_2O_3 was dissolved in a small quantity of water containing 5·2 g NaOH and the volume made up to 1000 ml, pH 9·6. It was stored in a CO_2 free atmosphere.

(C) Hydrazine sulphate 0·1 M was neutralized with NaOH and stored in a CO_2 free atmosphere.

(D) DPN 20 mg/ml was freshly prepared as required.

(E) Lactic dehydrogenase was used as purchased and contained 5 mg protein/ml.

Two ml buffer (B), 0·1 ml hydrazine sulphate (C), 0·2 ml sample (A), 0·2 ml DPN (D) and 1 drop enzyme solution (E) were mixed together, incubated at 22 °C for 30 min and the absorption read at 340 mμm against water using a 10 mm light path. The L(+) lactic acid in the samples was calculated by reference to lithium lactate standards. Allowance was made for the absorption of a blank in which water replaced the solution A. The range of lactic acid estimated was 5–20 μg/test.

Standard lithium lactate. A standard lithium lactate solution containing 4 mg (w/v) lactic acid/ml was prepared and diluted as required for each test. Using L(+) lactate and D(−) lactate, extracted respectively from cultures of *Streptococcus cremoris* NCDO 924 and *Leuconostoc mesenteroides* NCDO 523, as standards, the lithium lactate was found to contain equal amounts of both isomers.

RESULTS

Choice of medium. The media in general use in this laboratory contained measurable quantities of L(+) lactate and were unsuitable for examining lactic acid production by bacteria. A number of common media ingredients were examined for total lactic acid and the results are given in Table 1. Much of the lactate found in media came from the Lemco and smaller quantities from peptone and Yeastrel. The high value for lactate in Lemco is in agreement with the observation of Wood & Bender (1957) who reported 14·6% lactate in a sample of meat extract.

Media using amino acids or casein digest as nitrogen source are without lactate but will not support good growth of all strains of lactic-acid bacteria. However, by replacing Lemco with tomato juice and using small amounts of other lactate containing substances it was possible to make a medium (EPB) which supported growth of many lactic acid bacteria and in which the initial lactate content did not interfere with the estimation of the lactic acid formed by bacteria.

Table 1. *Lactic acid* (%, *w/v*) *in ingredients of laboratory media*

Evans peptone*	5·6	Bacto peptone†	0·67
Bacto casitone†	1·74	Bacto protone†	0·33
Oxoid tryptone‡	1·52	Casamino acids†	0·28
Oxoid peptone‡	1·26	Lemco‡	9·0
Proteose peptone†	1·2	Panmede§	1·26
Neo peptone†	1·4	Liver infusion†	0·9
Bacto tryptone†	0·94	Yeastrel‖	0·28
Soya peptone‡	0·96	Tomato juice	0·034

Estimates on single samples of only one batch of each product.

* Evans Medical Supplies, Liverpool.
† Difco product.
‡ Oxoid product.
§ Paines and Byrne Ltd. Greenford, Middlesex.
‖ Brewers Food Supply Ltd.

Changes in the ratio of L(+):D(−) *lactic acid during growth of some lactic acid bacteria.* The percentage of L(+) lactic acid in cultures growing in EPB are given in Table 2. Only the observations on the first and final samples are given but where there is an alteration in the percentage of L(+) acid intermediate values showed that the change was continuous. Fig. 1 shows the relationship of pH and the percentage L(+) lactic acid in 4 separate cultures of *L. acidophilus* NCDO 2 grown at different times and under slightly varying conditions. In each culture the proportion of L(+) lactic acid decreased with increased total lactic acid but the percentage L(+) acid for any given pH value varied considerably.

The pattern of lactic acid production in the 17 strains studied can be divided into 2 types. The first type (A) is formed by a group of cultures which includes the thermobacteria and one strain of *L. fermenti* and *Ped. cerevisiae*; in it the percentage of L(+) lactic acid is high initially and drops as more acid is formed so that when the pH is minimal D(−) lactic acid is often predominant. The amount of L(+) acid in this group never fell below 40%. The second type (B) of lactic acid production was shown by *Lactobacillus brevis, buchneri, cellobiosus, fermenti,* (one strain) *viridescens, plantarum* and *Ped. parvulus.* Here, the percentage of L(+) lactic acid remained almost constant throughout the incubation time and it was often below 50%. In strains of *L. plantarum* and *L. viridescens* the L(+) acid was usually below 40%.

Other strains of *L. plantarum* and *viridescens* were grown in EPB or yeast glucose litmus milk and were examined for lactic acids once only when acid production had stopped. In these cultures also the L(+) lactic acid content was low (Table 3) and it appears that this is a characteristic of these 2 species of lactobacilli. The other species giving the B type of lactic acid production formed more L(+) acid than either *L. plantarum* or *viridescens* and generally more than 40% of the acid was the L(+) isomer.

Most of the species showing the type A lactic acid formation grew at 37 °C while most of those showing the type B formation grew at 30 °C. *L. acidophilus* NCDO 2 was examined while growing at 30 °C and *L. plantarum* NCDO 82 while growing at 37 °C. The temperature of incubation did not influence the pattern of lactic acid formation, but there is an indication that the formation of L(+) acid may be favoured by incubation at 37 °C (Table 2).

Table 2. *Quantity of lactic acid isomers in cultures of some* DL *forming lactic acid bacteria grown in a broth medium*

Species of culture	NCDO no	Temperature of incubation, °C	Age of 1st sample, h	First sample pH	First sample L(+)+D(−) lactic acid, mg/ml	Final sample* pH	Final sample* L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total: First sample	L(+) lactic acid, % total: Final sample
1. *Ped. cerevisiae*	813	30	9·15	4·9	2·9	3·5	13·2	74	44·5
2. *Ped. cerevisiae*	991	30	9·15	5·2	1·5	3·6	11·17	71·6	40
3. *L. acidophilus*	2	37	15·45	5·0	3·35	3·8	9·7	76	43·5
4. *L. helveticus*	30	37	15·45	5·65	2·59	3·8	11·64	81	65
5. *L. jugurti*	87	37	27·00	5·4	1·69	4·15	6·8	68	45
6. *L. jugurti*	103	37	19·00	5·7	1·38	3·85	10·24	58	65
7. *L. fermenti*	215	37	12·15	5·2	2·07	4·1	5·3	81	65
8. *L. fermenti*	218	37	12·15	5·9	0·93	4·05	6·55	43	41
9. *L. brevis*	23	30	22·30	5·4	1·55	4·1	4·9	58	48
10. *L. buchneri*	1652	30	18·30	5·45	1·9	4·3	5·65	26·5	33·5
11. *L. cellobiosus*	927	30	14·30	5·4	2·32	4·6	7·49	37·5	42·0
12. *L. cellobiosus*	928	30	14·30	5·1	3·46	4·5	6·75	41·3	35·5
13. *L. viridescens*	1655	30	19·00	5·2	3·74	4·35	8·82	27	29
14. *L. plantarum*	82	30	11·45	5·5	0·85	3·7	9·77	5·9	32·5
15. *L. plantarum*	363	30	13·45	5·0	1·48	3·7	9·53	11·5	32·0
16. *Ped. parvulus*	1558	30	18·30	5·1	3·08	3·85	12·78	43	45
17. *Ped. parvulus*	1559	30	18·30	4·7	2·62	3·8	8·66	46	38·5
L. acidophilus	2	30	24·00	5·05	4·66	3·9	14·7	66	45·5
L. plantarum	82	37	10·30	5·0	1·65	3·6	15·35	54	42·5

* Final sample taken approximately 24 h after the first sample.

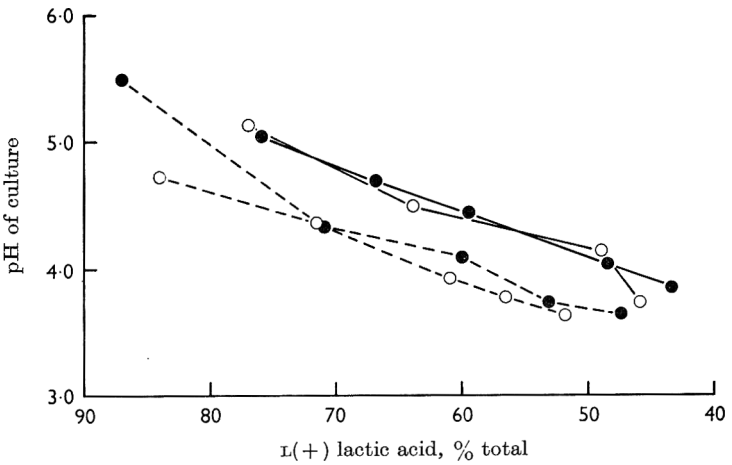

Fig. 1. The percentage of L(+) lactic acid at different pH values of cultures of *Lactobacillus acidophilus* NCDO 2 grown in Evans peptone broth. ●—●, grown under air, initial pH 6·7; ○—○, grown under H_2+CO_2, initial pH 6·7; ● - - - ●, grown under H_2+CO_2, initial pH 6·7; ○ - - - ○, grown under H_2+CO_2, initial pH 6·0.

The effect of pH on the ratio of L(+): D(−) *lactic acid produced by* L. acidophilus *NCDO* 2. As the conditions under which the cultures were growing appeared to influence the production of lactic acids, the effect of one variable, pH, was examined by growing *L. acidophilus* NCDO 2 at pH values of 6·7, 6·0 and 5·0. At pH 6·7 growth was slow, but at pH 5·0 it was so slow that the experiment was abandoned. At pH 6·0 the culture grew rapidly and acid was formed at the same rate as in the control. Table 4 shows the estimations of lactic acid in samples from cultures growing at pH 6·0 and 6·7. When the pH of a culture is maintained at 6·0 or 6·7 there is a drop in the percentage of L(+) lactic acid as the culture ages but the decrease is less than that in the control culture in which the pH is not maintained constant.

Table 3. *Percentage* L(+) *lactic acid formed by some cultures of* L. plantarum *and* L. viridescens *grown in Evans peptone broth and yeast glucose litmus milk*

	Evans peptone broth		Yeast glucose litmus milk	
Strain no.	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total
	L. plantarum			
NCDO 82	9·7	40	9·6	43
NCDO 343	8·25	41	9·8	40
NCDO 353	—	—	6·9	39
NCDO 363	—	—	9·0	33
	L. viridescens			
SMIA	—	—	5·9	13
NCDO 1655	—	—	7·8	34
S 40A	—	—	4·7	34
NCDO 403	—	—	3·5	20
NCDO 1179	4·2	29	5·5	35
NCDO 1180	4·1	15·5	4·8	24
NCDO 1613	—	—	5·4	37
NCDO 1615	—	—	5·4	26

It was found in this experiment that while the optical density of the culture continued to increase the plate colony count decreased, but microscopic examination did not show chaining or clumping of the cells. This problem was not further examined.

A comparison of the lactic acid in a culture of L. acidophilus *NCDO* 2 *and that formed by cell suspensions of the same organism*

Additional information on the formation of lactic acid was sought by studying cells harvested from cultures of different ages and grown under different conditions.

L. acidophilus NCDO 2 was grown in 3000 ml of EPB and samples of 250 ml were taken at intervals for the preparation of cell suspensions. Table 5 shows the amounts of L(+) and D(−) lactic acid formed in the broth and by the cell suspensions in tests starting at both pH 7·4 and 5·0. The ratio of L(+):D(−) acid formed by the cell suspensions was not appreciably affected by the pH at which the reaction started. During incubation the pH of the solution dropped, the fall was only slight (0·2–0·3 units) when the initial pH was 5·0 but greater (1·15–2·6) when it was 7·4. Since there are marked differences between the percentage L(+) lactic acid formed under

identical conditions but by cells harvested at different times, it seems that the ratio of the isomers formed is a function of the cells and not influenced by the pH of the external liquid.

Table 4. *Lactic acid production in cultures of* L. acidophilus *NCDO* 2 *grown in Evans peptone broth at constant pH*

	Culture kept at constant pH					Control culture				
Initial pH	Age of sample, h	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total	OD	Colony count $\times 10^{-6}$/ml	Age of sample, h	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total	OD	Colony count $\times 10^{-6}$/ml
6·6	13·15	2·31	91	—	—	10·30	2·18	87	—	—
	31·30	7·91	72·5	—	—	16·00	5·58	60	—	—
	55·0	13·50	67·5	—	—	37·15	10·6	47·5	—	—
6·0	10·30	2·61	85	53	45	10·30	3·25	84	62	54
	14·40	7·7	80	100	38	14·40	7·65	61	108	73
	17·40	11·45	73	120	38	17·40	9·05	56·5	120	48
	33·30	12·7	58·5	100	6	33·30	13·65	52	150	22

Each culture grown in 120 ml Evans peptone broth and 10-ml samples withdrawn at each sampling.

Table 5. *The formation of* L(+) *and* D(−) *lactic acid by cell suspensions of* L. acidophilus *NCDO* 2

			Original broth culture			Cell suspension starting pH 7·4		Cell suspension starting pH 5·2	
Age of sample, h	pH	OD	Colony count, $\times 10^{-5}$/ml	L(+)+D(−) lactic acid, mg/ml of original culture	L(+) lactic acid, % total	L(+)+D(−) lactic acid, mg/ml of test solution	L(+) lactic acid, % total	L(+)+D(−) lactic acid, mg/ml of test solution	L(+) lactic acid, % total
18·00	5·8	43	168	1·5	83	2·65	74·5	2·1	80·0
20·30	5·2	57	155	2·38	87	2·8	65·5	1·65	72·5
23·30	4·85	58	57	3·39	71	2·6	59·5	1·9	63·5
41·30	4·45	87	9	5·78	61·5	1·9	50·5	1·4	50·5

The culture was grown in 3000 ml EPB and 250-ml samples withdrawn at each sampling.

Cells formed during growth at constant pH were harvested from 120-ml amounts of culture. Cells were obtained from both young and old cultures (Table 6). Cells from cultures containing much L(+) lactic acid also form from glucose more of this acid than of the D(−) isomer. As in the earlier experiment, the percentage L(+) acid formed by the cell suspensions is less than in the corresponding culture, cells harvested later in the growth of the culture form more D(−) acid than those harvested earlier from the same culture, but when the culture is maintained at pH 6·0 cells from the mature culture form more L(+) acid than those harvested from the control culture in which the pH was allowed to fall.

DISCUSSION

The present knowledge of the lactic dehydrogenases of the lactic acid bacteria is limited to a study of *L. plantarum*. Dennis & Kaplan (1960) isolated 2 DPN dependent lactic dehydrogenases from the strain they examined while Snoswell (1959),

Table 6. *Comparison of the percentage of* L(+) *lactic acid formed by cultures of* L. acidophilus *NCDO 2 in Evans peptone broth and by cells harvested at the same time*

	Cultures kept at pH 6·0			Control culture initial pH 6·6		
*Age of sample, h	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total	L(+) lactic acid formed by cell suspension, % total	L(+)+D(−) lactic acid, mg/ml	L(+) lactic acid, % total	L(+) lactic acid formed by cell suspension, % total
9·30	2·52	87	68	1·9	90	75
16·15	11·45	77·5	63	9·45	57	52
33·30	12·7	58·5	67·5	13·65	52	51

* Three separate experiments. Each was stopped at the time indicated and the cells harvested.

working with a different strain, found 2 DPN independent enzymes. Neither paper reports any evidence of racemase activity. Dennis & Kaplan (1963) report racemase activities in a strain of *Clostridium butylicum*.

The ratio of L(+):D(−) lactic acid formed by cells of *L. acidophilus* NCDO 2 is influenced both by the age of the culture and by the pH of the medium from which the cells are harvested. Old cultures and low pH both favour the formation of D(−) lactic acid. No explanation of these observations is apparent. Other factors may help to determine which isomer is formed. In one experiment when *L. acidophilus* NCDO 2 was grown in a milk medium it was observed that the percentage of L(+) lactic acid increased from 64·5 after $16\frac{1}{2}$ h to 85 after 41 h incubation at 37 °C.

It is not possible yet to control the formation of L(+) and D(−) lactic acid in cultures but it is likely that broth cultures of *L. acidophilus* NCDO 2 in which the pH is high for any reason will contain predominantly L(+) lactic acid. The same is probably true of other strains of lactic acid bacteria showing the same type of lactic acid formation.

Kitahara & Obayashi (1955) suggested that the formation of D(−) and L(+) lactic acids in lactic acid bacteria forming both isomers could be used as an additional character in classification. Only a few strains of any species have been examined but the results appear to be useful in supporting separation into species using other criteria.

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A method of detecting dissimilation of citrate by lactic acid bacteria using *Streptococcus lactis* var. *diacetilactis* NCDO 1007

By ELLEN I. GARVIE

National Institute for Research in Dairying, Shinfield, Reading

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Summary. In suitable media the relationship between the citrate content and the acetoin formed by the growth of *Streptococcus lactis* var. *diacetilactis* NCDO 1007 is linear. An assay method for citrate is described which is based on this relationship. It has been used to assay the residual citrate in cultures of lactic acid bacteria and, therefore, to assess the ability of these bacteria to dissimilate citrate. Some suitable media for the test are described.

INTRODUCTION

Str. lactis var. *diacetilactis* converts citrate to acetoin via pyruvate. The reaction has been examined in some detail (Mizuno & Jezeski, 1961; Harvey & Collins, 1961, 1963).

The efficient conversion of citrate to acetoin by *Str. lactis* var. *diacetilactis* suggested that this organism might be used to detect residual citrate in bacteriological media after the growth of other lactic acid bacteria. The chemical methods available for citrate determination are either too tedious or otherwise not suitable for routine application in a bacteriological laboratory. The problem of estimating citrate bacteriologically was therefore investigated.

METHODS

Cultures. For the assay *Str. lactis* var. *diacetilactis* NCDO 1007 was selected as the most suitable strain because it formed large amounts of acetoin which remained constant if incubation of the fully grown culture was prolonged by as much as 24 h. It was maintained by daily subculture in litmus milk incubated at 22 °C.

Cultures examined for citrate dissimilation (Table 3) were all from the National Collection of Dairy Organisms and cultured under conditions known to give good growth.

Estimation of citrate dissimilation by lactic acid bacteria

The bacteria were grown in a medium containing citrate, the cells removed and the residual citrate estimated using *Str. lactis* var. *diacetilactis* NCDO 1007. Each test, therefore, required two media, a 'citrate-rich' medium in which the strains

under test were cultured, and an assay medium to which culture fluid from the 'citrate-rich' medium was added and in which NCDO 1007 was grown. Several different media were used successfully. The composition of the 'citrate-rich' medium was varied to obtain good growth of the species being examined and, for reasons discussed later, the assay medium was varied to partner the 'citrate-rich' medium (Table 2). Table 1 lists some of the media which were used, and Table 2 the combinations in which they were used and the bacteria tested in the 'citrate-rich' media.

Table 1. *Media used in the study of citrate dissimilation by bacteria*

Media for growing lactic acid bacteria ('citrate rich' media)

(A) (i) Skim-milk
(ii) Skim-milk + 0·3 % Yeastrel + 1·0 % glucose

(B) Evans peptone 1·0 %, Yeastrel 0·5 %, Lemco 1·0 %, $MgSO_4.7H_2O$ 0·02 %, $MnSO_4.4H_2O$ 0·005 %, pH 6·5
(i) Glucose 1·0 %, triammonium citrate 0·4 %
(ii) Glucose 0·2 %, triammonium citrate 1·0 %

(C) Evans peptone 1·0 %, Yeastrel 0·5 %, glucose 1·0 %, $MgSO_4.7H_2O$ 0·02 %, $MnSO_4.4H_2O$ 0·005 %, Tomato juice 25 %, triammonium citrate 1·0 %, pH 4·8

Media for estimating residual citrate with NCDO 1007. Assay media (final concentration in assay)

(X) Bacto tryptone 1·0 %, Yeastrel 0·5 %, Lemco 1·0 %, glucose 1·0 %, K_2HPO_4 0·5 %, $MgSO_4.7H_2O$ 0·02 %, $MnSO_4.4H_2O$ 0·005 %, pH 6·8

(Y) (i) Bacto tryptone 0·1 %, Yeastrel 0·05 %, glucose 0·1 %, pH 6·8
(ii) Bacto tryptone 0·5 %, Yeastrel 0·25 %, glucose 0·5 %, pH 6·8.

