Vol. 30, No. 1 February, 1963

THE JOURNAL OF DAIRY RESEARCH

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price:—TWENTY-FIVE SHILLINGS *Net*
(U.S.A. $4.25)

The Journal of Dairy Research

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

The subscription to the *Journal* is 60*s*. per volume, for orders placed in advance of publication, and covers three issues. Single copies may be purchased at 25*s*. each. Postage extra. Subscriptions may be sent to any bookseller or to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. The subscription price in the U.S.A. is $10.00. Enquiries should be sent to the Cambridge University Press, American Branch, 32 East 57th Street, New York 22, U.S.A.

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THE JOURNAL OF THE
SOCIETY OF DAIRY TECHNOLOGY
17 DEVONSHIRE STREET, LONDON, W.1. *Tel. Langham* 5059

The Journal is issued in quarterly parts, four parts constituting a volume. The parts are usually issued in January, April, July and October each year.

The Subscription rate to non-members of the Society, payable in advance, is £2. 2*s*. 0*d*. net per volume (post free): single numbers, 13*s*. 0*d*. net. Subscriptions and all business enquiries should be sent to the Secretary, Society of Dairy Technology, 17 Devonshire Street, London, W. 1.

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The occurrence and significance of blood in bovine milk

By C. G. RAMMELL

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(*Received* 17 *July* 1962)

Summary. Results are given of the examination for blood of 3214 milk samples from 935 cheese factory supplies. Over 75% of the milks contained less than 2·5 ppm. blood. Excessive blood was due to factors largely controllable by an efficient farmer, and so may be used as an indicator of poor dairy herd management and shed hygiene. No evidence could be obtained that the concentrations of blood likely to be found in commercial milk have any adverse effect on the keeping quality of whole milk or butter.

Because of a possible correlation between the blood content of milk and fat-oxidation in Cheddar cheese (Rammell, 1961*a*) a preliminary survey of milks for their blood content was made. Farming conditions and practices were also examined to see if these could be correlated with the occurrence of blood.

METHODS

Collection of milks. Samples (about 30 ml) were collected on the receiving stage of thirty-two cheese factories in the North Island of New Zealand by dairy instructors of the N.Z. Department of Agriculture. Most of the samples were examined at the laboratory within 4 h of being taken, but where a longer delay of up to 24 h was expected, the milks were preserved with 0·05% boric acid. Samples were collected intermittently over two dairy seasons.

Blood determination. Samples of milk (20 ml) were examined by the method described earlier (Rammell, 1961*b*).

Inspection of farms. Visits were made to twenty-four of the farms from which 820 milk samples were examined, and the dairy shed and milking routine inspected. Particular attention was paid to those factors that might be expected to result in blood being present in the milk.

RESULTS AND DISCUSSION

Blood content of milk

The blood content of the bulked milk from individual farms varied appreciably from farm to farm (Fig. 1). Some milk supplies consistently contained little blood (1*a*); others consistently contained appreciable amounts of blood (1*b*); and the remainder varied from day to day (1*c*, *d*).

The percentage distribution by blood content of all the 3214 milk samples examined from 935 suppliers during the two seasons is shown in Fig. 2.

The weight of milk delivered to a factory by each farmer was found from the factory records and used to calculate the weighted average blood content of the factory's milk supply on the day the milks were examined. Results obtained for one factory, some of whose sixteen suppliers' milk consistently contained appreciable amounts of blood, are shown in Fig. 3. There appears to be a tendency for the blood content to be highest at the peak of the season. Insufficient samples were obtained from other factories to see if this apparent trend was characteristic of all factories' supplies.

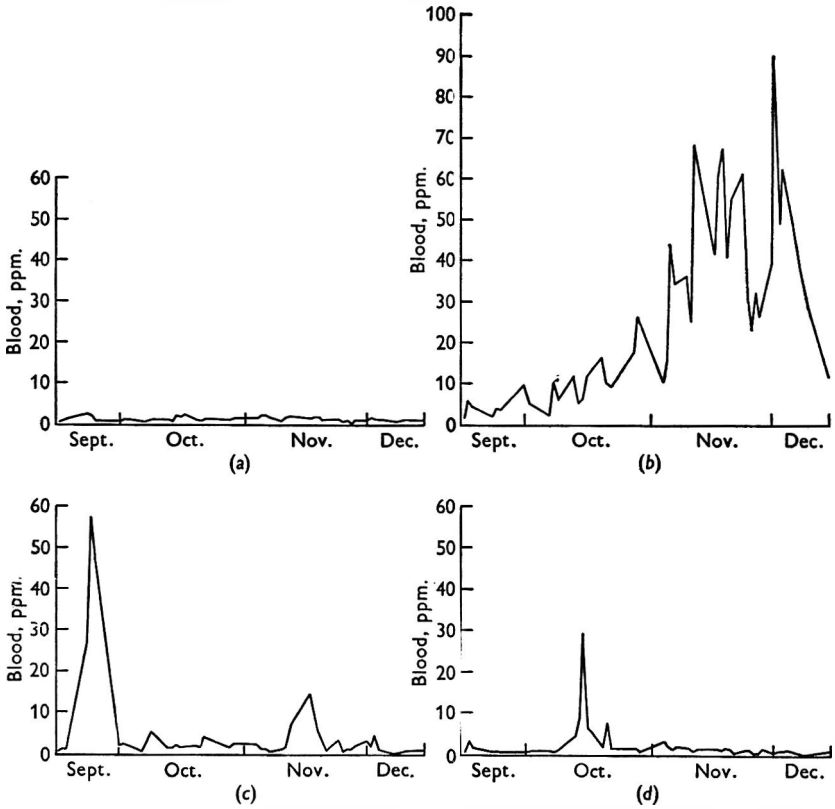

Fig. 1. Daily variation in the blood content of milk supplied to a cheese factory by four dairy farms (*a*, *b*, *c* and *d*). Forty-five milk samples were obtained intermittently from the bulked milk from each of the four farms throughout the period September to December 1959.

Causes of blood in milk

Teat damage. Apart from the blood sometimes present in the milk during the first few days after calving, blood entered the milk through damage to the teat. Factors applying throughout the season were cuts on the teats caused by other cows' horns, or farm implements and structures; and sores on the teats caused by faulty milking technique or inclement weather conditions. These wounds were sometimes aggravated by the entry of undesirable organisms into the wound, thereby prolonging the healing time, enlarging the damaged area on the exterior of the teat, or causing severe necrosis of the teat canal and sometimes loss of the quarter.

Milking machines and cups. Cows were machine-milked on all the farms inspected. There was no correlation between the incidence of blood and the type of milking machine or teat-cup inflations used. Although most of the machines possessed sight

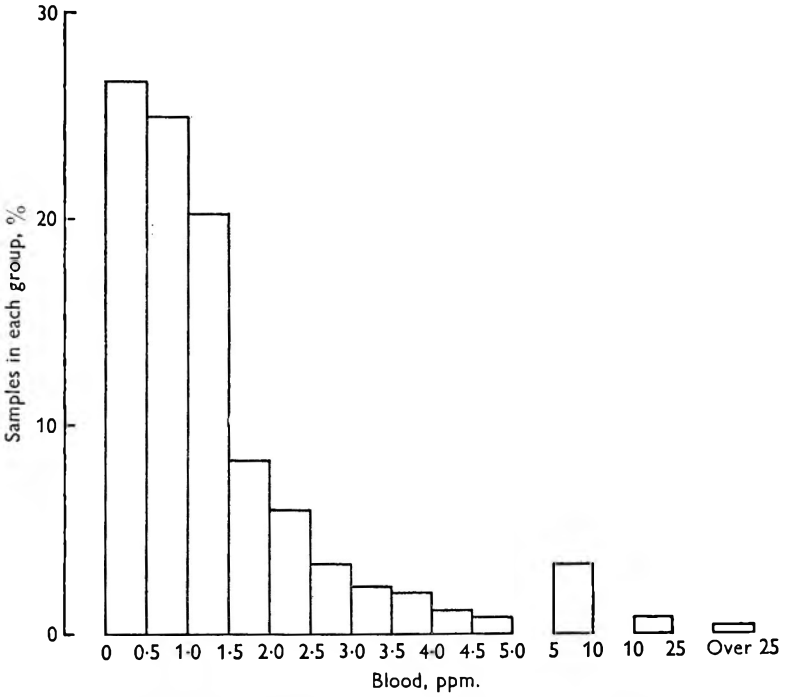

Fig. 2. Percentage distribution by blood content of 3214 milk samples obtained from 935 suppliers during two dairy seasons. The distribution is weighted to allow for differences in the number of times each supplier's milk was examined.

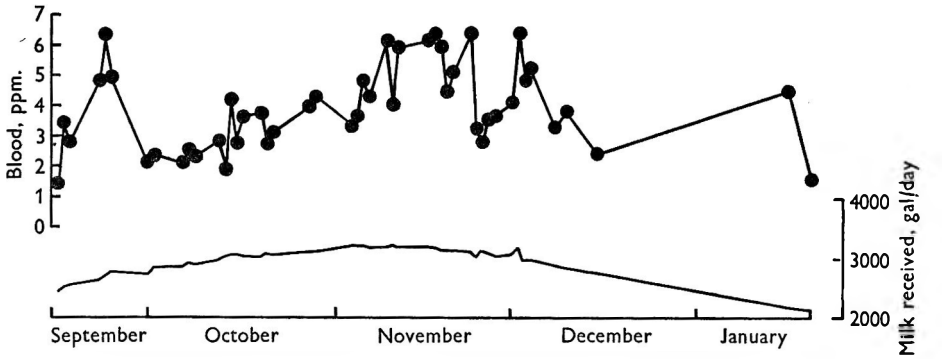

Fig. 3. Variation in the weighted average blood content of a cheese factory's milk supply throughout the period September 1959 to January 1960. The figure is based on the examination of 752 milk samples from the factory's sixteen suppliers.

glasses in the milk pipe, these were of little or no value for detecting blood in the milk passing through. Even when the cow's teat was bleeding profusely, blood was rarely seen in the milk, and then only for a fleeting moment. The inability to see

blood when present was due to the frothing that occurred in the sight glasses, and to the absence of a control sight glass through which blood-free milk passed.

The vacuum on most of the milking machines was closely controlled at 15 inHg by Ruakura vacuum regulators. No cases were observed of a high vacuum that might induce bleeding from the cow's teats.

Significance of blood in milk

Effect of blood on dairy produce. Apart from the possible effect of blood on fat oxidation in cheese (Rammell, 1961*a*) there are no reported references to its effect on other dairy products. Attempts in this laboratory to correlate excessive blood with off-flavours in whole fresh milk have failed. Bacterial spoilage always occurred before any oxidation of the fat, as judged by peroxide value and flavour, might otherwise have become apparent. Experiments in which up to 100 ppm. of haemolysed blood was added to commercially pasteurized milk also failed to produce any oxidized flavours attributable to the added blood. Under commercial conditions, of course, any blood present in milk would not normally be haemolysed, and so the effective concentration of haemoglobin would be negligible.

It is also unlikely that blood will affect the quality of those products in which the fat is separated from the milk. With butter, for example, 500–1000 ppm. of blood had to be added to the butter before any marked acceleration of fat-oxidation could be detected. Even though the blood content of commercial milk might originally be fairly high, much of it will be removed in the separator slime. Most of any blood remaining will then be separated along with the non-fat portion of the milk. Thus blood in milk should not accelerate fat-oxidation in commercial butter.

Blood as an indicator of dairy shed hygiene. Since most of the milks examined were virtually free from blood, its presence in milk must be considered abnormal. No evidence was obtained of any internal bleeding (other than that sometimes occurring early in lactation): all high levels of blood that were further investigated were found to be associated with external bleeding. On farms where the blood level was consistently high (e.g. Fig. 1*b*), the standard of dairy shed hygiene and herd management was always low. The almost universal use of milking machines in New Zealand probably contributes to the occasionally high level of blood found in suppliers' milks, for the careless farmer who is milking with machines is less likely to notice damaged teats than when hand-milking. The efficient farmer, however, pays due attention to the condition of his cows' teats, according them special treatment when damaged, and segregating any abnormal milk.

The examination of milk for its blood content might thus serve as an additional means of indicating the conditions under which the milk was produced. Experience here indicates that for a farmer to produce milk consistently free from blood, his milking technique and general herd management must be of a high standard.

Until reports on the blood content of milk in other countries are published, it is difficult to generalize on the occurrence and significance of blood in milk, but private communications suggest that the problem of blood in milk, like that of mastitis, is common throughout the world. It is possible that the problems of mastitis and blood in milk are often related, and that by paying attention to those factors responsible for blood in milk, the farmer will also reduce the incidence of mastitis in his herd.

The author gratefully acknowledges the assistance of Mr A. Bryant who prepared the line drawings; Mrs A. Splite and Miss Joan Vickers who analysed most of the milks for blood, and the Instructors of the N.Z. Department of Agriculture who assisted in obtaining milk samples and inspecting farms. The support and advice of Dr G. M. Moir throughout this work was much appreciated.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 7
With 2 plates

The action of rennin on casein

The disruption of the κ-casein complex*

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(*Received* 23 *July* 1962)

Summary. Approximately 30% of the nitrogen of κ-casein was soluble at pH 4·7 after the protein had been treated with rennin at pH 7 while approximately 10% was soluble in 12% trichloroacetic acid (TCA). The material soluble in 12% TCA appeared at a slower rate initially than did the nitrogen soluble at pH 4·7 but as the reaction proceeded it was released more rapidly.

Treating κ-casein with urea, or repeated precipitation of the protein at pH 4·7, caused the formation of material insoluble at pH 7, apparently para-κ-casein. Both treatments appeared to free the same soluble fraction as does rennin acting in low concentration or for a short time.

Low concentrations of rennin (0·07 μg/ml) released only part of the available soluble nitrogen from 2% solutions of whole casein at pH 7. Heating the reaction mixture appeared to restore the casein complex, the restoration being less complete the longer the reaction had proceeded.

It is suggested that κ-casein is not a single protein but a complex, and that the action of rennin is first to open the secondary bonds responsible for the stability of this complex.

Although rennin has been shown to possess a proteolytic activity comparable to that of trypsin and a specificity similar to that of pepsin (Fish, 1957), proteolytic cleavage of casein has not been demonstrated with any degree of certainty during its conversion into the calcium-sensitive form (para-casein) by the enzyme.

Alais, Mocquot, Nitschmann & Zahler (1953) reported the rapid release of peptide material, amounting to some 5% of the casein nitrogen, from whole casein when it was transformed into para-casein by rennin at pH 6·8. The reaction was distinct from the slower general proteolytic degradation of the casein at this pH, and was referred to as the primary action of the enzyme. The liberated material stemmed from

* This work, which was commenced in Berne, Switzerland, and continued in Melbourne, Australia, was reported in brief at a Food Science Conference held in September 1961 at the Division of Food Preservation, C.S.I.R.O., North Ryde, Sydney, Australia. This paper may be considered no. XV of the series: 'Das Lab und seine Wirkung auf das Casein der Milch'. No. XIV is Nitschmann, Hs. & Beeby, R. (1960). *Chimia*, **14**, 318.

the α-casein fraction, and later Wissmann & Nitschmann (1957) reported the appearance of phenylalanine as a new *N*-terminal residue in α-casein following treatment with rennin. This led to the conclusion that the primary action of the enzyme was one of specific limited hydrolysis.

A further advance was made when Waugh & von Hippel (1956) isolated κ-casein, a component of the α-casein complex. The peptide material freed during the primary attack by rennin on casein was shown to originate from this fraction (Waugh & von Hippel, 1956; Garnier, 1957; Wake, 1959). Wake (1959) found no difference between the *N*-terminal residues of κ-casein before and after treatment with rennin and concluded that no peptide bonds had been split. However, the quantity of α-amino groups found was in the range 0·12–0·16/mole per 10^5 g of protein which is so low as to make such a conclusion uncertain.

In the present study evidence is presented that the primary action of rennin on casein is more complex than has been assumed so far and that the first step consists in liberating a component from the κ-casein fraction by the rupture of secondary bonds only.

EXPERIMENTAL

Materials

All reagents were of analytical grade. The rennin was either crystalline or in a form in which crystallization could be induced by seeding. Stock rennin solutions were stored at 5 °C with thymol.

Whole casein was precipitated from raw skim-milk by slowly adjusting the pH to 4·6 with N-HCl. The precipitated protein was washed with distilled water and then extracted at pH 4·0 (acetic acid) for 5 h to remove proteolytic enzymes as described by McMeekin, Hipp & Groves (1959). The precipitate was dissolved by the addition of N-NaOH, taking care that the pH of the solution did not rise above 7·5, and the protein precipitated again with N-HCl. After four precipitations the casein was dried with absolute alcohol followed by ether, and ground. Casein solutions were prepared by mixing the dried protein with water and adding N-NaOH dropwise until solution was complete.

κ-Casein was prepared according to the procedure of McKenzie & Wake (1961) except that the urea step was omitted and the precipitated κ-casein dissolved at pH 7–7·5 by the addition of 0·1N-NaOH. After precipitation at pH 4·7 the protein was dissolved again at pH 7–7·5 and the solution freeze-dried. The yield was approximately 0·9 g/l of skim-milk.

Another method based on the procedure of Waugh & von Hippel (1956) and yielding 3–4 g of κ-casein per litre of skim-milk was developed in which the α-κ-complex was split in a dilute state to minimize interactions and consequent loss of κ-casein by co-precipitation with α- and β-casein. Whole casein was precipitated and protease removed as described above. After a second precipitation the protein was dissolved at pH 7 by the addition of N-NaOH, the concentration of protein adjusted to 2–3 %, and the solution cooled to 2 °C. Calcium chloride (80 %, w/v) was added slowly to give a final calcium concentration of 0·27 M, the pH being maintained at 7 by the addition of 0·1 N-NaOH. The mixture was held at 2 °C for 30 min after which it was warmed to 37 °C. The turbid supernatant was decanted from the precipitate

that had formed, centrifuged for 30 min at 20000 rev/min in a Spinco model L ultracentrifuge (rotor 21), and dialysed overnight against running water. The dialysed solution was reduced to 10 % of its original volume in a stainless steel evaporator at 30 °C and treated again with calcium as described. The turbid solution was then centrifuged for 45 min at 20000 rev/min. The clear supernatant was dialysed at 2 °C against 20 volumes of 0·1 M-NaCl for 24 h. The dialysis was repeated twice and the dialysed solution freeze-dried.

Thymol was added to all casein solutions during the preparation of whole casein and κ-casein.

METHODS

Casein solutions were treated with rennin at 25 °C.

Total nitrogen was determined by a micro-Kjeldahl procedure.

Portions were taken during the reaction with rennin and the casein precipitated by the addition of either a one-tenth volume of acetic acid–sodium acetate buffer (M with respect to each) or an equal volume of 24 % (w/v) TCA. The soluble nitrogen released by rennin was estimated in the filtrates by a modification of the method described by Brown, Duda, Korkes & Handler (1957). A sample of filtrate (0·1–0·5 ml) containing up to 5 μg N was digested in a test tube with 1 ml of acid reagent (10 ml of concentrated H_2SO_4 + 15 g K_2SO_4 made up to 100 ml with distilled water). The digestion was continued for $1\frac{1}{4}$ h after the water had evaporated. Five ml of distilled water was added to the cooled sample. When solution of the hydrated sulphate was complete 1 ml of phenolate reagent and 2 ml of saturated Na_3PO_4 were added and the contents of the tube mixed. One ml of 0·02 % sodium nitroprusside was then added and, after mixing, 1 ml of 0·1 M-sodium hypochlorite. The phenolate reagent contained 2·5 g of phenol + 16 g of NaOH per 100 ml and was adjusted so that 1 ml just neutralized (to methyl red) 1 ml of acid reagent. The colour was developed by heating in a bath at 100 °C for 5 min and when cool the optical density was measured at 625 mμ. Determinations were made in triplicate.

For paper electrophoresis an EEL unit was used at 4 °C. The strips were dried at 103 °C for 20 min and the protein bands stained by immersing for 20 min in 0·1 % bromphenol blue in ethanol containing 2 % $HgCl_2$.

The release of soluble nitrogen from κ-casein by rennin

Rennin (0·14 μg/ml) was added to a 0·5 % solution of κ-casein (pH 7) and the release of nitrogen soluble at pH 4·7 and in 12 % TCA determined. As Fig. 1 shows, the release of nitrogen soluble at pH 4·7 was initially considerably faster than the release of nitrogen soluble in 12 % TCA. As the reaction proceeded the nitrogen soluble in 12 % TCA accounted for a greater proportion of the nitrogen soluble at pH 4·7, the proportions being approximately 20 and 33 % respectively in the early stages and the end of the reaction.

The release of soluble nitrogen from κ-casein was accompanied by aggregation and eventually by precipitation of the para-κ-casein. These aggregates were difficult to disperse and persisted even at pH 10–11.

The soluble material released during the early stages of rennin action was isolated as follows. Rennin (0·14 μg/ml) was added to a 1 % κ-casein solution (pH 7) and

after 5 min the enzyme was inactivated by heating the mixture. When cool the solution was adjusted to pH 4·7 by the addition of 0·1 N-HCl, the precipitate removed by centrifugation (60000 *g*) and the supernatant freeze-dried after adjusting to pH 7 with 0·1 N-NaOH. Salt was removed by dissolving the freeze-dried material in distilled water and passing through a column of G 25 Sephadex (Porath & Flodin, 1959). The desalted solution was freeze-dried.

Para-κ-casein was prepared by allowing the reaction with rennin to proceed for 40 min. The protein was then precipitated at pH 4·7, filtered and washed. The precipitated para-κ-casein was titrated to pH 11 with N-NaOH, to break up the large aggregates, the pH reduced to 7 with N-HCl and the suspension freeze-dried.

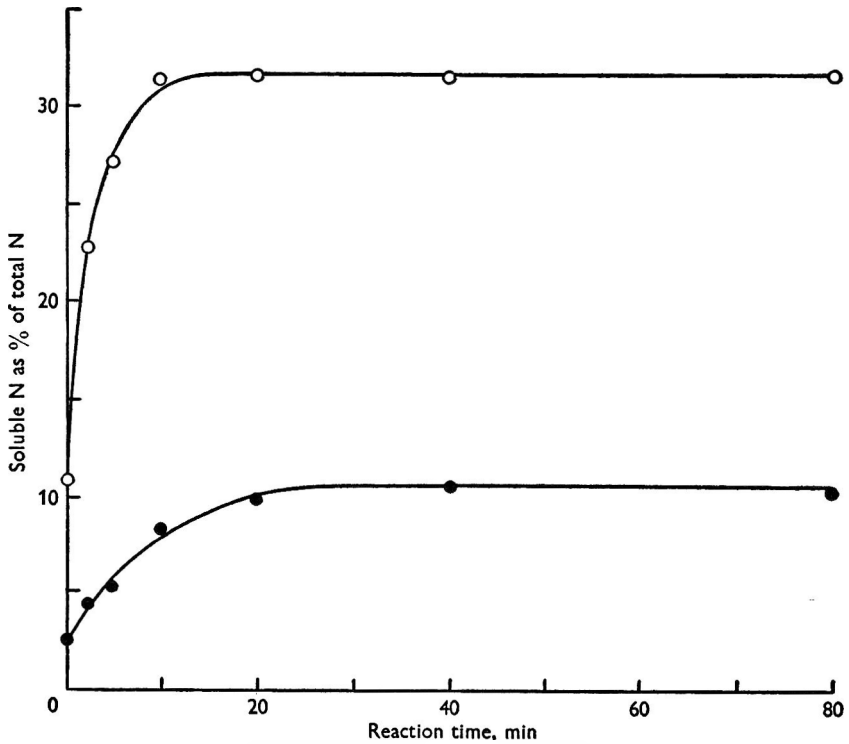

Fig. 1. The rate of release of soluble nitrogen in a κ-casein solution (0·5 %) containing rennin (0·14 μg/ml) at 25 °C and pH 7. ○, Nitrogen soluble at pH 4·7; ●, nitrogen soluble in 12 % TCA.

The effect of urea on κ-casein

In the method of McKenzie & Wake (1961) for the preparation of κ-casein the protein is precipitated from 50 % ethanol by ammonium acetate. The precipitate is dissolved in 6 M-urea and the urea removed by dialysis against 0·005 M-NaCl. When κ-casein was prepared according to this method a precipitate began to form 10 min after dialysis had commenced. At the completion of dialysis the precipitated protein was, like para-κ-casein, insoluble at pH 7 and only partially dispersed at pH 10–11. Rennin released no soluble nitrogen from this material at pH 7.

The cause of precipitation was traced to impure ammonium acetate which was found to contain acetic acid. This lowered the pH to approximately 5·0. When

κ-casein was dissolved in 6M-urea and the urea removed as above precipitation occurred after 24 h. This was apparently due to absorption of carbon dioxide by the solution, which caused the pH to drop to 5. This precipitate, however, dissolved readily at pH 7. The addition of rennin to the solution caused the release of soluble nitrogen and the formation of insoluble para-κ-casein. Thus the material with properties similar to para-κ-casein formed only when precipitation occurred before the bulk of the urea had had time to dialyse away.

The effect of urea in breaking down κ-casein into soluble and insoluble fractions was further studied. Two g of κ-casein was dissolved in 100 ml of 6M-urea which contained acetic acid–sodium acetate buffer (0·01M with respect to each). The solution was then dialysed for 24 h against 10 l of the acetate buffer (pH 4·7) at 2°C. The dialysis was repeated. The precipitate was collected by filtration, washed with distilled water and dispersed at pH 7. The fraction insoluble at pH 7 was collected by centrifugation, dispersed briefly at pH 11, the pH reduced again to 7 and the suspension freeze-dried. The filtrate was dialysed for 5 h against 10 l of 0·01M-NaCl, centrifuged 30 min at 60000*g* and freeze-dried. Salt was removed as described by Porath & Flodin (1959). The yield of the soluble fraction was approximately 0·4 g or 20%.