Table 2. *Media and methods for examining citrate dissimilation by lactic acid bacteria*

Method	'Citrate-rich' medium used for growing lactic acid bacteria	Bacteria tested	Incubation time, days	Assay medium used for NCDO 1007: Basal medium	Assay medium used for NCDO 1007: Vol. of culture fluid used, ml/ml assay media	Assay medium used for NCDO 1007: Additional citrate added, mg/ml
I	B (i)	Group N streptococci	5–7	X	0·4	1·5 and 3·0
II	B (ii)	Leuconostocs,		X	0·6–0·1	None
III	A (i) or A (ii)	Pediococci,		X	0·8	1·5 and 3·0
IV	A (i) or A (ii)	Lactobacilli		Y (i)	0·2	0·125 and 0·25
V	C	*Leuc.* acidophilic sp.	4–7	Y (ii)	0·6–0·2	None

Bacteria were grown in the 'citrate-rich' media at a suitable temperature and usually for several days to ensure that as much citrate as possible was utilized. The cultures were centrifuged, the supernatants (culture fluids) decanted and adjusted to pH 6·8 with N/1 NaOH. (In the case of media A (i) and A (ii), Table 1, the curd was spun down and the whey examined.)

The assay medium was prepared at 5 times the required strength and 1 ml measured into each of a series of test tubes. A measured volume of culture fluid was added to each tube and the volume made up to 5 ml with water, or with a standard solution of ammonium citrate. The tubes were sterilized by autoclaving (15 lb for 15 min). They were inoculated with 1 drop of $\frac{1}{100}$ dilution of a 24-h milk culture of NCDO 1007, and incubated at 30 °C overnight. A 'citrate-rich' medium

control was included in every batch, to determine the level of acetoin formed from the citrate in the 'citrate-rich' medium by the assay organism.

Acetoin production is not confined to *Str. lactis* var. *diacetilactis* and therefore it was necessary to determine the amount of acetoin added with the culture fluid to the assay medium with every strain tested. This blank value was estimated using the largest volume of culture fluid tested.

Estimation of acetoin and diacetyl. The method of Eggleton, Elsden & Gough (1943) was used. The red colour of the Voges–Proskauer test was either estimated on a Spekker absorptiometer (Hilger and Watts, London) using a green 604 filter or on a Unicam (SP 500) at 520 mμm with a 10-mm light path.

RESULTS

The estimation of acetoin and diacetyl. Estimations of aqueous solutions of acetoin or diacetyl showed that the linear relationship of molecular concentration to the light absorption of the pink diacetyl-creatine-α-naphthol complex was the same in each case. The minimum quantity of diacetyl which gave a pink colour was 3 μg.

The interference of organic materials in estimations of diacetyl or creatine was reported by Eggleton *et al.* (1943). In accordance with this observation it was found that when medium X was in the test solution the pink colour was reduced in intensity but the relationship between concentration and absorption was still linear. The decrease in colour intensity/μg diacetyl depended on the amount of medium X present and was calculated to be about 8% for 50 μg diacetyl and 0·1 ml medium X. However, the presence of 1 ml whey did not affect the colour intensity.

Acetoin production from citrate and pyruvate by Str. lactis *var.* diacetilactis *NCDO* 1007. NCDO 1007 was grown in medium X with different amounts of ammonium citrate and sodium pyruvate. After overnight incubation at 30 °C the cultures were examined for acetoin. The results are given in Fig. 1 and show there was an increase in acetoin with increasing amounts of both citrate and pyruvate. In neither case does the curve pass through the origin, and in confirmation of the observations of Harvey & Collins (1963) it appears that not all of the pyruvate is converted into acetoin. In this medium, 1 mg/ml of citrate or pyruvate are the minimum quantities which give detectable amounts of acetoin, when 0·05 ml culture is examined.

Citrate dissimilation by bacteria. The residual citrate after growth of various bacteria was determined using suitable combinations of media (Table 2). Methods I and III, Table 2, were tried adding citrate, as well as culture fluid from 'citrate-rich' media, or control media, to the assay medium to determine the effect of culture fluid or 'citrate-rich' medium on acetoin production in the assay. Figs. 2 and 3 show that NCDO 1007 formed the same amount of acetoin from citrate despite the differences in composition between the control and culture fluids and this was true of all the cultures examined by methods I and III (Table 3).

Figs. 2 and 3 also show the results obtained with 2 strains of *Leuconostoc dextranicum* (NCDO 517 and 529), neither of which formed detectable acetoin in the 'citrate-rich' medium at the level tested, despite the fact that one NCDO 529 dissimilated citrate. The results with a strain of *Str. lactis* var. *diacetilactis* NCDO 176 are also given. In this case, because acetoin was produced in the 'citrate-rich' medium, the

curve is almost the same as the control, but the acetoin added with the culture fluid did not affect further acetoin production from citrate by the assay culture. When the acetoin added with the culture fluid to the assay (i.e. blank) is taken into account it is clear that NCDO 176 had dissimilated all the citrate in the 'citrate-rich' medium.

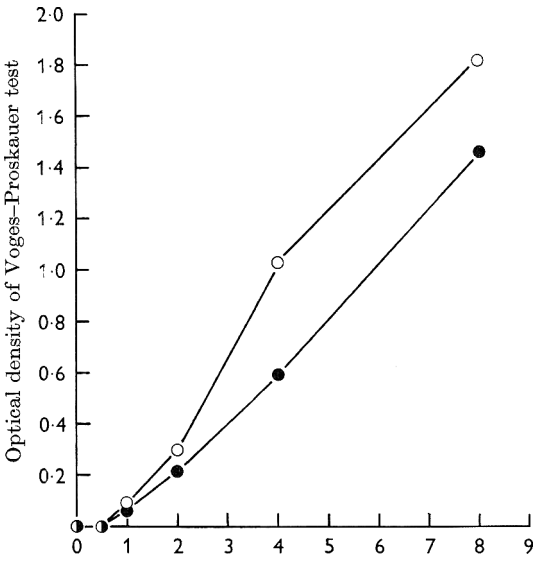

Fig. 1. Relationship between the concentration of pyruvate and citrate in medium X, and the light absorption of the pink compound from the acetoin formed by the growth of *Str. lactis* var. *diacetilactis* NCDO 1007. ○—○, sodium pyruvate; ●—●, triammonium citrate.

Fig. 4 shows the results obtained using method II (Table 2). In this case, because the 'citrate-rich' medium contained 10 mg/ml citrate, it was possible to assay 4 different volumes of culture fluid. The graph shows that NCDO 517 and the control gave virtually the same curve while NCDO 529 gave no acetoin at any level. NCDO 176 was not examined by this method but another strain of *Str. lactis* var. *diacetilactis* NCDO 615 was. It is obvious here that the only acetoin detected came from the culture fluid of the 'citrate-rich' medium of NCDO 615.

Methods IV and V have not been used so frequently as methods I, II and III but have also been found to be satisfactory.

Dissimilation of citrate by lactic acid bacteria. Table 3 gives the results obtained with a variety of lactic acid bacteria. The only doubtful result was obtained using a strain of *Str. cremoris* which consistently appeared to remove cnly small amounts of citrate from the 'citrate-rich' medium. With this type of result it is difficult to be sure if the concentration of citrate has been decreased or if some other factor is influencing acetoin production. In all other cases citrate was not found in the culture fluid added to the assay medium, or was so markedly reduced in quantity that the result was not in doubt.

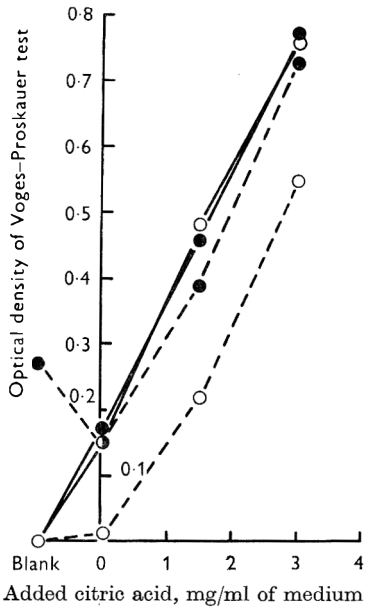

Fig. 2

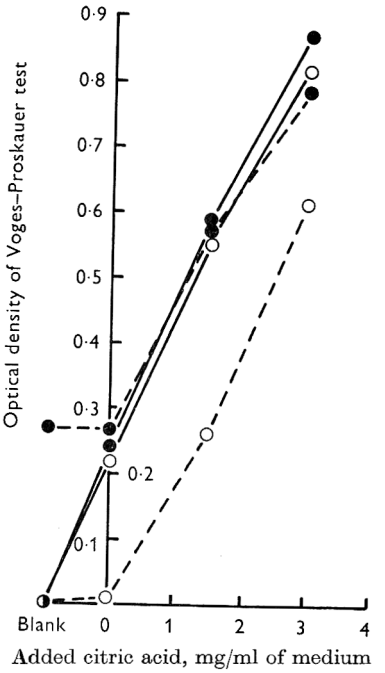

Fig. 3

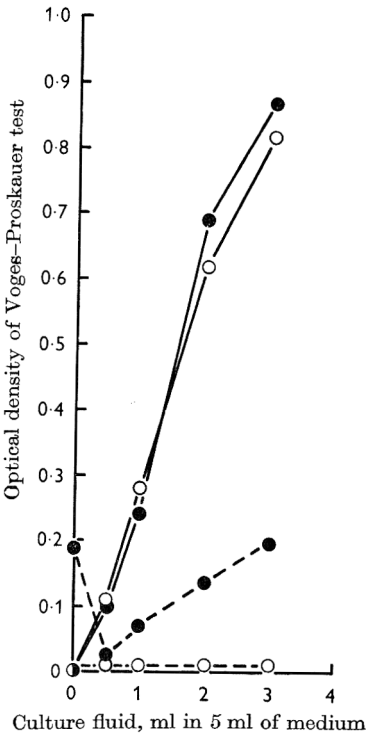

Fig. 4

Fig. 2. Estimation of citrate dissimilation by 3 strains of bacteria using method I. Citrate as well as culture fluid added to the assay medium. ●—●, 'citrate-rich' medium control; ○—○, culture fluid of *Leuc. dextranicum* NCDO 517; ● - - - ●, culture fluid of *Str. lactis* var. *diacetilactis* NCDO 176; ○ - - - ○, culture fluid of *Leuc. dextranicum* NCDO 529. All tubes contained the same volume of culture fluid.

Fig. 3. Estimation of citrate dissimilation by 3 strains of bacteria using method III. Citrate as well as culture fluid added to the assay medium. ●—●, 'citrate-rich' medium control; ○—○, culture fluid of *Leuc. dextranicum* NCDO 517; ● - - - ●, culture fluid of *Str. lactis* var. *diacetilactis* NCDO 176; ○ - - - ○, culture fluid of *Leuc. dextranicum* NCDO 529. All tubes contained the same volume of culture fluid.

Fig. 4. Estimation of citrate dissimilation by 3 strains of bacteria using method II. Blank value estimated at the 3-ml level. ●—●, 'citrate-rich' medium control; ○—○, culture fluid of *Leuc. dextranicum* NCDO 517; ● - - - ●, culture fluid of *Str. lactis* var. *diacetilactis* NCDO 615; ○ - - - ○, culture fluid of *Leuc. dextranicum* NCDO 529.

DISCUSSION

The basis of the methods described for the determination of citrate dissimilation by bacterial cultures can be used in any bacteriological laboratory and have the advantage that the conditions, under which citrate is broken down, can be varied without affecting the test method.

The results given were all determined using an optical measurement of the red colour, but for general purposes the result can be judged by eye. Apart from strains of *Str. lactis* var. *diacetilactis* the amount of acetoin formed in the 'citrate-rich' medium was very small in amount.

Table 3. *Citrate dissimilation by some lactic acid bacteria*

	No. of strains dissimilating or not dissimilating citrate† Method									
	I		II		III		IV		V	
Species*	+	−	+	−	+	−	+	−	+	−
Str. lactis	0	3	0	3	0	3	.	.	.	.
Str. lactis var. diacetilactis	5	0	1	0	5	0	.	.	.	.
Str. cremoris	(1)	3	(1)	3	0	4	0	1	.	.
Leuc. cremoris	5	(1)	5	0	6	0	.	.	.	.
Leuc. lactis	1	4	1	4	1	4	.	.	.	.
Leuconostoc (Group III)	1	7	1	7	1	7	1	6	.	.
Leuconostoc (acidophilic sp.)	.	.	.	.	.	.	.	.	18	1
Leuconostoc (Groups IV, V)	1	12	1	12	1	12	.	.	.	.
Leuconostoc (Group VI)	2	9	2	9	2	9	2	0	.	.
Ped. cerevisiae	0	5	0	5	0	5	0	3	.	.
Ped. parvulus	.	.	.	.	.	.	0	4	.	.
Pediococcus group III	.	.	.	.	.	.	0	5	.	.
Ped. halophilus	.	.	.	.	.	.	0	1	.	.
Pediococcus sp	.	.	.	.	.	.	1	0	.	.
Lactobacillus casei	2	0	.	.	2	0	.	.	.	.
L. planatarum	1	1	.	.	2	0	.	.	.	.
L. brevis	1	2	1	2	.	.	.	.	.	.
L. buchneri	0	3	0	3	.	.	.	.	.	.
L. viridescens	1	0	1	0	1	0	8	0	.	.
Lactobacillus sp	.	.	.	.	.	.	2	1	.	.

* Leuconostocs named according to Garvie (1960). Pediococci named according to Gunther & White (1961).

† Numbers in parentheses are doubtful results: *Str. cremoris* because the amount of citrate removed was slight, *Leuc. cremoris* because growth with this strain on this occasion was poor.

Certain precautions must be taken when altering either the 'citrate-rich' media or the assay media. It is advisable to plan these together so that there is enough citrate in the first medium for it to be assayed in the second without adding an excess of other organic matter which will interfere. Early in the work a 'citrate-rich' medium, which contained only 0·2% citrate, was used but the assays of the culture fluid were never satisfactory probably because large amounts of culture fluid had to be tested. The aim should be to have enough citrate in the 'citrate-rich' medium so that only a small volume of culture fluid is added to the assay medium.

Most of the culture fluids examined either showed all or none of the citrate had been removed. In order to achieve such clear-cut results the 'citrate-rich' medium should support vigorous growth of the strains for which it is used and incubation should be long enough for all the citrate to be metabolized. There should be no difficulty then in detecting those strains which dissimilate citrate.

Acetoin production by *Str. lactis* var. *diacetilactis* NCDO 1007 from a given amount of citrate is not constant when cultural conditions are varied. The composition of the medium in particular is important. In a casein digest medium (E. I. Garvie, unpublished) acetoin was detected after growth of NCDO 1007 in the absence of citrate in the medium. If this same medium was diluted with water before use the amount of acetoin in the absence of and from any given amount of added citrate was increased. Similarly, the addition of large amounts of various salts increased the

acetoin formed both in the casein digest medium and in medium Y. Fortunately, the differences between the composition of the 'citrate-rich' medium control and the 'citrate-rich' medium culture fluids have not been large enough to affect acetoin production by NCDO 1007, and therefore the test method has proved applicable to bacterial cultures grown in normal media and in the presence of a measured amount of citrate. Milk, where the initial amount of citrate is unknown, can also be used because comparison with the control is possible.

Whittenbury (1966) found that he was unable to use his method of detecting citrate dissimilation by strains of leuconostocs unless they fermented a pentose. This means that for leuconostocs the method is limited in its application, particularly as the most important species using citrate, *Leuc. cremoris*, fails to ferment either pentose.

It was surprising to find that so few of the leuconostocs tested attacked citrate, but most of the cultures were old laboratory strains and this may explain the result. However, all the strains of *Leuc. cremoris* and all but one of the acidophilic leuconostocs that were tested dissimilated citrate. Only a few strains of other species were found to be positive.

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The persistence of cloxacillin in the mammary gland when infused immediately after the last milking of lactation

By A. SMITH*, F. K. NEAVE and F. H. DODD
National Institute for Research in Dairying, Shinfield, Reading
and A. JONES and D. N. GORE
Beecham Research Laboratories, Brentford, Middlesex

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Summary. A series of experiments has been carried out in order to develop antibiotic preparations which, when infused into the udder at drying-off, eliminate established infection and prevent new infection from occurring in the dry period. It has been shown that the persistence of antibiotic in the dry udder is affected by the solubility of the antibiotic salt, the quantity of antibiotic infused and the base in which it is formulated. Whereas 0·2 g cloxacillin as the sodium salt infused in a 3 % aluminium monostearate in mineral oil base after the last milking of lactation persisted for less than 1 week, 0·5 and 1·0 g cloxacillin in the form of the benzathine salt, in a similar base, usually persisted for 3 weeks.

Secretions removed from the udders of dry cows inhibited the growth of *Bacillus subtilis* and *Sarcina lutea* but not 2 strains of staphylococci.

The dry period between consecutive lactations is particularly important in the control of udder disease. It is a time when many new infections occur and because few old infections spontaneously recover during this period, a higher proportion of cows is found to be infected at calving than at the preceding drying-off. Further, it is only during the dry period that the regeneration of damaged udder secretory issue occurs and when established infection can be eliminated without adding antibiotic to saleable milk. Based on these considerations, Neave, Dodd & Kingwill (1966) have proposed a control of udder disease in which the elimination of infection in the dry period is an essential part.

The advantage of therapy at drying-off has been reported by Johnson (1941), Dalling & Stableforth (1948), Schalm & Ormsbee (1949), Neave, Dodd & Lee (1951), Pearson (1951), Oliver, Dodd & Neave (1956) and Neave, Dodd, Kingwill & Oliver (1959), but it is evident from these publications that there is considerable scope for improving the effectiveness of this form of treatment.

Although many reports are available on the persistence of antibiotics in the lactating mammary gland, there are few reports on their persistence in the dry udder. Pearson (1951) and Uvarov (1960) infused penicillin preparations into the dry udder.

* On visit from Animal Husbandry and Dairy Research Institute, Irene, South Africa.

The antibiotic level declined very rapidly, but persisted at very low levels for at least 10 days (Pearson, 1951) or 21 days (Uvarov, 1960) when procaine penicillin was administered in a base containing aluminium monostearate.

In the non-lactating udder the antibiotic concentration will decrease because of hydrolysis in the udder secretion, and by diffusion and absorption into the body and blood circulation of the cow (Rasmussen, 1959). From the review by Albright, Tuckey & Woods (1961) it is clear that, at least in the lactating cow, antibiotics can sometimes be detected in the milk of non-infused quarters of cows receiving antibiotic udder infusion. When large doses of antibiotic are injected intravenously or intramuscularly small quantities of the antibiotic can usually be detected in the milk of the cow (Edwards & Haskins, 1953; Randall, Durbin, Wilner & Collins, 1954).

To be effective, antibiotic must diffuse throughout the whole udder to reach the foci of infection. Edwards (1964) has shown that areas of involution of mammary tissue in lactating cows prevent even distribution. On the other hand, Funke (1961) showed that diffusion through the udder in the dry period is similar to that of the normal lactating gland; furthermore, penetration of antibiotic into the tissue itself appeared to be improved when the cow was not lactating.

The experiments reported here are part of a programme to develop combined therapeutic and prophylactic preparations specifically for routine infusion of all udders at drying-off, with the aim that all animals calve free of udder infection. For this purpose it appears to be necessary to devise preparations which will maintain an antibiotic concentration in the udder for most of the dry period. In the experiments reported, cloxacillin, a semi-synthetic penicillin resistant to staphylococcal penicillinase, was used because, whilst streptococci and staphylococci are responsible for over 90 % of udder infections, only the staphylococci have proved difficult to eliminate.

Preliminary trials on the persistence of cloxacillin in quarters when infused at drying-off in a base containing 3 % aluminium monostearate in mineral oil showed that the less soluble benzathine cloxacillin persisted longer in the udder than the more soluble sodium cloxacillin. The dose of the benzathine salt used initially was equivalent to 0·2 g of the cloxacillin acid, which is comparable to the same dose as the sodium salt in the commercially available preparation (Orbenin L A, Intramammary Suspension, Beecham Research Laboratories, Great West Road, Brentford, Middlesex) for use in lactating cows. As this resulted in relatively low levels of the penicillin in the udder secretions the dose was increased to an equivalent of 1 g cloxacillin-free acid and later also tested at a level of 0·5 g.

In all the experimental preparations tested, the cloxacillin (in terms of free acid), was incorporated at a concentration of 15–20 % of the base.

METHOD

Udder quarters of cows were always infused after the last milking of lactation with experimental intramammary preparations of cloxacillin. At intervals in the dry period 5–8 ml of udder secretion were removed to determine the persistency of the antibiotic. Assays for cloxacillin were carried out on large plates with *B. subtilis* ATCC 6633 (Price & Boucher, 1954; Jones & Pay, 1961) or *Sarc. lutea* (Simpson & Lees,

1956) as test organism. The standard cloxacillin, 5·0 μg/ml and 20·0 μg/ml for *B. subtilis* and 1·25 μg/ml and 5·0 μg/ml for *Sarc. lutea*, and all dilutions of the samples were prepared in heat-treated milk free from antibiotic.