The effect of precipitation at pH 4·7 *on κ-casein*

Before treatment with rennin approximately 10% of the total nitrogen of κ-casein was soluble at pH 4·7 (Fig. 1). This soluble material was apparently not κ-casein because the solution developed no turbidity when treated with rennin at pH 7, while a κ-casein solution with half the nitrogen content (4 mg N/100 ml) became distinctly turbid under these conditions. Also the dilute κ-casein solution was turbid at pH 4·7 while the other preparation was clear.

Fifty ml of a 4% solution of κ-casein was adjusted to pH 4·7 with 0·1N-HCl and the precipitated protein separated by filtration. The precipitate was dissolved at pH 7–7·5 by the addition of 0·1N-NaOH and again precipitated at pH 4·7. After four precipitations the combined filtrates were centrifuged for 30 min at 60000*g*, adjusted to pH 7 with 0·1N-NaOH and freeze-dried. Salt was removed as described by Porath & Flodin (1959). The yield was approximately 0·3 g or 15%.

The precipitated κ-casein contained more material insoluble at pH 7–7·5 after each precipitation. The final precipitate was dispersed at pH 7 and centrifuged for 30 min at 60000*g*. The sediment was suspended at pH 7 and freeze-dried. The supernatant was freeze-dried, yielding approximately 0·8 g or approximately 40% of apparently unchanged κ-casein (paper electrophoresis).

The soluble and insoluble fractions from κ-casein

The insoluble fractions resulting from the treatment of κ-casein with urea and from repeated precipitation at pH 4·7 were compared with para-κ-casein by paper electrophoresis on Whatman 3MM paper in 0·01M-KOH–0·01M-KCl (pH 11·7). The patterns are shown in Plate 1. The three insoluble fractions had similar mobilities which were less than that of κ-casein, suggesting strongly that para-κ-casein had been formed by the treatment with urea and by precipitation at pH 4·7.

The soluble fractions obtained from κ-casein by treatment with rennin, with urea,

and by precipitation were compared by paper electrophoresis on Whatman 3MM paper in veronal buffer (ionic strength 0·02, pH 8·6). The three preparations gave similar patterns (Plate 2). The bulk of the material moved towards the cathode under these conditions in contrast to κ-casein which moved towards the anode.

The soluble fractions were analysed for total nitrogen (Kjeldahl), total phosphorus (Fiske & Subbarow, 1925), arginine (Macpherson, 1946) and sialic acid expressed as *N*-acetyl neuraminic acid (Warren, 1959). The results are given in Table 1.

Table 1. *Nitrogen, phosphorus, arginine and sialic acid contents of the soluble components obtained from κ-casein by treatment with rennin, urea or precipitation at* pH 4·7

Fraction freed from κ-casein by	% N	% P	% arginine	% sialic acid
(1) Rennin	13·6	0·8	2·9	3·8
(2) Urea	14·1	0·8	2·4	3·1
(3) Pptn. at pH 4·7	13·4	0·6	2·3	2·1

The soluble sample prepared by precipitation of κ-casein at pH 4·7 contained less sialic acid and phosphorus than the other two samples although the ratio of sialic acid to phosphorus was similar for each. This suggests that this sample may have contained other material deficient in both sialic acid and phosphorus. However, the rest of the data and their electrophoretic properties (Plate 2) indicated that the three samples were at least very similar. This fact, together with the formation of material closely resembling para-κ-casein during the precipitation of κ-casein or its treatment with urea, suggested strongly that the same material was split from κ-casein by these treatments as was released by the action of rennin. If the enzyme were to act by disrupting secondary forces rather than breaking covalent bonds then inactivating the enzyme might be expected to reverse the process and cause a decrease in the soluble nitrogen.

The effect of heating a rennin–casein mixture on the release of soluble nitrogen

Because aggregation accompanied the release of soluble nitrogen from κ-casein and the aggregates were difficult to disperse, whole casein was used to check the possibility of reversal of the release of soluble nitrogen. The slower appearance of nitrogen soluble in 12% TCA compared with that soluble at pH 4·7 indicated the likelihood of a breakdown of the released material after it had been freed. To minimize this effect a low concentration of rennin was used.

A 2% whole casein solution was treated with rennin (0·07 μg/ml) at pH 7 and duplicate portions taken at intervals to determine the rate of release of nitrogen soluble at pH 4·7. One series was heated for 4–5 min at 80 °C before precipitating the casein and another series not heated. The results (Fig. 2) show that this concentration of rennin rapidly released approximately 2% of the casein nitrogen. Heating prior to precipitation caused a decrease in the nitrogen content of the filtrates. The difference between heated and non-heated samples became less as the reaction proceeded.

When a higher concentration of rennin (1·14 μg/ml) was used, the soluble nitrogen in filtrates from heated and non-heated portions was 2·6 and 3·4% respectively after 5 min reaction and 4·9 and 5·1% respectively after 30 min. This figure of 5%

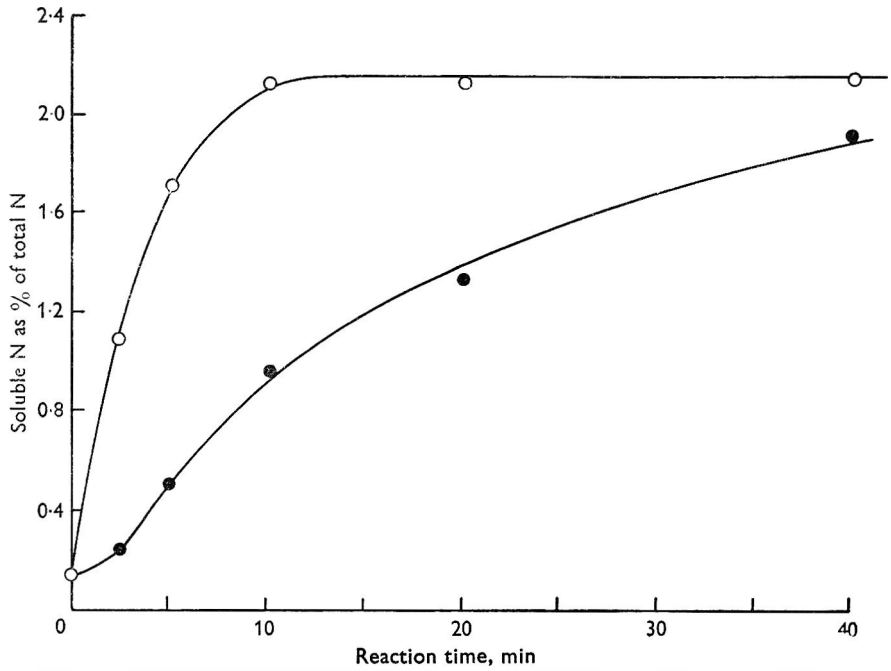

Fig. 2. The effect of heating to 80 °C for 5 min on the nitrogen soluble at pH 4·7 in a 2 % solution of whole casein containing 0·07 μg of rennin per ml (25 °C and pH 7). ○, Non-heated samples; ●, heated samples.

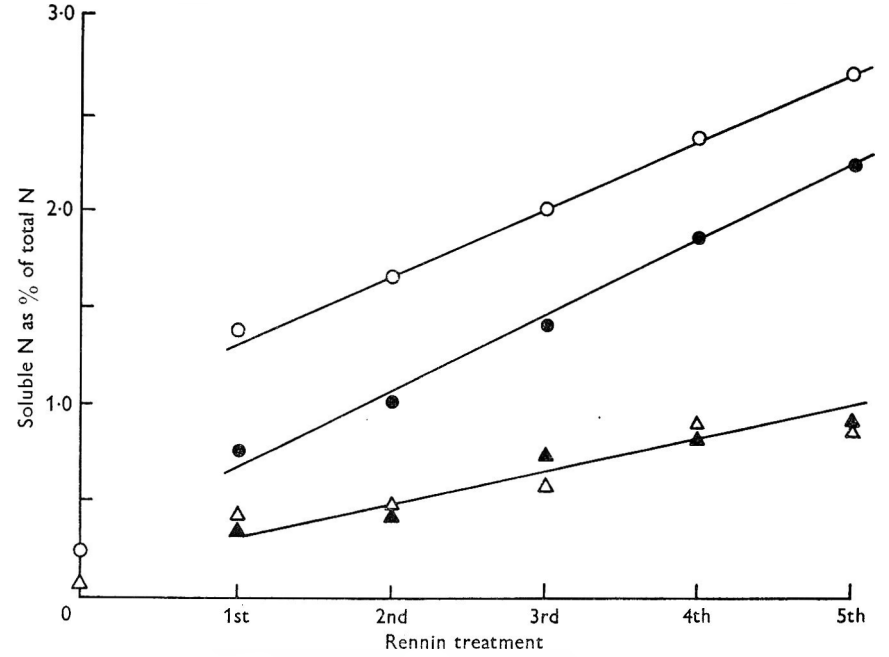

Fig. 3. The release of soluble nitrogen from whole casein by successive treatments of 10 min duration with rennin (0·07 μg/ml) at 25 °C and pH 7. ○, Nitrogen soluble at pH 4·7 before heating; ●, nitrogen soluble at pH 4·7 after heating; △, nitrogen soluble in 12 % TCA before heating; ▲, nitrogen soluble in 12 % TCA after heating.

agrees with that found by Alais *et al.* (1953) for the amount of soluble nitrogen that could be released from whole casein by rennin.

The lower content of soluble nitrogen in the heated samples was consistent with the expected recombination of the freed fraction with the casein. If this were so then it should be possible to release it again by adding more rennin.

To test this possibility a 2 % whole casein solution (pH 7) was treated with rennin (0·07 μg/ml), and after 10 min the enzyme was inactivated by heating as described. The mixture was cooled, fresh rennin added (0·07 μg/ml) and the reaction stopped again after 10 min. In this way the casein solution was given five separate treatments with rennin. Portions were removed before and after inactivation of the enzyme each time and the soluble nitrogen determined. In one series the casein was precipitated at pH 4·7 and in another with 12 % TCA. The results are given in Fig. 3. The amounts of nitrogen soluble in 12 % TCA released by each addition of enzyme were approximately equal and were little affected by inactivation of the rennin. In contrast to this the nitrogen soluble at pH 4·7 was always higher before heating than after, although the difference became less with increasing number of enzyme treatments. Extrapolation indicated that the two would coincide at 5 % of the casein nitrogen, which is the total amount of soluble nitrogen available for release (Alais *et al.* 1953 and present paper).

The amount of nitrogen soluble at pH 4·7 found after the fifth treatment was 2·3 % of the casein nitrogen. However, the sum of the amounts freed by the separate rennin treatments, which represents the total amount that had actually been released by the enzyme during the course of the experiment, was 4·7 % of the casein nitrogen. In a separate experiment in which the number of enzyme treatments was nine, the total nitrogen released was 8 % while the nitrogen content of the filtrate after the ninth addition of rennin was 4·3 % and fell to 3·9 % on heating.

DISCUSSION

The experiments in which whole casein was subjected to successive treatments with rennin show clearly that part of the material released by the enzyme is bound by the casein when the mixture is heated. Since a sufficient number of rennin treatments appears to set free more soluble nitrogen than is available for release, it is apparent that the portion that is bound on heating is released again during the subsequent enzyme treatments. This suggests strongly that the original casein complex is reformed under these conditions and, therefore, that covalent bonds are not broken in releasing the soluble material.

The difference between the nitrogen contents of the heated and non-heated samples represents the amount that recombines. However, as the rennin action proceeds, the nitrogen soluble at pH 4·7 loses its ability to re-form the casein complex (Figs. 2, 3), indicating that it is altered by the enzyme. Further evidence that this is so is obtained from the fact that the nitrogen soluble in 12 % TCA forms a constant proportion of the nitrogen soluble at pH 4·7 that is found after heating, i.e. the nitrogen that can no longer recombine with the para-casein. Also, the nitrogen which cannot re-form the casein complex appears initially at a slower rate than the total nitrogen released, but later it appears at a faster rate (Fig. 2).

That portion of the nitrogen released from casein by rennin which is soluble in 12% TCA was shown by Alais (1956) and Nitschmann, Wissmann & Henzi (1957) to consist almost exclusively of a single peptide with a molecular weight in the region of 8000 and containing sialic acid and other carbohydrate material. It seems most probable therefore that the glycopeptide is not released directly from the casein but is formed as a result of the degradation of a fraction that rennin first releases from the casein. Since the glycopeptide does not recombine with the para-casein on heating (Fig. 3), it is most likely formed by the rupture of a covalent bond; Garnier, Mocquot & Brignon (1962) have suggested an ester bond.

The experiments with κ-casein provide considerable substantiation for the above hypothesis. Precipitation of the protein at pH 4·7 or treatment with urea releases material which contains sialic acid and which is very similar to the material split off by rennin. At the same time insoluble material which markedly resembles para-κ-casein is formed. This indicates that κ-casein is not a single protein but a complex stabilized by secondary forces such as hydrogen bonds. The existence of such a complex would explain the electrophoretic heterogeneity observed in κ-casein preparations under disaggregating conditions (Libbey & Ashworth, 1961; Neelin, Rose & Tessier, 1962). In the presence of rennin the glycopeptide appears initially at a slower rate than does the nitrogen soluble at pH 4·7 but later it appears more rapidly (Fig. 1), again indicating that it is formed from material that is first released by the enzyme.

The first specific action of rennin on casein therefore appears to be the rapid disruption of the κ-casein complex by the opening of the secondary bonds responsible for its stability.

The authors wish to thank Mr G. Loftus Hills, Mr R. D. Hill and Dr N. Snow, Division of Dairy Research, C.S.I.R.O., for helpful discussion during the preparation of the manuscript. Thanks are also due to the Board of Directors, Theodor-Kocher Institute, Berne, for providing facilities for one of us (R. B.) at the Institute.

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EXPLANATION OF PLATES

PLATE 1

Paper-electrophoresis patterns of κ-casein and the insoluble fractions resulting from treatment of the protein with rennin, urea and precipitation at pH 4·7. The samples were run in KOH-KCl buffer at pH 11·7 ($\mu = 0{\cdot}02$) for 16 h at 150 V. A, κ-casein; B, fraction from treatment with rennin; C, fraction from treatment with urea; D, fraction obtained by precipitation at pH 4·7.

PLATE 2

Paper-electrophoresis patterns of κ-casein and the soluble fractions resulting from treatment of the protein with rennin, urea and precipitation at pH 4·7. The samples were run in veronal-acetate buffer at pH 8·6 ($\mu = 0{\cdot}02$) for 16 h at 200 V. A, κ-casein; B, fraction from treatment with urea; C, fraction obtained by precipitation at pH 4·7; D, fraction from treatment with rennin.

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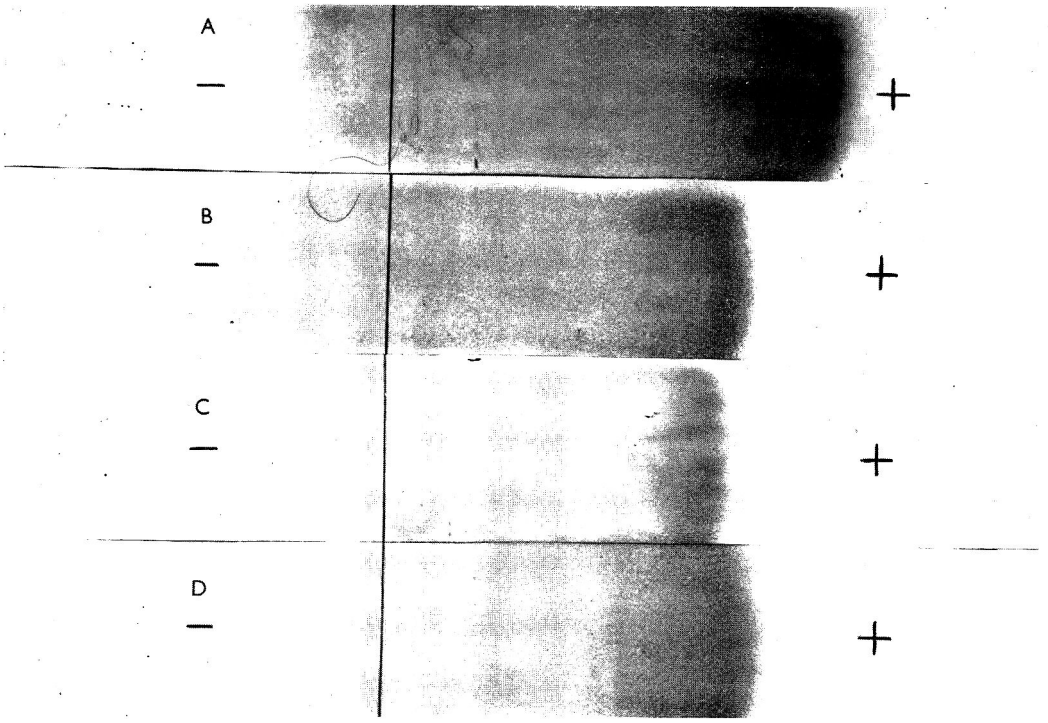
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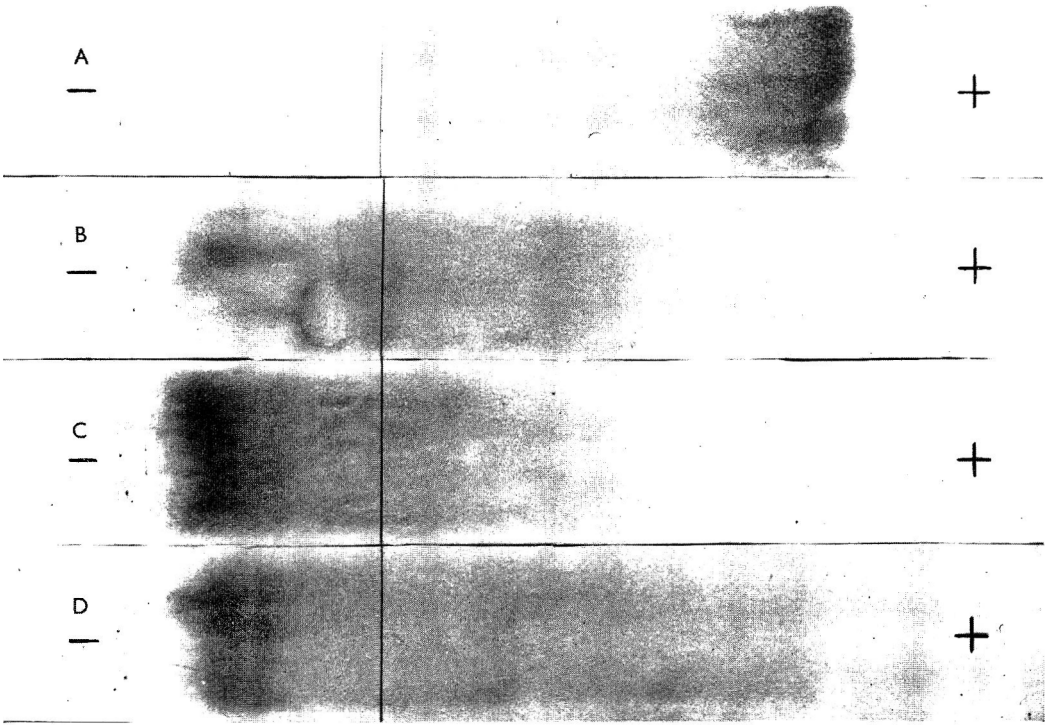
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J. Dairy Res. (1963), **30**, 17
With 2 plates

Action of rennet and other proteolytic enzymes on casein in casein-agar gels

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(*Received* 3 *August* 1962)

Summary. A series of precipitation zones appears when proteolytic enzymes diffuse through an agar gel containing 1% whole casein and 10–20 mM-calcium chloride. As the enzyme diffuses through the gel the casein stability changes giving first a precipitate which subsequently redissolves followed by a second precipitate which also redissolves. The first or outermost zone of precipitation occurs at the periphery of the area of the diffused enzyme. Using a high enzyme concentration all four zones, two of precipitation and two of subsequent clearing, are visible in about 24 h. The proportion of the total nitrogen which occurs as non-protein nitrogen (N.P.N.) in the first or outermost precipitation zone is similar to that found after the first stage of rennet action on casein solutions.

The nature of the chemical and physical changes which take place when a gel or curd is formed in milk by the action of proteolytic enzymes on casein is still mainly obscure. It is well established that release of N.P.N. by the enzyme precedes gel formation (Alais, Mocquot, Nitschmann & Zahler, 1953) and that this N.P.N. is released mainly from one specific casein fraction, κ-casein (Wake, 1959). The κ-casein appears to maintain the stable colloidal micelle system in milk by forming a complex with the less stable casein components (Waugh & von Hippel, 1956).

The colloidal protein system and the formation and structure of the gel are influenced by calcium ion concentration and temperature. Temperature dependence is shown by the increased stability of milk to the action of rennet below 10–15 °C (Berridge, 1942). This seems to be a consequence of the high activation energy required for the aggregation of rennet-treated κ-casein solutions at temperatures of 10 °C or below (Cheeseman, 1962).

During an attempt to develop an agar diffusion assay for rennet interesting phenomena were observed which may have some bearing on the behaviour of casein during and after rennet action. The experiments were designed to make use of the fact that under suitable conditions casein remains soluble in the presence of moderate concentrations of calcium until it is modified by rennet, when it becomes insoluble. The enzyme was allowed to diffuse into a gel containing soluble casein and a zone of precipitation was expected. In fact two zones were observed. This seemed worth further investigation.

The following have been the subjects of short experiments:

(1) The formation of zones of precipitation by rennet, pepsin and trypsin in agar gels containing whole casein with and without added calcium chloride.

(2) The liberation of N.P.N. from casein by rennet in the gel.

(3) The effect on zone formation when purified fractions of casein were used in place of whole casein.

(4) The effect of temperature on zone formation.

(5) The modification of the zones in the presence of sodium dodecyl sulphate, a detergent which will interact with and cause dissociation of protein aggregates.

EXPERIMENTAL

Agar assay plates were prepared in the manner used for antibiotic assays. The medium contained agar 1 %, sodium acetate 0·1 M, sodium caseinate (prepared from lyophilized acid-precipitated whole casein) 1 %, calcium chloride 10 mM. The pH was adjusted finally to 5·7 before addition of the agar. A final pH of 6·1 was also used on occasion, and 20 mM-calcium chloride sometimes in place of 10 mM. Undiluted commercial rennet was put into wells cut in the agar plates and the enzyme was allowed to diffuse for 48 h at room temperature. For experiments on the possibility of developing a method of assay, dilutions of the commercial rennet solutions up to 1/1000 were used.

When experiments were done without calcium chloride in the agar the plates were flooded after the diffusion period with a solution of calcium chloride (20 mM). Solutions of pepsin (Armour Pharmaceutical Co.) and trypsin (British Drug Houses Ltd.) were prepared by dissolving the crystalline enzymes in the buffer used for making up the agar.

The liberation of N.P.N. by enzyme as it diffused through the casein-agar gel was determined by extracting excised portions of the gel with a relatively small volume of trichloroacetic acid (TCA) of sufficient concentration to give a final concentration of 2 % in the mixture. The soluble nitrogen was determined by the micro-Kjeldahl method. The rate of release of N.P.N. from casein in solution was determined under conditions approximating to those in the agar gels, namely, 1 % casein, 0·1 M-acetate buffer at a final pH of 5·7 and 20 mM-calcium chloride or no calcium chloride. The solutions were preserved with 0·001 M-thiomersal. This concentration was shown to have no effect on the clotting of milk. Undiluted commercial rennet (0·125 ml) was added to 20 ml of the casein solution and the N.P.N. determined from time to time over a period of 52 h.

α-Casein was prepared by the urea method of Hipp, Groves, Custer & McMeekin (1952) and would therefore be expected to contain κ-casein. β-Casein was supplied by Dr R. Aschaffenburg of this Institute. κ-Casein was prepared by the method of Cheeseman (1962). The α- and β-caseins were tested in the absence of calcium at a final pH of 6·1 since solution of these fractions was difficult at pH 5·7. The κ-casein, however, remained soluble at pH 5·7 and was therefore tested by the first method, namely with 10 mM-calcium chloride in the agar and a final pH of 5·7.

An indication of the effect of temperature on the process was obtained by allowing diffusion to occur at 4 °C for 48 h. After observation the effect of allowing the plates to come to room temperature was noted.

The effect of sodium dodecyl sulphate (S.D.S.) on the appearance of the zones was determined by cutting a radial channel across zones which had already been formed as a result of diffusion for 48 h, and filling this channel with a concentrated solution of S.D.S. in 0·1 M-acetate buffer at the appropriate pH. The S.D.S. was then allowed to diffuse overnight at room temperature.

RESULTS

(1) Diffusion of rennet through agar containing whole casein.

Two zones of precipitation were produced in the form of rings separated by a circular zone of relatively clear agar (Pl. 1 (i)). The mean diameters of all the zones increased with the time of diffusion and with the concentration of the enzyme. The rectilinear relation between the zone diameter and the enzyme dilution is illustrated in Fig. 1.

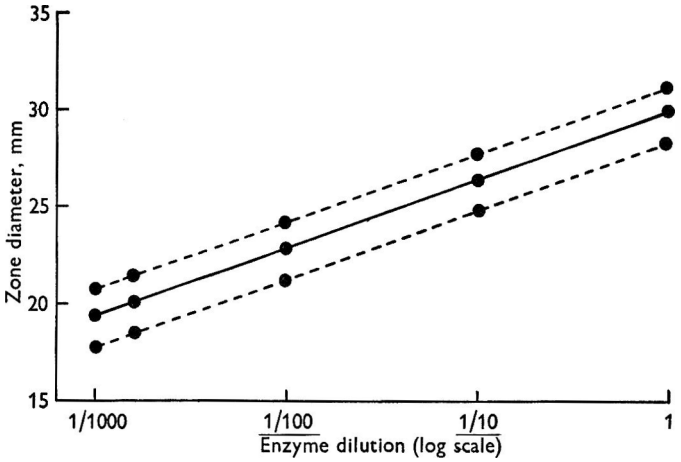

Fig. 1. Assay of rennet by diffusion through whole casein-agar gel. The relationship is shown between zone diameter, after 16·5 h at room temperature, and enzyme dilution. Five levels of dilution of commercial rennet solution and five replicates per dilution were used. ●——●, Fitted regression line; ●- - -●, 95 % confidence limits for a single diameter at a given dilution.