In the first experiment udder secretion samples taken 1 week or later in the dry period inhibited the growth of *B. subtilis* due to the presence of natural inhibitory substances in the secretion. In further trials udder secretion was heated to 75 °C for 5 min. This treatment reduced the natural inhibitory substances of the dry period secretion sufficiently to prevent inhibition of growth of *B. subtilis* or *Sarc. lutea* on plates.

Samples were also tested for the presence of cloxacillin with *Staphylococcus aureus* Oxford, which was insensitive to the natural inhibitory substances of most dry-period secretions, and with the penicillinase producing strain of *Staph. aureus* 'm' which was not affected by any of the dry-period secretions that were examined. Single layer seeded nutrient agar in Petri dishes was used. With the Oxford strain, 10-mm holes were cut in agar; with the 'm' strain, fish spine cups filled by capillary action were placed on the agar (Lightbrown & Sulitzeanu, 1957). Results were reported as positive (+) when zones of inhibition were obtained. Samples were not preheated before testing.

Cloxacillin could be detected but not precisely estimated at the low levels of 0·5 μg/ml with *B. subtilis*, 0·2 μg/ml with *Sarc. lutea*, 0·3 μg/ml with *Staph. aureus* 'm' and 0·1 μg/ml with *Staph. aureus* Oxford.

RESULTS

Cloxacillin levels in the udder secretion following the infusion into the lactating udder after the last milking of lactation of 1 *g cloxacillin as the benzathine salt in various bases*

Six cows were used in this experiment; one udder quarter of each was left untreated as a control, whilst the other 3 each received the equivalent of 1 g of cloxacillin as the benzathine salt in one of the following preparations: (*a*) water, (*b*) silicone (MS 550 (Midland Silicones Ltd., Barry, Glamorgan) or (c) 3 % aluminium monostearate in mineral oil base.

The silicone base was used because it is very water repellent and might be expected to release antibiotic slowly over a long period; the aqueous base was used for comparison. The 3 % aluminium monostearate base is generally used in the treatment of lactating cows as a slow release base for antibiotics.

Samples were taken for assay at 16 h and 1, 2, 3 and 4 weeks after infusion and assayed using *B. subtilis* as the test organism.

The cloxacillin concentrations in udder secretion at various times after infusion are shown in Table 1.

After the first week all samples from untreated quarters inhibited the growth of *B. subtilis*, due to the presence of natural inhibitory substances in the secretions. This inhibition was equivalent to 2–5 μg cloxacillin/ml secretion. The secretion from an infused quarter was, therefore, only regarded as definitely containing the antibiotic if the inhibition zones were larger than those given by the corresponding control secretion. Any antibiotic which may have diffused into the control quarters was not detectable at 16 h or thereafter.

Table 1. *Cloxacillin concentrations (μg/ml) in udder secretions following infusion of 1 g cloxacillin as the benzathine salt in various bases after the last milking of lactation*

Base used for infusion cloxacillin	No. of quarters treated	Interval after infusion: 16 h Mean	16 h Range	16 h No. quarters containing antibiotic*	1 week Mean	1 week Range	1 week No. quarters containing antibiotic	2 weeks Mean	2 weeks Range	2 weeks No. quarters containing antibiotic	3 weeks Mean	3 weeks Range	3 weeks No. quarters containing antibiotic	4 weeks Mean	4 weeks Range	4 weeks No. quarters containing antibiotic
(*a*) Aqueous	6	541	260–1000	6	5·2	2·5–8·1	5	4·7	4·4–5·3	1	3·0	2·6–3·7	0	3·6	3·3–4·0	0
(*b*) Silicone	6	127	28–225	6	20	5·0–7·0	6	4·6	4·1–5·2	1	3·1	2·6–4·0	0	3·7	3·3–4·5	0
(*c*) 3 % aluminium monostearate	6	18	6–29	6	35	21–60	6	13	4·2–27	4	6·9	2·3–26	1	3·4	3·1–4·4	0
(*d*) No infusion (control)	6	No inhibition		—	4·6†	3·9–5·0	0	4·1	3·6–4·2	0	3·0	2·4–3·8	0	3·4	2·7–4·2	0

* The secretion of an infused quarter was regarded as containing the antibiotic only if the inhibition zones were larger than those given by the corresponding control secretion.

† Inhibition of growth of the test organism caused by natural substances and not due to antibiotic in the dry period secretion, expressed as cloxacillin equivalent.

The greatest persistency was found with the benzathine cloxacillin in the 3 % aluminium monostearate base. The anticipated slower release of cloxacillin from the silicone base did not occur. Two weeks after infusion the secretion from 4 out of 6 quarters infused with antibiotic in 3 % aluminium monostearate base still contained antibiotic at levels detectable above the natural inhibitory substances in the secretion. In comparison, antibiotic could only be detected 1 week after infusion in one quarter of those infused with the antibiotic in an aqueous or silicone base.

After modification of the assay method to overcome the difficulty arising from the natural inhibitory properties of the secretion, the experiment was repeated for benzathine cloxacillin in the 3 % aluminium monostearate base only.

Cloxacillin levels in the udder secretion following the infusion into the lactating udder after the last milking of lactation of 1 *g cloxacillin as the benzathine salt in a* 3 % *aluminium monostearate base*

In this experiment 1 g of cloxacillin, as the benzathine salt, in a 3 % aluminium monostearate base was infused into 3 quarters of each of 6 cows after the last milking of the lactation. One quarter of each cow was left untreated. Secretions were sampled at 1, 2, 3 and 4 weeks after infusion; at 5 weeks all the secretion was removed from each quarter as a final sample.

Samples of secretions were tested for the presence of cloxacillin with *Staph. aureus* Oxford and *Staph. aureus* 'm' and the preheated samples with *B. subtilis* as test organism.

The cloxacillin content of the udder secretions at weekly intervals after infusion are indicated in Table 2. Unheated control samples from the uninfused quarters taken throughout the test period did not inhibit either of the strains of staphylococci, and *B. subtilis* was not affected by a heat-treated control.

Cloxacillin could be detected in all samples taken 1 week after infusion. The mean level was 23 μg/ml with a range of 8–46 μg/ml. After two weeks 4 pre-heated samples did not give any inhibition zones on *B. subtilis* plates, but all inhibited both strains of *Staph. aureus*.

Three weeks after infusion 6 samples did not inhibit growth of *Staph. aureus* 'm' but 1 week later the secretions from 3 of these quarters were again inhibitory. One quarter was not sampled at 3 weeks, but was still positive when tested again a week later.

It therefore seems probable that antibiotic persisted in 15 out of 18 quarters for a period of at least 3 weeks in levels high enough to suppress the growth of the penicillinase-producing strain of *Staph. aureus* 'm'. Four weeks after infusion, antibiotic could still be detected in 12 of the 18 quarters, but after 5 weeks only one quarter remained positive.

Similar results were obtained with the penicillin-sensitive Oxford strain, but because of its greater sensitivity, antibiotic could be detected in 9 of the 18 quarter samples 5 weeks after infusion.

After the completion of this experiment a large-scale field trial was organized to test the efficiency of infusing the 1 g benzathine cloxacillin preparation at drying-off in reducing the level of udder infection at calving. In this, it was compared with a standard commercially available product containing 0·2 g as sodium cloxacillin, both

Table 2. *The persistence of cloxacillin* in udder secretions following infusion of 1 g cloxacillin as the benzathine salt in a 3 % aluminium monostearate base after the last milking of lactation*

Interval after infusion	Cow 1. Quarters 1	2	3	Cow 2. Quarters 1	2	3	Cow 3. Quarters 1	2	3	Cow 4. Quarters 1	2	3	Cow 5. Quarters 1	2	3	Cow 6. Quarters 1	2	3	Mean	Range	Quarters positive	Quarters positive in at least one test
1 week																						
Cloxacillin level, μg/ml	21	26	21	23	20	23	21	14	17	36	35	46	8	22	14	29	29	12	23	8–46	18/18	—
Inhibition *Staph. aureus* 'm'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	18/18	18/18
2 weeks																						
Cloxacillin level, μg/ml	5	10	5	25	19	20	9	16	9	0*	0	0	1	7	3	7	4	0	8	0–25	14/18	—
Inhibition *Staph. aureus* 'm'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	18/18	—
Inhibition *Staph. aureus* Oxford	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	18/18	18/18
3 weeks																						
Cloxacillin level, μg/ml	Not tested, levels too low for assay																					
Inhibition *Staph. aureus* 'm'	−	+	−	+	+	+	+	+	+	+	+	−	−	−	−	+	+	Not	—	—	11/17	—
Inhibition *Staph. aureus* Oxford	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	tested	—	—	15/17	15/17
4 weeks																						
Inhibition *Staph. aureus* 'm'	+	+	+	+	+	+	+	+	−	+	−	+	−	−	−	+	−	+	—	—	12/18	—
Inhibition *Staph. aureus* Oxford	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	—	—	15/18	15/18
5 weeks																						
Inhibition *Staph. aureus* 'm'	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	—	—	1/18	—
Inhibition *Staph. aureus* Oxford	−	+	−	+	+	+	+	+	+	−	−	+	−	−	−	+	−	−	—	—	9/18	9/18

* The concentration is recorded as zero when it fell below 0·5 μg/ml.

preparations using the same base (Smith, Dodd, Neave & Brander, 1966). Before this trial was carried out the persistence in the dry udder of these 2 products was tested in a separate experiment.

Cloxacillin levels in the udder secretion following the infusion ofter the last milking of lactation of 0·2 g cloxacillin as the sodium salt or 1 g as the benzathine salt both in 3 % aluminium monostearate base

Six cows were infused after the last milking of lactation with cloxacillin in a 3 % aluminium monostearate base either as the sodium salt equivalent to 0·2 g or as the benzathine salt equivalent to 1 g of cloxacillin. The left-fore and left-hind quarters of each cow received the benzathine salt and the right-fore and right-hind quarters the sodium salt. Udder secretions were removed 2, 4, 6 and 8 days after infusion and tested for the presence of cloxacillin with *Staph. aureus* Oxford as test organism. Samples of secretions were also pre-heated and cloxacillin levels assayed using the *B. subtilis* and *Sarc. lutea* methods.

The results (Table 3) show clearly that the antibiotic levels of the secretion were markedly higher with the 1 g benzathine cloxacillin than with the 0·2 g sodium cloxacillin preparation.

After infusion of the benzathine salt, the levels of cloxacillin in the mammary gland remained relatively high during the test period.

Of the 12 quarters infused with the sodium salt, 2 had a cloxacillin level of less than 1 μg/ml when tested 2 days after infusion. After 6 days this number increased to 9 and after 8 days antibiotic could not be detected in the secretions from any of the quarters using *B. subtilis* as the test organism, but on *Staph. aureus* Oxford plates all but 3 samples gave small inhibition zones.

These results suggest that therapeutically effective levels of cloxacillin in the udder secretion would be limited to a few days, and certainly less than 1 week, using the 0·2 g sodium salt suspension. Preceding experiments suggested that the level of cloxacillin would be maintained for about 3 weeks when the antibiotic at the higher dose levels was incorporated as the benzathine salt.

Effect of dosage level, solubility of salt, and the properties of the base on the persistence of the antibiotic during the dry period

Because of the success of the 1 g benzathine cloxacillin product in eliminating infection (Smith *et al.* 1966), further experiments have been carried out on antibiotic formulations for dry period therapy.

Udder quarters were infused after the last milking of lactation with cloxacillin preparations as follows:

(*a*) 1 g as benzathine salt in a 3 % aluminium monostearate base (6 quarters);
(*b*) 0·5 g as benzathine salt in a 3 % aluminium monostearate base (7 quarters);
(*c*) 0·5 g as benzathine salt in 5 % aluminium monostearate base (7 quarters);
(*d*) 0·5 g as benzathine salt in high viscosity mineral oil $\eta_{37\,^{\circ}C} = 65 \times 10^3$ cP base (6 quarters);
(*e*) 0·5 g as benzathine salt in low viscosity mineral oil $\eta_{37\,^{\circ}C} = 14 \times 10^3$ cP base (6 quarters);
(*f*) 1 g as sodium salt in a 3 % aluminium monostearate base (6 quarters).

Table 3. *Cloxacillin concentrations, μg/ml, in udder secretions following infusion of either* 1 *g as the benzathine or* 0·2 *g as the sodium salt in the* 3 % *aluminium monostearate base after the last milking of lactation*

Infusion	No. of quarters	Assay organism		Days after infusion 2	4	6	8
1 g cloxacillin as benzathine salt	12	*B. subtilis*	Mean	66	42	45	42
			Range	35–155	15–112	11–138	5–154
		Sarc. lutea	Mean	67	34	34	49
			Range	31–117	10–80	8–107	7–123
0·2 g cloxacillin as sodium salt	12	*B. subtilis*	Mean	5	2	*	0
			Range	*–12	*–4	*–2	*
		Sarc. lutea	Mean	5	2	*	*
			Range	*–12	*–4	*–2	*
		Staph. aureus Oxford		Number of quarters showing inhibition 12	12	12	9

* Trace of inhibition of assay organism but level of antibiotic not precisely estimated.

Udder secretion was removed from each quarter 16 h, 2, 4, 6, 8, 14, 21 and 28 days after infusion. The presence of cloxacillin was tested with *Staph. aureus* Oxford and the pre-heated samples were assayed with *B. subtilis* for secretion obtained up to 8 days after infusion and with *Sarc. lutea* for samples taken at 14, 21 and 28 days after infusion.

Table 4 shows that the antibiotic levels in the secretion after the infusion of 1 g cloxacillin as the benzathine salt in 3 % aluminium monostearate base (*a*) confirmed results of previous experiments on this preparation. Antibiotic could still be detected although at low levels, in all infused quarters 28 days after infusion. When the dose of antibiotic was reduced to 0·5 g, cloxacillin as benzathine salt in the same base (*b*) the antibiotic persistence was similar to the larger dose but at lower levels during the first few days of the trial. An increase in the aluminium monostearate content of the base to 5 % (*c*) did not affect the levels or persistency of the antibiotic to any marked degree.

With the benzathine salt prepared in the mineral oil base of either high or low viscosity (*d* and *e*) and the sodium salt in the 3 % aluminium monostearate base (*f*) the cloxacillin levels were initially high, and were eliminated from nearly all quarters in less than 8 days.

DISCUSSION

A characteristic of the penicillins is that a specific amount of penicillin is bound to the bacterial cell wall within a few minutes of contact, and the concentration needed to inhibit growth is that required to saturate the penicillin-binding sites (Cooper, 1956; Park, 1963). There is no additional binding at higher penicillin concentrations (Mass & Johnson, 1949; Cooper & Rowley, 1949). In mastitis therapy, this has been confirmed by Jepsen (1950) and Tucker (1954), who demonstrated that in the lactating cow the antibacterial effect is not in proportion to the dose level. However, prolonged maintenance of an effective antibiotic concentration improves the therapeutic results (Schofield, 1946; Murname, 1946; Spencer, Kraft & Underbjerg, 1947; Brander *et al.* (1964).

The period required for the complete clearance of infection will presumably vary greatly depending on the extent of the diseased tissue, the number, size and location of any abscesses, the nature of the abscesses, whether active or quiescent and the characteristics and state of the infecting organisms.

It would be expected that penetration of an abscess would be achieved most readily with a high concentration of antibiotic but a low level of antibiotic maintained for a long time may be just as effective antibiotically as a high initial level followed by a low level that persists.

With an appropriate preparation, it should be possible to maintain therapeutic levels in the udder secretion for most of the dry period which normally lasts 4–12 weeks. Three factors have been demonstrated to be important in maintaining antibiotic in the udder during the dry period; the base in which the antibiotic is infused; the solubility or molecular size of the antibiotic and the dose level.

The nature of the base was the most important factor determining the pattern of release of the antibiotic into the udder secretion (Table 4). The most persistent antibiotic concentrations were obtained using a base consisting of 3 % aluminium

Table 4. *Persistency of cloxacillin in the udder secretions following infusion of either the sodium or benzathine salt in different bases into the udder after the last milking of lactation*

(The presence of cloxacillin is indicated either as μg/ml of udder secretion or by the number of quarters from which the secretion inhibited the growth of *Staph. aureus* Oxford.)

Dose and formulations	Quarters infused	Interval after infusion: 16 h	2 days	4 days	6 days	8 days	14 days	21 days	28 days
(*a*) 1 g as benzathine salt in 3 % aluminium monostearate base	6								
Mean level		42	31	37	41	32	10	2·3	—
Range		9–69	9–76	14–65	15–74	16–65	5–23	0·4–6·5	*–2
Quarters inhibiting *Staph. aureus* Oxford		6	6	6	6	6	6	6	6
(*b*) ½ g as benzathine salt in 3 % aluminium monostearate base	7								
Mean level		22	16	26	30	23	11	2·4	—
Range		11–39	8–31	5–47	9–63	6–53	2–23	0·5–6·2	*–3
Quarters inhibiting *Staph. aureus* Oxford		7	7	7	7	7	7	7	5
(c) ½ g as benzathine salt in 5 % aluminium monostearate base	7								
Mean level		18	18	12	23	29	10	1·7	—
Range		7–57	8–23	5–23	10–40	9–70	2–22	0·4–6·4	*–0·7
Quarters inhibiting *Staph. aureus* Oxford		7	7	7	7	7	7	7	7
(*d*) ½ g as benzathine salt in high viscosity paraffin base	6								
Mean level		65	42	11	—	—	—	—	—
Range		16–130	9–106	< 1–25	0	0–*	0	0	0
Quarters inhibiting *Staph. aureus* Oxford		6	6	6	5	6	3	0	0
(*e*) ½ g as benzathine salt in low viscosity paraffin base	6								
Mean level		227	15	—	—	—	—	—	—
Range		220–390	10–26	0–0·5	0	0	0	0	0
Quarters inhibiting *Staph. aureus* Oxford		6	6	1	0	0	0	0	0
(*f*) 1 g as sodium salt in 3 % aluminium monostearate base	6								
Mean level		166	54	29	14	11	—	—	—
Range		49–376	20–100	6–46	7–21	1–26	0–2	0	0
Quarters inhibiting *Staph. aureus* Oxford		6	6	6	6	6	5	0	0

* Trace of inhibition of assay organism but level of antibiotic not precisely estimated.

monostearate in mineral oil. Nevertheless, the release pattern could be still further improved, and in this respect the nature of the base is likely to be the most important factor.

The effective persistency of the cloxacillin in the 3% aluminium monostearate base was extended by increasing the dose level of the sodium salt from 0·2 to 1 g and further extended by incorporating the antibiotic as the less soluble benzathine salt when it could be detected in most quarters for at least 3 weeks.

For reasons which have been discussed above, these experiments were mainly concerned with determining conditions which would give high persistency of antibiotic in the dry udder. However, it cannot be assumed that these conditions will eliminate infection more effectively than those resulting in a high initial level but persisting for only a few days. This aspect is being investigated in a field experiment currently in progress.

We are grateful to Dr J. G. Franklin and Mr H. M. Underwood for determining the optimum heat treatment for neutralizing the inhibition properties of dry period udder secretion and to Mrs P. B. Smith and Miss L. M. Cleverly for doing the assays.

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The persistence of penicillin G in the mammary gland when infused immediately after the last milking of lactation

By A. SMITH,* F. K. NEAVE
National Institute for Research in Dairying, Shinfield, Reading
and A. JONES
Beecham Research Laboratories, Brentford, Middlesex

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Summary. Penicillin G at a dose level of 1 and 5 million units infused in a quick release base or an aqueous solution into quarters of cows after the last milking of the lactation persisted in infused quarters for about 4 days. From 1 h after infusion penicillin was detected in the venous blood and from 2 h after infusion in non-infused quarters.

In a previous publication (Smith, Neave, Dodd, Jones & Gore, 1966) the problems associated with an effective antibiotic preparation suitable for infusing into the udders of cows on drying-off have been discussed.

The experiment described in this paper, which is part of the same study, was designed to investigate the persistence of penicillin G when infused into lactating quarters after the last milking of lactation, and to measure the level of pencillin at this time in the secretion of the non-infused quarters and in the blood serum. Doses of 1 and 5 million units were used, which is very much larger than those normally used for udder infusion.

METHOD

Three cows (170, 961 and 937) in their 1st, 2nd and 4th lactations with body weights of 485, 453 and 634 kg were used. All were free from udder infection. To determine the amount of milk that would accumulate in their udders at the start of the dry period they were not milked for 24 h before the last milking of the lactation. At this final milking the cows were milked with an individual quarter milking machine and the penicillin G infused. The quantities of milk removed at the last milking of lactation are given in Table 1 and the penicillin G preparations and doses in Table 2.

For 6 days after infusion samples of venous blood and intra-mammary secretion were removed for penicillin G assay. The sampling programme is shown in Table 3. The blood samples were taken from the jugular vein and the intra-mammary secretion samples (5 ml) expressed from the teat.