(2) Diffusion of enzymes through agar containing whole casein.

(*a*) *Rennet.* In the absence of added calcium only the inner zone of precipitation was apparent. The outer zone appeared after the agar was flooded with calcium chloride solution (Pl. 2 (i*a*, *b*)).

(*b*) *Pepsin.* The results here were similar to those with rennet, the differences being merely quantitative (Pl. 2 (ii*a*)).

(*c*) *Trypsin.* Three rings of precipitation appeared after diffusion and an additional outer ring was seen only after calcium had been added (Pl. 2 (ii*b*)).

(3) Diffusion of rennet through agar containing purified fractions of casein.

The so-called α-casein produced two zones of precipitation even before calcium had been added (Pl. 2 (iii*a*)). The β-casein showed only a slight opacity extending from the centre to a position probably corresponding to the intermediate clear zone produced in whole casein (Pl. 2 (iii*b*)). Washing with calcium chloride solution did not produce any new rings. The κ-casein produced zones similar to those obtained with whole casein under the same conditions.

(4) The effect of temperature on zone formation.

At 4 °C only the outermost zone of precipitation appeared in casein-agar containing calcium chloride. The area within this zone was similar in appearance to that obtained with β-casein. However, on allowing the casein-agar to come to room temperature

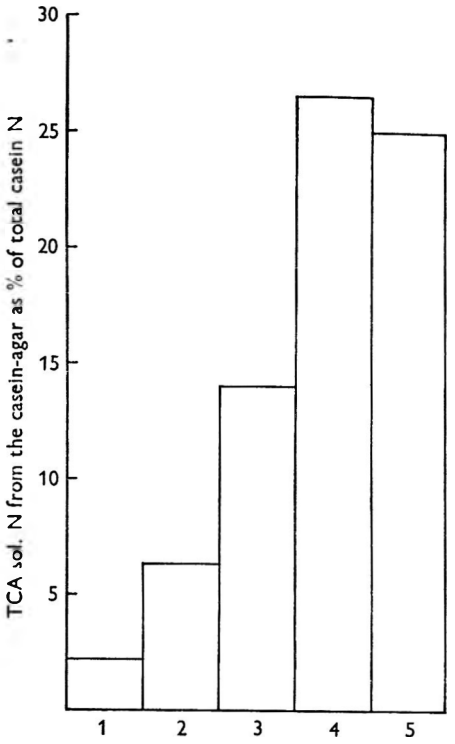

Fig. 2. Non-protein nitrogen (2 % TCA soluble) in the zones obtained by rennet action in whole casein-agar gels. The values shown are averages from three separate experiments. The columns numbered 1–5 correspond to: (1) the unattacked portion of the gel; (2) the first or outer precipitation zone; (3) the small intermediate clear zone; (4) the inner precipitation zone; (5) the inner clear zone.

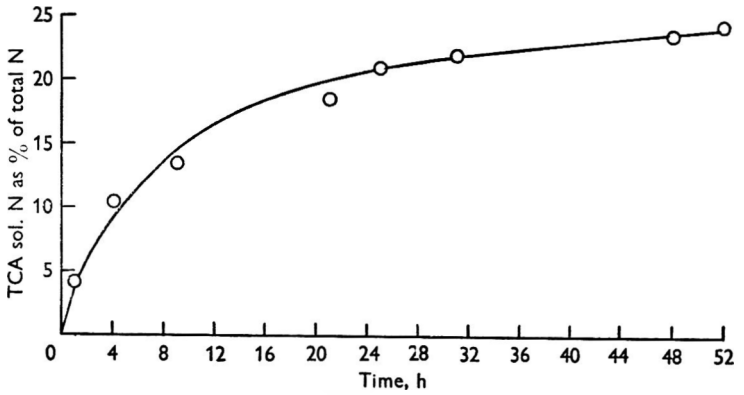

Fig. 3. Increase in 2 % TCA soluble nitrogen during the action of rennet on whole casein solutions.

the inner slightly opaque area changed giving rise to the inner precipitation zone and the innermost clear zone. This reaction was irreversible.

(5) The modification of the zones by sodium dodecyl sulphate.

The complex patterns produced when sodium dodecyl sulphate was allowed to diffuse into the zones are shown in Pl. 1 (ii*a*, *b*, *c*). There were three areas of precipitation but they did not quite coincide with the positions of the original zones.

(6) The concentration of N.P.N. present in the various zones.

These results are shown in Fig. 2 from which it is clear that a relatively large proportion of N.P.N. was found in the inner zones and that even the outermost zone contained a significant proportion.

(7) Release of N.P.N. from whole casein in solution.

The release of N.P.N. from solutions of casein is shown in Fig. 3. The rate of increase after incubation for 24 h was relatively small.

DISCUSSION

The use of a diffusion assay for rennet does not appear to be attractive. The sensitivity of the method is poor (see Fig. 1) and in this respect does not compare favourably with the milk-clotting technique (cf. Berridge, 1952) which remains the preferred assay method.

Several zones of precipitation during the diffusion of reagents through a gel suggest Liesegang rings. Here, however, the conditions are more complex than the diffusion of a pair of ions into another salt solution, for we have not only the enzyme itself diffusing outwards but products of its activity also. Some of these are smaller molecules and will diffuse more quickly than the enzyme. Although the amount of N.P.N. obtained from the outermost zone would suggest that this zone corresponds to the first stage of enzyme action, consideration must be given to the possible contribution of products of enzyme digestion which may have diffused from regions in which the enzyme is more concentrated.

Casein solutions with similar protein, calcium and electrolyte concentrations and at similar pH values to those of the gels are almost clear. Addition of enzyme brings about only a very slight cloudiness, no precipitation occurs, nor is there any other visible change. The maximum amount of N.P.N. release is, however, similar to that obtained from the casein-agar gels. The special conditions existing in the gel must therefore be responsible for the insoluble zones.

It is already known that as the enzyme alters the protein the sensitivity of the product to the calcium ion concentration changes. It must be emphasized that the appearance of the zones in *whole* casein-agar gels is dependent upon the concentration of calcium and other electrolytes. If the calcium concentration is too high then the whole plate is opaque: if too low then no zones appear.

Although several hypotheses come to mind to explain the zone formation probably the most attractive is one involving interaction between the agar and the casein. The presence of charged groups on the agar molecule could possibly lead to interaction with the altered casein giving rise to complexes of varying degrees of calcium sensitivity. Under these conditions the release of N.P.N. could proceed as the analyses suggest or there could be a release from inner zones with faster-diffusing smaller

molecules being responsible for the observed N.P.N. figures in the outer zones. That the agar may have some influence is suggested by the fact that a concentration of about 1 % agar appears essential for the clear development of the two precipitation zones. At higher and lower agar concentrations the intermediate clear zone is less well marked and the two zones of precipitation tend to merge into a single zone.

The failure of the inner zone of precipitation to appear until the temperature is raised suggests that the modified casein is kept in solution by β-casein which is more soluble at lower temperatures. The zones of clearing and precipitation formed by sodium dodecyl sulphate suggest at least three different modifications of the casein, although the zones from the S.D.S. do not quite coincide with those produced by the enzymes.

At this stage it is impossible to say whether the explanation given above is correct. However, there can be no doubt that the observations reported are associated with changes induced in the caseins by the enzymes.

The author is grateful to Dr N. J. Berridge for his interest and encouragement, to Miss Z. Hosking for the analysis of the rennet assay results and the provision of the data for Fig. 1, and to Miss K. V. I. Carmichael and Miss S. E. Hunt for technical assistance.

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EXPLANATION OF PLATES

PLATE 1

(i) Zones of precipitation and clearing obtained when rennet diffuses through 1 % whole casein-agar gel. The gel contained 0·1 M-acetate buffer (pH 5·7) and $CaCl_2$ at a concentration of 20 mM. Undiluted commercial rennet (0·125 ml) was placed in the well and the gel was photographed after 48 h at room temperature.

(ii) Effect of sodium dodecyl sulphate on the zones produced by rennet action. (*a*) In the absence of additional calcium only the innermost S.D.S. precipitated zone is plainly seen. (*b*) After washing with $CaCl_2$ solution the other two S.D.S. precipitated zones become visible. (*c*) A diagram showing the extent of the three S.D.S. precipitated zones shown in (*b*).

PLATE 2

Zones of precipitation and clearing obtained when rennet, pepsin and trypsin diffuse through 1 % casein-agar gels.

(i) (*a*) Action of rennet in whole casein-agar gels in the absence of calcium. (*b*) Same gel after flooding with 20 mM-$CaCl_2$ solution.

(ii) (*a*) Action of pepsin in whole casein-agar gels. (*b*) Action of trypsin in whole casein-agar gels.

(iii) (*a*) Action of rennet on α-casein-agar gel at pH 6·1 in the absence of calcium. (*b*) Action of rennet on β-casein-agar gel at pH 6·1 in the absence of calcium.

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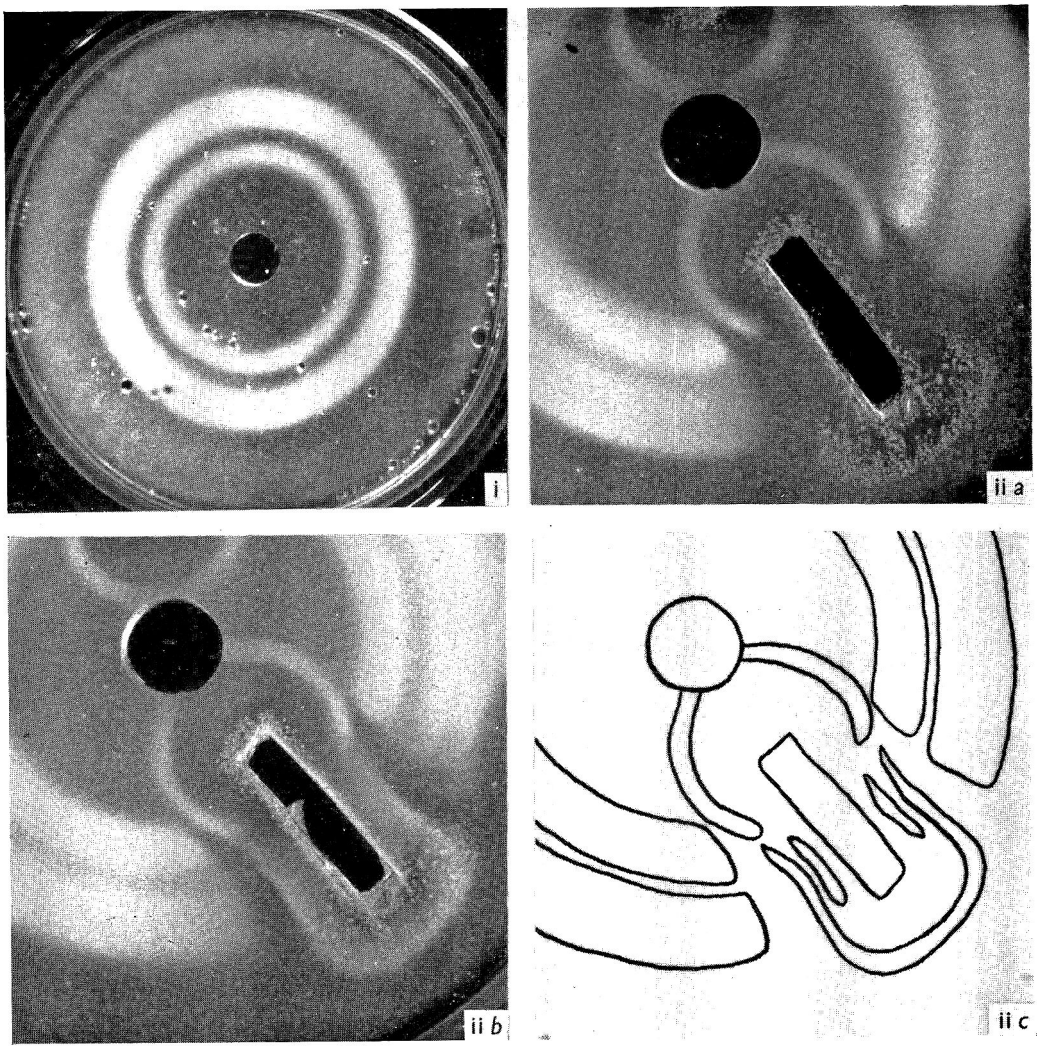
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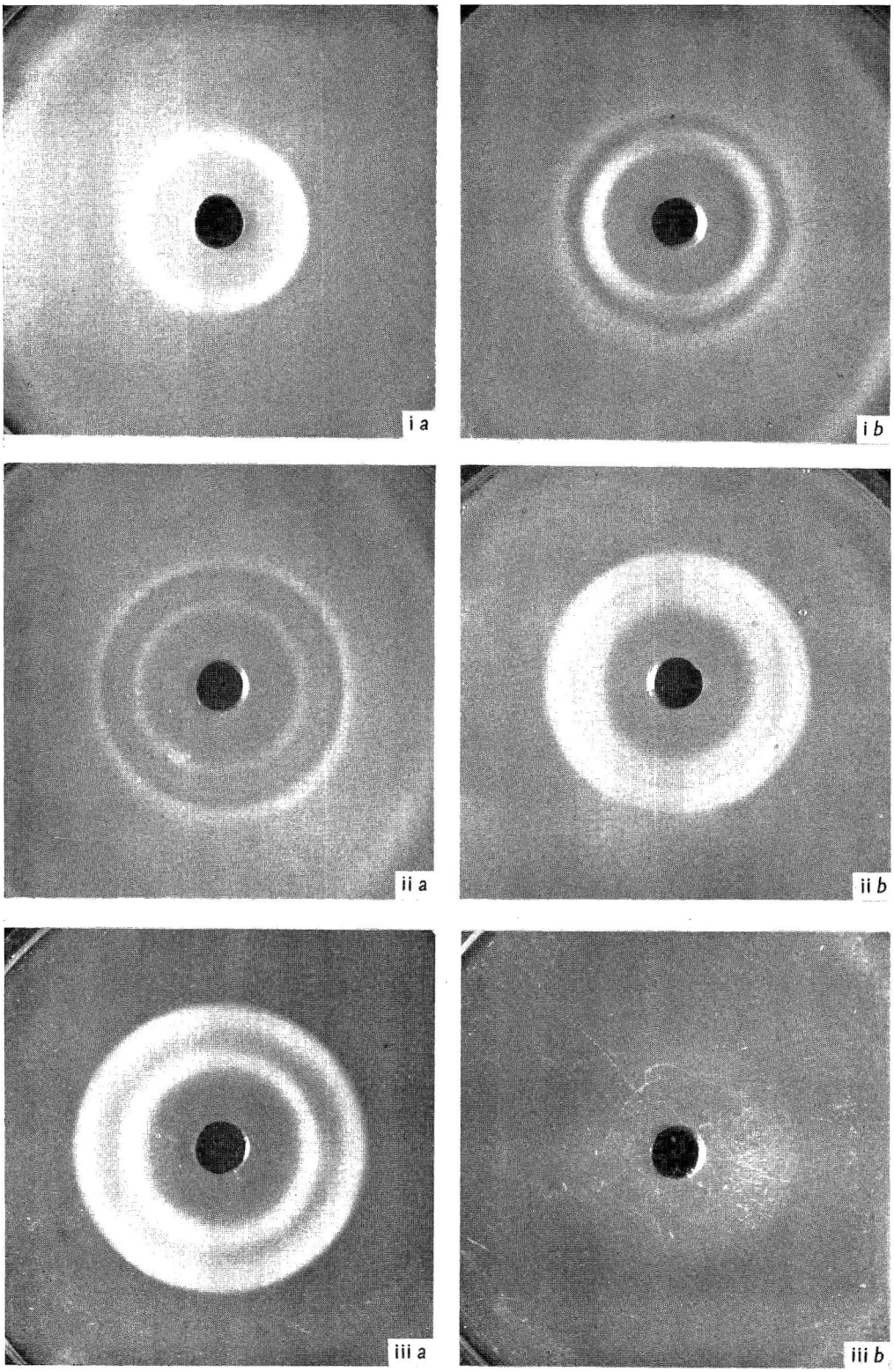
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J. Dairy Res. (1963), **30**, 23

The relationship between milk yield, composition and tissue damage in a case of subclinical mastitis

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(*Received* 10 *August* 1962)

Summary. A chemical, cytological and bacteriological examination of the milk from each quarter of a cow suffering from subclinical mastitis, but giving 5–6 gal of milk per day, was made at weekly intervals during the first 84 days after parturition. The infection, caused by micrococcal and staphylococcal bacteria, resisted repeated treatments with various antibiotics administered via the teat canals and also intramuscularly, although *in vitro* the bacteria were susceptible to all the antibiotics used. The cow was slaughtered and a histological examination made of the udder in an attempt to establish the cause of the continuing infection and to assess the extent of tissue damage. Two quarters each contained large abscesses in the upper level of the udder and these could have acted as reservoirs of infection; no cause was established for the other two quarters and it can only be assumed that reinfection occurred from the two abscessed quarters.

The amount of active lesions in the lobules of all the quarters was small, 1–6 %, but half or more of all the lobules were involuted, although only a minority appeared to have involuted as a result of infection. In the two abscessed quarters there was extensive damage to the duct system, 23 and 31 % showing lesions.

When the milk contained an abnormally large number of cells the chemical composition was also abnormal, containing less lactose (and hence less solids-not-fat) and having a nitrogen distribution in which there was more blood serum albumin and globulin and less casein than usual. Cell content and chemical composition were better indicators of tissue damage than the presence of mastitis organisms. It is estimated that the solids-not-fat content of the milk of the whole udder as a result of the infections was considerably lower than it would otherwise have been (8·0 instead of 8·8 %) and that the daily loss in milk yield was about 9 lb.

In a study of the chemical composition of the milk of a group of cows throughout lactation it was found that the colostrum from all four quarters of one cow was heavily infected with staphylococci in the first sample taken 5 h after calving. The cell count of this sample and those taken soon afterwards was also abnormally high, containing between $1–2 \times 10^6$ cells/ml, of which 50–90 % were polymorphs. Penicillin was administered via each teat, but numerous staphylococci were found in the subsequent samples. The cow showed none of the clinical signs of mastitis and by the seventh day of lactation was producing 55–58 lb of milk per day. Despite repeated treatment with preparations of penicillin, streptomycin and oxytetracycline, by both

intramammary and intramuscular injection, the infection persisted in the milk of each quarter for the first 84 days of lactation although, *in vitro*, the bacteria were susceptible to all the antibiotics used. It was clear, therefore, that treatment was ineffective because the antibiotics were not reaching the source or sources of infection. Although not a common occurrence, similar conditions had been encountered sufficiently frequently in the past to make it worthwhile to slaughter the animal for a histological examination of the udder, and it was decided to do this while the cow was still in full milk. It was thought that this might help to supply needed information linking chemical and cytological analyses of the milk with a histological examination of the tissue that produced it. The early chemical examinations of the milk showed that the solids-not-fat (S.N.F.) and lactose contents were appreciably lower, and the nitrogen distribution considerably different in the milk from three quarters than from the fourth. The following account gives the histological findings at slaughter and details of the chemical composition and cell count of the milk of each quarter at weekly intervals from parturition until slaughter on the 84th day.

EXPERIMENTAL

The cow was $8\frac{1}{2}$ years old when she began her seventh lactation and in earlier lactations she had been relatively free from any form of mastitis. When she was dried-off at the end of the previous lactation, the milk did not contain any pathogenic bacteria.

She had been well fed before calving, and after the first week in milk was fed a simple ration which provided approximately 120% of the Woodman standard of feeding. The cow was milked twice a day and the weight of the milk recorded. Milk samples from the whole udder were taken on 6 days of each week and on 1 day a quarter-milking bucket was used at successive afternoon and morning milkings. The two samples from each quarter were mixed in proportion to their afternoon and morning yields and analysed for total solids, fat, lactose, total nitrogen, total albumin nitrogen, β-lactoglobulin and non-protein nitrogen. The chemical methods employed have already been described (Waite, Castle & Watson, 1959). These samples were also used for determining the cell count of the milk by the method of Blackburn, Laing & Malcolm (1955), and foremilk samples taken before using the quarter-milking bucket provided material for bacteriological examination by a method already described (Blackburn, 1956).

After slaughter, the udder was detached and examined by the method of McFarlane, Blackburn, Malcolm & Wilson (1949) and Blackburn (1952) in which the frozen udder is subjected to multiple horizontal slicing. Before freezing, 10 ml of aqueous light green dye solution was injected via the teat canal of the two fore quarters to show clearly the demarcation between fore and hind quarters. Four or five blocks of tissue were taken from each of four levels in each quarter for histological examination as indicated in Fig. 1. Each block was typical of the region from which it was taken and the extent of any pathological condition seen in the sections made from each block has been used to calculate the approximate amount of tissue change at each level and, from these, in the whole quarter.

The lesions in the udder have been arbitrarily divided into those found in the

lobules and those found in the ducts. The acute lesion in the lactating lobule takes the form of a cellular exudate into the acini which appears to cause the affected lobule eventually to involute and lose its acini. The severity of the acute lesion in the lactating lobule was judged mild, moderate or severe by the amount of exudate present in the acini. Healthy lobules also involute as lactation proceeds but involution as a result of inflammation can be distinguished by the presence of cellular exudate in the associated small ducts and this, if present, was noted. In the tissue examined, the proportion of lobules involuted as a result of inflammation was recorded and, in addition, the proportion of lactating lobules to all involuted lobules.

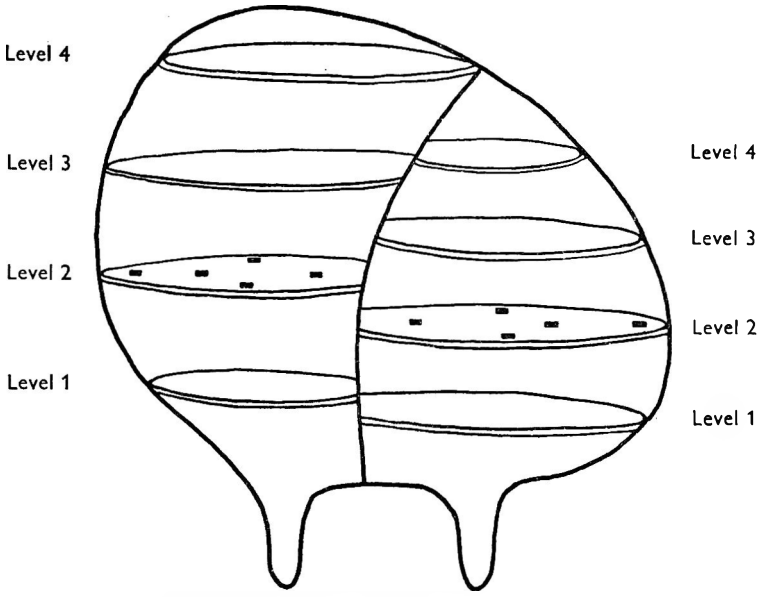

Fig. 1. Diagram of half udder showing the levels and locations from which blocks of tissue were taken for histological examination.

The lesions in the ducts occur between the epithelial layer and the elastic tissue surrounding the duct and take the form of subacute inflammation, i.e. the formation of granulation tissue. The severity of these lesions was judged mild, moderate or severe according to the amount of granulation tissue present. The term periductal fibrosis has been used to describe the healed subacute lesion and it was graded mild, moderate or severe according to the amount of fibrous tissue present.

RESULTS

Bacteriology and cell count of the milk

Two types of bacteria were present in the milk of all the quarters at most samplings, coagulase negative micrococci and coagulase positive staphylococci. Their incidence is given in Table 1 where coagulase negative micrococci are designated by the letter m and coagulase positive staphylococci by the letter S; the figure preceding the letter is the number of colonies growing on blood agar in 18 h at 37 °C from 0·01 ml of

milk. The cell count of the mixed evening and morning milk from the quarter, and the antibiotic treatments are also recorded in Table 1.

For the purpose of the original study for which the group of cows was being used it was essential to keep udder infection to a minimum and when the first sample from all the quarters of this cow was found to be heavily infected, penicillin (100 000 units) was injected via the teat canal. In the samples a week later the bacteria were present in three of the four quarters and further treatment with streptomycin (250 mg) was given, but numerous bacteria were still present at the next sampling although the bacteria cultured *in vitro* were susceptible to the antibiotics used. In an endeavour to reach the sites of infection via the blood stream an intramuscular injection of streptomycin (3×10^6 units) was next given, but again with little success. Seven out of eight samples taken at the two succeeding weekly samplings were heavily infected with coagulase positive staphylococci, and oxytetracycline (425 mg) was therefore

Table 1. *The numbers of colonies* of coagulase negative micrococci* (m) *and of coagulase positive staphylococci* (S) *and the cell count of the milk from each of the four quarters in the first* 80 *days of lactation*

	Left fore		Left hind		Right fore		Right hind		
Days calved	Colonies/ 0·01 ml	Cell count, 10^{-6}/ml	Colonies/ 0·01 ml	Cell count, 10^{-6}/ml	Colonies/ 0·01 ml	Cell count, 10^{-6}/ml	Colonies/ 0·01 ml	Cell count, 10^{-6}/ml	Treatment† (all quarters)
7	>30m	0·82	>30m	0·05	>30S	0·01	>30m	0·06	Penicillin
14	0	0·41	>30m	0·94	>100S	1·22	>30S	0·07	Streptomycin
21	30m	1·12	30m	1·16	30m	2·62	30m	0·01	—
28	0	0·24	10m	0·43	>30S	6·95	8m	0·01	Streptomycin intramuscularly
35	0	0·01	>30S	0·64	>30S	3·66	>30S	0·02	—
42	>30S	3·30	>30S	0·12	>30S	3·68	>30S	0·04	Oxytetracycline
49	>30m	3·64	0	0·19	>30S	4·36	0	0·59	—
56	0	2·82	11m	0·04	>30S	1·06	0	0·10	Streptomycin intramuscularly
65	0	2·50	0	1·03	>30m	4·12	>30m	0·33	—
71	7S	3·76	0	0·11	>30m	0·94	0	11·0	—
78	>30S	—	12m	—	>30S	—	>30S	—	—
80	6S	5·84	8m	0·40	0	3·28	>30S	1·84	—

* When grown on blood agar for 18 h at 37 °C from 0·01 ml milk.