The large plate method of assay was used with *B. subtilis* and *Sarcina lutea* as test organism (Price & Boucher, 1954; Simpson & Lees, 1956). Udder secretions taken

* On visit from Animal Husbandry and Dairy Research Institute, Irene, South Africa.

from the infused quarters from 12 to 96 h were assayed using *B. subtilis*. All other samples of secretion and blood were assayed with *Sarc. lutea*. Penicillin G could be quantitatively assayed to levels as low as 0·01 μg/ml with *Sarc. lutea*.

Table 1. *The milk production (g) of the individual quarters of the cows at the last milking of lactation after a milking interval of 24 h*

Quarter	Cow 170	Cow 961	Cow 937
Right fore	520	700	700
Right hind	1275	1630	1350
Left fore	275	750	525
Left hind	1125	1675	1930

Table 2. *The quantities of penicillin G infused into the lactating udder immediately after the last milking of the lactation (i.e. at drying-off)*

Cow no.	Base	Right fore-quarter	Right hind-quarter	Left fore-quarter	Left hind-quarter
170	*Quick release	599 mg (1 000 000) units	—	2993 mg (5 000 000) units	—
961	Quick release	—	—	2993 mg (5 000 000) units	599 mg (1 000 000) units
937	Water	—	—	2993 mg (5 000 000) units	—

* Oil emulsion as in Orbenin quick acting intramammary suspension, Beecham Research Laboratories, Great West Road, Brentford, Middlesex.

Table 3. *Programme of sampling venous blood and secretion from infused and non-infused quarters*

Time interval after infusion, h	Venous blood	Udder secretion	
		Non-infused quarters	Infused quarters
0 (before infusion)	+	+	+
1	+	+	.
2	+	+	.
3	+	+	.
4	+	+	.
5	+	+	.
6	+	+	.
9	+	+	.
12	+	+	+
15	+	+	.
18	+	+	.
24	+	+	+
30	+	+	.
36	+	+	+
48	+	.	+
72	+	.	+
96	.	.	+
120	.	.	+
144	.	.	+

RESULTS

The results in Table 4 indicate clearly that, even in the unmilked udder, there is a rapid decline in the concentration of penicillin in the secretion, and that virtually all of the penicillin had either been destroyed or had diffused from the udder within 5 days. The rate of decline in the concentration of antibiotic is illustrated in Fig. 1 where the concentrations are expressed as a percentage of the level of antibiotic found after 12 h.

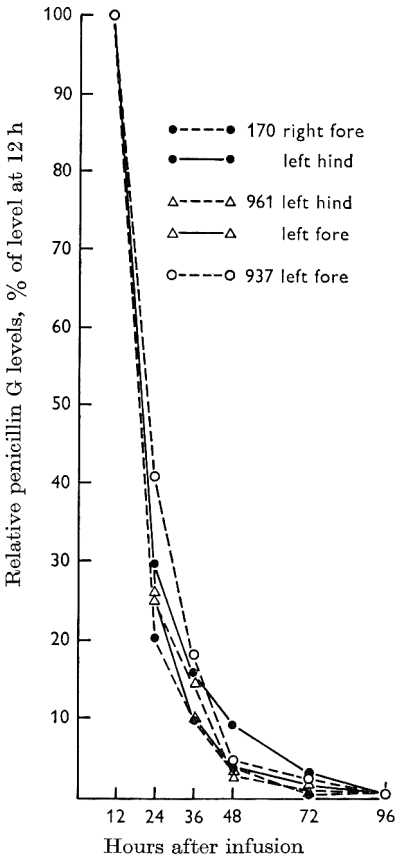

Fig. 1. Penicillin G levels in infused quarters. Levels at 12 h post-infusion taken as 100%. ● - - - ●, 170 right fore-quarter; ●—●, left hind-quarter; △ - - - △, 961 left hind-quarter; △—△, left fore-quarter; ○ - - - ○, 937 left fore-quarter.

The penicillin G levels of the secretions from the quarters given the higher level of antibiotics were 3 times greater at 12 h, but persisted not more than 24 h longer than those given the lower dose. Fig. 1 indicates that the patterns of the decline in antibiotic levels of the secretions were similar for both dose levels. Only one cow (937) was infused with the penicillin in aqueous solution; with this cow the maximum recorded concentration was much higher than that in the infused quarters of the other cows, and this higher level was maintained for 4 days.

The penicillin G levels in the non-infused quarters are presented in Table 5. In relation to the extremely high dosage of penicillin, the transfer of penicillin to non-

infused quarters was very low and could not be detected until 2 h after the infusion. With cow 937 infused in one quarter only and with the penicillin in aqueous base the antibiotic was detected only in 3 samples all from one of the non-infused quarters and at a very low concentration.

Table 4. *Penicillin G levels (μg/ml) of secretion from infused quarters*

	Cow 170		Cow 961		Cow 937
	Right fore	Left fore	Left hind	Left fore	Left fore
Dose infused (mg) ...	599	2993	599	2993	2993
Time interval after infusion, h					
Control (before infusion)	0	0	0	0	0
12	700	2200	390	1000	8500
24	140	660	100	260	3500
36	70	350	55	100	1500
48	32	200	20	45	550
72	8	65	5	8	200
96	1·3	10	1·5	2·3	15
120	< 0·01	< 0·01	0·2	0 1	0·4
144	< 0·01	0·05	0·4	< 0 01	0·4
312	None detected				

Table 5. *Penicillin G levels (μg/ml) in non-infused quarters following infusion into one or more quarters of the same udder after the last milking of the lactation*

Sample intervals, h after	Cow 170		Cow 961		Cow 937		
infusion	Right hind	Left hind	Right fore	Right hind	Right hind	Right fore	Left hind
1	None detected						
2	0·12	0·08	0·02	1·30	None	None	None
3	0·07	0·12	0·06	0·04	None	None	None
4	0·10	0·10	0·10	0·04	None	None	0·05
5	0·10	0·04	0·10	0·07	None	None	0·05
6	0·08	0·11	0·07	0·07	None	None	0·02
9	0·09	0·08	0·08	0·07	None	None	None
12	0·07	0·05	0·05	0·04	None	None	None
15	0·04	0·04	0·04	0·04	None	None	None
18	0·02	0·02	0·02	0·02	None	None	None
24	0·03	0·04	0·03	0·03	None	None	None
30	None	0·02	0·01	0·01	None	None	None
36	None	0·01	None	None	None	None	None

From 1 h after infusion penicillin was detected in the venous blood. The highest concentratons were found within 2 h of infusion but there were measurable quantities in the blood for 24–48 h (Table 6). At all times the concentration of penicillin in the blood was higher than in the secretion of the non-infused quarters.

DISCUSSION

The results indicate that after infusion of penicillin G, in either aqueous solution or in a quick release base, the penicillin levels of the udder secretion decline rapidly even when milking is discontinued. The dose levels were very much higher than those

normally used in intra-mammary infusion yet the antibiotic fell in all quarters to less than 0·4 μg/ml within 5 days of the infusion. The level of antibiotic in the udder secretion of the quarters infused with 2993 mg reached a level about 3 times as high as in those quarters infused with 599 mg. The difference was maintained for 4 days, after which the antibiotic had virtually been eliminated from the quarters.

Table 6. *Antibiotic* content of blood serum after infusion of penicillin G into the udder following the last milking of the lactation*

Interval from infusion to sampling, h	Penicillin G, μg/ml		
	Cow 170	Cow 961	Cow 937
Control (before udder infusion)	0·06	< 0·01	0·02
1	1·00	1·00	0·06
2	0·80	1·00	0·30
3	0·50	0·70	0·10
4	0·60	0·60	0·10
5	0·50	0·70	0·10
6	0·40	0·50	0·10
9	0·40	0·30	0·10
12	0·30	0·10	0·20
15	0·20	0·10	0·20
18	0·10	0·08	0·20
24	0·08	0·05	0·20
30	0·06	0·05	0·20
36	0·05	0·07	0·10
42	0·01	0·10	0·20
48	0·07	0·07	0·10
72	0·03	0·02	0·04

* Antibiotic activity of blood serum is expressed as μg penicillin G/ml. This figure includes the natural bacterial inhibitory properties of blood serum. The control values taken before infusion of the antibiotic suggest that the inhibitory property of the blood would not materially affect the results.

From the results it is estimated that less than 30 % of the infused penicillin G was present in the infused glands 24 h after infusion. Since *in vitro* studies have shown that penicillin G does not lose more than 20–30 % of its potency in 2 days in milk at 37 °C, most of the antibiotic must have diffused into the udder tissues. The samples taken from the venous blood and other infused quarters indicated that a considerable quantity of antibiotic had passed from the infused quarters. Within 1 h of infusion of penicillin into 2 quarters of each of 2 cows the blood levels had reached 1·0 μg/ml of blood serum and the antibiotic could be detected for about 36 h. In these 2 cows the antibiotic was not detected in the secretion of the non-infused quarters until 2 h after infusion; the levels were much lower than in the blood but it was detectable for about the same length of time.

It is not possible from this experiment to estimate the quantity of the antibiotic diffusing from the udder for it is known that the breakdown of free penicillin in the blood is rapid. Watts & McLeod (1946), Kelly & Bell (1947) and Morse (1948) have shown that a single intravenous infection of penicillin G will persist for only about 1 h. Since in the experiment described a blood level greater than 0·5 μg/ml was maintained for at least 5 h it would appear that a substantial quantity of the antibiotic diffused into the blood within a few hours of the infusion. In humans a constant infusion of

about 60 mg/h is required to maintain a blood level of about 0·1 μg/ml (Goerner & Geiger, 1945; Smith & Harford, 1945).

The results obtained with cow 937, infused in only one quarter with an aqueous solution of antibiotic, are interesting in that the antibiotic was retained longer in the infused quarter. With this cow, the maximum level detected was 4 times greater than in any quarter of the other 2 cows, the antibiotic persisted for at least 24 h longer, and there was much less antibiotic found in either the venous blood or in the secretion of the non-infused quarter. This rather surprising result may either be due to infusing the antibiotic in aqueous solution, or be peculiar to the particular cow used.

These results reinforce the conclusion drawn from the previous experiment that in developing antibiotic preparations for use in dry cows where it is desirable to maintain a therapeutic dose level for several weeks, the antibiotic must be released slowly from the preparation by formulating the antibiotic in a base from which it diffuses slowly, and possibly by using an antibiotic of very low solubility.

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Investigations on the use of the Milko-tester for routine estimation of fat content in milk

By M. F. MURPHY and T. C. A. McGANN
The Agricultural Institute, Fermoy, Co. Cork, Ireland

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Summary. Results of studies on the operation of the Milko-tester Mark II for the estimation of fat in both herd and individual milks are presented. Milko-tester readings were compared to the means of duplicate Gerber results.

It was found that, for 894 herd milks, a direct Milko-tester reading ($G = M$) had a standard error of estimate of $\pm 0{\cdot}071$, whereas regression equation ($G = 1{\cdot}02\,M - 0{\cdot}075$) derived for the same data gave $\pm 0{\cdot}070$. For 1133 individual milks, the equation ($G = 1{\cdot}03M - 0{\cdot}186$) also had an accuracy of $\pm 0{\cdot}070$, while the direct Milko-tester reading gave $\pm 0{\cdot}107$. However, a simple subtraction of 0·077 from each Milko-tester value (i.e. $G = M - 0{\cdot}077$) for individual milks reduced the standard error to $\pm 0{\cdot}071$.

It is concluded that the Milko-tester is satisfactory for herd milks, particularly for milks up to 3·8 % fat. For individual milks a special regression relationship is necessary to achieve comparable accuracy.

A recent development in the field of fat estimation in milk has been the introduction of the Milko-tester (manufactured by Foss Electric, Hillerød, Denmark). This instrument is claimed by the makers to be capable of determining fat percentage in milk in 35 s with a standard deviation (compared to Röse Gottlieb) of $\pm 0{\cdot}06$ and $\pm 0{\cdot}10$ in the range 0–6 and 6–8 % fat, respectively. It is also claimed that addition of preservative to the milk does not influence the result.

The principle of the method involves turbidity measurement at 600 mμ in a flow-through cuvette. Prior to the photometric measurement the milk is heated, homogenized and clarified with ethylenediaminotetraacetic acid (versene solution) in the instrument.

The present work was planned to study the accuracy and suitability of the Milko-tester in comparison to the Gerber method for the estimation of fat in both individual and herd milks on a routine basis. The possibility of improving the method of operation and the accuracy of the Milko-tester was also investigated.

METHODS

Operation of the Milko-tester Mark II

Senft, Grochowalski & Cieslar (1965) have described the manufacturer's method of operation of the Milko-tester. A modification to this procedure, which we introduced in place of the recommended zero adjustment, was to insert a standard milk

after every 5 readings and then adjust the scale reading to the correct fat percentage, if necessary, using the fine adjustment screw. This modification had the same effect as a zero adjustment, but had the added advantages of being faster to perform (since flushing with the versene solution was obviated) and, more important, detecting certain faults which give rise to incorrect fat readings without causing any zero drift (e.g. homogenizer malfunction or air in the versene syringe.)

Standard milk

The standard milk was made up twice weekly from the bulked milk of a herd of over 100 cows and preserved by the addition of 1 Lactab (manufactured by Thompson and Capper Ltd., Liverpool, England, and containing $\frac{3}{16}$ grains mercuric chloride and $\frac{13}{16}$ grains potassium dichromate) per 250 ml milk. The fat percentage of this milk was taken as the mean of quadruplicate Gerber determinations.

Milk samples

Herd milk samples (894) were taken from a 10 % random selection from 3400 herds supplying milk in a liquid milk area. All samples were preserved similarly to the standard milk and analysed for fat percentage after 2 days.

Individual milk samples (1133) were selected from a herd containing 100 cows of mixed breed (mainly Friesian × Shorthorn). Each sample was a composite of evening and the following morning milk and was analysed on that day without the addition of preservative.

The investigations were performed in the summer months during normal lactation.

Chemical analysis

Duplicate Gerber (Irish Standard Specification, 1955) and single Milko-tester fat determinations were made on each sample of milk. The means of the duplicate Gerber estimations were compared to single Milko-tester readings, since the aim of the studies was to determine the performance of the Milko-tester under practical routine conditions. The capacity of the Irish Standard milk pipette is 10·80 ml.

RESULTS

Reproducibility of methods

The standard errors of the mean of duplicates determined under practical routine conditions were found to be as follows: (*a*) Gerber $\pm 0{\cdot}027$ ($n = 2120$); (*b*) Milko-tester $\pm 0{\cdot}016$ ($n = 220$).

Herd milks

The fat content of the 894 herd milks ranged from 1·79 to 4·95 % (Gerber). Linear regression equations (Table 1) relating Gerber (G) and Milko-tester (M) were derived for the entire data and for the data segregated into the following ranges of fat: up to 3·30; 3·31–3·80 and greater than 3·80 %. The standard error of estimate and of the regression coefficient were calculated for each equation (Steel & Torrie, 1960).

The regression coefficients calculated for each of the equations were all highly significant ($P < 0{\cdot}001$).

The standard error of estimate of each of the separate equations was less than ± 0·070 for milks containing up to 3·80 % fat, whereas above 3·80 % it was ± 0·081.

By reducing the regression coefficient of the overall equation ($G = 1{\cdot}02M - 0{\cdot}075$) to unity and correspondingly correcting the regression constant at the mean Milko-tester value (3·440), the relationship $G = M - 0{\cdot}006$ was obtained. This equation is for practical purposes identical to $G = M$ (i.e. a direct Gerber:Milko-tester comparison). This phenomenon is consistent with the virtual identity of the overall mean fat percentage values (cf. Table 1). It is noticeable, however, that with increasing fat percentage there was a trend towards slight but progressive underestimation by the Milko-tester.

Table 1. *Linear regression of mean Gerber value (G) on single Milko-tester value (M) for preserved herd milks in selected fat ranges*

Fat range (Milko-tester), %	No. of values	Mean Gerber value (G)	Mean Milko-tester value (M)	Average difference ($M-G$)	Regression equation	S.E. of estimate	S.E. of regression coefficient
All values	894	3·436	3·440	+0·004	$G = 1{\cdot}02M - 0{\cdot}075$	±0·070	±0·006
Up to 3·30	306	3·045	3·053	+0·008	$G = 1{\cdot}02M - 0{\cdot}063$	±0·069	±0·017
3·31–3·80	472	3·525	3·530	+0·005	$G = 0{\cdot}98M - 0{\cdot}051$	±0·067	±0·022
> 3·80	116	4·110	4·091	−0·019	$G = 1{\cdot}03M - 0{\cdot}096$	±0·081	±0·014

Table 2. *Preserved herd milks. Accuracy of overall equation ($G = 1{\cdot}02\,M - 0{\cdot}075$) and the direct Gerber:Milko-tester comparison ($G = M$) in selected fat ranges*

Fat range (Milko-tester), %	S.E. of estimate	
	$G = 1{\cdot}02M - 0{\cdot}075$	$G = M$
All values	±0·070	±0·071
Up to 3·30	±0·069	±0·070
3·31–3·80	±0·067	±0·067
> 3·80	±0·082	±0·086

Table 2 gives the accuracy of both the overall equation and $G = M$ in each of the 3 ranges of fat. The overall equation was as accurate as the separate equations in their respective ranges (cf. Table 1). Similarly, $G = M$ was practically equal in accuracy to the overall equation for the entire data combined and for the low and medium fat ranges. However, above 3·80 % the standard error of estimate of $G = M$ was ± 0·086 compared to ± 0·081 for the separate equation ($G = 1{\cdot}03M - 0{\cdot}096$) for that range.

Fig. 1 gives the distribution of differences for the overall equation and for the direct Gerber:Milko-tester comparison. It is seen that both distributions are similar. However, in the case of $G = 1{\cdot}02M - 0{\cdot}075$ a slightly higher proportion of values fell within ± 0·05 and ± 0·10 % fat.

The correlation coefficients calculated for $G = 1{\cdot}02M - 0{\cdot}075$ and $G = M$ were almost identical, being + 0·974 and + 0·973, respectively.

Individual milk

The fat content of the 1133 individual milks ranged from 2·08 to 7·0 % (Gerber). Table 3 summarizes the results of regression analysis (similar to that for the herd

milks) on the entire data and on the data segregated into the following ranges: up to 3·20; 3·21–3·90 and greater than 3·90 %. These ranges, which differ from those for herd milks, were chosen because of the greater spread in fat values for individual milks.

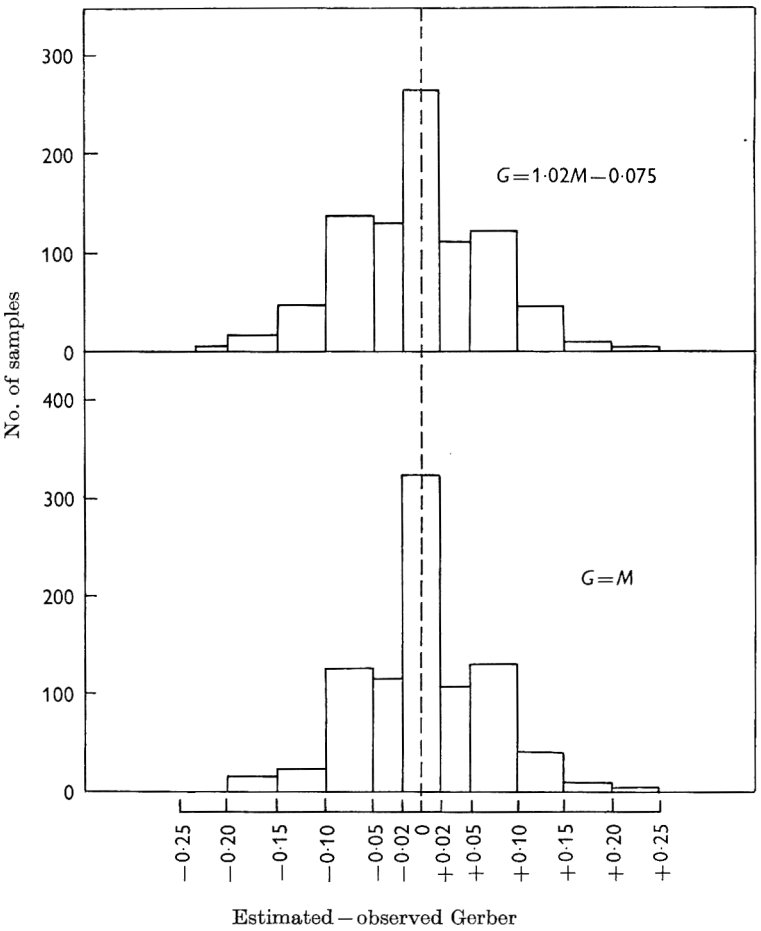

Fig. 1. Preserved herd milks. Distribution of fat differences for the direct Gerber:Milko-tester comparison ($G = M$) and the overall regression equation ($G = 1{\cdot}02\,M - 0{\cdot}075$).