† Intramammary treatment started 24 h after the milk sample had been taken and was repeated after a further 24 and 48 h.

introduced into each teat. This treatment also had limited success and was followed by a final intramuscular injection of streptomycin (3×10^6 units) together with intramammary penicillin in both fore quarters a fortnight later. It was now obvious that the cow was unsuitable for the original purpose and it was decided to let the infection take its course for a few weeks before slaughtering the animal for udder inspection.

The cell count, in general, had been high in those milk samples which contained either type of bacteria. This was particularly so in the milk from the right fore quarter, a quarter later found to be abscessed, and to a lesser extent in the left hind quarter. On the other hand, the right hind quarter had much the same high incidence of infection of both types but, until the 49th day of lactation, the milk contained very few cells. In the left fore quarter, which also at slaughter was found

to be abscessed, the cell count of the milk had, except at one sampling, been uniformly high although antibiotic treatment showed slightly more temporary effect in this quarter than in any other.

The cell counts of the milk from the fully milked quarter agreed more with the bacteriological findings than cell counts made on the fore milk. In a previous study (unpublished) we found that when counts were less than 100 000/ml or greater than a million the agreement between the cell counts of fore milk and quarter milks was close, but in the middle range of counts there were frequently considerable differences and this finding applied to the present results.

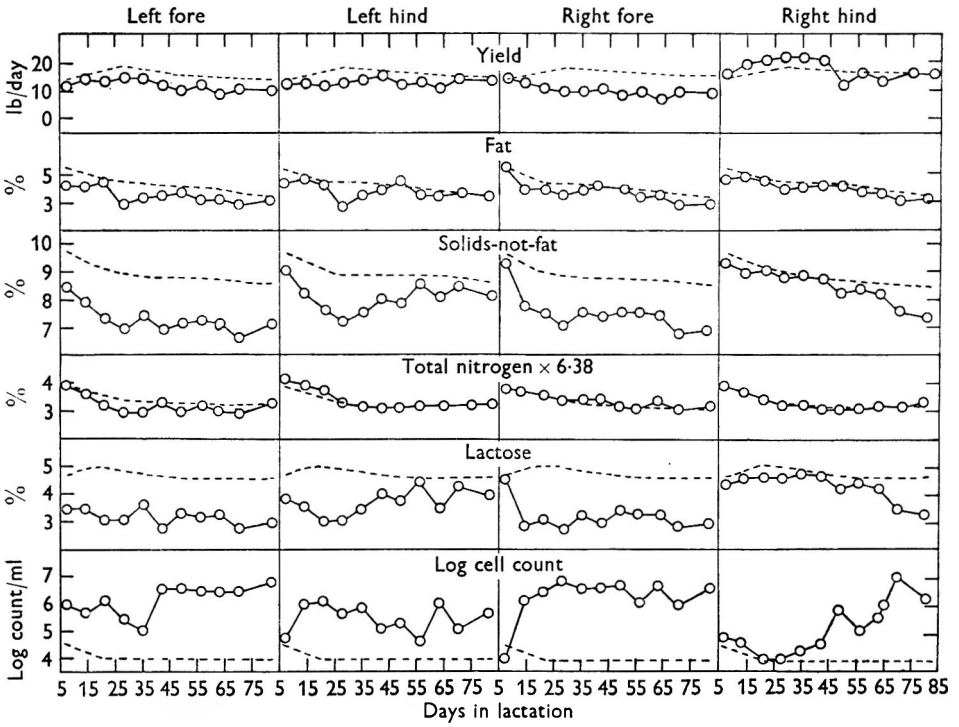

Fig. 2. Yield, chemical composition and cell count of the milk of each quarter. (The broken line represents typical values for milk from a healthy quarter.)

A differential count of polymorphs, epithelial cells and lymphocytes was made on all milk samples taken and, as previously reported (Waite & Blackburn, 1957), a close positive relationship between the total and polymorph cell counts ($r = 0{\cdot}651$, $P < 0{\cdot}001$) was found.

Previous studies (Waite & Blackburn, 1957) have shown that the total cell count of milk from a healthy quarter is frequently well below 100 000/ml. It can be seen therefore that, with the exception of the right hind quarter for the first 42 days, almost all the samples taken contained abnormally high counts and this was also true for the percentage of polymorph cells in the total counts.

Chemical analyses. In the first few months of lactation marked changes normally occur in the chemical composition of cow's milk and these must be borne in mind when considering the additional effect of disease. The values for daily milk yield, the

percentages of fat, S.N.F. and lactose, together with the total nitrogen content for the milk from each quarter are shown in Fig. 2. Since cell count and chemical composition are related (Rensburg, 1947; Waite & Blackburn, 1957) the values for the total count of the same samples as analysed chemically are also included in this figure. For comparison with a healthy udder and also to give some measure of the stage of lactation effect, the analyses of milk substantially free from cells and pathogenic bacteria from a cow in the same group are shown in Fig. 2 by a broken line.

Milk yield. Only in the right hind quarter did the yield rise appreciably after calving, whereas in both the quarters later found to contain a large abscess (left and right fore) the yield declined prematurely.

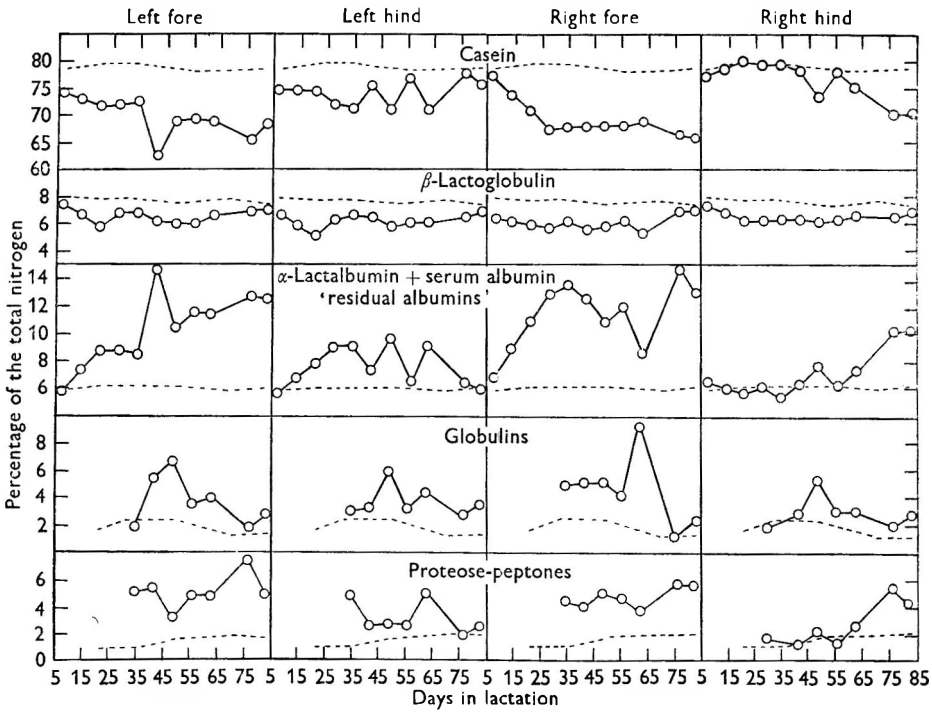

Fig. 3. The nitrogen distribution in the milk of each quarter. (The broken line represents typical values for milk from a healthy quarter.)

Fat. The fat percentage of the milk from the left quarters was somewhat lower and more variable during the first 50 days than from the right quarters but it is unlikely that these differences were the effect of disease.

Solids-not-fat. The rate of fall in the S.N.F. percentage was abnormally high in the milk from all the quarters except the right hind, and it will be noticed that the cell count in the milk from both left quarters and the right fore quarter was abnormally high, frequently containing a million per ml or more for most of the time. The general level of S.N.F. in the two abscessed quarters (left and right fore) was about 7·5 % whereas in the right hind quarter the S.N.F. level for the first 42 days was 8·8–9·0 % but declined more rapidly thereafter as the cell count rose to very high values.

Total nitrogen. The curves showing the total nitrogen content of the milk were

similar in shape to that from the healthy quarter shown for comparison (broken line). There were, however, marked changes in the distribution of the nitrogen between the various proteins in the milk from quarters which suffered damage. These are given more fully below.

Lactose. The rapid decline in S.N.F. levels in the milk of three of the quarters was entirely the result of abnormally low lactose values and hence the S.N.F. and lactose curves showed considerable similarity. A comparison of the curves for lactose and total cell count for each quarter indicated a strong inverse relationship and the correlation coefficients between lactose percentage and log total cell count for the four quarters were negative and all significant; viz. left fore, $-0{\cdot}595$ ($P < 0{\cdot}05$); left hind, $-0{\cdot}683$ ($P < 0{\cdot}02$); right fore, $-0{\cdot}814$ ($P < 0{\cdot}01$) and right hind, $-0{\cdot}672$ ($P < 0{\cdot}02$).

Nitrogen partition. The distribution of the total nitrogen is shown graphically in Fig. 3, where the nitrogen content of each compound is given as a percentage of the total. Comparative values for milk from a healthy quarter are again shown by a broken line. The chemical changes associated with the presence of bacteria and large numbers of cells in the milk were a decrease in the proportion of casein nitrogen, a marked increase in the α-lactalbumin plus serum albumin fraction and increases in the proteose peptone and globulin contents. The amount of β-lactoglobulin fraction in all the quarters was a little lower than in the milk from the healthy quarter shown for comparison, although this may have been the result of a difference between animals. It can be seen that all the chemical changes mentioned had taken place to the greatest extent in the milk from the two abscessed quarters (left and right fore).

Histological examination of the udder after 84 *days in milk*

Left fore quarter. A thin-walled (1 mm) staphylococcal abscess measuring $2{\cdot}5 \times 2{\cdot}0 \times 1{\cdot}0$ cm was found in level 4 (Fig. 1) and it was in tissue adjacent to the abscess that the most severe and extensive lesions were found. The extent of tissue damage, in the terms defined above, is given in Table 2. In level 1, 4 % of the ducts showed severe fibrosis. This quarter consistently contributed 23 % of the total yield of the udder, i.e. 12–13 lb of milk per day, but at slaughter on the 84th day of lactation only about a third of the lobules were lactating; of the non-lactating lobules at least half were judged to have involuted as a result of inflammation, cellular exudate being present in the associated small ducts. Table 2 shows that a further 6 % of lobules had lesions of some degree of severity but had not at that time involuted. Of the ducts, some 77 % were normal and most of the lesions were mild except in the neighbourhood of the abscess.

Left hind quarter. There was very little damaged tissue in this quarter. At level 4, 5 % of the lobules had mild inflammation and 2 % of the ducts at level 1 had a mild degree of fibrosis. The proportion of the total yield contributed by this quarter was lower than normal for the first 5 weeks of lactation; it was lower also than would be expected relative to the other hind quarter, being no more than that of the left fore quarter, but thereafter the yield increased slightly to 14 lb, producing 28 % of the total. At slaughter, slightly more than half the lobules were involuted but of these only a negligible amount showed evidence of inflammation.

Right fore quarter. A spherical thick-walled (2 mm) staphylococcal abscess of 1·5 cm diameter was found in level 4 and 30 % of the adjacent ducts showed moderate fibrosis. The extent of tissue change in the quarter is given in Table 3, which shows that 95 % of the lobules and 70 % of the ducts were undamaged.

Inflammation in the lobules and ducts resulted in the presence of cellular exudate in about a quarter of the ducts at all levels examined, with most in levels 1 and 4, and also in the small ducts within the lobules. About 40 % of the lobules in this quarter were lactating, although its daily milk yield of about 10 lb represented only 16–19 % of the total yield of the udder.

Table 2. *Tissue damage in the left fore quarter*

Tissue	Level in udder (see Fig. 1)	Extent of lesions (% of tissue examined) Mild	Moderate	Severe		Total
Lobules	1	1	0	0		
	2	1	0	0		
	3	1	0	0		
	4	1	10	10		
Quarter	(Average)	1	2·5	2·5	=	6
Ducts	1	18	0	(4)*		
	2	36	0	0		
	3	10	0	0		
	4	0	22	2		
Quarter	(Average)	16·0	5·5	1·5	=	23

* Fibrosed.

Table 3. *Tissue damage in the right fore quarter*

Tissue	Level in udder (see Fig. 1)	Extent of lesions (% of tissue examined) Mild	Moderate	Severe		Total
Lobules	1	5	—	—		
	2	5	—	—		
	3	7	—	—		
	4	3	—	—		
Quarter	(Average)	5	—	—	=	5
Ducts	1	20	30	—		
	2	20	—	—		
	3	20	—	—		
	4	—	(30)*	—		
Quarter	(Average)	15	15	—	=	30

* Fibrosed.

Right hind quarter. Tissue damage in this quarter was small, 97 % of all ducts and 95 % of the lobules were estimated to be normal. Lesions in the ducts were confined to level 1, where 11 % showed moderate inflammation and 1 % were severely fibrosed. A few lobules (3 %) in each level had mild lesions, and in level 3 some 5 % showed severe inflammation. There was cellular exudate in a small proportion of ducts and

lobules at all four levels, the highest amounts occurring in level 4. Approximately half the lobules in this quarter were lactating and half were involuted, the quarter producing about 20 lb of milk daily, or 35 % of that from the whole udder.

DISCUSSION

The histological findings show the damage caused by bacterial infections of the udder, leading to involution of lobules, to be lesions in both lobules and ducts and to active abscesses in two quarters. These in turn gave rise to large numbers of cells in the milk and a marked disruption of the normal lactation pattern of the main milk constituents. The small proportion of lobules (1–6 %) showing active lesions was probably the reason for the lack of the clinical signs of mastitis, i.e. heat, pain or swelling in the udder, yet in two quarters the tissue damage was extensive and in all quarters a high proportion of lobules was no longer milk-producing. The presence of bacteria alone was clearly insufficient to arrive at a sound assessment of possible tissue damage. Numerous bacteria of both micrococcal and staphylococcal types in the right hind quarter produced only a very small number of lesions and for 42 days did nothing to upset normal milk production in quantity or quality. The presence of cells in the milk and the chemical evidence would appear to be more indicative of tissue damage.

Lesions in udder tissue apparently allow transudation of blood constituents into the milk secreted by healthy tissue (Aschaffenburg & Drewry, 1959; Lecce & Legates, 1959; Weigt, 1959). This is shown by a change in the relative amounts of the various milk proteins and by a fall in the lactose content. In the milk from healthy quarters the albumin fraction of the protein normally accounts for about 14 % of the total nitrogen (Waite *et al.* 1959) and of this fraction β-lactoglobulin contributes a little more than half (about 8 %). The residual albumins consist mainly of α-lactalbumin together with a protein essentially similar to blood serum albumin, the latter normally contributing only about 2 % of the total N. It can be seen from Fig. 3 that in the milk from the two abscessed quarters the proportion of the residual albumin rose to as high as 15 %, and if, as mentioned by Aschaffenburg & Drewry (1959), the amount of α-lactalbumin remains materially unchanged in mastitis milk, this would represent an increase in the blood serum albumin fraction from 2 to 11 % of the total nitrogen. It may be noted that in the right hind quarter the residual albumin fraction was normal until the 42nd day and that the later abnormal values appeared at the same time as large numbers of cells in the milk. In the milk from all quarters there was also a greater than normal globulin content when the cell count was high. A rise in the immune globulin content has been found to result also from streptococcal mastitis (Weigt, 1959).

The rise in the proportion of the total nitrogen found in the albumin and globulin fractions naturally results in a corresponding fall in the proportion contributed by the casein fraction and this is clearly evident in the present results (Fig. 3), particularly when compared with the values for milk from a healthy quarter. The low level of the lactose contents of three of the quarters and ultimately of the fourth is further evidence of the effects of tissue lesions on the chemical composition of the milk and, since lactose is the major constituent of the S.N.F., the total S.N.F. content, too, had

abnormally low values for most of the time, with a mean value for the milk from the whole udder for 80 days of 8·0 %. If infection had not been present this figure would probably have been about 8·8 %, the average value for the milk of the right hind quarter before the bacteria, which had been present in the milk from parturition, finally caused tissue damage.

It is possible to make an approximate estimate of the loss of milk as a result of these infections. In a completely healthy udder the hind quarters normally secrete very similar amounts of milk and each of them contributes about 30 % of the total yield; the fore quarters also secrete similar amounts, each contributing about 20 % of the total. Taking the yield of the right hind quarter during the first 42 days as being normal, the probable yield of the other three quarters, if they had been healthy, was calculated. The total probable loss during the first 42 days amounted to about 560 lb of milk, or some 13 lb/day, and over the whole period of 80 days to about 760 lb, or 9 lb/day. Most of this milk was lost from the left hind quarter and, to a lesser extent, from the right fore. These estimates would be somewhat too high if there had been any compensatory increase in yield from the right hind quarter as a result of the early infection of the right fore quarter, but they serve to indicate the magnitude of the possible loss of milk caused by subclinical mastitis in a high-yielding cow. It was surprising to find that more than half the lobules in each quarter were involuted, either as a result of disease or natural change, since this is the condition that has been found in udders much nearer the end of lactation (Blackburn, 1952). We do not know of any reports for completely healthy udders in early lactation and insufficient is established about the relationship between the number of lactating lobules and milk yield to explain this apparent anomaly.

In the previous lactation of this cow pathogenic bacteria had been present for a few days in the milk of both fore quarters 2–3 months before the end of lactation but had yielded to antibiotic treatment, leaving the milk free from mastitis organisms. Since these organisms were present in the colostrum 5 h after parturition it would appear that the infection described was contracted during the dry period.

The presence of two abscesses in the upper part of the udder suggests that the antibiotics had not reached them, since the bacteria in the milk were susceptible to treatment. These abscesses would act as reservoirs of infection for the rest of the quarter in a manner similar to that from a botryomycosis lesion (Blackburn, 1959). The reason for the continued presence of bacteria in the milk from the two hind quarters is not clear and it must be assumed that re-infection occurred from one or other of the abscessed quarters, possibly at milking time or from an external source. The lack of tissue damage in the right hind quarter for 42 days, despite the presence of numerous micrococci and staphylococci in the milk suggests that these organisms took this time to cause mastitis. Davidson (1961) has recently concluded that this condition is not uncommon but that tissue damage will usually result if the organisms persist more than a short time. We would agree with Crossman, Dodd, Lee & Neave (1950) who concluded that 'one type of organism may infect more than one quarter of the same udder, causing little or no irritation in one gland and marked abnormalities in the other'.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 35

The effect on the performance of growing pigs of the level of meal fed in conjunction with an unrestricted supply of whey

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(*Received* 21 *August* 1962)

Summary. 1. Results are reported of an experiment made under commercial conditions on the effect of the level of meal fed in conjunction with an unrestricted supply of whey on the performance and carcass quality of pigs.

2. All the pigs received the same meal mixture and had continuous access to an unrestricted supply of whey. The four treatments were: (1) 3 lb meal per pig per day reduced to 2 lb at 13 weeks of age; (2) 3 lb meal per pig per day throughout; (3) 2½ lb meal per pig per day throughout; and (4) as (1) but the daily allowance of meal increased to 2½ lb/pig at 20 weeks of age. There were five pens of nine group-fed pigs on each treatment, involving a total of 180 pigs. The pigs were on experiment from 8–9 weeks of age to bacon weight. Comprehensive carcass measurements were made on all the pigs.

3. Mean differences in rate of growth, efficiency of food utilization and carcass quality between the four groups of pigs were small and were not statistically significant. The significant differences in the proportions of meal and of whey consumed by the pigs on the different treatments during the growing period are discussed in relation to relative prices of the two foods. It is concluded that where unrestricted whey feeding is to be used, the feeding system employed in treatment 3 could be recommended in most of the environmental and economic situations likely to be encountered in practice.

4. The superior performance of the pigs in the experiment compared with that in earlier trials is discussed in relation to the stock improvement work and concurrent improvements in housing and management carried out during the intervening years on the Cow and Gate farm in Dorset where the experiments were conducted.

In experiments on the Dorset farm of Cow and Gate Farms Ltd. in 1954–55 (Braude, Clarke, Mitchell, Cray, Franke & Sedgwick, 1957), a reasonably satisfactory performance was obtained by giving growing pigs unrestricted access to whey together with a daily allowance per pig of 3 lb of a sow and weaner meal. When the daily allowance of meal was reduced from 3 lb to 2 lb/pig at 13 weeks of age, the pigs took an average of 12 days longer to reach bacon weight, but they consumed approximately 26 % less meal and 28 % more whey; also their carcasses were less fat

and the commercial grading results were accordingly better. It was concluded, therefore, that when the requirement was for the maximum quantity of whey and minimum amount of meal to be used, consistent with a reasonable performance and carcass quality, the latter feeding system was the one of choice.

Subsequent experiments using this system of feeding (Braude, Mitchell, Cray, Franke & Sedgwick, 1959*b*), showed that either 10% white fish meal or 15% dried skim-milk should be considered the minimum amount of these protein supplements that should be included in the basal meal fed with unrestricted whey. It was shown also that reduction of the daily meal allowance to 1 lb or less had a marked adverse effect on the growth rate of the pigs.

Since these early trials were carried out, attempts to improve the stock by selection and the use of superior boars have been continuously in progress in the Cow and Gate herd. The main object of the experiment reported here was to investigate whether in the light of this and other concurrent improvements in housing and management, the daily meal allowance of 3 lb/pig, reduced to 2 lb at 13 weeks of age, given with an unrestricted supply of whey, remained the feeding system of choice, and at the same time to obtain a measure of the absolute performance of these genetically improved pigs as compared with similarly fed pigs in previous trials.

In addition, the possibility that there might be some advantage in increasing the level of meal-feeding in the later stages of the growing period was investigated. This was based on the observation made in previous trials that the rate of growth of pigs given 2 lb meal/day with unrestricted whey either declined as they got older (Braude *et al.* 1957; Braude, Mitchell, Cray, Franke & Sedgwick, 1958) or increased only very slightly (Braude, Mitchell, Cray, Franke & Sedgwick, 1959*a*; Braude *et al.* 1959*b*). This was in contrast to the marked and continuously increasing rate of growth throughout the growing period of similar pigs given an all-meal diet (Braude, Clarke, Mitchell, Cray, Franke & Sedgwick, 1958; Braude *et al.* 1959*a*). It was considered that this difference in growth curves might be due to the pigs not having the capacity to drink sufficient whey in the later stages of the growing period to meet their requirements and that an increase in the daily meal allowance might, therefore, be desirable during this period.

EXPERIMENTAL

Treatments and diets

For the first 10 days after being put into the experimental pens all the pigs were fed the same creep pellets that they had received from 3 weeks of age, and had access to an unrestricted supply of whey which they had been fed since birth. They were then gradually changed over to the experimental meal mixture shown in Table 1 so that they were receiving this only plus unrestricted whey on the 14th day after being put into the pens. The four experimental treatments based on differences in the amounts of basal meal given were as follows:

Treatment 1. 3 lb meal per pig per day reduced to 2 lb at the 13th week of life.

Treatment 2. 3 lb meal per pig per day throughout.

Treatment 3. $2\frac{1}{2}$ lb meal per pig per day throughout.

Treatment 4. 3 lb meal per pig per day reduced to 2 lb at the 13th week of life and then increased to $2\frac{1}{2}$ lb per pig per day at the 20th week of life.

The alterations in the daily allowances of meal in treatments 1 and 4 were made gradually over a period of one week, the reductions from 3 to 2 lb being started when the pigs averaged 12 weeks of age and being completed when they were 13 weeks of age, while the increase from 2 to 2½ lb on treatment 4 was started at an average age of 19 weeks and completed at the age of 20 weeks.

The experimental period started from the time the selected pigs entered the Danish fattening house after weaning at approximately 8–9 weeks of age, and continued to slaughter at an average liveweight of 204 lb. All pigs were given the creep pellets or experimental basal meal *ad lib.* from the start of the trial up to the daily maximum of 3 lb/pig on treatments 1, 2 and 4, and 2½ lb/pig on treatment 3.

Table 1. *The percentage composition of the basal meal given to all the pigs*

Barley meal	55
Weatings	35
White fish meal	10
Vitamealo 'Supercon' special supplement*	3½ lb/2000 lb

* Agricultural Food Products Ltd., containing in 3½ lb: 14 g oxytetracycline, 4 200 000 i.u. vitamin A, 1 050 000 i.u. vitamin D_3. Other B vitamins and trace minerals were included in this commercial product.

PIGS

All the pigs used were reared with their dams on pasture and were weaned at 8–9 weeks of age, weaning being done on one day in each week. Both sows and litters had access to unrestricted whey during the suckling period. In addition, proprietary creep pellets containing an antibiotic were provided from 3 weeks of age. All the litters were cross-breds, the majority being from cross-bred sows containing Large White, Landrace or Wessex Saddleback blood. The sires of the litters were either pure Large White or pure Landrace. As far as possible litters were chosen from sires that had produced at least twenty litters in the herd and whose performance was such that the boars had been given a classification of high quality. All the pigs were injected with swine erysipelas vaccine at 5–6 weeks of age.