The regression coefficients calculated for each of the equations were all highly significant ($P < 0{\cdot}001$).

The standard error of estimate of the separate equations for the 3 ranges increased with increasing fat content. It was less than $\pm 0{\cdot}070$ for milks up to 3·90 % fat, whereas above this fat percentage it was $\pm 0{\cdot}083$—a pattern similar to that observed for herd milks.

By reducing the regression coefficient of the overall equation ($G = 1{\cdot}03M - 0{\cdot}186$) to unity and correspondingly correcting the regression constant at the mean Milko-tester value (3·644), the relationship $G = M - 0{\cdot}077$ was obtained. This equation, which is materially different from that for herd milks, shows that the Milko-tester considerably overestimates the fat percentage of milk from individual cows. This

effect is clearly demonstrated by the substantial difference (+0·081) between the overall mean fat percentage values (cf. Table 3). As in the case of the herd milks, the Milko-tester tended to give progressively lower deviations from Gerber with increasing fat content.

Table 3. *Linear regression of mean Gerber value (G) in single Milko-tester value (M) for preserved individual milks in selected fat ranges*

Fat range (Milko-tester), %	No. of values	Mean Gerber value (*G*)	Mean Milko-tester value (*M*)	Average difference (*M* − *G*)	Regression equation	S.E. of estimate	S.E. of regression coefficient
All values	1133	3·563	3·644	+0·081	$G = 1{\cdot}03M - 0{\cdot}186$	±0·070	±0·004
Up to 3·20	166	2·907	3·000	+0·093	$G = 0{\cdot}97M - 0{\cdot}004$	±0·057	±0·022
3·21–3·90	721	3·491	3·578	+0·087	$G = 1{\cdot}05M - 0{\cdot}251$	±0·066	±0·013
> 3·90	246	4·219	4·270	+0·051	$G = 1{\cdot}00M - 0{\cdot}046$	±0·083	±0·012

Table 4. *Unpreserved individual milks. Accuracy of different equations in selected fat ranges*

Fat range (Milko-tester), %	S.E. of estimate		
	$G = 1{\cdot}03M - 0{\cdot}186$	$G = M - 0{\cdot}077$	$G = M$
All values	±0·070	±0·071	±0·107
Up to 3·20	±0·058	±0·059	±0·109
3·21–3·90	±0·067	±0·068	±0·110
> 3·90	±0·084	±0·087	±0·097

Table 4 gives the standard errors of estimate in the 3 ranges of fat percentage and on the entire data combined, of the following equations: (*a*) overall equation ($G = 1{\cdot}03\ M - 0{\cdot}186$), (*b*) the equation calculated from the overall equation with a regression coefficient of unity ($G = M - 0{\cdot}077$) and (*c*) the direct Gerber : Milko-tester comparison ($G = M$).

The overall equation was virtually as accurate as the separate equations for the 3 fat ranges. The standard error of estimate of $G = M - 0{\cdot}077$ was also almost equal to that of the overall equation in the low and medium fat ranges. However, for milks containing more than 3·90 % fat, the accuracy of $G = M - 0{\cdot}077$ diminished to ±0·087 compared to ±0·083 and ±0·084 for the separate and overall equations for the same range of fat, respectively, In the case of $G = M$ the standard error of estimate was considerably higher and varied from ±0·097 to ±0·110 in the 3 fat ranges.

Fig. 2 shows distributions of differences for the 3 equations in Table 4. Both $G = 1{\cdot}03M - 0{\cdot}186$ and $G = M - 0{\cdot}077$ show similar distribution patterns with approximately equal numbers of positive and negative deviations. On the other hand, for $G = M$ some 85 % of all samples show positive deviations from Gerber. The correlation coefficients between predicted value and Gerber result for the first-mentioned equations were almost identical (+0·983 and +0·982, respectively), whereas for $G = M$ the value was +0·950.

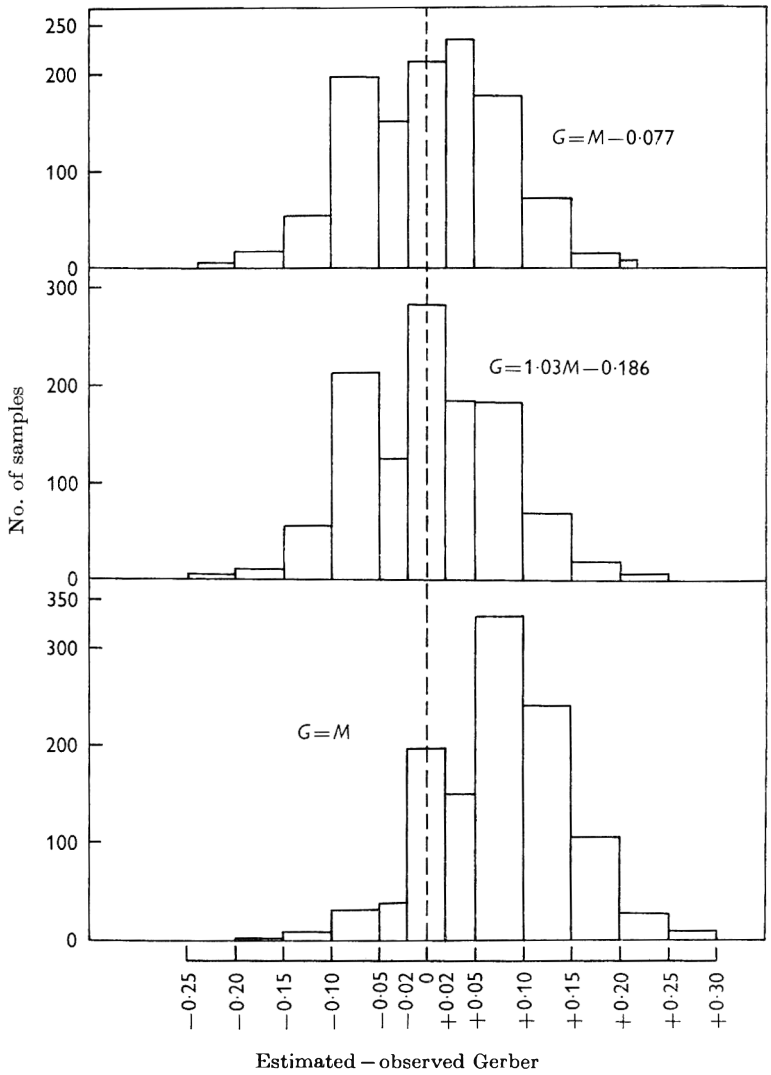

Fig. 2. Unpreserved individual milks. Distribution of fat differences for the direct Gerber: Milko-tester comparison ($G = M$), the overall regression equation ($G = 1{\cdot}03M - 0{\cdot}186$) and the simplified equation ($G = M - 0{\cdot}077$).

DISCUSSION

The regression equations which were derived for herd milks were very similar to the direct Gerber:Milko-tester comparison ($G = M$) and very closely resembled it when their regression coefficients were reduced to unity and the regression constants correspondingly corrected at the mean Milko-tester value. In practice the relationship $G = M$ was as accurate as the derived relationships.

The standard error of estimate of $G = M$ for herd milks in the 3 ranges of fat examined varied from $\pm 0{\cdot}067$ to $\pm 0{\cdot}086$ (i.e. coefficient of variation 1·9–2·3 %) with an overall value of $\pm 0{\cdot}071$. This accuracy of the Milko-tester is somewhat lower than claimed by the manufacturers, i.e. $\pm 0{\cdot}058$, in comparison to the Röse

Gottlieb method (Brems, 1965). However, the accuracy of the Gerber method is considerably less than that of Röse Gottlieb, a factor which could contribute to a higher standard error of estimate in the Gerber:Milko-tester comparisons. Schmidt (1966) has reported a standard deviation of $\pm 0{\cdot}04$ % fat from 10234 comparisons between Milko-tester and Gerber tests and from 7000 similar results Černá & Písecký (1966) claimed a standard deviation of $\pm 0{\cdot}06$ % fat. This level of accuracy compares favourably with that of a number of other routine estimation methods used for large-scale milk analysis (Booy, Klijn & Posthumus, 1962; Goulden, 1964; McGann & Murphy, 1965).

It is concluded that, for herd milks, a direct Milko-tester reading is sufficiently accurate for the routine estimation of low and medium fat milks. In the case of milks over 3·8 % fat, the Milko-tester was found to be less satisfactory, being less accurate and showing a tendency to underestimate the fat content. That this defect could be rectified, in part at least, by additional calibration of the instrument with high fat milks is borne out by the reduction obtained in the standard error of estimate (from $\pm 0{\cdot}086$ to $\pm 0{\cdot}081$) when the separate equation for values over 3·8 % fat was introduced in place of $G = M$.

In marked contrast to the results for herd milks, the regression equations calculated for individual milks differed considerably from $G = M$ and were appreciably more accurate than it. This effect is largely due to the fact that the Milko-tester, on the whole, considerably overestimates the fat content in individual milks (cf. Fig. 2). In fact, by simply modifying $G = M$ to $G = M - 0{\cdot}077$, a relationship was obtained which approximated closely in accuracy to that of the overall regression equation both for the entire data combined and for the results in each of the 3 ranges of fat separately.

It is concluded that, for individual milks, it is necessary to correct the actual Milko-tester reading by means of a regression equation or by making a simple subtraction of 0·077 (in practice 0·08) from each reading.

Since a correction is essential, it may be desirable to prepare a special scale for individual milks based on the overall equation or preferably on the separate equations for ranges. The use of the separate equations in place of $G = M$ did, in our investigations, reduce the standard error of estimate by 47·7, 40·0 and 14·4 % in the low, medium and high ranges of fat, respectively. By employing $G = M - 0{\cdot}077$, in place of $G = M$, an overall improvement in the standard error of estimate of 33·6 % was effected. The latter approach is attractive because it can be achieved by an appropriate adjustment to the standard milk setting.

It is noteworthy that exactly the same standard error of estimate ($\pm 0{\cdot}070$) was obtained for the overall regression formulae for herd and individual milks. Furthermore, it is seen that the results for the high fat samples for both types of milk exhibited similar patterns—both show reduced accuracy and a tendency towards underestimation with increasing fat content (evidenced in the case of the individual milks by the decreasing average difference with increasing fat content, cf. Table 3).

More than one factor may account for the difference in the findings between herd and individual milks. The differences could possibly be explained by (*a*) the bigger range in fat globule size in individual milks which could reflect itself in a less uniform degree of homogenization in the Milko-tester; (*b*) individual protein differences (both

type and overall amount) which may give rise to varying levels of clarity after addition of the clearing agent (versene solution); (*c*) influence of mastitis (some 50 % of the herd tested in our investigations were affected to some degree by mastitis); (*d*) presence of naturally occurring colouring substances, e.g. carotene; (*e*) the effect of the preservative added.

In our experiments a preserved milk was used to standardize the Milko-tester for use with unpreserved individual milks. Because of its immediate importance, the effect of preservative was studied in some detail. Duplicate Milko-tester readings were performed on 17 individual milks, split into preserved and unpreserved samples. The average difference between mean unpreserved and preserved values was only +0·006 % (which is not measurable on the instrument). This confirms the manufacturers claim and the finding of Černá & Písecký (1966) that the addition of preservative does not affect the Milko-tester reading.

Before recommending the Milko-tester for general use with individual milks, a fuller understanding of the factors influencing the technique would be desirable.

Favourable features of the Milko-tester include (*a*) the fact that no pungent or corrosive reagents are required; (*b*) relatively high speed of operation (we worked at a rate of approximately 75 tests per hour per operator); and (*c*) the high degree of reproducibility of the instrument.

The main disadvantage of the Milko-tester is its complexity which lends itself to the development of mechanical faults, often of a subtle nature. Such faults can lead to undetected erroneous results.

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The effect of concentrating milk on the fat retention property of the cheese curd

By L. A. MABBITT and G. C. CHEESEMAN
National Institute for Research in Dairying, Shinfield, Reading

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Summary. Concentration of milk results in a considerable increase in retention of fat by the rennet curd formed under conditions of disturbance. The fat retention property is not conferred by concentration *per se* but by some effect, probably involving surface phenomena, associated with the process.

Changes in composition of the fat globule membrane due to incorporation of milk protein accompany the improvement in fat retention. It is suggested that casein bound firmly to the globule during the concentration process, forms strong links with the coagulated micelles of the curd.

Introduction

The general advantages of a continuous process of cheese-making are apparent but a major difficulty in devising a continuous process of producing curd is that the coagulum has to be formed under conditions of movement or disturbance. This fortunately results in a curd which has rapid drainage properties (Ubbels & van der Linde, 1962) but unfortunately the fat and casein losses in the whey are high. The same phenomenon can be observed when rennetted milk is stirred up to or beyond the point of coagulation (Cheeseman & Chapman, 1966). However, the continuous curd-making process of Hutin & Stenne (see Hutin & Stenne, 1966), although involving considerable disturbance during coagulation, for some reason results in remarkably small fat losses in the whey (Brissenden, 1966). The originators of the method consider that the 3-fold concentration of the milk which precedes coagulation and is a novel feature of the process, confers remarkable properties on the milk, which minimizes losses of fat and, indeed, also of protein. These properties persist even if the concentrated milk is diluted back almost to its original volume before coagulation.

It is generally assumed that the fat globules are normally trapped mechanically in the interstices of the curd structure. Since the size of the casein micelles is known to increase when milk is concentrated (Hostettler, Imhof & Stein, 1965) it might be expected that the resulting change in curd structure would alter the fat-retaining properties of the curd. On the other hand, if substantial chemical or physico-chemical bonds between fat globules and the casein coagulum are involved, another explanation would be possible.

The object of the present work was to investigate the factors which determine the extent of fat retention in curd and to discover why it is improved by concentrating the milk. For this purpose the conditions used in the evaporation and their effect on the properties of the cream were investigated.

MATERIALS AND METHODS

Milk

Bulked milk (mixed morning and evening milk) from the Institute's herd of Friesian cows was used. The contents of the bulk storage tank were held at 4 °C with intermittent stirring.

Milk concentration

A laboratory climbing film evaporator was used; the jacketed climbing film column (130 × 1 cm) was heated by circulating water at 80 °C and low pressure (about 0·5 cmHg) was applied by an oil pump. The mean temperature of the milk emerging from the column was 34 °C ± 2 depending on vacuum conditions and rate of milk flow. In any one experiment 600 ml of milk was usually concentrated to 200 ml. This took approximately 20 min during which the milk circulated about 10 times round the apparatus. After each pass the milk was held in a vertical column (140 × 3 cm) at ambient temperature.

Fat losses from disturbed curd

The milk (100 ml) was stirred at 100 rev/min in a beaker at 40 °C, 0·3 ml rennet extract (Hansens Ltd.) was added and stirring continued. Coagulation began after about 2 min and stirring was continued for a further 10 min. The resultant curd slurry was filtered through 2 layers of fine cheese cloth and the fat content of the whey determined by the Gerber method. Under these exaggerated conditions of disturbance the fat content of the whey from curd made from untreated whole milk (fat content of 3·4 % ± 0·2) was about 2·0 % but from concentrated-rediluted milk only about 0·5 %. All determinations were done in duplicate.

Preparation of cream

Cream was prepared from fresh bulked raw milk by separation in a Lister cream separator (Model 15 mechanically driven) set to give 40–45% fat with the milk at 37–40 °C. About 100 ml cream was washed 3 times to remove residual milk using 2 l buffered sucrose solution (Hayashi & Smith, 1965) for each wash and separating each time by the cream separator.

In some experiments the cream was separated by centrifuging about 225 ml quantities of milk at 40 °C in 250-ml containers at 250 *g* for 20 min. With the rediluted concentrated milk it was necessary to have a further centrifuging for 20 min to obtain a good yield of cream. These conditions kept free-fat formation to a minimum. The cream obtained fom 900 ml of milk was washed 3 times in washes of 200 ml and centrifuged at the same speed for 20 min to recover the cream after each wash.

Preparation of fat globule membrane

The soluble membranous material was removed from the washed cream using the sodium deoxycholate (DOC) treatment (Hayashi & Smith, 1965). In some instances the DOC solution contained urea (8 M) to facilitate removal and solution of any casein which might have been adsorbed on the fat globules. This material, containing the high molecular weight lipoprotein of the membrane, after dialysis against 0·01 M Tris-chloride buffer (pH 8·5) containing 0·1 M sodium chloride, to remove

the sodium deoxycholate, was concentrated either by ultra-centrifugation or by dialysis against polyethyelene glycol (MW 6000) solutions in water. Portions of the concentrated solution were then dialysed against the appropriate buffer systems used for the ultracentrifugation and electrophoresis, respectively.

Preparation of solutions

Milk dialysate was prepared by dialysing 1 vol of raw skim-milk against 3 vol of distilled water for 24 h at 4 °C. The dialysate was concentrated by rotary evaporation to a quarter of its volume to give approximately the same salt concentration as in milk. Casein micelles were obtained from skim-milk by centrifuging at 40000 ***g*** for 80 min. They were washed by resuspension in milk dialysate, recovered by centrifuging and suspended in milk dialysate to give the same volume as the original milk. Casein-free milk was prepared from skim-milk by removing the micelles as above and removing the residual soluble casein by acid precipitation at pH 4·6. The serum was neutralized and dialysed against milk dialysate to obtain the correct salt balance.

The solutions used to make 'creams' with washed butterfat were prepared as follows: casein micelles were resuspended in a solution containing 0·01 M-NaCl and 0·01 M-$CaCl_2$ and in order to allow for the losses during preparation the volume was made up to 80 % of the original volume of the skim-milk. Sodium caseinate solutions were prepared by acid precipitation from skim-milk, the precipitate was washed once and redissolved in water by the addition of 0·1 N-NaOH to pH 6·5 and the volume made up to 80 % of the original volume of the skim-milk. Milk serum obtained after centrifuging out the casein micelles and removing soluble casein was used after neutralizing to pH 6·5 with 0·1 N-NaOH.

Preparation of emulsified butterfat

Washed butterfat at 50 °C was mixed with the appropriate volumes of separated milk, casein micelles, sodium caseinate solutions or milk serum and the mixture treated in a cream emulsifier until the globule size judged microscopically was of the same order as found in milk.

Analytical procedures

The sedimentation characteristics of the membrane material were obtained using a Spinco Model E analytical ultracentrifuge. Patterns were obtained in 0·0125 M Tris-chloride buffer, pH 8·5, containing 0·1 M sodium chloride using two 4° sector cells in the An-D rotor, one of the cells having a 1° positive wedge.

Electrophoresis was carried out at 4 °C in polyacrylamide gels (5 %, w/v) containing 0·005 M Tris-citrate buffer pH 8·6 and 8 M urea.

The gels, 1·5-mm thick and 18·5 × 15 cm, were used in a horizontal position and connexion between the gel and the buffer and electrode compartments containing 0·1 M Tris-citrate buffer pH 8·6 was by lint wicks saturated in buffer. After electrophoresisis the gels were stained with 0·1 % amido-black solution (water–methanol–acetic acid, 7·5:2·5:1·0 (v/v) containing 0·1 % (w/v) amido-black).

Nitrogen was determined by the method of Lang (1958).

Fat globule size distribution in milk and in rediluted concentrated milk was determined using a Coulter counter. The milks were diluted 500-fold with 0·9 % (w/v) aqueous NaCl before examination.

RESULTS

Factors in concentrating process affecting fat retention by curd.

Time of treatment (degree of concentration)

Bulk milk was concentrated in the evaporator and at suitable intervals representative samples were withdrawn, diluted to their original volume, and the fat content of the whey, after making disturbed curds, was determined. The fat content of the whey decreased with time of treatment in the evaporator, to a minimum of about 0·5 % (Fig. 1) when the concentration was about 3-fold. Dilution of the concentrated milk to its original volume followed by a second concentration and redilution effected a further decrease in fat loss to about 0·3 %.

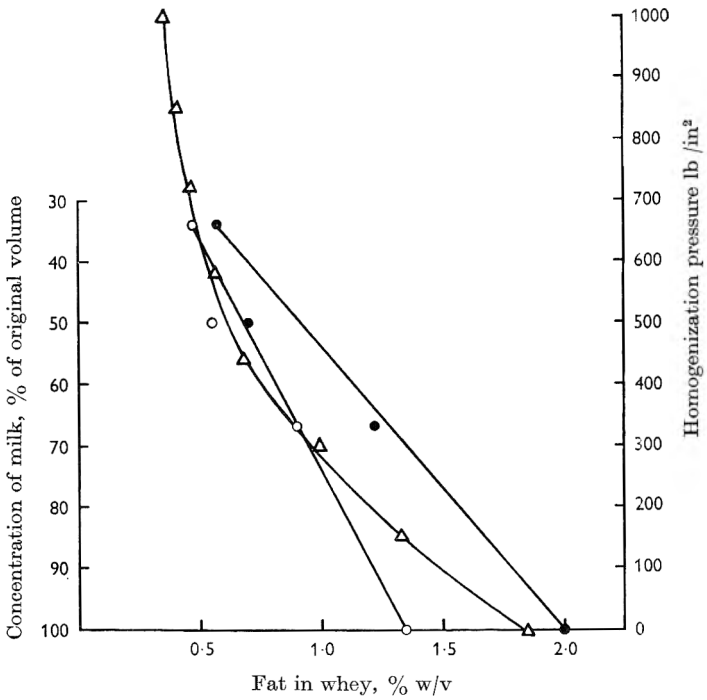

Fig. 1. Effect of concentrating or homogenizing milk to various degrees on the fat retention of the curd produced by rennetting under disturbed conditions. ○, ●, effect of concentrating (2 expts); △, effect of homogenizing.