After weighing at weaning, the pigs were selected for experiment in the way described by Braude *et al.* (1957) and brought into the Danish-type fattening house described below.

There were five pens of nine pigs on each of the four treatments, making a total of 180 pigs for the experiment. The four treatments were randomly allocated to the pens within each of the five replicates. The first replicate was started on 16 April 1959 and the fifth replicate on 9 June 1959.

Housing

The Danish fattening house previously described by Braude *et al.* (1957) was used. Before the experiment began, however, raised insulated concrete floors with a greater slope were installed in each of the pens, replacing the wooden platforms and straw bedding used previously.

Records

All pigs were individually weighed once weekly in the morning. The meal given to each pen of pigs was weighed, and the whey given recorded in gallons. Each week, coinciding with the weighing day, refusals of meal and whey were determined so that records were available of the total weekly food consumption of each pen of pigs throughout the experiment.

In the determination of the total food consumption of each pen of pigs, an estimate of the proportion of the food eaten by those pigs which either died or had to be taken off the experiment (see later) was made and deducted from the total. To obtain an over-all figure of food consumption per lb of gain, the whey consumed was converted to a 12 % moisture basis, equivalent to the approximate moisture content of the meal portion of the ration. Using figures of 6·24 % for the dry-matter content of whey and 10·3 lb for the weight of 1 gal of whey, the 12 % moisture equivalent for the whey in pounds was obtained by multiplying the figure for whey in gallons by 0·72895.

Carcass grading

When each pig reached a minimum liveweight of 200 lb at the once-weekly weighing, it was removed from the pen and sent to slaughter without further feeding. All the pigs were sent to the same local bacon factory and were killed soon after arrival the same day. All carcasses were commercially graded and, in addition, carcass measurements were taken as described in detail by Mitchell & Sedgwick (1960).

Health of the pigs

During the experiment nine of the total of 180 pigs (5 %) died or had to be withdrawn. Of the three pigs that died one had severe pneumonia, one acute enteritis and the cause of death of the third, which was on experiment for only one week, was unknown. All but one of the six pigs taken off the experiment were withdrawn because they failed to grow normally, probably owing to pneumonia. The remaining pig had a protruding rectum.

As in previous trials at East Farm signs of virus pneumonia were observed in the pigs and the routine described previously (Braude *et al.* 1957) to control the associated secondary complications of this disease was again successfully applied. All pigs were sprayed with benzene hexachloride twice at 7–14-day intervals shortly after they entered the fattening house as a precaution against sarcoptic mange, and were given a vermifuge containing piperazine when approximately 10 weeks old. At the beginning of June, an outbreak of swine fever occurred in a group of pigs on another part of the farm, and as a precaution all the pigs on experiment were injected with crystal violet vaccine.

RESULTS

Treatment means for daily liveweight gain, total consumption of meal and whey per pig and per lb liveweight gain, dressing percentage and carcass measurements, together with their standard errors, are given in Table 2. The standard errors were calculated from randomized block analyses of variance on the pen means, no adjustments being made for variation in either initial liveweight or cold dead weight

Table 2. *Treatment means, with their standard errors, for liveweight gain, efficiency of food conversion and carcass measurements of pigs on experiment from approximately 8 weeks of age to bacon weight. All pigs had access to an unrestricted supply of whey*

	Treatment no. and daily meal allowance/pig					
	1	2	3	4		
	3 lb reduced to 2 lb at 13 weeks of age	3 lb all through	2½ lb all through	3 lb reduced to 2 lb at 13 weeks of age and increased to 2½ lb at 20 weeks of age	Standard error of means†	Significance of treatment mean square‡
umber of pigs	44	44	41	42	—	—
ıitial weight, lb	44·1	44·1	44·3	44·2	—	—
ınal weight, lb	203·6	203·5	204·1	203·7	—	—
ays on experiment	131·5	126·1	124·2	127·0	—	—
aily gain, lb	1·23	1·28	1·30	1·27	0·018	N.S.
ɔtal consumption of food/pig:						
Meal, lb	278·2	360·6	304·0	289·9	—	—
Whey, gal	400·0	270·4	347·5	363·9	—	—
Meal + 88 % dry-matter whey, lb	569·9	557·7	557·3	555·2	—	—
ɔnsumption of food/lb gain:						
Meal, lb	1·75	2·26	1·90	1·82	0·029	***
Whey, gal	2·51	1·69	2·18	2·28	0·042	***
Meal + 88 % dry-matter whey, lb	3·57	3·50	3·49	3·48	0·031	N.S.
ɾessing percentage	76·4	76·8	76·6	76·2	0·34	N.S.
ıɾcass length, mm	808·0	813·6	811·9	813·8	2·9	N.S.
ıoulder back fat, mm	48·5	48·6	48·6	48·4	1·4	N.S.
ɔin back fat 2, mm	30·9	30·4	30·2	30·0	0·56	N.S.
ɜlly thickness, mm (average of 3 points)	41·3	40·8	41·2	40·9	0·25	N.S.
ye muscle:						
Breadth, mm	81·9	82·0	82·0	82·5	0·28	N.S.
Depth, mm	40·3	40·6	40·6	40·8	0·30	N.S.

† Based on 12 degrees of freedom.
‡ N.S. $P > 0{\cdot}05$, ***$P < 0{\cdot}001$.

Table 3. *Commercial grading results (percentages)*

		Final grade										Carcass length, mm			
ɾreat-ment no.	No. of pigs	AA+	AA	A	Total A and above	B+	B	Total B+ and B	C	F	Grade A on shoulder	Grade A on loin	800 and above	775–799	Below 775
1	44	34·1	11·4	4·5	50·0	36·4	2·3	38·7	6·8	4·5	84·1	52·3	68·2	25·0	6·8
2	44	40·9	2·3	2·2	45·4	43·2	2·3	45·5	6·8	2·3	84·1	45·5	77·3	18·2	4·5
3	41	41·5	2·4	0·0	43·9	39·0	2·5	41·5	12·2	2·4	83·0	48·8	78·0	19·5	2·5
4	42	50·0	9·5	2·4	61·9	23·8	0·0	23·8	11·9	2·4	85·7	61·9	76·2	21·4	2·4

(Barber, Braude & Mitchell, 1957). The commercial grading results are summarized in Table 3, and in Table 4 the pattern of food consumption over 7-day periods during the experiment is shown. In Fig. 1 the average growth curves of the pigs on the four treatments are given from approximately 8 weeks of age up to the time when the first pigs went to slaughter.

(a) Daily liveweight gain and efficiency of food utilization

Differences between treatment means were small, and neither for daily liveweight gain nor efficiency of food conversion (meal plus 88 % dry matter whey/lb gain) were the treatment mean squares significant at the 5 % level. There were, however, large differences in the proportions of meal and of whey consumed by pigs on the different treatments, the higher the level of meal given, the lower the amount of whey con-

Table 4. *Mean consumption of whey and of meal plus* 88 % *dry-matter whey per pig per day over 7-day periods during the experiment up to the time when the first pigs went to slaughter*

	Treatment no. and daily meal allowance/pig							
	1		2		3		4	
	3 lb meal reduced to 2 lb at 13 weeks of age		3 lb meal all through		2½ lb meal all through		3 lb meal reduced to 2 lb at 13 weeks of age and increased to 2½ lb at 20 weeks of age	
Approximate age of pigs in middle of period, weeks	Whey, gal	Meal plus 88 % dry-matter whey, lb	Whey, gal	Meal plus 88 % dry-matter whey, lb	Whey, gal	Meal plus 88 % dry-matter whey, lb	Whey, gal	Meal plus 88 % dry-matter whey, lb
9	0·6	2·3	0·7	2·3	0·7	2·4	0·7	2·2
10	0·9	3·0	0·9	3·0	1·0	3·2	0·9	3·0
11	1·0	3·4	0·9	3·3	1·1	3·3	0·9	3·2
12	1·1	3·8	1·1	3·6	1·5	3·6	1·2	3·7
13	1·5	3·4	1·2	3·9	1·7	3·7	1·5	3·4
14	2·1	3·5	1·6	4·1	2·0	4·0	1·9	3·4
15	2·4	3·8	1·6	4·2	2·3	4·2	2·3	3·7
16	2·8	4·1	1·8	4·3	2·7	4·5	2·9	4·1
17	2·9	4·1	1·9	4·4	2·8	4·5	3·1	4·2
18	3·9	4·8	2·4	4·8	3·2	4·9	3·6	4·7
19	3·8	4·8	2·5	4·8	3·2	4·8	3·6	4·6
20	4·1	5·0	2·8	5·1	3·7	5·2	4·0	5·2
21	4·4	5·2	3·2	5·3	3·9	5·3	4·1	5·5
22	4·4	5·2	3·4	5·4	4·1	5·5	4·2	5·6
23	4·9	5·6	3·5	5·5	4·3	5·6	4·3	5·6
24	4·9	5·6	3·3	5·4	4·3	5·6	4·4	5·7

sumed by the pigs. Thus, pigs on treatment 2, given the highest level of meal (3 lb meal/day all through) consumed significantly less whey per lb liveweight gain than the pigs on each of the other three treatments ($P < 0{\cdot}001$). Pigs given the next highest level of meal (2½ lb meal/day all through) on treatment 3 consumed significantly less whey per lb liveweight gain ($P < 0{\cdot}001$) than the pigs on treatment 1 given the lowest level of meal (3 lb meal reduced to 2 lb at 13 weeks of age). Similarly, the pigs on treatment 4 which had their daily meal allowance increased from 2 to 2½ lb at 20 weeks of age consumed significantly less whey per lb liveweight gain ($P < 0{\cdot}01$) than the pigs on treatment 1 which continued on the 2 lb level of meal to slaughter. The growth curves in Fig. 1 show that on all treatments the pigs maintained a fairly steady rate of growth throughout (apart from a fall immediately following the reduction in meal allowance from 3 to 2 lb, which is discussed later) with no tendency

to fall off in the later stages of fattening. A slight increase in average rate of growth was apparent when the meal allowance of the pigs on treatment 4 was increased from 2 to 2½ lb per pig per day.

(*b*) *Dressing percentage and carcass measurements*

All treatment differences for these variables were small and none was statistically significant.

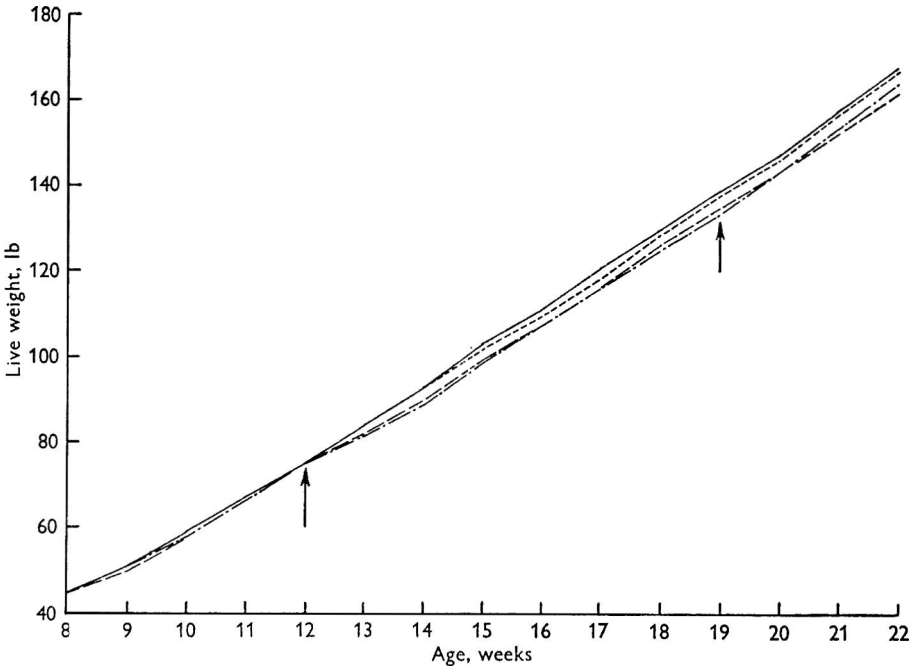

Fig. 1. Average growth curves of the four groups of pigs up to the time that the first pigs went to slaughter. — — — —, Treatment 1; - - - - -, treatment 2; ————, treatment 3; — · — · — ·, treatment 4. ↑, points where changes in daily meal allowance on treatments 1 and 4 were started (see text).

(*c*) *Commercial grading results*

As shown in Table 3, the grading results were satisfactory except for the measurement of back fat over the loin. There were no consistent differences in grading results between treatments with the exception that the mean percentage of carcasses grading A over the loin, and in consequence of final grade A carcasses, was somewhat higher for the pigs on treatment 4 than for those on the other three treatments. The commercial grading of fat thickness over the loin was made according to the following grouping: a loin fat thickness of 30 mm or below was grade A, of 31–35 mm grade B, of 36–39 mm grade C and of 40 and above grade F. Although there were no significant differences between treatments in the *mean* loin fat measurements given in Table 2, there was a possibility that the distribution of individual measurements was such that when grouped into the four grades given above, the proportion of pigs falling into each grade differed significantly from treatment to treatment. In order to test this hypothesis, contingency χ^2 tests were applied to the numbers of pigs falling into

each of the four grades for loin fat thickness, according to the individual loin fat measurements made, and also to the numbers in grades A and in B to F for each treatment. The results of both tests showed that the proportion of pigs falling into each grade did not differ significantly from treatment to treatment, and it may be concluded, therefore, that it is unlikely that any differences in the commercial grading results given in Table 3 were due to treatment effects.

(*d*) *Pattern of food consumption during the experiment*

The similarity of the weekly figures in Table 4 for meal plus 88 % dry-matter whey consumed by the four groups of pigs shows that the relationship observed between total meal consumption and total whey consumption (see Table 2) was evident throughout the experiment. It is apparent, however, that following changes in the level of meal fed, there was an interval of a few weeks before the pigs adjusted their voluntary intake of whey to compensate fully for the altered dry-matter intake from the meal portion of their ration. Thus, when the daily meal allowance of the pigs on treatments 1 and 4 was reduced from 3 to 2 lb at 13 weeks of age, their daily intake of total solids fell below that of the pigs on treatment 2 (3 lb meal/day all through) and remained about $\frac{1}{2}$ lb/day less for the following 2–3 weeks.

Similarly, when the meal allowance of the pigs on treatment 4 was increased from 2 to $2\frac{1}{2}$ lb/day at 20 weeks of age, their daily intake of total solids rose above that of the group of pigs on treatment 1 which were kept on the 2 lb meal/day level, and remained so for 2–3 weeks. Both of these relative changes in the intake of total solids were reflected as small changes in the mean rate of growth of the pigs concerned (see Fig. 1).

The peak mean daily consumption of whey per pig was reached by all treatment groups during about the 23rd–25th weeks of life, and for treatments 1, 2, 3 and 4 it was 5, $3\frac{1}{2}$, $4\frac{1}{4}$ and $4\frac{1}{2}$ gal respectively. These figures represent means of all pens on each treatment, and there was considerable variation in actual mean peak consumption between the different pens of pigs on the same treatment.

DISCUSSION

The results obtained in this experiment show that in growth rate, efficiency of food utilization and carcass quality, there was little to choose between the four levels of meal-feeding compared. Such differences in treatment means that occurred were all relatively small and statistical analyses indicated that none was likely to be the result of real treatment effects.

The results obtained in the earlier experiment carried out in 1954–55 (Braude *et al.* 1957) differed in that the pigs given 3 lb meal/day throughout grew significantly faster and were also distinctly fatter than pigs given 2 lb meal/day only from 13 weeks of age. In comparing the two experiments it is of interest to consider the striking differences in absolute performance of the animals involved.

In the earlier trial pigs given, for example, 3 lb meal/day reduced to 2 lb at 13 weeks of age in conjunction with an unrestricted supply of whey, grew at a mean rate of 1·11 lb/day with an efficiency of food utilization of 4·14 lb meal plus 88 % dry-matter whey/lb gain, whereas in the present experiment the corresponding figures for the

similarly fed pigs (treatment 1) were 1·23 and 3·57 respectively. Also the mean carcass length of the pigs in the earlier trials was only 774 mm, compared with 808 mm in the present trial, although mean back fat thickness was similar in the two groups. Mean figures for daily gain and efficiency of food utilization of pigs fed in the same way in other experiments carried out on the Cow and Gate farm in 1955 and 1956 were 1·10 and 4·28 (Braude, Mitchell, Cray, Franke & Sedgwick, 1958), 1·14 and 3·83 (Braude *et al.* 1959*a*) and 1·18 and 4·10 (Braude *et al.* 1959*b*), which again emphasize the markedly superior performance of the pigs in the experiment reported here. There can be little doubt that the stock improvement work and other improvements in housing and management continuously carried out in the Cow and Gate herd, as mentioned earlier, have been primarily responsible for these much improved results, and the general observation can be made that where such marked differences in the absolute performance of the pigs in different experiments occur, differences in treatment response are not to be unexpected. The results obtained in the present experiment also show conclusively that with the right stock and management it is possible to obtain a very reasonable performance from pigs on an unrestricted whey feeding system.

Table 5. *Total food costs during the growing period on the four experimental treatments using whey prices of* 1*d.*, 2*d.* *or* 2½*d./gal and meal prices of* £25 *or* £35*/ton, and the figures for total consumption of food given in Table* 2 (*shillings/pig*)

	Treatment no. and daily meal allowance/pig							
	1		2		3		4	
	3 lb reduced to 2 lb at 13 weeks of age		3 lb all through		2½ lb all through		3 lb reduced to 2 lb at 13 weeks of age and increased to 2½ lb at 20 weeks of age	
Prices of meal/ton ...	£25	£35	£25	£35	£25	£35	£25	£35
Prices of whey/gal								
1*d.*	95	120	103	135	97	124	95	121
2*d.*	129	154	126	158	126	153	125	151
2½*d.*	145	170	137	169	140	167	141	166

Although increasing the daily meal allowance from 2 to 2½ lb/pig at 20 weeks of age resulted in some increase in the total daily intake of dry matter, which was reflected in a small acceleration in the rate of growth of the pigs, one must conclude from the absence of any significant difference in the performance over the whole growing period of these pigs (treatment 4) and of those that continued on the 2 lb/day level to slaughter (treatment 1), that increasing the meal allowance in the later stages of the growing period to this extent, at least, is unlikely to result in any measurable effect on overall performance.

Since the only major difference observed between the four groups of pigs was in the proportions of meal and of whey consumed, it follows that the decision as to which of the four meal-feeding systems should be selected can be based largely on the question of differences in total food costs between the four systems. In order to illustrate the effect of variations in the relative prices of meal and of whey on total

food costs per pig on the different treatments, the figures in Table 5 have been calculated. The food consumption data in Table 2 were used in the calculations and in order to cover a fairly wide range, prices of 1*d*., 2*d*. and 2½*d*./gal for whey and of £25 and of £35/ton for meal were used, although it is realized that at a price as high as 2½*d*./gal *unrestricted* whey feeding is unlikely to be considered.

When the meal price is at the lower figure of £25/ton, it is seen that if the price of whey is also low (1*d*./gal or less) the system of feeding using the most meal and the least whey (treatment 2) would have the highest food costs, with little difference between the other three feeding systems. When the price of whey is increased to 2*d*./gal with meal remaining at £25/ton, the system of feeding with the highest food costs becomes that on which least meal and most whey is consumed (treatment 1) with little difference between the other three feeding systems. Increasing the price of whey further to 2½*d*./gal results in treatment 1 becoming even more unfavourable, and the point is reached when the system using most meal and least whey (treatment 2) becomes the one with the lowest food costs with treatments 3 and 4 intermediate. On the other hand, when the meal price is at the higher figure of £35/ton, total food costs on treatment 2 remain unfavourably high at each of the three whey prices shown in Table 5. As the whey price is increased from 1*d*. to 2½*d*./gal the trend is for the lowest food cost system to change from that using most whey (treatment 1) to the intermediate systems (treatments 3 and 4). From the practical aspect, it is noteworthy that the food costs on the intermediate meal-feeding systems (treatments 3 and 4) are at no point in the range of meal and whey prices included in Table 5 ever substantially higher than those on the actual least cost system. Hence in a situation where meal and whey prices are tending to be continually fluctuating, it would probably be advantageous in the long run to use one of the intermediate systems (treatments 3 or 4) all the time rather than to keep changing to the actual least cost feeding system in step with the changes in meal and whey prices.

Treatments 2 and 3 have the advantage over treatments 1 and 4 that they involve no changes in the daily meal allowance per pig once the levels of 3 and 2½ lb respectively are reached at 9–10 weeks of age. Moreover, although under the conditions of this experiment treatments 1 and 4 would have the lowest food costs when the whey price was low (1*d*. gal or less), the possibility should be borne in mind that under less favourable environmental conditions, the set-back observed in growth rate following the reduction in the daily meal allowance from 3 to 2 lb/pig, could be more prolonged and lead to more serious adverse effects on the pigs than occurred in this experiment. If this were so, one of the two treatments involving no change in the meal allowance might, in practice, prove the most profitable even when the whey price was relatively low and the meal price high.

To sum up, therefore, it would appear that when all the factors discussed above are taken into consideration, the feeding system that could be recommended in most of the environmental and economic situations likely to be encountered in practice is that used in treatment 3 (2½ lb meal per pig per day throughout the growing period).

Comparison of the pattern of whey intake in this experiment, which was carried out during the summer, with that in other trials on whey feeding on the Cow and Gate farm, some carried out in the summer and some in the winter, adds further support to the observation that on the Cow and Gate farm the daily intake of whey

at a given age tends to be higher during the winter months of the year than during the summer (Braude *et al.* 1959*b*). At first sight this would seem to be the opposite of the expected effect of seasonal temperature changes on whey intake. However, as there is evidence indicating that the acidity of the whey used tends to be lower in the winter than in the summer, it is possible that this is the major factor which determines the level of whey voluntarily consumed by the pigs.

The authors thank Dr R. Braude and Mr A. S. Cray for their advice in this work, and the assistance of Mr A. Franke, Farm Manager at East Farm, is gratefully acknowledged. Thanks are also due to Mr P. Prideaux of Messrs C. and G. Prideaux, Ltd., Bacon Curers, Motcombe, Shaftesbury, Dorset, for his valuable contribution in taking all the carcass measurements of the experimental pigs. The help of Patricia Cooke with the statistical analysis of the carcass data is also gratefully acknowledged.

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Printed in Great Britain

Studies on the properties of New Zealand butterfat

VI. Comparison of the properties and vitamin A potencies of butterfats produced by clover-fed and ryegrass-fed dairy cows

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(*Received* 24 *August* 1962)

Summary. In an indoor feeding trial with three sets of monozygotic twin cows during late spring the effects of H_1 short rotation ryegrass and white clover as feeds for the cows on the characteristics and the carotene and vitamin A contents of the butterfat were compared. The butterfat from the cows fed ryegrass had a lower iodine value and refractive index, higher Reichert and saponification values, and lower carotene and vitamin A contents than the butterfat from the cows fed clover. The carotene and vitamin A contents of the blood of the cows fed ryegrass were also lower than those of the cows fed clover.

In a subsequent outdoor feeding trial with four sets of monozygotic twins the feeding of pure H_1 short rotation ryegrass or of perennial ryegrass was compared with the feeding of mixed pastures containing a high but varying proportion of clover. The results for the spring and early summer months were similar in direction but smaller in magnitude than those from the indoor feeding trial. In the result for the autumn months there was a reversal of the relationship for the characteristics and the carotene and vitamin A contents of the butterfat and for the carotenoid contents of the blood.

It is suggested that the lack of consistency between the spring and autumn results could have been due to differences in the stage of growth of the ryegrass fed and that butterfat characteristics may be influenced more by the stage of maturity than by the botanical composition of the pasture.

The regular pattern of seasonal change in the characteristics of butterfat in all districts in the North Island of New Zealand reported by Cox & McDowall (1948) and of the carotene and vitamin A contents reported by McDowell & McDowall (1953) were shown, in the previous paper in this series (McDowall, 1962), not to be associated with stage of lactation. These properties are known to be markedly influenced by the type of feed consumed by the cows, and it was concluded that the seasonal fluctuations must be related to alterations in the character of the pastures which form the main source of nutrients for New Zealand dairy cows. The same conclusion had also been reached by McGillivray (1961) in relation to the seasonal changes in the vitamin A and carotenoid contents of the butterfat and blood of pasture-fed cows. The pasture alterations could be due to changes in the botanical composition with progress of the

season, or to changes in the chemical composition of one or more of the pasture species present, or to a combination of these two factors.

The most notable fluctuations reported by Cox & McDowall (1948) and McDowell & McDowall (1953) were the sharp fall in iodine value and in carotene and vitamin A contents during the late spring–early summer period. This coincides with the upsurge of clover as a dominant constituent of the pasture. Sears, Goodall & Newbold (1942, 1948), for example, found that with clover–ryegrass associations in New Zealand pastures the percentage of clover in the sward increased from 10 to 15 in late winter to 50 or more in summer. McGillivray (1952) has suggested that the lower vitamin A potency of New Zealand butterfat in the summer months may be associated with the high proportion of clover in the summer pastures, and Worker & McGillivray (1957) reported that diets high in clover tended to depress the total vitamin A potency, the iodine value, the oleic acid content and the tocopherol content of the fat.

In trial I of the present investigation butterfats from cows fed indoors on clover were compared with butterfats from cows similarly fed on ryegrass. In trial II butterfats from cows grazed on clover-dominant pastures were compared with butterfats from cows grazed on pure stands of ryegrass. In both trials estimations were made also of the carotenoid and vitamin A contents of blood samples from the cows in the two groups.