Temperature of treatment

The jacket temperature of the climbing film column was normally 80 °C and at the vacuum used the temperature of the milk as it left the climbing film column was 34 °C ± 2; the temperature of the boiling milk at the bottom of the column gave a similar reading. It was, however, possible that local temperatures in the climbing film were considerably higher and high enough to cause protein denaturation. The temperature of the jacket was therefore decreased to 50 °C in one experiment. The temperature of the milk was now 24 °C as it left the climbing film column and, to avoid buttering, the milk returning to the holding tube had to be warmed to about 40 °C by passing it through a condenser at 50 °C. Concentration under these condi-

tions was fully effective in conferring the fat conservation property, and the idea that protein denaturation due to high temperatures was involved in the phenomenon was dismissed.

Concentration

To check whether concentration *per se* was essential in order to obtain fat conservation, milk was processed in the evaporator, but as evaporation proceeded, water was continuously mixed with the circulating milk as it left the climbing film column so that the total volume of the milk remained constant. The treatment was terminated when the volume of distillate would have represented a 3-fold concentration had the milk not been continuously diluted. The fat retention by the resulting curd was equal to that obtained in curd from milk concentration in the usual way (Table 1). It could be argued that local concentration effects were sufficient to confer the fat conservation property despite the continuous addition of water. The property was, however, also conferred when the milk was first diluted 3-fold and then concentrated to its original volume. Concentrating the milk was not, therefore, the essential feature of the process as far as fat conservation was concerned.

Table 1. *Effect of various treatments of* 2 *milks in evaporator on fat conservation property*

Treatment	Fat in whey from disturbed curd, % w/v	
	Milk A	Milk B
None (control)	2·25	1·78
Concentrated to $\frac{1}{3}$ original volume	0·40	0·45
Volume kept constant	0·52	0·45
Diluted 3-fold before concentrating to original volume	—	0·31

Formation of fat retention property

Influence of curd structure

Because of the known influence of concentrating milk on the size of casein micelles (Hostettler *et al.* 1965) it was possible that the resulting changes in gel structure would modify the fat retention ability of the curd. This hypothesis was tested by concentrating skim-milk 3-fold in the evaporator and then carefully stirring back the cream which had been removed. After dilution to the original volume no fat conservation could be detected. Modification of casein micelles or other subtle changes involving protein or salts in the absence of fat could not therefore be involved in the mechanism of fat conservation.

Role of the fat globule

The preceding experiments had demonstrated that the milk fat had to be present during concentrating if enhanced fat retention was to result. Further experiments showed that if the cream was removed from concentrated-rediluted milk by centrifuging and then stirred gently into skim-milk which had not been concentrated, the resulting whole milk had excellent fat retention properties (Table 2). The increased

fat retention of curd from concentrated-rediluted milk is, therefore, carried by the fat globules.

It was possible that the concentration process was causing some decrease in mean fat globule size. This was checked by comparing the size distribution of globules in milk with those in concentrated-rediluted milk using a Coulter counter. A decrease in mean globule size from 3·5 to 2·75 μm was observed after concentrating, and there was a general 'sharpening up' of the distribution curve. It seemed possible that such changes might explain the phenomenon of the increased fat retention since it is known that homogenization does have this effect (King, 1953). The fat retention of curds from milk treated in a homogenizer at different pressures was therefore determined (Fig. 1) and was found to increase progressively as the pressure was raised; at a pressure of 400 lb/in^2 the fat retention was as good as that obtained after 3-fold concentration of milk and even at the relatively low pressure of 160 lb/in^2 a pronounced increase in fat retention was observed. The globule size distribution in the 'homogenized' samples was not determined, but microscopic examination showed little difference between the globule sizes in these samples and in concentrated-rediluted milk.

Table 2. *Typical results of experiments showing that the increased fat retention property of curds from concentrated-rediluted milk is carried by the fat globules*

	Milk	Fat in milk, %, w/v	Fat in whey from disturbed curd, % w/v
A	Untreated	3·65	1·55
B	Concentrated and rediluted	3·55	—
C	Cream from B + untreated skim-milk	3·0	0·5
D	Cream from A + skim-milk from B	4·90	2·4
	Untreated skim-milk used for C	0·12	—
	Concentrated rediluted skim-milk used for D	0·81	—

The changes in size of the fat globules as a result of the treatment in the evaporator, or in the homogenizer at low pressure, seemed to be too small to explain on the basis of globule size alone the large effect on the fat retention by the curd. It was, therefore, deduced that in both cases the effect was caused by some change in composition of the existing fat globule membrane and this aspect was investigated for concentrated-rediluted milk.

Influence of concentration on composition of fat globule membrane

It has been suggested (e.g. Hayashi & Smith, 1965) that a least some, if not most, of the outer membrane of the fat globule is easily removable by such physical methods as stirring, concentration and homogenization at low pressures. Even washing of cream with water causes high lossess of soluble material from the membrane (Erickson, Dunkley & Smith, 1964).

The property of increased fat retention may, therefore, be due to removal of the outer membrane, or part of the outer membrane, with resulting changes in the

character of the membrane surface. Further, the removed material may be replaced by other milk protein or lipoprotein which would affect the membrane properties. If it is only removal of the membrane or part of the membrane that results in enhanced fat retention by disturbed curd, then cream that has been washed in water several times should, when added back to skim-milk show the retention property. This, however, did not occur and the loss of membrane material does not, in itself, therefore confer the property.

Table 3. *Effect of concentrating and rediluting milk on rate of creaming*

	Fat left in milk after centrifuging, % w/v	
	Untreated milk	Concentrated-rediluted milk
After 1st centrifuging (250 *g*)	1·36	1·85
After 2nd centrifuging (250 *g*)	0·40	1·42
After 3rd centrifuging (40,000 *g*)	0·00	0·02

Table 4. *Analysis of washed cream prepared from concentrated-rediluted and untreated whole milks and the casein content of the washings*

Expt. no.*		Total membrane N, mg	Total fat, g	Membrane N, mg/g fat	% of total casein in washes		
					1	2	3
1	Concentrated	28·4	17·1	1·7	82	15	3
	Untreated	35·6	43·4	0·8	95	4	1
2	Concentrated	32·5	14·0	2·3	83	13	4
	Untreated	40·1	43·7	0·9	95	4	1
3	Concentrated	42·9	19·5	2·2	—	—	—
	Untreated	46·6	22·4	2·1	—	—	—
4	Concentrated	41·1	23·6	1·7	—	—	—
	Untreated	45·0	21·5	1·8	—	—	—

* In expts. 1 and 2 the cream was prepared from 2 l. milk using the cream separator; in expts. 3 and 4 the cream was prepared from 900 ml milk using a laboratory centrifuge for separation.

The effect of concentration upon the creaming properties of the rediluted concentrated milk is very marked, as is shown from the residual fat in the milk after various periods of centrifugation (Table 3). The fat content of the cream from the concentrated-rediluted milk as first isolated on the separator was less than half of that of the control cream, and the volume obtained varied between 60–90 % of that from untreated milk. At each wash the fat content of the cream from concentrated milk increased as the milk constituents were removed until the value was the same as that of the control cream. There was a corresponding decrease in volume of cream recovered. The nitrogen content of the washes was determined to see if the casein and non-casein contents were similar from the 2 creams. The casein in each wash was recovered by addition of 0·1 N-HCl until the pH was 4·6 . The residual nitrogen in the washes gave comparable values for both control cream and cream from concentrated milk, however, the casein contents of the second and third washes were comparatively higher for the cream from concentrated milk. The results for casein content of the creams are shown in Table 4 and suggest that the casein is, in some

way, linked with the fat globules of milk after concentrating so that its removal by washing the cream is less effective.

In the first 2 experiments shown in Table 4 the nitrogen:fat ratio for the control cream was about half of the value obtained for the other samples, this may have been due to some loss of membranous material by the treatment on the cream separator and may reflect the instability of the fat globule membrane. Furthermore, it would suggest that the modified membrane obtained after concentration is more stable.

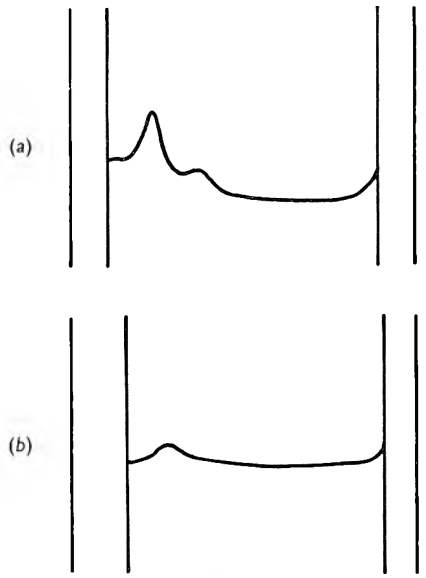

Fig. 2. Sedimentation patterns obtained on the ultracentrifuge of soluble material from the fat globule membranes. Conditions as in text. Sedimentation is towards the right. (*a*) Material from concentrated-rediluted milk after 70 min at 42040 rev/min; (*b*) material from untreated milk after 38 min at 42040 rev/min. The solution of the material from the concentrated-rediluted milk was twice as concentrated, in mg N/ml, as the solution from the untreated milk.

Qualitative analysis of membrane

The water-soluble material associated with the fat globule membrane in the washed cream was investigated by analytical ultracentrifugation and gel electrophoresis. The ultracentrifuge patterns are shown in Fig. 2 and indicate that the material from both samples was heterogeneous. Material from the control cream had an $S_{obs.}$ value at the peak of distribution of about 6·5*S*. The 2 peak regions of the material from the concentrated milk had $S_{obs.}$ values of about 4·7*S* and 6·8*S*, respectively. This evidence indicates that the soluble material from the membrane obtained from the cream of concentrated-rediluted milk contains a significant proportion of particles with lower sedimentation rates; the electrophoresis patterns (Fig. 3) also demonstrate the presence of 2 extra protein components, probably α_s-casein and β-casein. The material in common in the 2 preparations stained poorly and had low electrophoretic mobility under the conditions used (Fig. 3*b*, *c*).

Constituents of milk involved in fat conservation property

Washed cream samples were mixed with appropriate volumes of either separated milk, milk dialysate + casein micelles, or milk serum, and the mixtures concentrated

3-fold in the evaporator. The concentrates were then diluted to the original volume and the cream recovered by centrifuging. The creams were tested for fat retention property by stirring back into skim-milk and making disturbed curds in the usual way. As seen from Table 5, treatment with both separated milk and casein micelles conferred good fat retention properties, treatment with milk serum had less effect.

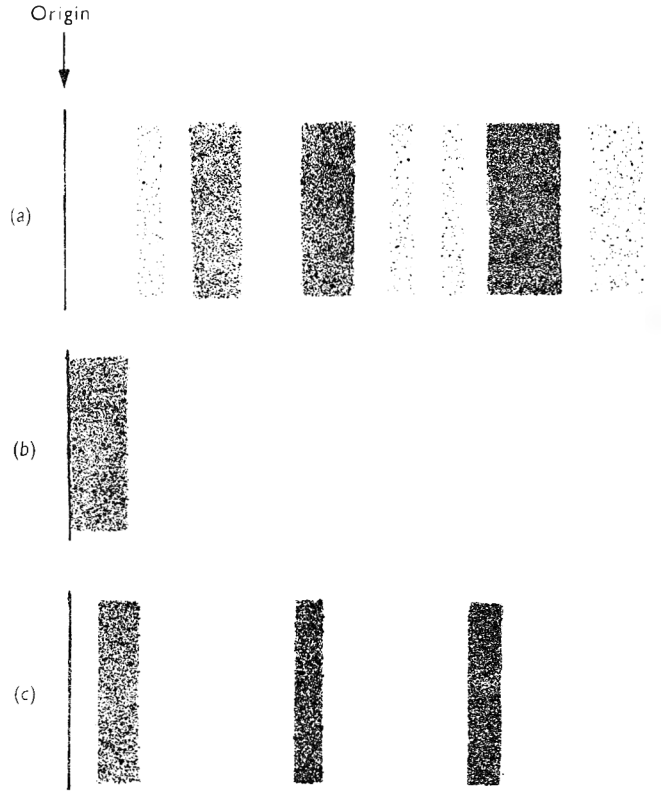

Fig. 3. Electrophoresis pattern obtained in polyacrylamide gels of the soluble material from the fat globule membrane. Voltage, 300 V for 4 h; current, 45 Ma. (*a*) Whole casein; (*b*) material from untreated milk; (*c*), material from concentrated-rediluted milk.

Table 5. *Effect of treating washed cream in the climbing film evaporator in the presence of various milk fractions* and the effects of various milk fractions used in the preparation of emulsions of butterfat on the fat conservation property*

	Fat in whey, % of total fat		
Milk fraction†	Using cream	Using butterfat‡	
Separated milk (control, not concentrated)	63·5		
Separated milk	9·6	8·1	10·0
Casein micelles	1·6	5·6	12·8
Sodium caseinate	—	62·2	37·5
Milk serum	30·4	4·2	22·6

* After concentration treatment the cream was recovered and incorporated in untreated separated milk. Disturbed curds were then made.

† See Methods for preparative procedures.

‡ Results of 2 separate experiments.

'Creams' made from butterfat emulsified in separated milk, micelles and milk serum gave creams which had good fat retention properties but those made in sodium caseinate did not (Table 5).

DISCUSSION

Although treatment of milk in a climbing film evaporator results in high fat retention in the curd produced by rennetting the rediluted milk (Hutin & Stenne, 1966), we have shown that the concentration of milk constituents is not in itself the cause of the effect.

The high fat retention property results mainly from modifications to the composition of the fat globule membrane and the results indicate that the incorporation of casein micelles or whey protein into the membrane is responsible, Since the ratio membrane-N/fat in the fat globule is not appreciably changed after concentration and redilution of the milk, it is deduced that the original membrane material is lost before new protein is incorporated. Although the combination of fat globule with casein micelles is very efficient in conferring the fat conservation property it is not understood why sodium caseinate does not produce a similar effect (Table 5).

Evidence that the fat globule membrane may consist of at least 2 layers of lipoprotein, of which the outer layer is more easily removed, has been presented by Hayashi & Smith (1965). Our results are consistent with a hypothesis that the fat conservation property is formed in 2 stages: (1) the outer layer of lipoprotein is removed, and (2) casein or other milk protein is joined to the globule, perhaps to the inner membrane.

The removal of the original membrane material may occur to some extent in the evaporator but it is also known to occur when milk is cooled and stirred (Greenbank & Pallansch, 1961). Thus, the efficiency with which the fat conservation property is conferred may partly depend on the previous history of the milk.

The factors involved in the second stage, the combination of fat globule and milk protein, are not understood, Preliminary results strongly suggest that the reaction is a surface phenomenon which takes place in the climbing milk film of the evaporator. The alteration in the nature of the fat globule membrane is reflected in the change in creaming properties of the rediluted concentrated milk and it is interesting to note that homogenization, which is known to reduce creaming rate, also confers the fat retention property.

Since incorporation of casein in the globule membrane results in high fat retention in the curd it is suggested that, after rennetting, the casein in the membrane is linked to the casein of the coagulum by chemical bonds and the fat globules therefore become an integral part of the curd. Until now it has been generally assumed that in normal curd the fat globules are mechanically trapped, It now seems more probable that weak but significant forces normally occur between the lipoprotein of the normal globule membrane and the micelle structure of the curd, and that these forces are considerably increased when casein is incorporated into the membrane by treatment in the evaporator.

The Hutin & Stenne (1966) process claims to result not only in higher yields of cheese fat but also in higher yields of protein. Although the results presented here show that the concentration of the milk constituents is not essential for the increased

fat retention of the curd, the effect of concentrating the milk on yield of protein was not investigated.

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The C-terminal sequence of amino acid residues of κ-casein isolated from buffalo's milk

By M. H. ABD EL-SALAM* and W. MANSON
The Hannah Dairy Research Institute, Ayr

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Summary. When κ-casein from buffalo's milk was treated with carboxypeptidase A (EC 3.4.2.1),4 amino acids, valine, threonine, serine and alanine were released from the protein in a manner consistent with the view that they originate in the C-terminal sequence of a single peptide chain. The amounts produced suggest a minimum molecular weight for buffalo κ-casein of approximately 17000, in agreement with the value calculated from the phosphorous content on the basis of the presence of 2 phosphorus atoms/molecule. A comparison is made with the C-terminal sequence reported for bovine κ-casein.

The casein complex of buffalo's milk may be separated satisfactorily into 4 main constituents by application of procedures designed originally for the fractionation of casein derived from cow's milk. These 4 components, like those from bovine casein, are distinguishable electrophoretically and they may be considered as being analogues of the α-, β-, γ- and κ-caseins of cow's milk. Further comparison of properties of caseins from the 2 species, e.g. in respect of their behaviour on electrophoresis (Aschaffenburg & Sen, 1963) and on treatment with proteolytic enzymes (Ganguli, Prabhakaran & Iya, 1964), has however revealed differences which suggest that the corresponding fractions from the 2 species although analogous, may nevertheless differ significantly in structure. In this paper the C-terminal sequence of amino acid residues of κ-casein from buffalo's milk is reported.

MATERIALS AND METHODS

Preparation of κ-casein

Freshly skimmed-milk obtained from individual buffaloes of the herd of the Bahtim Agricultural Station, U.A.R., was adjusted to pH 4·5 at 25 °C by addition of N-HCl and the precipitated casein washed free from contaminating whey proteins with water. It was then dissolved in 6·6 M-urea and the κ-casein present isolated following a procedure which was essentially that described by Zittle & Custer (1963) for the preparation of the κ-component of bovine casein. It was stored at 4 °C as a freeze-dried powder. Prepared in this way, κ-casein contained 0·36 % phosphorus as determined by the method of Allen (1940) and exhibited a maximum light absorption

* Present address: The Dairy Research Unit, National Research Centre, Cairo, U.A.R.

at 278 mμ, $E^{1\%}_{1\,cm} = 11{\cdot}0$. The corresponding values reported for bovine κ-casein were 0·30 and 12·2 %, respectively, (Zittle & Custer, 1963). The preparation of buffalo κ-casein was subjected to starch gel electrophoresis at pH 9·2 in the buffer system described by Aronsson & Grönvall (1957). Before application to the gel, which was made 6 M with respect to urea and 0·25 % (v/v) with respect to mercaptoethanol, the protein was first allowed to stand for 1 h at 20 °C in a 1 % (v/v) solution of mercaptoethanol in buffer. After electrophoresis at 4–5 mA/cm² (170–200 V) for 16 h at 4 °C, this preparation behaved essentially as a single protein.

Determination of C-terminal sequence

The buffalo κ-casein was digested with carboxypeptidase A (EC 3.4.2.1, Sigma Chemical Co., London). The enzyme preparation had been treated with diisopropylfluorophosphate to eliminate possible activity arising from contamination with trypsin or chymotrypsin. The digestion was performed in a total volume of 10 ml at 25 °C and at pH 8·0, with 50 mg samples of κ-casein. An enzyme:substrate ratio of 1:500 was employed, and since the substrate itself was found to provide sufficient buffering action, no additional buffer was incorporated in the reaction mixture. Samples (1·0 ml) were withdrawn from the digestion mixture at regular intervals and immediately acidified to pH 3·0 by addition of 0·1 N-HCl to stop further enzyme action. The amino acids released by the action of carboxypeptidase A were then separated from the remaining constituents of the digestion mixture by passage of these samples through a column of Sephadex G–25, 1·6 cm diam. and 25 cm long, prepared from a suspension in 0·1 M-NaCl adjusted to pH 3·0 with 0·1 N-HCl. The column was washed with this acidified NaCl solution and the eluate was collected in 2·0-ml fractions. It was found in preliminary experiments with standard amino acid mixtures, that under these conditions only fractions 16, 17, and 18 contained amino acids as judged by their reaction with ninhydrin (Moore & Stein, 1954), and that furthermore a recovery of 96 %, based on the colour yield with this ninhydrin reagent, was obtained. Accordingly, the 16–18th fractions from the digestion samples were combined and reduced in volume to 3·0 ml by evaporation at room temperature *in vacuo* over anhydrous $CaCl_2$. Quantitative amino acid analyses were then performed on these extracts using an Automatic Amino Acid Analyser (Evans Electroselenium Ltd., Halstead, Essex, England) following the procedure of Spackman, Stein & Moore (1958). As an additional check, fractions 15 and 19 from each sample were treated with ninhydrin but in no instance was the presence of amino acids detected.