EXPERIMENTAL

Trial I. Indoor feeding

Three pairs of monozygotic twins were used. The cows had all calved between 15 July and 18 August 1956. The intervals between calving dates of the twin mates were 37, 33 and 6 days, average 25 days. To overcome the difficulties referred to by McGillivray (1957*a*) in indoor feeding of cows accustomed only to outdoor grazing, the cows were fed cut leafage of mixed pasture in a concrete yard for some days before commencement of the trial. From 15 October to 15 November they were kept indoors and were fed cut plant material at 11 a.m., 5.30 p.m. and 6.30 a.m., as shown in Table 1.

Table 1. *Feeding schedule for trial I*

Period	Date	No. of days	Plant material fed
Pre-experimental	15–23 Oct.	8	Cut mixed pasture to all cows
Experimental	23 Oct.–12 Nov.	20	Cut clover to one member of each twin pair, cut ryegrass to twin mate
Post-experimental indoors	12–15 Nov.	3	Cut mixed pasture to all cows
Post-experimental outdoors	15–26 Nov.	11	Outdoor-grazed mixed pastures for all cows

Plant material used. Pure stands of New Zealand certified white clover (*Trifolium repens*, high HCN) and short rotation ryegrass (*Lolium hybrid* H_1) were grown until

tall enough to be suitable for cutting. At commencement of cutting the clover was 6–8 inches high, and at completion of the trial it was just starting to flower. The ryegrass was 12–16 inches high and was tending to maturity. The plant material was cut once daily, at 10 a.m., i.e. 1 h before the first feeding of the cows on the freshly cut leafage. The quantity fed to each cow was weighed, and the residue remaining after 1 h was removed and weighed.

The cut material was subjected to botanical analysis daily. Samples were taken also for estimation of dry-matter content.

Sampling and analysis. One-day composite samples of milk were taken from each cow twice weekly during the pre-experimental period, then daily for the first 8 days of the experimental period, and thereafter at 2-day intervals. Samples of blood were taken from all cows at weekly intervals.

Analysis of milks, preparation of butterfats from the 1-day composite milk samples, and analysis for butterfat characteristics were carried out as described by McDowall & Patchell (1958). Carotenoids and vitamin A were estimated in butterfats and blood plasmas by the method of Thompson, Ganguly & Kon (1949). Pasture samples were taken at bi-weekly intervals for estimation of carotene and tocopherol by the method of Worker (1957, 1958).

Trial II. Outdoor grazing

Four pairs of monozygotic twins were used. The cows had all calved between 28 August and 21 September 1957. The intervals between calving dates of the twin mates were 0, 24, 17 and 15 days, average 14 days.

Plant material used. The clover was mainly white clover of the same species as used in trial I (*Trifolium repens*, high HCN). The ryegrass was either H_1 short rotation as used in trial I or perennial ryegrass (*Lolium perenne*). Fields of the two ryegrasses were prepared and maintained in pure stands by use of sulphuric acid sprays and nitrogenous fertilizers to suppress the clover growth. Because of the lack of a suitable treatment which would suppress the ryegrass growth and not affect the clover, it was not found possible to maintain a pure clover stand. The clover swards thus contained some ryegrass. With each transfer of the clover-fed cows to a fresh 'clover' pasture an estimate was made of the proportion of clover present (Fig. 3).

All the cows were grazed together on a mixed pasture for a uniformity control period of 2 weeks, before segregation of the two groups for separate feeding on clover and ryegrass. One cow of each pair was grazed on clover-dominant pasture while the twin mates were alternated between H_1 short rotation and perennial ryegrass swards, at times to suit the availability of the grass in the desired condition (Fig. 3). The clover was grazed at a height of 6–8 inches, and the ryegrasses at heights of 12–18 inches. Twice daily, after the morning and evening milkings, the cows were moved and confined by an electric fence to a fresh area of clover or ryegrass. This area was varied, according to the nature of the sward, to ensure that the pasture was grazed uniformly short and the possibility of selective grazing thus reduced to a minimum. The trial extended from mid-September until early in March. From time of commencement until the first week in December the rainfall was adequate to maintain plant growth. Thereafter some spray-irrigation was necessary at intervals to supplement the rain-

fall. Throughout the trial it was possible to feed clover at a relatively uniform state of maturity. The ryegrasses available from commencement until early December were, however, considerably more mature than those available during the second part of the trial.

Sampling and analysis. Two-day composite samples of milk were collected from each cow twice weekly for analysis for fat, total solids and protein contents, and for preparation of a butterfat sample for examination as in trial I. Samples of blood were taken at weekly intervals for analysis as described in trial I. In addition, tocopherol was estimated in blood plasmas using the method of Worker (1958). Bi-weekly pasture samples were examined for carotene and tocopherol as described in trial I.

RESULTS

Trial I

Intake of plant material by cows

After the first few days of initiation into feeding on cut foliage the two groups of cows reached during the pre-experimental period a uniform average intake of mixed pasture, varying from 130 to 150 lb of green material daily. During the experimental period of 20 days the average daily intakes of clover and ryegrass were 162 and 127 lb respectively. The average dry-matter content of the clover was 12·6% and of the ryegrass 19·6%. The average daily intakes of dry matter were therefore 20·4 lb for the clover-fed group and 23·9 lb for the ryegrass-fed group. During the post-experimental control period of indoor feeding on mixed pastures the daily consumption of green plant material was uniform within the range 170–200 lb for both groups of cows.

Milk and butterfat analytical results

Yield and composition of milk. The average daily milk yields from the two groups of cows were not appreciably altered by the transfer from outdoor grazing to indoor feeding (Fig. 1, graph A). The average daily yield of the clover-fed group fell during the experimental period whereas that of the ryegrass-fed group was fully maintained. There was no change in the composition of the milk attributable to the change from outdoor to indoor feeding and there were no appreciable differences in the fat, solids-not-fat and protein contents of the milks during the experimental period. The milk yield and milk-composition results thus support the observations that the health of the cows was not affected by the indoor feeding. The trial therefore can be accepted as giving a valid comparison between butterfats from clover and ryegrass feeding.

Iodine value and refractive index (*Fig.* 1, *graphs B and C*). Before the beginning of experimental feeding, these values were showing the usual seasonal fall as found by Cox & McDowall (1948). For the butterfat from the cows fed on ryegrass this fall continued for some days, whereas for the butterfat from the cows fed clover there was a tendency for these values to rise. The results indicate, therefore, that the seasonal fall in iodine value of butterfat delivered to commercial dairy factories in October–November is not due to the increased clover growth which occurs at this time and that some other factor is involved.

Saponification value and Reichert value (*Fig.* 1, *graphs D and E*). The changes in these values were, as usual, complementary to the change in iodine value and refractive index, i.e. with change to feeding on clover the normal seasonal rise in these values was interrupted.

Softening point (*Fig.* 1, *graph F*). During the latter part of the experimental period there was a divergence in softening points, the values for the butterfats from the

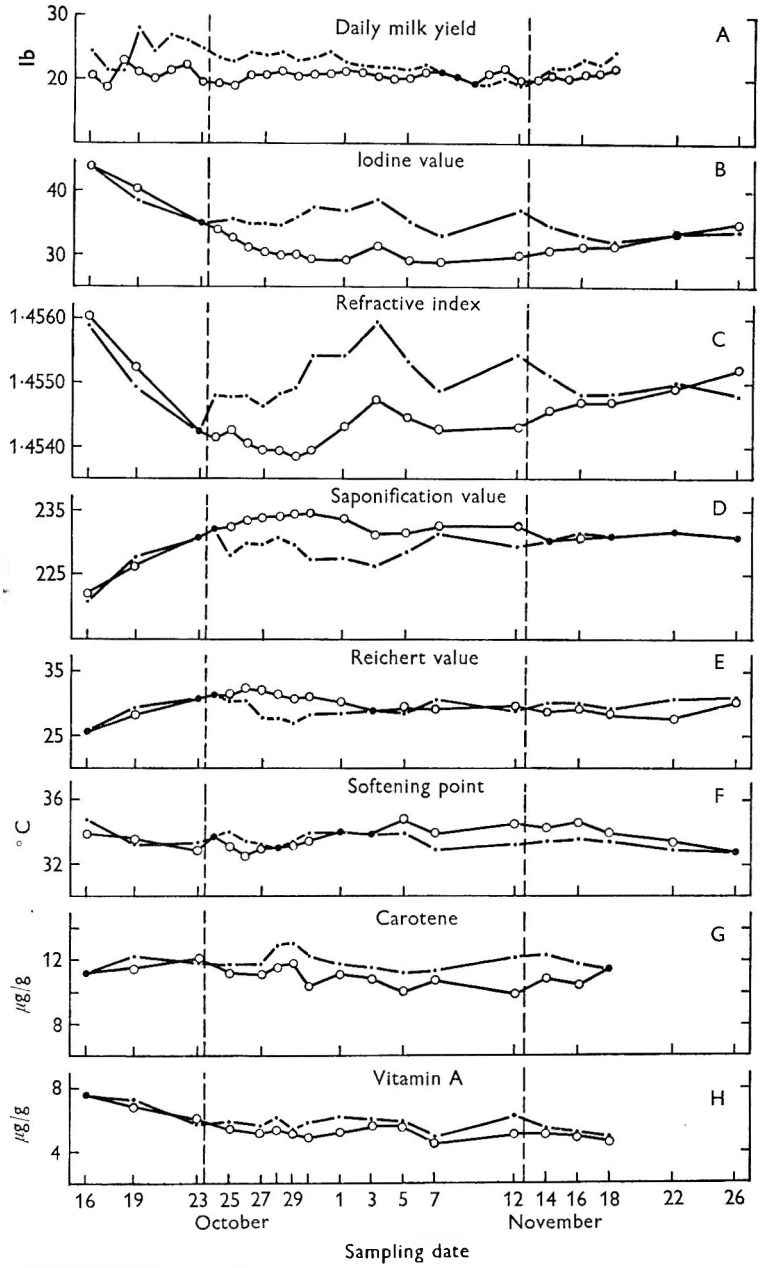

Fig. 1. Average milk yields, butterfat characteristics, and carotene and vitamin A contents of butterfat from cows fed either white clover (·—·) or short rotation ryegrass (○—○) (trial I).

ryegrass-fed cows being slightly higher than for the clover-fed cows. The change did not occur until some time after the divergence in iodine values of the butterfats.

Carotene and vitamin A (*Fig.* 1, *graphs G and H*). In the pre-experimental period the carotene and vitamin A contents of the butterfats were not significantly different. After commencement of the experimental feeding of clover and ryegrass there was a divergence between these values for the butterfats from the two groups. Both carotene and vitamin A contents were lower in the butterfats from the cows receiving ryegrass. There was a lag of several days in the appearance of the full difference in carotene content after the change to experimental feeding, and a similar lag in the disappearance after the return of the two groups to feeding on the same mixed

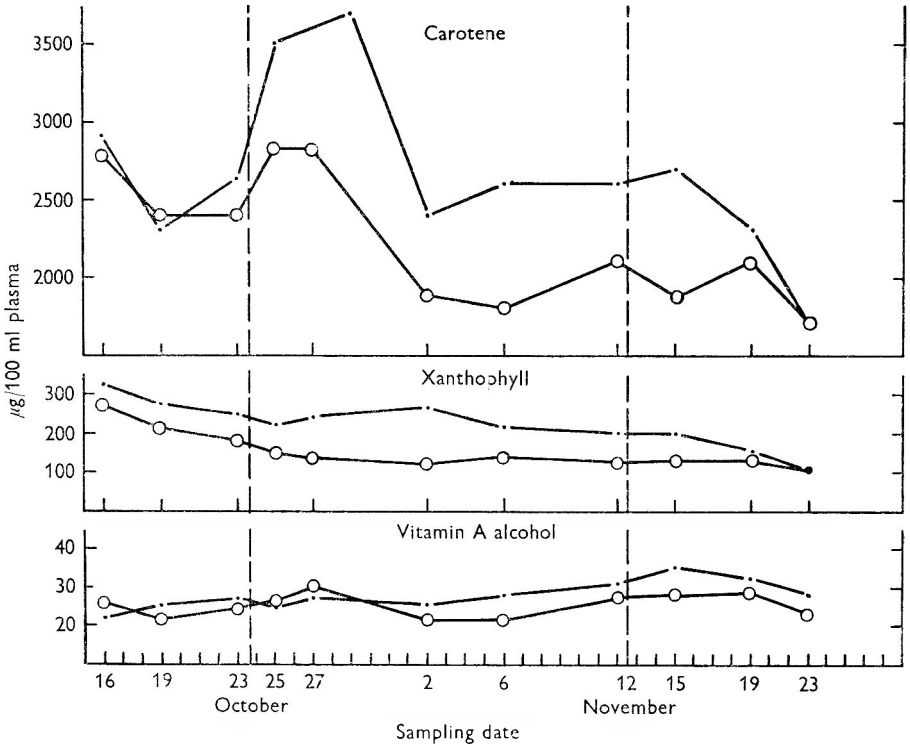

Fig. 2. Average carotenoid and vitamin A alcohol contents of the blood of cows fed either white clover (·—·) or short rotation ryegrass (○—○) (trial I).

pasture. The changes in xanthophyll content of the butterfat (not shown in Fig. 1) were similar in character to the changes in carotene content. The results indicate that the seasonal fall in carotene and vitamin A contents of butterfat in late spring, like the changes in iodine value referred to above, is not due to the increase in proportion of clover in the pasture at that time.

Carotenoid and vitamin A in the blood (*Fig.* 2)

During the experimental period the carotenoid content of the blood was lower for the cows fed on ryegrass. The effect on the vitamin A alcohol was in similar direction but of smaller magnitude. There was a delay in its appearance and it persisted

throughout the post-experimental period. The values found in butterfat were thus reflexions of the changing carotenoid and vitamin A contents of the blood.

Carotene and tocopherol in the pastures

The carotene and tocopherol content of the clover, ryegrass and mixed pastures remained relatively uniform throughout the trial. Average values, together with standard deviations, on a dry-matter basis, were: for carotene, clover 360 ± 40 μg/g, ryegrass 350 ± 70 μg/g, mixed pastures 375 ± 25 μg/g; and for tocopherol, clover 225 ± 60 μg/g, ryegrass 260 ± 60 μg/g, mixed pastures 225 ± 25 μg/g. The average daily carotene intake was thus approximately 3·3 g for the clover-fed cows and 3·8 g for the ryegrass-fed cows.

Trial II

Since the cows were grazed on uncut pastures, no estimate could be made of feed intake; but the relatively uniform body weights, milk yields and fat, solids-not-fat and protein contents of the milk, and the general observations of the condition of the animals during the trial, indicated that their health and general well-being was unaffected by the experimental treatment.

Butterfat analytical results

Iodine value and refractive index (*Fig.* 3, *graphs A and B*). During the period from beginning of October to December these values were not markedly affected by feeding of pasture containing up to 70% of clover. On two occasions during this period, however, namely from 14 to 20 October and from 4 to 21 November, they were distinctly lower in the fat produced by the cows fed on ryegrass. After the beginning of December, on the other hand, there was a rise in iodine value and refractive index of the butterfat from the cows fed on ryegrass and during the whole of January and February these butterfats had higher iodine value and higher refractive index than those from the cows fed on the clover-dominant pastures although the butterfat from these later cows showed the customary autumn rise in iodine value (Cox & McDowall, 1948).

Saponification and Reichert values (*Fig.* 3, *graphs C and D*). The initial divergence in iodine value and refractive index of the butterfats, referred to above, was not accompanied by any clearly defined change in Reichert value and saponification value. During the autumn months there was the customary fall in these values for both groups, but both saponification value and Reichert value were considerably lower in the butterfat from the cows feeding on pure ryegrass than in that from the cows feeding on clover-dominant pasture.

Softening point (*Fig.* 3, *graph E*). Throughout the trial there were no consistent differences in the average softening point values for the two butterfats. The absence of a consistent direct relationship between iodine value and softening point of New Zealand butterfat has already been referred to by Cox & McDowall (1948).

Carotenoids and vitamin A (*Fig.* 3, *graphs F and G*). During the spring the butterfat of the ryegrass-fed cows had a lower carotene content and a slightly lower vitamin A content than the fat from the clover-fed cows, but the effect was reversed in the autumn period.

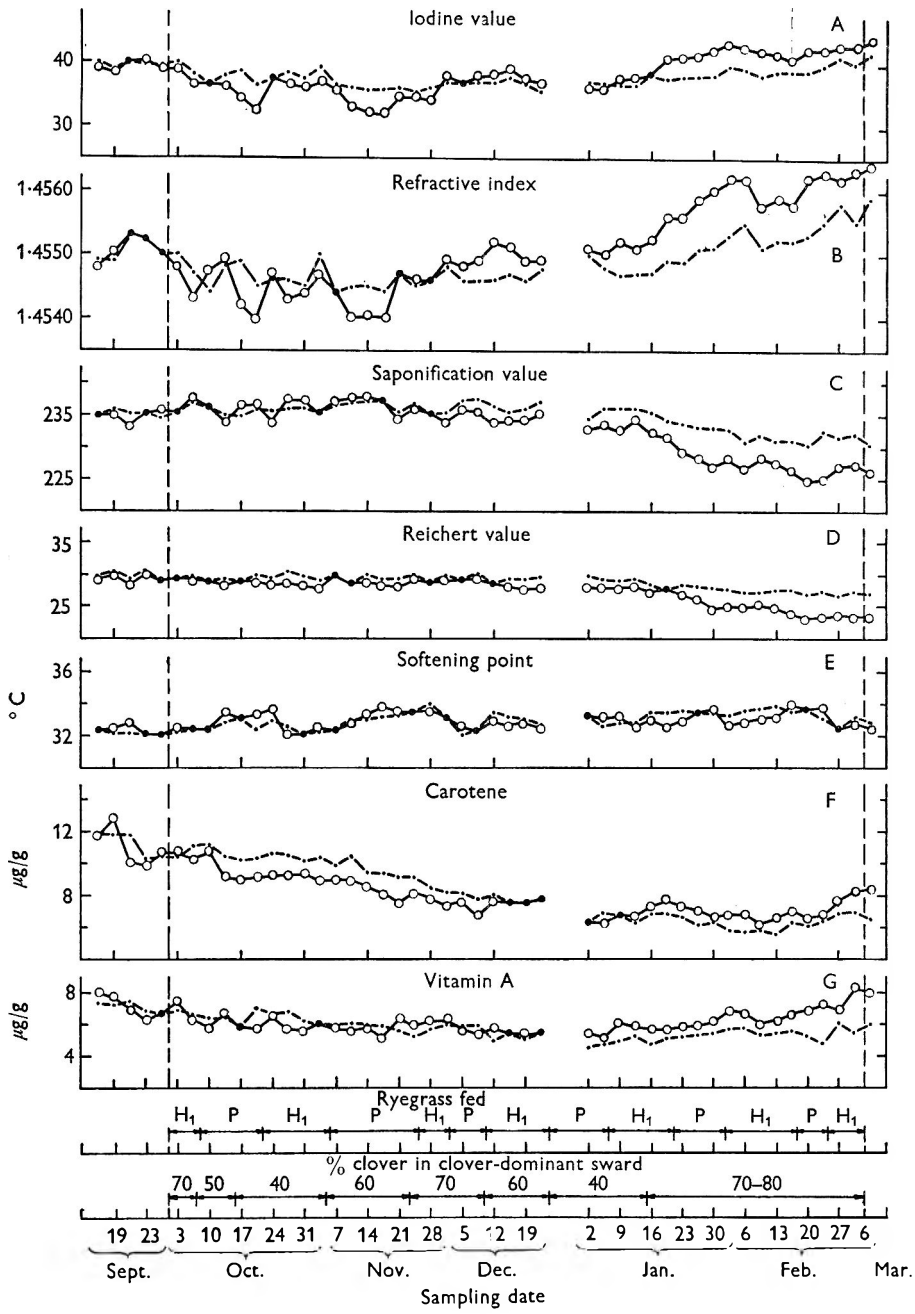

Fig. 3. Average characteristics and carotene and vitamin A contents of butterfats from cows fed either perennial (P) and short rotation (H_1) ryegrass (○—○) or white clover-dominant pasture (·—·) (trial II).

Carotenoids and vitamin A in the blood (Fig. 4)

During the spring the average carotene content of the blood from cows feeding on pure ryegrass was lower than that of the blood from the cows feeding on high-clover pasture, but, like the carotene values for the butterfat, the relationship was reversed during the autumn period. The average xanthophyll content of the blood showed a similar cross-over between the spring and the autumn feeding. There was no significant difference in the average vitamin A alcohol or tocopherol contents of the bloods from the two groups of cows during the spring period. Vitamin A alcohol was slightly higher in the blood of the ryegrass-fed cows during the autumn. Results for tocopherol are not available for the autumn period.

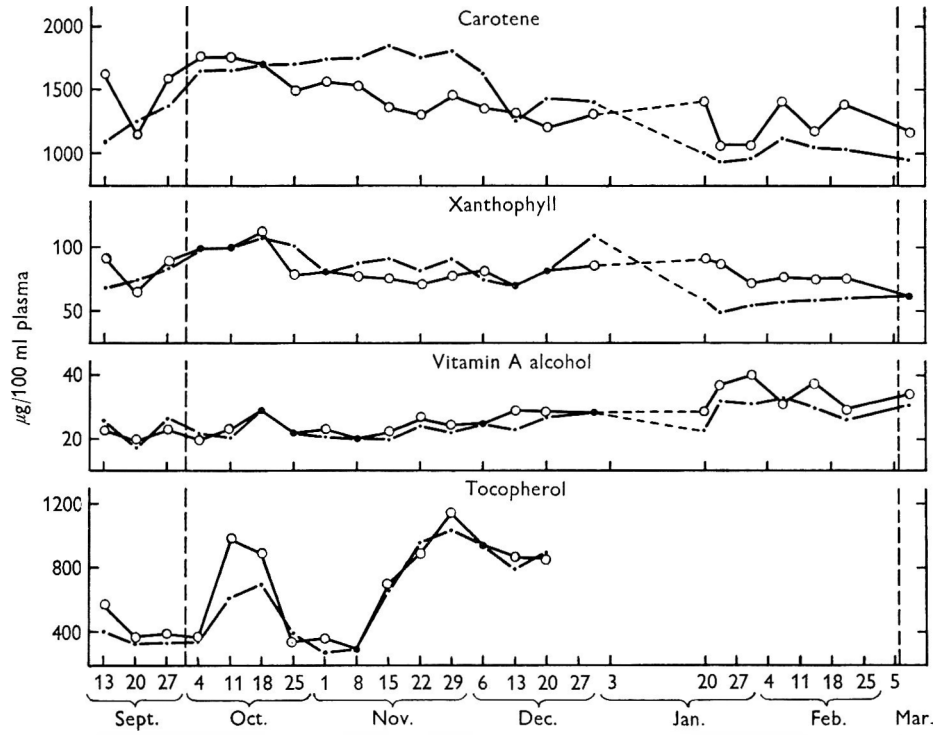

Fig. 4. Average carotenoid, vitamin A alcohol and tocopherol contents of the blood of cows fed either perennial and short rotation ryegrass (○—○) or white clover-dominant pasture (·—·) (trial II).

Carotene and tocopherol in the pastures

The carotene and tocopherol content of the pasture fluctuated more widely than in trial I. Average contents on a dry-matter basis were: for carotene, clover 440 ± 90 μg/g, ryegrass 470 ± 110 μg/g; and for tocopherol, clover 270 ± 80 μg/g, ryegrass 300 ± 70 μg/g. The fluctuations, however, were of an erratic nature and, as previously reported for carotene (McGillivray, 1956), there was no evidence of a seasonal trend in the carotene and tocopherol content of the pastures.

DISCUSSION

The effect of feeding white clover and ryegrass during the spring months on butterfat characteristics and on the vitamin A and carotenoid content of the butterfat and blood was clearly much more pronounced in trial I than in trial II. This is not unexpected, however, since in trial I the cows were fed indoors on cut leafage from virtually pure stands of clover and H_1 short rotation ryegrass. In trial II the clover sward contained some ryegrass and since the cows grazed uncut pastures there was opportunity, despite the precautions taken, for some selective grazing of this ryegrass in preference to the clover. The alternation of the other group of cows between H_1 short rotation ryegrass and perennial ryegrass may also have affected the results obtained in trial II, although no clearly defined differences associated with the feeding of the two types of ryegrass are apparent in the data presented in Figs. 3 and 4.

The cross-over in the relationships between spring–early summer and autumn results in trial II for almost all the analyses, both of the butterfat and the blood, is of considerable interest. The relationships between the iodine value and the carotene and vitamin A contents of the butterfats from the clover- and the ryegrass-fed cows during the autumn confirm the findings of Worker & McGillivray (1957) that the feeding of white clover during the autumn resulted in a marked lowering of iodine value and of carotene and vitamin A contents of the butterfat as compared with ryegrass feeding.

The results presented here, and those obtained by Worker & McGillivray (1957), thus clearly establish that the characteristics of the butterfat and the carotene and vitamin A contents of the butterfat and blood may be influenced by botanical composition of the pasture fed, but they indicate also that the relative effects of clover and ryegrass are dependent on the time of the year at which the comparison is made. The changing botanical composition of typical New Zealand pastures (Sears *et al.* 1942, 1948) would not therefore, in itself, explain the seasonal changes in the characteristics of butterfat (Cox & McDowall, 1948) and in the carotene and vitamin A contents of butterfat (McDowell & McDowall, 1953) and blood (McGillivray, 1957*b*).

The emergence of clover in the early summer as the dominant pasture species in many parts of New Zealand coincides with decreasing iodine values and carotene and vitamin A contents of commercial butterfat, but the results presented here show that increasing proportion of clover in the pasture prior to mid-summer produces an opposite effect on the butterfat. On the basis of this trial and the earlier investigation of Worker & McGillivray (1957) it appears, however, that the effect of clover is reversed late in the season and a high proportion of clover in dairy pastures during the summer and early autumn could perhaps contribute to the relatively lower iodine values and carotene and vitamin A contents of New Zealand butterfats at that time.