The absence of tryptophan from the digestion mixture was confirmed separately by examination of the supernatant liquid obtained after acidification of the reaction mixture to pH 4·5 and removal of the precipitated protein by centrifugation. No selective light absorption at 278 mμ indicative of the presence of an aromatic amino acid, could be detected.

RESULTS AND DISCUSSION

The results obtained from a typical digestion of κ-casein from buffalo's milk by carboxypeptidase A are shown in Table 1. It is apparent that under the influence of this enzyme, 4 amino acids have been split from the protein. The order and manner

of their release is well-defined and indicates clearly the presence at the C-terminal end of a single peptide chain of the sequence –Ala.Ser.Thr.Val OH. This being so, it is possible to estimate the molecular weight of the primary unit of κ-casein from the amounts of these amino acids liberated, particularly from the amounts of valine and threonine produced since no increase in either was observed during the last hour of the digestion. On this basis a value of approximately 17000 was obtained. When consideration is given to the phosphorus content of 0·36 %, it is apparent that this value is in good agreement with that of 17200 calculated for the presence of 2 phosphorus atoms/molecule of κ-casein. A molecular weight of between 18000 and 20000 has recently been suggested for the primary unit of bovine κ-casein (Swaisgood & Brunner, 1963).

Table 1. *Amino acids released from buffalo κ-casein by action of carboxypeptidase A*

	Incubation time, min				
	15	30	60	180	240
Amino acid	Amino acids released (moles/17000 g protein)				
Valine	0·41	0·72	0·83	1·03	0·97
Threonine	0·04	0·14	0·42	1·06	1·06
Serine	0·00	0·07	0·29	0·72	0·92
Alanine	0·00	0·00	0·00	0·39	0·49

The findings of this investigation, while indicating a general similarity in the structures of bovine κ-casein and that from buffalo's milk, differ in detail from those of earlier investigations of bovine κ-casein. Thus, treatment of bovine κ-casein with carboxypeptidase A has been reported to release the 4 amino acids reported here as being produced from buffalo's κ-casein (Jollés, Alais & Jollés, 1962), and a similar result was obtained from the glycopeptide prepared by the action of rennin (EC 3.4.4.3) on bovine κ-casein (Jollés, Alais & Jollés, 1961). However, the sequence of their release from each of these sources was stated to be valine, alanine, threonine and finally serine. While this differs from that found for buffalo's κ-casein in respect of the position occupied by alanine, it is perhaps significant that this sequence was apparently deduced from experiments with bovine κ-casein in which the enzyme action extended over only 1 h at the end of which a single determination was made of all 4 amino acids liberated, whereas in the present study the enzyme:substrate ratio was adjusted to enable the sequence of release to be studied over a much longer period. Further work is, therefore, needed to confirm that the κ-caseins from cow's and buffalo's milk differ in their C-terminal sequence of amino acid residues.

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A compound responsible for metallic flavour in dairy products

II. Theoretical consideration of the mechanism of formation of oct-1-en-3-one

By R. A. WILKINSON and W. STARK
Division of Dairy Research, C.S.I.R.O., Melbourne, Australia

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Summary. Possible mechanisms are considered for the formation of a metallic flavour component, oct-1-en-3-one, in a vacuum-packed butterfat-antioxidant-synergist mixture, and in washed cream and butter with free access to atmospheric oxygen. The oct-1-en-3-one is likely to be derived from linoleic or arachidonic acids, or both. A complex of reactions linking oxygen, catalysts, substrate, radicals, and antioxidants is discussed. Pathways for the production of intermediates giving rise to oct-1-en-3-one are suggested for systems with free and with limited access to oxygen. These mechanisms would involve secondary oxidations with formation of polyfunctional monomers and would be competing for oxygen with those giving rise to polymers. The reasons why they compete to a significant extent are discussed in relation to the detailed environmental conditions including lipid reactivity, effective oxygen pressure *in situ*, steric effects at phase boundaries and the role of acidity and of ionic intermediates.

Among the increasing number of compounds now being identified as contributors to off-flavours in oxidized lipids, perhaps one of the most significant is oct-1-en-3-one. This applies particularly to dairy products where, in certain storage flavour defects described as 'fishy-oily', 'trainy', 'metallic' and 'oxidized', it has been shown (Stark & Forss, 1962) that oct-1-en-3-one is responsible for the metallic component of the flavour. Some workers describe this as the 'oxidized' component. (El-Negoumy & Hammond, 1960; El-Negoumy, Miles & Hammond, 1961; Hammond, El-Negoumy & de Puchal, 1961).

The importance of the discovery of oct-1-en-3-one in lipids lies first in the fact that it is a very powerful tainter and its contribution to off-flavours, especially those of the 'fish-oil' type, is probably greater than any other single component isolated from flavour distillates. Secondly, it marks the recognition of a new class of compound deriving from early oxidation in lipids.

Oct-1-en-3-one is always a minor quantitative constituent of the volatile flavour fraction and does not accumulate with even a greatly increased degree of oxidation. This characteristic, as well as the conditions which are known to influence the formation of metallic flavour in oxidizing lipids, is taken into account in the mechanisms for the formation of oct-1-en-3-one which are suggested in this paper.

Sources of metallic flavour

Metallic flavour was first isolated as a fraction of a fish-oil flavour, which developed in a commercially prepared butterfat to which nordihydroguaiaretic acid (NDGA) and citric acid dissolved in propylene glycol had been added as an antioxidant, and which had then been stored in vacuum-sealed cans. The final concentrations of NDGA and citric acid in the fat were 0·005 and 0·01 %, respectively. Pont, Forss, Dunstone & Gunnis (1960) made an extensive study of the development of the flavour and concluded that a combination of factors was responsible. These were the presence of a dissolved acid (citric or lactic), NDGA and a restricted air supply.

Pont *et al.* (1960) drew attention to the similarity of both the fish-oil flavour in the butterfat and the fish-oil flavours in butter made from washed cream treated with copper and ascorbic acid, to that in salted butter made from acid cream. Forss, Dunstone & Stark (1960*b*), therefore, analysed fish-oil flavours developed in washed cream in the presence of 5 ppm. copper and 50 ppm. ascorbic acid. (Without the ascorbic acid the same flavour developed, but more slowly.) A comparison of the gas chromatograms of the volatile carbonyls stripped from butterfat containing NDGA and citric acid and those from butter made from washed cream (Forss *et al.* 1960*b*) gave the following results: (i) the same components occurred in both flavours but in different relative amounts, mainly in respect of unsaturated aldehydes; (ii) the oct-1-en-3-one occurred as a minor quantitative component in both, and to about the same extent; (iii) the quantity of this compound found immediately after churning remained almost unaltered on holding the butter for 9 days.

Metallic flavour has also been induced in safflower oil, containing approximately 70 % linoleate, emulsified with 0·5 % water containing copper and ascorbic acid at pH 1·5 (Stark & Forss, 1962). El-Negoumy *et al.* (1961) and El-Negoumy, de Puchal & Hammond (1962) isolated very small amounts of an 'oxidized' flavour component from linoleate esters as well as from butterfat. They concluded that it was identical with the metallic component isolated in these laboratories, and that linoleate was its precursor.

Lea (1943, 1960) found that fishy odours in dehydrated meat developed strongly only in sealed containers with limited oxygen supply. A greater incidence of oxidized flavour in 'vacuum-capped' milk than in 'air-packed' milk was reported by Brown, Tracy & Prucha (1936).

Conditions of formation

The complex relationships between the factors influencing the paths of oxidation in such food lipid systems were discussed by Wilkinson (1964) with particular attention to the functions of catalyst, acidity, oxygen-tension and phase relationships. Possible mechanisms for the formation of oct-1-en-3-one were suggested and will be related here to the particular conditions prevailing in the washed cream butter and in butterfat with NDGA-citric acid.

The systems differ considerably in some physical and chemical aspects. These differences are mainly the presence in the butterfat of an added phenolic-type antioxidant, a restricted oxygen supply and a condition of homogeneity, whilst in the washed cream, and the butter made from it, there is a higher concentration of phospholipid, apparently free oxygen supply and interfaces of lipid, water and

protein. However, in both systems there is a metal ion catalyst and a proton donor which accelerates development of the flavour.

In cream and butter, protein and the phospholipid of the fat-globule membrane probably serve to facilitate the catalytic functions of the important copper ions by 'fixing' them close to the ideal site for catalysis of lipid oxidation, while at the same time by chelation they increase the intrinsic catalytic activity of the metal. The aqueous phase favours this chelation by providing the polar ionic medium for reaction, and is essential for maintenance of the structure of the lipo-protein complex of the membrane.

The exact role of NDGA is not clear but while it is normally antioxidant there are reasons to believe that NDGA-citric acid in propylene glycol added to butter oil could have pro-oxidant properties. The reasons are:

(i) The antioxidant properties of NDGA decrease rapidly as the pH is lowered from pH 6·5 until at pH 3·0 a concentration of 0·005 % has no antioxidant effect in the oxidation of phospholipid (Stull, Herreid & Tracy, 1951).

(ii) Traces of cupric ion, frequently observed in this laboratory to be an impurity in the citric acid could be almost immediately reduced by NDGA. Flitman & Frieden (1957) reported the rapid reduction of cupric ion by NDGA in aqueous acetone buffered at pH 7·2. However, the effects of (*a*) possible sequestration of cupric ion by citric acid and (*b*) low pH values were unknown.

We therefore studied the rates of reaction of NDGA and cupric ion in 33 % aqueous ethanol both with and without citric acid. The final concentrations of copper and citric acid were 8×10^{-6} M and 7×10^{-3} M, respectively, representing approximately a 500-fold excess of citric acid over the theoretical sequestrant amount. NDGA was added in 200-fold excess and the pH was 3·6 in all cases.

The formation of cuprous copper was detected with cuproin (2,2′-biquinoline) and measured spectroscopically at 543 mμ in a Beckman DK 2 spectrophotometer using a time-drive attachment.

Results showed that the rate of reduction of cupric ion at room temperature and pH 3·6 was not influenced by the presence of citric acid, the reaction being 99 % complete in 33 sec from time of mixing. The action of the NDGA in reducing the cupric ion complexed by citrate appears analogous to that by which ascorbic acid reduces cupric ion complexed by protein (Frieden, 1958). When citric acid was dispersed in the fat as a powder, and not prepared as a concentrated solution in propylene glycol, the off-flavours were not apparent (Pont *et al.* 1960).

(iii) Phenolic antioxidants are known to show pro-oxidant activity and this has been explained by Privett & Quackenbush (1954), using a concept later refined by Privett (1961), on the basis of their reactions with hydroperoxides to produce further radicals.

$$AH + ROOH \rightarrow A\cdot + RO\cdot + H_2O$$

or

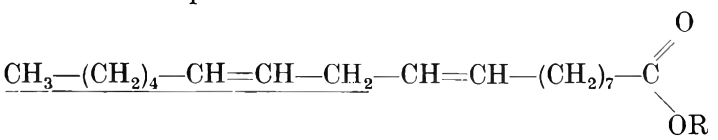
$$AH + ROOH \rightarrow A\cdot + R\cdot + H_2O_2$$

The phenomenon is normally observed at high antioxidant concentration and high oxygen pressure but as is discussed later could occur under the conditions in which metallic flavour is formed.

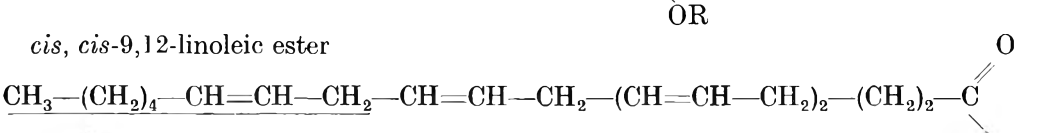
Parent substance of oct-1-en-3-one

The systems in which metallic flavour develops may also differ in the unsaturated fatty acid from which the oct-1-en-3-one is derived. It seems probable, because of the amyl structure $CH_3(CH_2)_4$— in oct-1-en-3-one, that either 9,12-linoleic esters or 5,8,11,14-arachidonic esters, or both, are the parent substance. Analysis of milk lipids (e.g. Scott, Herb, Magidman & Riemenschneider, 1959; Smith & Lowry, 1962) shows that these are the only readily oxidizable unsaturated esters of known double bond location which can retain the amyl radical after primary oxidation. The non-conjugated tetraene in butterfat has been shown by Bosworth & Sisson (1934) to be arachidonic acid.

It is possible that other more highly unsaturated esters such as pentaene and perhaps hexaene acids (Shorland & Johannesson, 1951) but of unknown double bond location, may be the source of oct-1-en-3-one. However, the discussion of mechanisms which follows will apply equally well should such esters contain within their structure the underlined portion of the esters shown.



cis, cis-9,12-linoleic ester



all *cis*-5,8,11,14-arachidonic ester

Arachidonic ester oxidizes more rapidly than linoleic ester and, because of its high concentration in phospholipid (approximately 10 % of total fatty acids (Smith & Lowry, 1962)), it would appear the more likely precursor in products retaining their fat globule membrane structure. In butterfat, linoleic ester concentration is approximately 15 times that of arachidonic which occurs to the extent of only 0·1–0·2 % (Smith & Lowry, 1962; Schaffer & Holm, 1950).

General considerations

Before considering in detail the possible pathways of oxidation, it is desirable to view in some perspective the complex of reactions which we believe can link the activities of catalyst, antioxidant, substrate, molecular oxygen, radicals and intermediate compounds. These are set out schematically in Fig. 1. The catalytic reactions have already been discussed in detail (Wilkinson, 1964) whilst those leading to polymer formation are discussed below.

It will be seen that the metal ions besides reacting with the hydroperoxides also react with other constituents in ways which will influence the course of oxidation. These include the following:

(1) Radical-forming electron-transfer reactions between oxygen and metal ion.

(2) A chain reaction leading to lowering of antioxidant concentration, with consequent lowering of oxygen tension.

(3) Catalysis of formation of the keto group.

The predominant radical species at low oxygen pressure will be RO·, R· and ·OH or, more probably, their complexes with copper ions; e.g. Cu^{+}—OH is the likely oxidant species under the described conditions rather than the free radical ·OH since the lifetime of this radical is extremely short. It is, however, used in the latter form for convenience. At high oxygen pressure the extremely low activation energy for the reaction $R\cdot + O_2 \rightarrow RO_2\cdot$ will ensure the predominance of $RO_2\cdot$. Concentration of R· is related to the reactivity of the olefin.

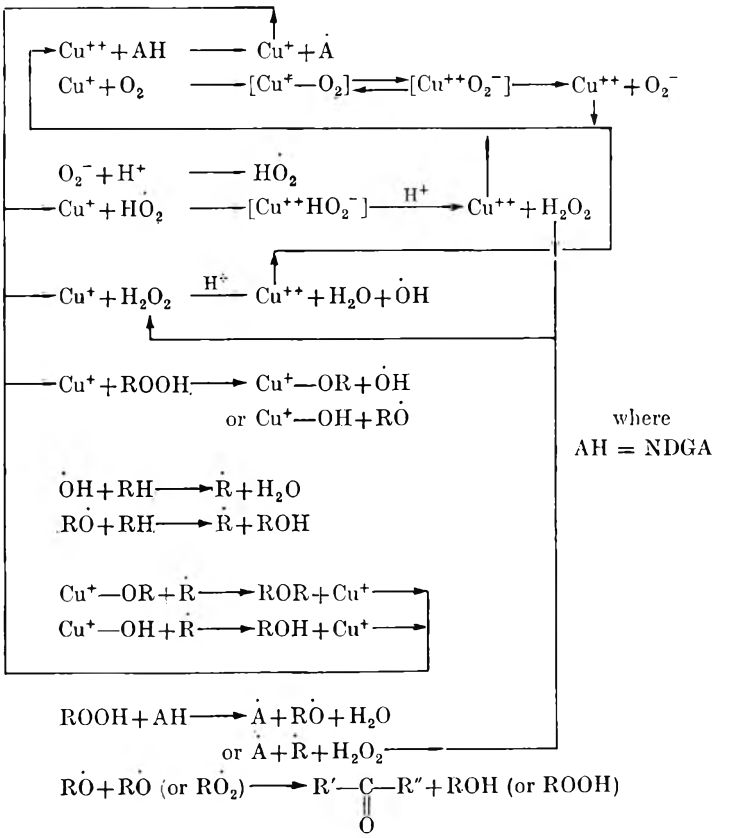


Fig. 1.

Suggested mechanisms

Any suggested mechanism of formation of oct-1-en-3-one must taken account of the various conditions experienced in the experimental systems, in particular both free and limited access of oxygen. These two conditions are considered separately in the mechanisms suggested.

The proposed mechanisms are based upon secondary oxidation of the initial monomeric oxidation products. Such secondary oxidations lead mainly to the formation of dimers or polymers, and the proportion of such volatile multi-functional products as oct-1-en-3-one will therefore tend to be small.

(i) *Secondary oxidations of lipid with free oxygen access*

Primary oxidation at carbon atom 13 in linoleate, or 15 in arachidonate, would result in the following structure:

$$R'—R''—CH\overset{t}{=}CH—CH\overset{c}{=}CH—R'''$$

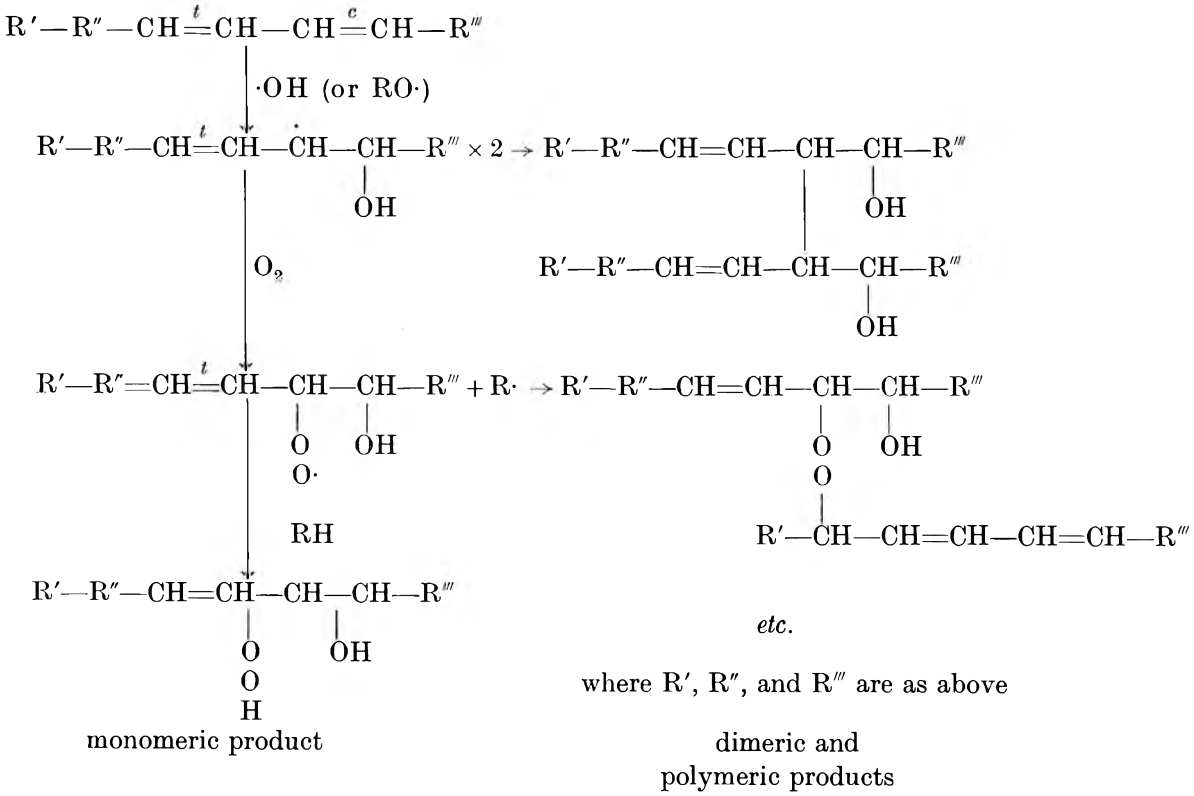
where R′ is $CH_3—(CH_2)_4—$

R″ is either —CH(OOH),

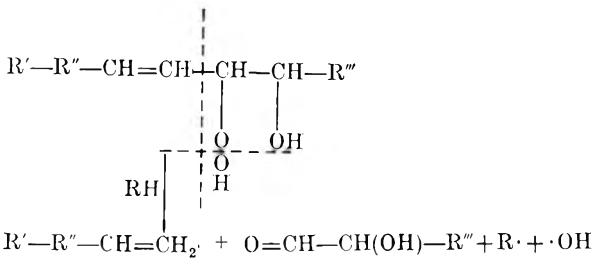
—CH(OH),

or —C=O

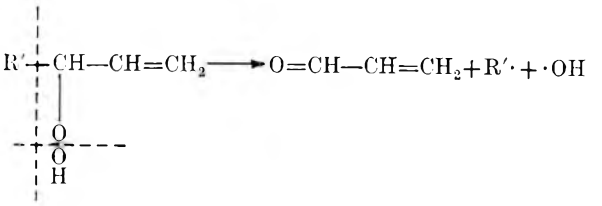
R‴ is the remainder of the ester chain. Secondary oxidation may occur over the conjugated carbon-carbon structure of the primary product, particularly in linoleate which no longer contains activated methylene groups. Attack by a radical at the *cis* double bond creates a new radical site to which in turn molecular oxygen may add with eventual formation of a hydroperoxy-group or polymers:



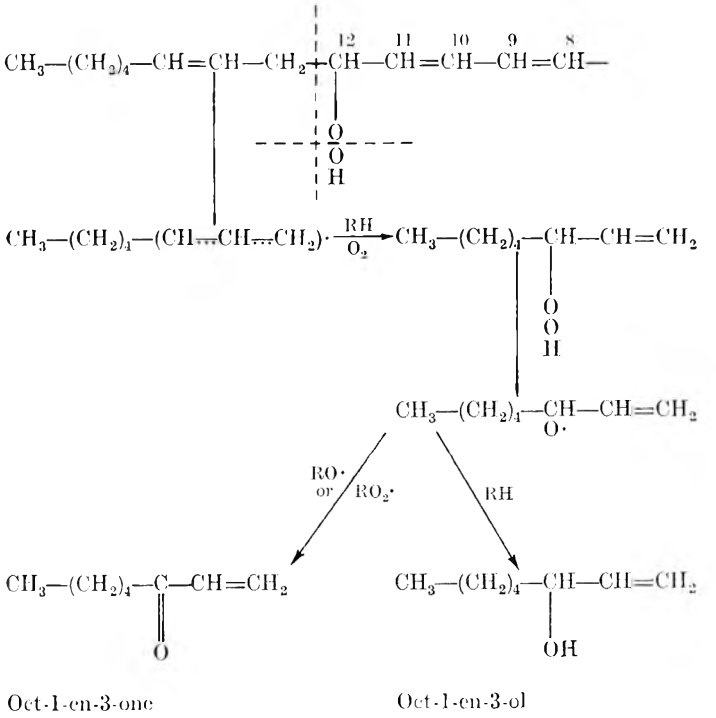
Decomposition of the monomeric product with cleavage of the C—C chain at the 10–11 bond in linoleate, or the 12—13 bond in arachidonate, followed by propagating hydrogen exchange reaction with substrate lipid, may then result in the formation of oct-1-en-3-one or oct-1-en-3-ol, the latter compound being responsible for the mushroom flavour reported by Hoffmann (1962) and Stark & Forss (1964):



If R″ is —CH(OOH) it may be dehydrated or reduced to a keto or hydroxy group before or after the chain cleavage shown. Decomposition *after* the chain cleavage may also lead to further fragmentation with formation of propenal, which has been isolated from mildly oxidized lipids (Forss, Dunstone & Stark, 1960*a*).



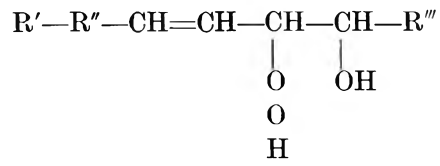
Another mechanism by which oct-1-en-3-one or oct-1-en-3-ol may be formed under these conditions relates specifically to arachidonic esters and therefore to oxidizing membrane lipid, provided the C_{12} mesomeric radical may be formed along with the preferred C_{15} species.



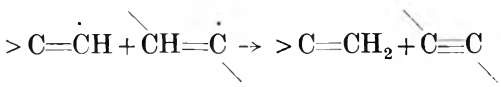
The vinyl hydroperoxide formed is identical with that of the previous mechanism and is therefore open to the same possible reaction of propenal formation.

(ii) *Secondary oxidation of lipids with restricted oxygen access*

Alternative reactions of the intermediate

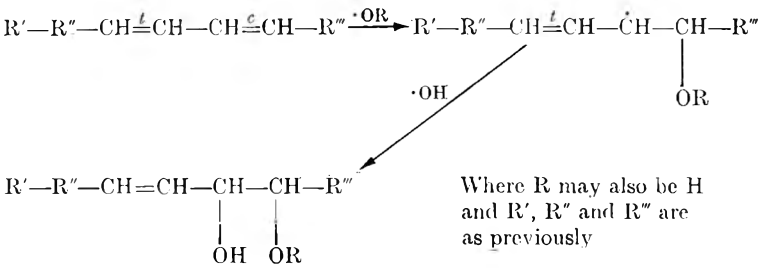


must be considered when such a structure may be formed under conditions of continuously decreasing oxygen pressure. Formation in the initial stages of oxidation at high partial pressure of oxygen may be followed at a later stage by decomposition under low oxygen pressure conditions. Thus, radicals of the type R′—R″—CH=$\dot{C}$H, derivable from the above structure, may undergo disproportionation as follows:



Although such a reaction may be swamped in the presence of oxygen by the formation of new terminal hydroperoxides, Merritt, Forss, Angelini & Bazinet (unpublished) have isolated small amounts of hept-1-yne and oct-1-yne together with a number of alk-1-enes from autoxidized butterfat containing copper ion.

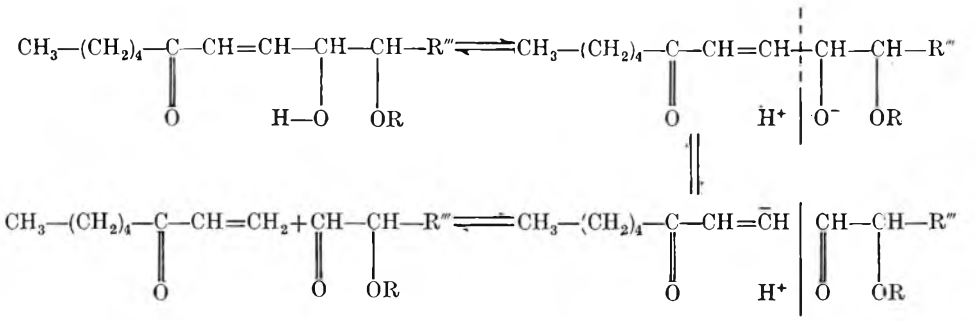
Secondary oxidation may take place without involving the addition of molecular oxygen.



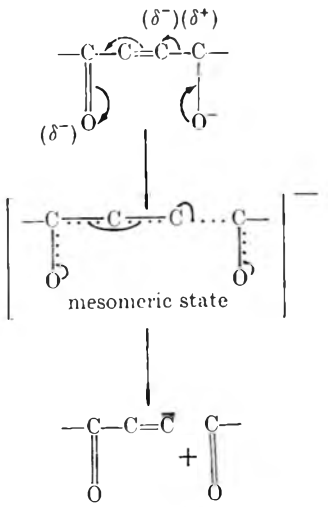
Double radical addition, which may be either 1,2 or 1,4 to the conjugated diene system with the formation of hydroxylated intermediates such as the 1,2 type shown above, is also likely with metal catalyst and low oxygen pressure. Here, the chance of addition of oxygen to the new free radical site, created by the addition of the first radical, is decreased, whilst the adding radicals are generated by the metal-catalysed reactions shown earlier. Both of these reactions are of a termination type and thus the quantity of intermediate will be limited.

The extent of decomposition of hydroxylated intermediates may well depend on the acidity factor, which appears to strongly favour metallic flavour formation. Acids are known to catalyse decomposition of hydroperoxides (Privett, 1959), but these primary products of oxidation would not appear likely to be converted to oct-1-en-3-one without the involvement of some secondary oxidation of the lipid chain.

Whilst the majority of the volatile products of oxidation, particularly carbonyls, can be explained by homolytic peroxide-cleavage mechanisms, sight should not be lost of a number of possible ionic decompositions of polarized (oxidized) fatty esters, particularly where 'abnormal' acidic or basic reagents are involved. One such mechanism by which it is conceivable that oct-1-en-3-one may be formed from hydroxylated keto derivatives of linoleate or arachidonate in aqueous media is shown in the nett reaction:



The reaction is essentially a reversed aldol condensation subject to the influence of a conjugated keto group. Such reactions are known to be ionic in nature (Ingold, 1957), but the extent of decomposition will be dependent upon the absolute acidity since, although reactions of this type are reversible, aldol condensation is catalysed by acids as well as alkali. Shemyakin & Shchukina (1957) have shown that many hydroxylated or unsaturated compounds are a potential source of carbonyls via ionic elimination reactions provided substituent groups on β-carbon atoms are sufficiently capable of polarizing the C—C bond to which the O—H group becomes attached. In the mechanism above, the polarizing substituent is not directly attached to the β-carbon atom but is so placed as to allow its electron-attracting influence to be transmitted via a conjugated olefinic bond. Thus a mesomeric effect replaces an inductive effect in a simple carbonyl-forming elimination reaction. The mechanism is, nevertheless, basically the same. Formation of the mesomeric anion and its decomposition may be conveniently represented thus:



The carbanion resulting from the cleavage of the C—C bond may attack any neighbouring molecule capable of donating a proton, especially hydroxylic solvents, acids etc.

For structures of the type

$$R'—R''—CH=CH—\underset{\displaystyle OH}{\underset{|}{CH}}—\underset{\displaystyle OR}{\underset{|}{CH}}—R'''$$

in which R'' is —CH(OH), decomposition is unlikely, the electron displacement effect being reversed so that oct-1-en-3-ol could not arise by the route suggested for the ketone.

The dielectric constant of the medium will be the most influential environmental factor governing reactions of this type. They are more likely to occur in dairy products which retain some, if not all of their lipoprotein structure in a substantially unaltered state, dispersed in the aqueous phase, e.g. milk, cream, washed cream and the serum phase of butter. Nevertheless, even in a non-polar medium such as butterfat, solubilization of citric or other acids with a polar glycol could assist ionic reactions.

DISCUSSION

While the reactions presented above may be plausible pathways for the formation of oct-1-en-3-one under the observed conditions, there are many known alternative and competing pathways for the progress of lipid oxidation. In particular, since secondary oxidation in olefins tends to lead predominantly to the formation of dimers and polymers, (Privett, 1961; Kummerow, 1961) it remains to be considered why sufficient diversion takes place to form even small amounts of oct-1-en-3-one and particularly why this happens mainly in the early stages of oxidation.

Polymers may be formed by extensive oxidation under conditions of free oxygen access. They are then characterized by ether or peroxide links. They may also be formed competitively with primary hydroperoxides by restricting oxygen access (Bateman, 1954) and they then contain mainly carbon-carbon or ether links.

Since, in the two main propagation reactions of autoxidation

$$R\cdot + O_2 \xrightarrow{\text{fast}} RO_2\cdot \qquad —— (a)$$

$$RO_2\cdot + RH \longrightarrow ROOH + R\cdot \qquad —— (b)$$

under conditions of unlimited O_2 concentration, $RO_2\cdot$ is much greater than R·, polymerization through termination reactions occurs mainly by 2 $RO_2\cdot \rightarrow$ non-radical end products. However, the rate of reaction (*b*) is an expression of the reactivity of the olefin RH, so that when $[O_2]$ is small polymerization by the reaction 2 R· → R—R is also observed. In addition, the radicals RO· and ·OH formed by the copper-catalysed reactions discussed earlier will enter the polymer-forming termination expressions.

If the reactivity of RH is high, e.g. in arachidonate, then dehydropolymerization occurs, as explained by O'Neill (1954), through the reactions

$$RO\cdot + RH \rightarrow ROH + R\cdot$$

and $$\cdot OH + RH \rightarrow H_2O + R\cdot$$

$$2R^{\cdot} \rightarrow R—R.$$

Where the reactivity of RH is low and $[O_2]$ is also low, double radical addition to monomers (e.g. primary reaction products of linoleate) may become the significant termination reaction and thus provide intermediates decomposing to form oct-1-en-3-one.

Secondary oxidation of initial monomeric oxidation products would be facilitated if the hydroperoxides retained their monomeric identity, usually denoted by ROOH. During the early stages of oxidation [ROOH] is low and the monomeric identity will predominate (Bateman, 1954). Decomposition of these monomers catalysed by metal ions as shown in Fig. 1, follows a unimolecular pattern, the radicals and/or ions produced being capable of both secondary oxidation and acceleration of the propagation reactions.

However, over the period in which oct-1-en-3-one formation appears to reach a static maximum, the peroxide value continues to rise, indicating that the rate of formation of ROOH is greater than its rate of decomposition. At these higher concentrations the dimeric hydrogen bonded form

$$\mathrm{ROOH \ldots \underset{H}{\overset{}{O}}OR}$$

will become significant (Bateman, Hughes & Morris, 1953), except perhaps in lipoprotein where steric effects may restrict its formation. Thermal decomposition of these dimers has been shown by Bateman (1954) to be a bimolecular reaction in the sense that 2 molecules are required to be bonded before the dimeric unit decomposes.

$$2\,\mathrm{ROOH} \rightleftharpoons [\mathrm{ROOH} \cdots \underset{H}{\mathrm{O}}\mathrm{OR}] \rightarrow \mathrm{RO_2\cdot} + \mathrm{RO\cdot} + \mathrm{H_2O}$$

The $RO_2\cdot$ radical can be reconverted to ROOH by hydrogen abstraction reactions or can form polymers, but the powerful oxidant ·OH is lost to the stable end product H_2O.

Of more significance than the results for thermal decomposition, however, are those of metal ion catalysed decomposition. Bawn, Pennington & Tipper (1951) and Bawn (1953) have shown that dimers, readily detected at concentrations greater than 0·1 M by infra-red spectroscopy (Bateman & Hughes, 1952), appear to decompose via the two basic reactions (*a*) and (*b*) below:

$$\mathrm{ROOH} + \mathrm{M}^{(n+1)+} \rightarrow \mathrm{RO_2\cdot} + \mathrm{H^+} + \mathrm{M}^{n+} \quad (a)$$

$$\mathrm{ROOH} + \mathrm{M}^{n+} \rightarrow \mathrm{RO\cdot} + \overline{\mathrm{O}}\mathrm{H} + \mathrm{M}^{(n+1)+} \quad (b)$$

$$2\,\mathrm{ROOH} \rightarrow \mathrm{RO_2\cdot} + \mathrm{RO\cdot} + \mathrm{H_2O} \quad (c)$$

The sum of these two reactions, (*c*), is seen to be exactly that for the bimolecular thermal decomposition of hydroperoxide. Changes of decomposition order therefore appear to be mainly a function of the state of molecular association of the hydroperoxide. The presence of other molecules with which monomeric hydroperoxide may form hydrogen bonds are also known to favour decomposition of the lower order (Bateman *et al.* 1953).

In butterfat, ester carbonyl oxygen may act in this way and therefore favour unimolecular decomposition of peroxide.

Lipids oxidizing with free access to oxygen will be degraded mainly via the bimolecular mechanism. This, perhaps, offers an explanation of why oct-1-en-3-one formation does not appear to increase with greatly increased hydroperoxide concentration, i.e. if oct-1-en-3-one is derived from multiply-oxidized monomers the probability of its formation is lessened as peroxide monomer concentration is decreased by dimer formation.

Restriction of oxygen supply is therefore seen to be an effective way of limiting bimolecular decomposition in favour of multiple oxidation of primary oxidation products. Its effectiveness will be greater where steric effects are not capable of restricting dimer formation, e.g. in bulk oxidation of butterfat.

It has been assumed so far that lipid oxidation in cream and butter takes place under relatively high oxygen pressure, but it seems quite possible that the high reactivity of the polyunsaturated phospholipids could lead to a localized oxygen deficiency and therefore rate-limiting conditions for continued oxidation. Bateman (1954) has shown that the higher the reactivity of olefin, the higher the pressure of oxygen at which the rate becomes sensitive to it—even linoleate ester at 45 °C becomes pressure dependent at O_2 pressures of 100–150 mmHg, i.e. just below one atmosphere of air. Arachidonate must be expected to show O_2-pressure dependence well above one atmosphere of air even at lower temperatures. As a result, diffusion-controlling conditions, e.g. existence of phase boundaries, will then influence the identity of the end product.

As a corollary, the lower the reactivity of the olefin, the lower the pressure at which the rate becomes O_2-pressure dependent and the lower the pressure at which termination reactions of the carbon-carbon type become significant. Thus, in single-phase oxidation of butterfat, linoleate will be expected to show termination characteristics at air pressures below one atmosphere while arachidonate should show these effects at air pressures even higher than atmospheric.

However, when these esters occur in phospholipid in butter or cream, spatial arrangement in the lipoprotein complex may restrict their ability to form polymers. Evidence of spatial effects has already been shown by Bergström, Blomstrand & Laurell (1950) in aqueous emulsions of linoleate where formation of micelles was considered to be responsible for the absence of polymers in samples oxidized to a level of 2 mole O_2/mole linoleate.

Thus, we may conclude that

(i) in single-phase oxidation of butterfat, radical-addition termination-type secondary oxidation of linoleate is favoured by restricted air supply (with a consequent limitation on the amount of dimeric form of hydroperoxide) and the presence of metal ions and other pro-oxidants which yield the adding radicals. Polymer formation by arachidonate is restricted by its negligible concentration in butterfat;

(ii) in lipoprotein complex, where steric effects probably prevent polymer formation of both linoleate and arachidonate

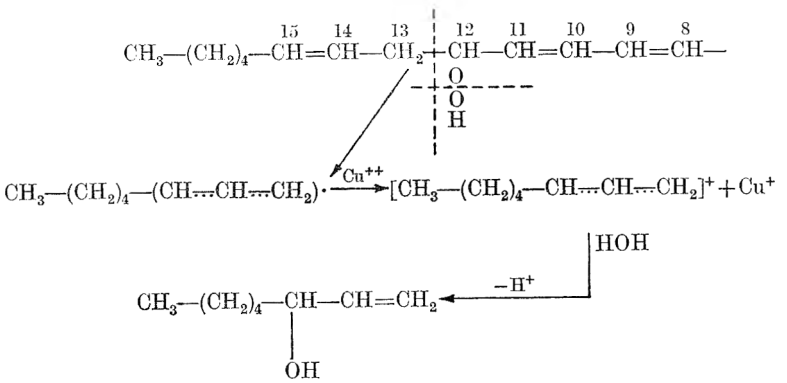
(*a*) if $[O_2]$ is not limited, secondary oxidation in arachidonate will probably proceed through its active methylene groups remaining after primary oxidation, which would not give rise to oct-1-en-3-one, whereas linoleate must oxidize over its (probably) conjugated carbon-carbon diene structure giving cyclic peroxides or hydroxy- or

alkoxy- secondary hydroperoxides which, as has been shown above, are possible precursors.

(*b*) if $[O_2]$ is limited, as we have seen it may well be locally as the result of the high reactivity of the highly unsaturated phospholipids, secondary oxidation may be restricted to radical addition in both linoleate and arachidonate, particularly where high metal ion concentration in the phospholipid catalyses peroxide decomposition and is thus a source of RO· and ·OH radicals. Both the intermediates resulting from such radical addition may give rise to oct-1-en-3-one.

Alternatively, with arachidonate, hydrogen exchange reactions of RO· and ·OH radicals with the remaining active CH_2 groups could produce lipid radicals to react with neighbouring protein or solvent (water) molecules, again decreasing the chance of oct-1-en-3-one formation. That such reactions with proteins may occur is indicated by the work of Pokorny (1963) who demonstrated that lipid derivatives may be formed with casein and albumen and of Desai & Tappel (1963) on linoleate derivatives of cytochrome C.

Reactions with water molecules may be exemplified by the possible formation of oct-1-en-3-ol from arachidonate in a hydrated system



This type of reaction was shown by Kochi & Rust (1962) to be an important synthetic method for allylic alcohols and ethers.

If these conclusions are correct, and secondary oxidations of monomers form an essential step in the mechanisms, linoleate may be a more likely parent substance than arachidonate.

Thus, although it may appear initially that oct-1-en-3-one has its origin in distinctly different mechanisms in the two systems, the same types of active intermediates may occur in both. Confirmation or otherwise of these theoretical suggestions must await a long-needed advance in our knowledge and understanding of multiple oxidation and loss of unsaturation of monomeric esters under the complex conditions in dairy products.

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