A notable feature of trial II was that while the clover was grazed at a fairly uniform stage of maturity throughout the trial, the ryegrass available during the late spring–early summer period was considerably more mature than that used during the latter part of the trial. This suggests that stage of maturity of the pasture might affect the characteristics and vitamin content of the butterfat more than botanical composition of the sward. The experimental basis for the subsequent findings of McDowall, McGillivray & Hawke (1961), that stage of maturity of ryegrass, which constitutes a

large proportion of New Zealand dairy pastures, is an important factor, will be presented in two subsequent papers of this series.

The authors are indebted to the late Prof. W. Riddet for his stimulating interest in this work, to Messrs M. R. Patchell, J. P. Hobbs and J. Fraser for their supervision of the feeding and milking of the cows, to Mr D. C. Collis for the sampling of the milks and creams, and to Mr C. J. G. Baker and Miss Fay Frecklington for assistance with the analytical work.

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Printed in Great Britain

Studies on the properties of New Zealand butterfat

VII. Effect of the stage of maturity of ryegrass fed to cows on the characteristics of butterfat and its carotene and vitamin A contents

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(*Received* 24 *August* 1962)

Summary. In two outdoor feeding trials with monozygotic twin cows during the spring and early summer months the effects of mature and immature H_1 short rotation ryegrass on the composition of the butterfat produced and on the carotene and vitamin A contents of the butterfat and the blood were compared. In both trials the characteristics investigated were markedly affected by the stage of maturity of the grass. The butterfat from cows receiving immature ryegrass was more unsaturated and had higher carotene and vitamin A contents than that from cows receiving mature ryegrass. The blood of cows fed immature ryegrass also contained more carotene than the blood of cows fed mature ryegrass.

It is suggested that variation in the stage of maturity of New Zealand dairy pastures throughout the year is an important factor contributing to the seasonal variations in the characteristics and the carotene and vitamin A contents of commercial butterfat. The influence of stage of maturity is probably associated with the quantity and nature of the pasture lipids.

In a previous publication in this series (McDowall & McGillivray, 1963) it has been shown that the seasonal changes in the characteristics of New Zealand butterfat reported by Cox & McDowall (1948) and the changes in carotene and vitamin A contents reported in the butterfat by McDowell & McDowall (1953) and in the blood of dairy cows by McGillivray (1957) are not primarily attributable to changes in the proportion of clover present in the clover–ryegrass associations typical of many New Zealand dairy pastures. The indications were that the stage of growth of the ryegrass was a more important factor controlling the properties of the butterfat. The present report covers an investigation of the effect of state of maturity of ryegrass fed to cows on the characteristics of the butterfat and the carotene and vitamin A contents of the butterfat and the blood. A preliminary report of these findings has already been published (McDowall, McGillivray & Hawke, 1961).

EXPERIMENTAL

Preparation and feeding of the pastures

The pastures were on sandy loam overlying gravel. Both pastures were adequately top-dressed with nitrogenous fertilizers to promote good growth. Natural rainfall was supplemented with irrigation as required.

The mature pasture was obtained by allowing pure stands of H_1 short rotation ryegrass (*Lolium* hybrid H_1) to grow until they had become long and somewhat stalky. The immature pasture was obtained by grazing other stands of the ryegrass closely in the late winter. When grazed the stands of mature ryegrass were 12–16 inches high and were fibrous, with appreciable stalk at times, especially in trial II. The immature ryegrass stands were all in a young, succulent condition, 3–4 inches high.

With both pastures cows were moved to a fresh area twice daily, after the morning and evening milkings. The cows were confined to these areas by means of electric fences. The areas were adjusted to ensure that the cows were adequately fed but that they had little opportunity for selective grazing.

Cows used

In trial I four pairs of monozygotic twins were used and in trial II five pairs.

In trial I all cows calved between 14 July and 12 August and in trial II between 11 and 29 July. The intervals between calving dates of the twin mates were 1, 2, 3 and 18 days, average 6 days, for trial I, and 1, 8, 10, 11 and 11 days, average 8 days, for trial II. In trial I the cows were grazed together on a mixed pasture for 3 weeks before commencement of the experimental feeding, and in trial II for 2 weeks. During the post-experimental periods the cows were again grazed together on mixed pasture, predominantly ryegrass and clover.

Sampling and analysis

(*a*) *Pastures.* In trial II samples of the foliage were taken at intervals for estimation of carotene content, by the method of Worker (1957), and for estimation and examination of the lipids present (Hawke, 1963).

(*b*) *Blood.* Samples of blood were taken from the cows for estimation of carotenoid and vitamin A alcohol contents. The method used was that described by Thompson, Ganguly & Kon (1949).

(*c*) *Butterfat.* Composite milk and butterfat samples were taken over a 2-day period for each sampling date. In trial I samples were taken twice weekly, and in trial II they were taken once weekly except for 3 weeks after transfer of the cows from the mixed pasture to the ryegrass grazing and for 1 week after completion of the ryegrass grazing, when they were taken twice weekly.

Analysis of the milks for fat, solids-not-fat and protein contents, preparation of butterfats and analysis for butterfat characteristics and vitamin content were as previously described (McDowall & Patchell, 1958; Thompson *et al.* 1949).

RESULTS

Throughout both trials average body weights, milk yields and fat, solids-not-fat and protein contents of the milk remained relatively uniform for the two groups of cows. This, and general observations on the condition of the cows, indicated that their health and general well-being were unaffected by the experimental treatments.

The results of the analysis of the butterfats and of the bloods of the twin mates showed the same general relationship for all pairs of twins in each trial. Average results for each group are shown in Fig. 1 (trial I) and Fig 2 (trial II).

Iodine value and refractive index (*Figs.* 1 *and* 2, *graphs A and B*). In both trials the butterfats from the cows receiving mature ryegrass had a lower iodine value and a lower refractive index than those from the cows receiving immature ryegrass. The average differences over the experimental period in trial I were 2·9 and 0·0004 and in trial II, 5·3 and 0·0008. The greater difference in trial II as compared with trial I may be attributed to the more advanced state of maturity of the 'mature' grass in this trial. On return of the twin pairs to mixed pasture the iodine value and refractive index differences were no longer evident. The fall in iodine value in the butterfats from the groups fed mature ryegrass during September–October was similar to, although it occurred somewhat earlier than, the fall recorded by Cox & McDowall (1948) for fat from commercial butters.

Saponification and Reichert values (*Figs.* 1, 2, *graphs C and D*). Butterfats from cows fed mature leafage had a higher saponification value, particularly in trial II, than that from cows fed immature ryegrass. The difference between the Reichert values for the butterfats from the two groups of cows was in the same direction but was very small. During the experimental period of trial II the average differences in saponification values and Reichert values were 3·2 and 0·5 respectively.

Softening point (*Figs.* 1, 2, *graph E*). The change of maturity of the grass did not cause any consistent difference in the softening points.

Carotene and vitamin A. Butterfat from cows on the mature ryegrass had lower carotene and vitamin A contents than butterfat from cows on immature ryegrass (Figs. 1, 2, graphs F and G). In trial I the effect of feeding was more evident in the carotene content than in the vitamin A content, whereas in trial II the differences were of the same order for carotene and vitamin A. In both trials there was a delay in the appearance of the difference after the segregation of the group to the different types of ryegrass growth, and a delay in disappearance of the difference after the twin mates were reunited for grazing on a common composite pasture. The delay was greater in trial II than in trial I, and was more evident for the carotene values than for the vitamin A values.

The average differences in carotene and vitamin A contents of the butterfat during the experimental periods of trial I were 1·5 and 0·7 μg/g, and of trial II 1·1 and 1·8 μg/g respectively. The stage of maturity of the ryegrass fed thus affected the average total vitamin A potency of the butterfats to the extent of about 5 i.u./g in trial I and 10 i.u./g in trial II.

In trial I the carotene and xanthophyll contents of the blood of cows fed on the immature ryegrass were higher than those of the blood of cows fed on mature ryegrass, and, as with the carotene and vitamin A contents of the butterfats, the differences

persisted for some time after the twin pairs had been reunited. In trial II there was a similar difference in the carotenoid content of the blood but the differences persisted for a shorter time after the twin mates were reunited on a common pasture.

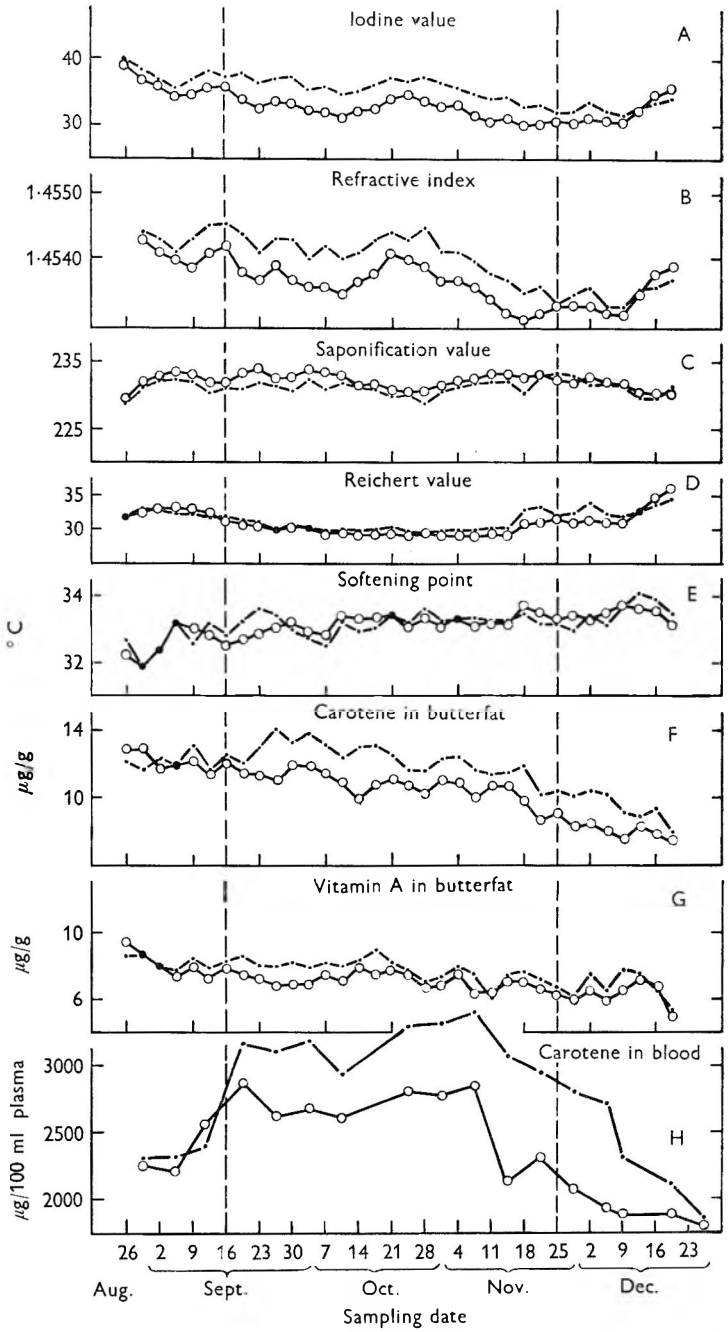

Fig. 1. Average characteristics and carotene and vitamin A contents of the butterfat and carotene content of the blood from cows fed mature (○—○) and immature (·—·) short rotation ryegrass (trial I).

Since in each trial carotene and xanthophyll contents of the blood were closely correlated, only carotene results are shown (Fig. 1, graph H; Fig. 2, graph J).

During the experimental periods of both trials the average difference in carotene contents of the blood between the two groups of cows was 450 μg/100 ml of plasma.

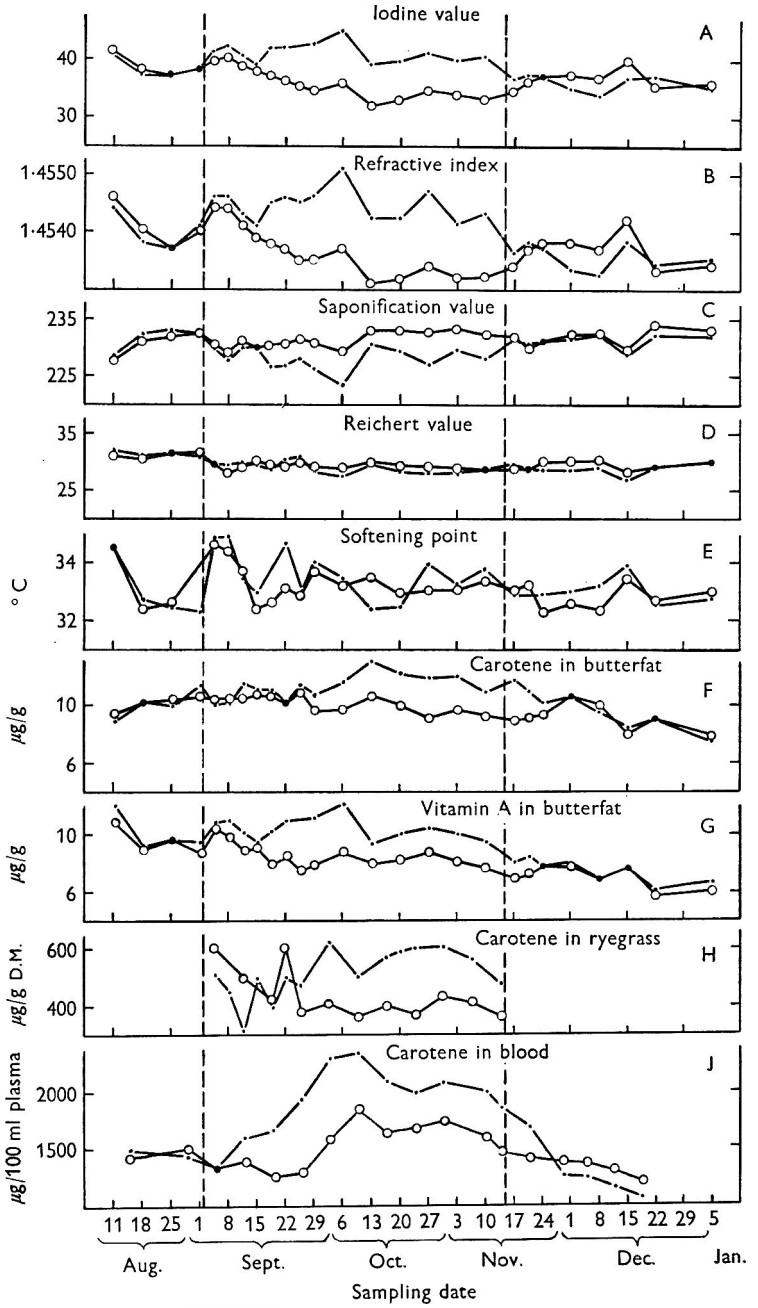

Fig. 2. Average carotene content of the pastures and average characteristics and carotene and vitamin A contents of the butterfat and carotene content of the blood from cows fed mature (○—○) and immature (·—·) short rotation ryegrass (trial II).

The experimental treatments had no appreciable effect on the vitamin A alcohol content of the blood. In both trials this averaged 24 μg/100 ml of plasma (range 21–32).

The carotene contents of ryegrass samples taken at intervals during trial II are shown in Fig. 2, graph H. The average carotene content of the mature ryegrass was 440 μg/g and of the immature ryegrass 580 μg/g.

DISCUSSION

The results of both trials indicate that the stage of maturity of the pasture fed to cows may markedly affect the characteristics of the butterfat and the carotene and vitamin A content of the butterfat and blood. The direction and magnitude of the effect suggests that the regular seasonal pattern of change noted by Cox & McDowall (1948), McDowell & McDowall (1953) and McGillivray (1957) is associated with the influence of climatic conditions and of the natural growth cycle of H_1 ryegrass on the state of the foliage in the pasture. This would apply particularly to the rapid fall in iodine value and in carotene and vitamin A contents of butterfat and in carotene content of blood in late spring and early summer when it is usual for the plants to become more mature. It would also apply to the rises in these values in the autumn and winter coincident with the appearance of new growth due to autumn rains. These findings are in line with the general experience of butter factory operators that in the autumn when there is rapid growth of grass following a warm rain there is a marked softening of the butterfat, and some alteration in the system of cream processing in the factory is necessary for the making of butter of satisfactory physical properties.

The average differences in characteristics of the butterfats and in carotene contents of the blood associated with the stage of maturity of the ryegrass in the trials reported here are somewhat less than the seasonal changes regularly observed. It is difficult to arrange for pastures typical of spring and summer herbage to be available at the same time, and in the present trials the average differences in maturity were not as great as would generally be encountered between normal summer (mature) and spring or autumn (immature) ryegrass. The butterfat from cows grazing typical dairy pastures may also be affected by the proportion of clover (McDowall & McGillivray, 1963) and possibly of other pasture species in a sward, as well as by environmental conditions.

As will be reported in a subsequent paper in this series (Hawke, 1963), there were marked differences in the nature and amount of lipids in the ryegrasses of different stages of maturity fed in the present trials. The lipid fraction from the immature ryegrass was more highly unsaturated than that from mature leafage, and the maturing of the ryegrass was also associated with a decrease in total lipid material. These changes in lipids with increasing maturity of the pastures are in agreement with the findings of Brouwer (1944). It is well recognized that the feeding of unsaturated lipids to cows affects the characteristics of the butterfat and particularly its oleic acid content. In the present investigation the divergences observed in iodine value and the compensatory changes in other butterfat characteristics may result directly from the varying nature and total intake of lipids by cows grazing mature or

immature ryegrass. Alternatively other differences in composition and the differences in physical character of the leafage of the ryegrass could affect the microbial metabolism of the rumen and hence the degree of hydrogenation of unsaturated dietary lipids or the extent of synthesis of butterfat precursors in the rumen.

The effect of stage of maturity of the ryegrass on the carotene and vitamin A contents of the butterfat and on the carotene content of the blood is of considerable interest. Variations in the carotene intake of cows are the most usual cause of variations in the vitamin A potency of milk. The differences in contents of carotene in the blood and butterfat found in this investigation thus could be merely reflexions of the differences in the carotene contents of the mature and immature ryegrass (Fig. 2, graph H). There is, however, strong evidence to suggest that other factors are involved. A number of workers have attempted to relate carotene intake to butterfat vitamin A potency and, although lower figures have been quoted, it is now generally agreed that to produce butterfat of maximum potency, the pasture must contain at least 260 μg/g in the dry matter (Hibbs, Krause & Monroe, 1949), giving a daily intake of the order of 2·5 g carotene (Wiseman, Shepherd & Cary, 1949). Below this level there is a fairly direct relationship between carotene intake and butterfat vitamin A potency, but above it additional carotene causes no increase in the quantities of carotene or vitamin A in the fat. In trial II the minimum carotene content recorded was 320 μg/g in the dry matter. The carotene intakes throughout the trial were therefore in the region where variations in intake would not be expected to influence carotene and vitamin A contents of the butterfat. This is borne out by the fact that during mid September there was a divergence in the vitamin A contents of the butterfat and the carotene contents of the blood although there was no significant differences in the carotene contents of the two types of ryegrass; and the steady increase during late September and early October in the carotene content of the blood of the group of cows fed mature ryegrass was not interrupted by the sudden fall in carotene content of the grass from 600 to 380 μg/g. It appears therefore that the divergences in carotene and vitamin A contents of the butterfat and carotene contents of the blood cannot be explained in terms of varying carotene intake from the pastures. This applies also to the seasonal variation in vitamin A potency of New Zealand butterfats, since there is no evidence of any comparable seasonal trend in the carotene content of typical dairy pastures (McGillivray, 1956).

The close correlation between the seasonal variations in iodine values and the variations in carotene contents of New Zealand butterfats (McDowell & McDowall, 1953) suggests that the same factor may influence both the characteristics of the fat and its carotene content. Investigating possible variations in pasture composition which might influence carotene absorption at high intake levels, McGillivray, Thompson & Worker (1958) found that both the quantity and the nature of the fat in the diet had a considerable effect. Within the limits of the digestive capacity of the animals, increased lipid intakes resulted in better utilization of dietary carotene. Carotene utilization also increased markedly with the degree of unsaturation of the vehicle.

Established relationships between carotene intake and vitamin A potency of the butterfat (Hibbs *et al.* 1949; Wiseman *et al.* 1949) may thus be influenced by the quantity and nature of the dietary lipids. Despite the high carotene intakes from

both types of pasture, better carotene utilization and hence higher carotene vitamin A contents of the butterfat could therefore be anticipated from the immature than from the mature ryegrass.

The above results establish that stage of maturity of H_1 short rotation ryegrass can have a marked effect on the characteristics and carotene and vitamin A content of butterfat and on the carotene content of the blood of cows. There is evidence that the effect, not only on fat characteristics but also on vitamin A potency, is associated with the quantity and nature of the lipid present in the ryegrass at different stages of maturity. It would seem likely that changes in the stage of maturity of the pastures fed to dairy cows throughout the year are to a large extent the cause of the seasonal changes in the characteristics and the vitamin A potency of New Zealand butterfat. The results, however, relate only to one type of ryegrass whereas under practical farming conditions pastures contain a variety of grasses, clovers and weeds. The effects of stage of growth of these pasture species on butterfat properties still remain to be established and further experiments in this connexion are now being undertaken. It would also be anticipated that the properties of commercial butterfats would be affected by the general practice on New Zealand dairy farms of using supplementary feeds such as choumoellier, turnips, green maize, hay and silage during periods of low pasture availablility.

The authors are indebted to Mr D. C. Collis for supervision of the feeding and milking of the cows and for the sampling of the milk and creams; and to Mr C. J. G. Baker and Miss Fay Frecklington for assistance with the analytical work.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 67

Studies on the properties of New Zealand butterfat

VIII. The fatty acid composition of the milk fat of cows grazing on ryegrass at two stages of maturity and the composition of the ryegrass lipids

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(*Received* 5 *September* 1962)

Summary. The lipid content of short rotation ryegrass at two stages of growth was followed at weekly intervals for several months in two spring–early summer seasons. Short succulent ryegrass consisting entirely of leaf tissue contained more lipid (mean, 8·1 % of the dry weight) than mature ryegrass which contained appreciable stalk (mean, 5·1 % of the dry weight). The lipid from the new growth contained fatty acids with appreciably higher proportions of linolenic acid, which was balanced mainly by lower proportions of linoleic and palmitic acids.

When monozygotic twin milking cows were grazed on the short rotation ryegrass grown to two stages of maturity, it was found that the fatty acid composition of the milk fat from the two groups was different. The milk fat of cows grazed on the new growth contained higher proportions of oleic acid and other C_{18} acids, whilst the proportions of myristic and palmitic acids were lower. The total proportions of the short-chain fatty acids were not greatly different, although in the group on new growth butyric acid was present in higher proportions and hexanoic and octanoic acids in lower proportions. The higher unsaturation of the fatty acids in the milk fat of this group of cows may be related to the higher levels of unsaturated fatty acids in the young ryegrass diet and to the extent to which these unsaturated fatty acids are hydrogenated in the rumen.

When Hansen & Shorland (1952) investigated the seasonal changes which occur in the composition of the fatty acids of New Zealand butterfat they found that the major differences throughout a season were: (i) a regular increase in the content of C_6–C_{14} saturated acids up to November, and thereafter a slow decline to the end of the season; and (ii) a variation of the C_{18} unsaturated acids in the inverse direction to that of the C_6–C_{14} constituents. Similarly, McDowell (1953) found that seasonal changes in iodine value were closely related to changes in the oleic acid content of butterfat. Thus these workers were able to explain in chemical terms the regular seasonal pattern of the changes in iodine, saponification, and Reichert values, observed earlier by Cox & McDowall (1948).

In the other papers of the present series (McDowall, 1962; McDowall & McGillivray, 1963*a*, *b*), some of the factors which possibly cause these regularly recurring seasonal fluctuations have been investigated, using paired feeding of monozygotic twins

(McDowall & Patchell, 1958). It was found that regular differences in the physical and chemical constants of the butterfat, particularly iodine values, were obtained when cows were grazing on short rotation ryegrass at different stages of growth (McDowall & McGillivray, 1963*b*). The present report which formed part of an earlier preliminary communication (McDowall, McGillivray & Hawke, 1961), is of a parallel investigation of this dietary effect on the composition of the fatty acids of butterfat.

There have been many studies of the effect of dietary lipids on the composition of the milk fat of ruminants (Hilditch, 1956). The much smaller effect of unsaturated dietary lipids on the lipids of ruminants than on the lipids of non-ruminants is due to the hydrogenation of these lipids in the rumen (Shorland, Weenink & Johns, 1955; Reiser & Reddy, 1956). When linseed and other oils which contain high proportions of fatty acids such as linoleic and linolenic acids are fed, the main result is an increase in the oleic acid content of the butterfat, balanced by a reduction in the contents of palmitic, myristic and stearic acids (Hilditch, 1937). Further, Mattsson (1949) found that during the feeding of linseed fodder which contained no conjugated acids, the conjugated diene in the milk fat increased from 0·5 to 6·7 %, while little or no increase occurred in the amounts of linoleic and linolenic acid. Despite the highly unsaturated nature of grass lipids (Shorland, 1944), it was reasoned that the lipid in the diet under normal grazing conditions would not lead to variations in fatty acid composition of the butterfat of the bovine (Hansen & Shorland, 1952). However, Brouwer (1944) had earlier proposed that the oleic acid content of butterfat depended on the degree of unsaturation of the fatty acids in grass.

Studies of ruminant digestion have revealed many hitherto unknown interrelationships involving changes in microbial metabolism within the rumen. For instance, recent observations by Shaw, Ensor, Tellechea & Lee (1960) indicate that rations producing high proportions of propionate in the rumen led to milk and body fat with high iodine values, and it was suggested that under these conditions the hydrogenation of the small proportions of unsaturated fatty acids in the diets was less efficient. The grass lipids represent only a small proportion of the dry weight of grass but their variable fate during digestion could influence the nature of such products of metabolism as milk fat. The present investigation provides information on the lipids of the ryegrass which was grazed at two stages of growth (McDowall & McGillivray, 1963*b*) and on the fatty acid composition of the butterfats obtained from these paired feeding experiments.

EXPERIMENTAL

Maintenance of ryegrass pastures and of cows

The full grazing conditions for the monozygotic twin pairs were described in the preceding paper in this series (McDowall & McGillivray, 1963*b*). Pure pastures of short rotation ryegrass (New Zealand Certified no. 1 hybrid) were grazed at two stages of maturity—one stage ('new growth') consisted of short (3–4 in) succulent leaves grown over a period of 2–3 weeks and the other stage ('mature growth') consisted of long (approximately 12 in) fibrous grass with appreciable stalk. One cow of each twin pair in the experiment was grazed on each pasture in such a way that a new area was available for grazing each day.

Extraction of lipid from ryegrass

At weekly intervals about 5 kg of fresh grass was collected from the areas to be grazed that day. The grass was chopped into short lengths, mixed and then 100 g was immediately macerated in ethanol with a Waring Blendor. The resulting slurry was warmed to about 55 °C to complete deactivation of lipolytic enzymes and then filtered through a sintered funnel. The fibrous residue was washed on the funnel several times with small volumes of ethanol and diethyl ether and transferred to Soxhlet thimbles. Soxhlet extraction was carried out for 8 h with diethyl ether. Solvents were distilled from an all-glass apparatus before use. Diethyl ether was first refluxed over ferrous sulphate.

The ethanolic and ether extracts were evaporated to dryness separately, and each residue extracted with ether. These ether extracts were combined and shaken with distilled water to remove water soluble impurities which are mainly carbohydrate in nature. To avoid the formation of stable emulsions, shaking was not vigorous in the first two extractions, but in the third and final extraction vigorous shaking was used and the emulsions which formed were broken by centrifugation. The washed ether solution was evaporated to dryness and the residue extracted with anhydrous ether. The ether soluble material thus obtained was freed of ether and weighed, to give a measure of the lipid content of the grass. All solvents were evaporated at moderate temperatures in a rotary evaporator. The final traces of solvent were removed *in vacuo*.

The dry weight of the grass was determined on separate samples (50 g) by heating in an air-oven at 110 °C for 18 h.

Milk fat samples

Composite milk fat samples were prepared by combining equal quantities of the 2-day fat samples obtained from five pairs of monozygotic twin cows grazing on each type of ryegrass pasture as described by McDowall & McGillivray (1963*b*). For each milk fat sample analysed for fatty acids a grass lipid sample which corresponded in sampling date was chosen for similar analysis.

Preparation of samples for gas-liquid chromatographic analysis

The grass and milk lipids were saponified by refluxing with ethanolic KOH for 3 h, and the non-saponified material removed by ether extraction. The fatty acids obtained from milk lipids were steam-distilled in a micro-Kjeldahl apparatus to separate the fatty acids into two fractions (Hawke, 1957). The steam-volatile acids were titrated with alkali and a slight excess added before concentration of the soaps to a small volume on a rotary evaporator. The non-volatile portion consisting of C_{10} acids and above (Hawke, 1957), and the total fatty acids obtained from grass were then converted to methyl esters by refluxing with 1 % H_2SO_4 in methanol (Hilditch, 1956). The fatty acids of grass were weighed before conversion to methyl esters (Table 1).

Separations by gas-liquid chromatography

Volatile fatty acids. The procedure was the same as previously described (Hawke, 1957) except that the sample for analysis was acidified immediately prior to chromatography. A concentrated solution of sodium salts was taken up in a small hypo-

dermic syringe and one or two drops added to a mixture of equal parts by weight of anhydrous $KHSO_4$ and Celite 545, in a small platinum boat held at the gas inlet end of the chromatographic column. After acidification in this way, the boat was quickly pushed into the heated region of the column by means of an attached wire. The nitrogen supply was then connected.

Methyl esters. The methyl esters were analysed by gas-liquid chromatography using a β-ionization detector and argon as carrier gas (Pye Instruments Ltd., Cambridge, England). To identify the methyl esters, R_F values were determined on two types of column (Hawke, Hansen & Shorland, 1959). The columns (4 ft long and 4 mm internal diameter) consisted of (*a*) 20 % Apiezon M on Celite 545 (48–85 mesh) held at 200 °C, and (*b*) 20 % succinic acid diethylene glycol on Celite 545 (48–85 mesh) held at 150 °C.

The proportions of each component were determined by measurement of peak areas.

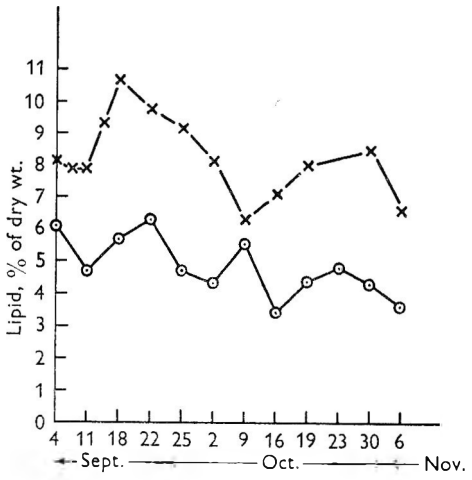

Fig. 1

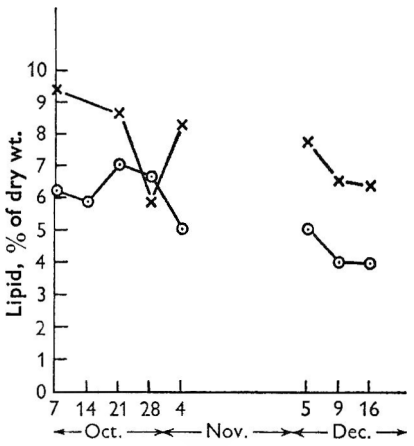

Fig. 2

Fig. 1. The lipid content of short rotation ryegrass (New Zealand Certified no. 1 hybrid) in the spring and early summer of 1959. ×, New growth; ⊙, mature growth.

Fig. 2. The lipid content of short rotation ryegrass (New Zealand Certified no. 1 hybrid) in the spring and early summer of 1960. ×, New growth; ⊙, mature growth.

RESULTS

Lipid content and fatty acid composition of ryegrass

Although the total lipid content of the grass varied considerably from week to week (Figs. 1, 2) two clearly defined trends were apparent. First, the lipid content of the new grass was greater than that of the mature grass and secondly, the lipid content of both types of growth showed a general decrease from September to December. Unseasonal drought conditions in November 1960 forced an interruption in the experiments (Fig. 2) and an exception to the above-mentioned differences in lipid levels in the two types of grass occurred when difficulties were being experienced in maintaining pasture differences prior to the break in the experiment. In the samples selected for analysis of fatty acids it was found that approximately one-half

of the total lipid consisted of fatty acids (Table 1) and in each case a higher yield of fatty acids was obtained from the lipid extracted from new growth. The analysis of fatty acids in the 1959–60 series of experiments (Tables 1 and 2) are on combined extracts obtained from grass sampled in two successive weeks.

Chromatography of the methyl esters prepared from two types of ryegrass on polyester columns gave good separations of the unsaturated components and enabled the proportions of each of the major components to be measured. The proportions calculated from these and similar recording traces (Table 2) show the highly unsaturated nature of the fatty acids of ryegrass and in particular the high proportions of linolenic acid (58·6–79·1 moles %) and linoleic acid (6·8–12·8 moles %). Higher proportions of linolenic acid were present in new growth and, because of the higher lipid content at this stage of growth (Figs. 1, 2), the total amount of this unsaturated fatty acid in the grass was therefore appreciably higher. The other main differences were the lower proportions of palmitic and linoleic acids in the new growth. Brouwer (1944) also found that two trends during the maturation of grass were decreased fatty acid content and increased proportions of saturated acids.

Table 1. *Yields of fatty acids from total lipid extracts of ryegrass*

Date of collection of grass	Total lipid, % of dry wt.		Fatty acids, % by wt. of total lipids	
	New growth	Mature growth	New growth	Mature growth
25. ix. 59	9·15	4·70	52·8*	49·1*
2. x. 59	8·07	4·44		
16. x. 59	7·05	3·41	55·1*	40·3*
19. x. 59	8·00	4·42		
7. x. 60	9·45	6·26	53·8	51·4
4. xi. 60	8·34	5·04	78·7	58·8

* Fatty acids determined on combined extracts.

The finding that the samples collected on 4 November 1960 show only slight differences in composition is again probably due to the difficulties in maintaining the ryegrass at different stages of maturity in this period of the experiment.

Fatty acid composition of milk fat

The fatty acid composition of the milk fat from the groups of cows grazing on the two ryegrass pastures is given in Tables 3 and 4. Small proportions of C_{20} saturated and C_{14} and C_{20} unsaturated acids were also found but are not included in the tables. The amounts of C_{10} acids were not determined in either the volatile or the non-volatile fraction of milk fat but these acids are of minor importance. Excluding the samples taken on 5 November 1960, the major differences in composition were in the proportions of myristic, palmitic and oleic acids and these changes would account for the differences in the iodine value of the milk fat from the two groups (McDowall & McGillivray, 1963*b*). In total contribution, the C_{18} acids, both saturated and unsaturated, formed a higher proportion of the non-volatile acids in the milk fat of the cows grazing on the grass with the higher lipid content, although the proportions of oleic acid were the most affected. The same cows gave milk fat with lower proportions

Table 2. *Composition of the fatty acids of short rotation ryegrass, moles % of methyl esters*

	25 Sept.–2 Oct. 1959			16–19 Oct. 1959			7 Oct. 1960			4 Nov. 1960		
Acid	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.
Saturated												
C_{12}	tr	0·5	+0·5	0·24	0·47	+0·23	0·41	0·58	+0·17	0·41	0·62	+0·21
C_{14}	0·5	1·1	+0·6	0·48	0·94	+0·46	0·81	1·04	+0·23	0·61	0·93	+0·32
C_{16}	11·2	15·2	+4·0	9·95	13·61	+3·66	12·40	21·96	+9·56	13·68	13·93	+0·25
C_{18}	0·5	1·1	+0·6	0·73	0·94	+0·21	0·83	1·04	+0·21	1·43	2·17	+0·74
Unsaturated												
$C_{16}^{1=}$	0·9	1·6	+0·7	1·22	1·17	−0·05	1·65	2·28	+0·63	2·04	0·93	−1·11
$C_{17}^{1=}$	0·9	0·5	−0·4	—	—	—	—	—	—	—	—	—
$C_{18}^{1=}$	1·4	3·1	+1·7	1·46	3·29	+1·83	1·24	1·66	+0·42	2·24	2·17	−0·07
$C_{18}^{2=}$	9·4	13·6	+4·2	6·80	11·74	+4·94	9·09	12·83	+3·74	8·37	9·28	−0·91
$C_{18}^{3=}$	75·2	63·3	−11·9	79·12	67·84	−11·28	73·55	58·61	−14·94	71·22	69·99	−1·23

* Difference taking new growth as 0.

Table 3. *Composition (moles %) of the volatile fatty acids (VFA) of milk fat of cows grazing on ryegrass at two stages of growth*

	25 Sept.–2 Oct. 1959			16–19 Oct. 1959			7 Oct. 1960			4 Nov. 1960		
Acid	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.
C_4	41·18	25·43	−15·75	47·62	30·00	−17·62	34·29	21·05	−13·24	54·67	36·11	−8·56
C_6	26·46	32·20	+5·74	28·53	35·00	+6·47	43·80	57·90	+14·10	31·06	41·67	+10·61
C_8	32·36	42·37	+10·01	23·80	35·00	+11·20	21·91	21·05	−0·86	14·29	22·22	+7·93
m-equiv. VFA/g fat												
	1·05	0·98	—	1·05	1·48	—	1·10	0·91	—	1·31	1·10	—

* Difference taking new growth as 0.

Table 4. *Composition of the non-volatile fatty acids of milk fat of cows grazing on ryegrass at two stages of growth, moles % of methyl esters*

	25 Sept.–2 Oct. 1959			16–19 Oct. 1959			8 Oct. 1960			5 Nov. 1960		
Acid	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.	New growth	Mature growth	*Diff.
Saturated												
C_{12}	0·4	tr	−0·4	0·6	1·3	+0·7	2·5	2·3	−0·2	1·0	1·6	+0·6
C_{13}	—	—	—	—	—	—	—	—	—	—	—	—
C_{14}	5·7	9·3	+3·6	8·6	12·7	+6·1	11·1	13·1	+2·0	11·6	11·0	−0·6
C_{15br}	0·9	1·1	+0·2	1·0	2·0	+1·0	0·6	1·1	+0·5	1·2	2·4	+1·2
C_{15}	0·9	1·1	+0·2	0·8	1·3	+0·5	1·2	1·4	+0·2	1·2	1·6	+0·4
C_{16}	25·5	29·4	+3·9	25·7	31·9	+6·2	33·3	35·5	+2·2	36·0	37·3	+1·3
C_{17br}	0·9	0·8	−0·1	0·4	0·3	−0·1	0·8	0·9	+0·3	0·5	0·9	+0·4
C_{17}	tr	0·8	+0·8	0·2	0·3	+0·1	0·6	0·9	+0·3	0·5	0·9	+0·4
C_{18}	16·0	15·9	−0·1	17·5	13·7	−3·8	19·1	17·7	−1·4	21·1	15·8	−5·3
C_{20}	0·6	0·6	0·0	0·4	tr	−0·4	—	—	—	—	—	—
Unsaturated												
$C_{16}^{1=}$	3·2	2·5	−0·7	2·1	2·6	+0·5	1·2	1·6	+0·4	1·5	3·7	+2·2
$C_{18}^{1=}$	41·5	33·8	−7·7	38·0	25·4	−12·6	26·6	22·0	−4·6	20·5	24·2	+3·7
$C_{18}^{2=}$	3·8	3·3	−0·5	2·9	5·2	+2·3	2·1	2·9	+0·8	1·1	tr	+1·1
$C_{18}^{3=}$	0·6	0·6	0·0	1·9	3·3	+1·4	0·9	0·7	−0·2	2·9	tr	−2·9

* Difference taking new growth as 0.

of myristic and palmitic acids. Although the proportions of total short-chain fatty acids are not greatly different in the two groups (Table 3) the proportions of individual acids are strikingly dissimilar, there being appreciably more butyric acid and less hexanoic and octanoic acids in the milk fat from cows grazing on new growth.

Taking the two sets of analysis together this means that the proportions of saturated fatty acids of intermediate chain length (C_6–C_{16}) are greater in the milk fat obtained from the cows grazing on the mature pasture, with correspondingly lower levels of butyric and oleic and other C_{18} acids.

DISCUSSION

The finding that the group of twin cows grazing on grass with the higher lipid content secreted milk fat with higher proportions of unsaturated fatty acids, may be interpreted as a further instance of the effect of highly unsaturated dietary lipid on the composition of milk fat. If the amount of unsaturated lipid in the diet is increased, as in experiments where cows were given up to 500 g of linseed oil per day in addition to the normal grass diet (McDowall, Reid & Patchell, 1957), the increased unsaturation of the milk lipids which resulted may be reasoned to be due to decreased over-all hydrogenation in the rumen. This hydrogenation when studied *in vitro* has been found to be a stepwise process from linolenic to stearic through linoleic and oleic acid (Shorland *et al.* 1955).

Variations in the degree of hydrogenation are likely to be important in milk fat synthesis because of the resultant variations in the unsaturation of the lipid absorbed into the blood. On the basis of experiments with tritium-labelled stearic acid it has been suggested that dietary lipids might contribute up to about 27 % of the total fatty acids in milk (Glascock, Duncombe & Reinius, 1956), the balance being synthesized in the mammary gland from acetate and a C_4 molecule, most likely β-hydroxybutyrate (Popják, French, Hunter & Martin, 1951).

Although the amounts of lipid in the grass are different at different stages of growth and subsequent events may be explained on the basis of greater lipid intake, it is likely that this effect may be reinforced or counteracted by the effects of other compositional changes such as soluble sugar content, which are known to bring about changes in the microbial metabolism in the rumen. Shaw *et al.* (1960), for example, found that small amounts of readily digestible material added to a ration greatly increased the proportions of propionate in the rumen, increased the level of volatile fatty acids in the rumen and increased the unsaturation of the body fat. These workers also referred to unpublished work in which similar rations gave greatly increased iodine values in the milk fat. These observations suggested to Shaw *et al.* (1960) that changes in microbial metabolism affect not only the short-chain fatty acids but also the extent of hydrogenation of long-chain fatty acids.

Short rotation ryegrass used in the present experiments has been found by other workers (Johns, 1962) to yield higher proportions of propionate in the rumen of sheep than perennial ryegrass. Similar changes in the proportions of volatile fatty acids have been found to be related to increased digestibility and to the presence of small amounts of readily fermentable constituents (Balch & Rowland, 1957; Ensor, Shaw & Tellechea, 1959; Balch, 1960; Tilley, Deriaz & Terry, 1960). It is possible that

differences in digestibility of the short and mature growths compared in the present experiments may give similar differences in fermentation products. The work of Shaw *et al.* (1960) suggests that lower digestibility of mature growth would be likely to lead to lowered hydrogenation activity in the rumen when mature growth was grazed.

In the absence of intake measurements, it is not possible to calculate the intake of the various dietary components. However, the body weights, milk and milk-fat yields (McDowall & McGillivray, 1963*b*) of the two groups showed similar trends over the experimental periods, which suggest that the intakes of the two groups might not have been greatly different. An assessment of the intake of lipid can be made by assuming a daily consumption of 20 lb of grass on a dry-weight basis. In terms of total lipid, the group on new growth would have ingested about 720 g of lipid/day, whereas the group on mature growth would have ingested about 450 g of lipid/day. These figures are calculated on an 8 and 5 % lipid content of new and mature growth respectively. In terms of fatty acids, which are the important constituents in the present discussion, the intake is reduced to approximately half this value, i.e. 360 and 225 g/day respectively. The nature of grass and clover lipids has been re-investigated recently (Weenink, 1961) and it has been established that the acetone soluble lipids are mainly galactolipids which yield a lower proportion of fatty acids on saponification.

Consequently it is difficult to decide whether differences in dietary lipid levels are causing the changes in milk lipids or whether factors such as digestibility and fermentation and consequential changes in volatile fatty acids are of overriding importance. From our present knowledge of dietary effects on microbial hydrogenation activity within the rumen (Shorland *et al.* 1955; Shaw *et al.* 1960), these two factors would be complementary in their effect on the fatty acid composition of milk fat.

A further complication is that although the two groups of cows yielded milk fat with appreciable differences in unsaturation, the major proportional changes in fatty acid composition involve oleic, palmitic and myristic acids, i.e. acids of different chain length. In this connexion it is interesting to note that Garton, Lough & Vioque (1961) obtained some evidence for limited conversion of stearic acid to palmitic acid when rumen contents were incubated with linseed oil.

The author gratefully acknowledges the expert assistance of Miss J. T. Barnett in carrying out much of the experimental work, and is indebted to Dr F. H. McDowall, The Dairy Research Institute (N.Z.), for the invitation to participate in these investigations. The work was aided by a grant from the New Zealand D.S.I.R.

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Printed in Great Britain

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Studies on the κ-casein complex

I. The release of sialic acid-containing material by rennin

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(*Received* 3 *September* 1962)

Summary. When κ-casein was treated with rennin at neutral pH all the sialic acid contained in the protein was found in the glycopeptide released by the enzyme. From the known molecular weight of the glycopeptide and its proportion in κ-casein, a molecular weight of the order of 50000 was calculated for the protein.

Alais (1956) and Nitschmann, Wissmann & Henzi (1957) reported that the enzyme rennin released a large peptide when it acted on whole casein at pH 6·8. This peptide had a molecular weight in the region of 8000, contained sialic acid together with other carbohydrate material, and had the somewhat unusual characteristic of being soluble in 12% trichloroacetic acid (TCA).

The discovery of κ-casein by Waugh & von Hippel led to the finding that rennin acted specifically on this fraction of the casein complex, releasing soluble nitrogen (Waugh & von Hippel, 1956; Garnier, 1957; Wake, 1959). The same glycopeptide as that released from whole casein was found by Nitschmann & Beeby (1960) and Jollés & Alais (1960) in that portion of the soluble nitrogen from κ-casein that was not precipitated by 12% TCA. The amount of nitrogen soluble in 12% TCA found after rennin had acted on κ-casein was 8–10% of the κ-casein nitrogen (Wake, 1959; Tsugo & Yamauchi, 1960; Beeby & Nitschmann, 1963). However, molecular weights as low as 16000 have been reported for κ-casein (Waugh, 1958). If the glycopeptide accounted for all the nitrogen soluble in 12% TCA that was released, either the molecular weight of this peptide must be considerably less than 8000 or the molecular weight of κ-casein considerably greater than 16000. The latter appeared the more likely since a molecular weight of 8000–9000 is indicated for the glycopeptide from its amino acid composition (Nitschmann & Beeby, 1960; Jollés & Alais, 1960).

The present studies were undertaken to determine whether the sialic acid of the glycopeptide accounted for all of the sialic acid of κ-casein, and if it did, to use this fact to estimate the molecular size of κ-casein.

EXPERIMENTAL

Materials and methods

κ-Casein was prepared by the procedure described by Beeby & Nitschmann (1963). The preparation contained 14·6% nitrogen and 1·8% sialic acid, calculated as *N*-acetyl neuraminic acid.

The κ-casein solutions were treated with crystalline rennin at 25 °C and pH 7. Portions were removed at intervals and the protein precipitated by the addition of either a one-tenth volume of sodium acetate–acetic acid buffer (M with respect to

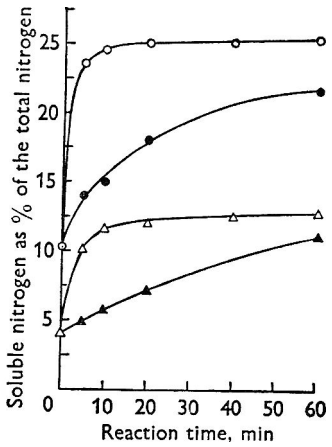

Fig. 1

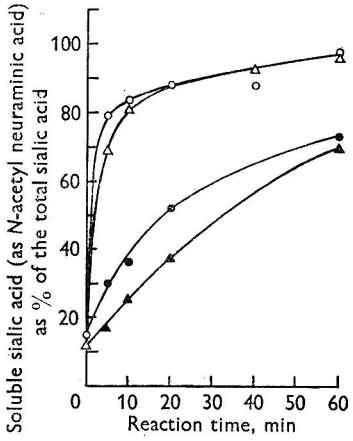

Fig. 2

Fig. 1. The release of soluble nitrogen in a 0·6 % κ-casein solution treated with rennin at 25 °C and pH 7. ○, Nitrogen soluble at pH 4·7, enzyme concentration 1·4 μg/ml; ●, nitrogen soluble at pH 4·7, enzyme concentration 0·14 μg/ml; △, nitrogen soluble in 12 % TCA, enzyme concentration 1·4 μg/ml; ▲, nitrogen soluble in 12 % TCA, enzyme concentration 0·14 μg/ml.

Fig. 2. The release of soluble sialic acid in a 0·6 % κ-casein solution treated with rennin at 25 °C and pH 7. ○, Sialic acid soluble at pH 4·7, enzyme concentration 1·4 μg/ml; ●, sialic acid soluble at pH 4·7, enzyme concentration 0·14 μg/ml; △, sialic acid soluble in 12 % TCA, enzyme concentration 1·4 μg/ml; ▲, sialic acid soluble in 12 % TCA, enzyme concentration 0·14 μg/ml.

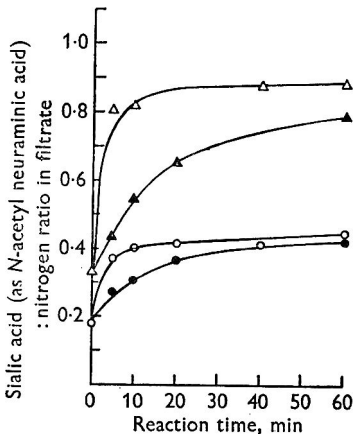

Fig. 3. The change with time of the sialic acid : nitrogen ratio of the soluble material released from κ-casein (0·6 %) by rennin at 25 °C and pH 7. ○, pH 4·7 filtrate, enzyme concentration 1·4 μg/ml; ●, pH 4·7 filtrate, enzyme concentration 0·14 μg/ml; △, 12 % TCA filtrate, enzyme concentration 1·4 μg/ml; ▲, 12 % TCA filtrate, enzyme concentration 0·14 μg/ml.

each), or an equal volume of 24 % (w/v) TCA. The mixtures were filtered and the nitrogen in the filtrates determined by a semi-micro Kjeldahl procedure.

The sialic acid content of each filtrate was determined by the method of Warren

(1959) after first neutralizing to pH 5–6 and dialysing overnight against a large excess of distilled water. The nitrogen content of each dialysed solution was determined and used to calculate the sialic acid content of the original filtrate. This procedure was made necessary by the interference of high concentrations of acetate buffer with the liberation of bound sialic acid and the loss of sialic acid when heated with appreciable quantities of TCA.

The release of soluble nitrogen and sialic acid from κ-casein by rennin

Fig. 1 shows the rate of release of soluble nitrogen in a 0·6 % κ-casein solution when it was treated with rennin (0·14 or 1·4 μg enzyme/ml). The corresponding release of sialic acid is given in Fig. 2, while Fig. 3 demonstrates the changes in the ratio between sialic acid and soluble nitrogen. With the higher concentration of enzyme the κ-casein solution became rapidly turbid and resembled skim-milk after 20–30 min. The turbidity of the solution treated with the lower concentration of rennin, on the other hand, was only slightly increased after 1 h.

After 60 min reaction with 1·4 μg/ml of rennin a sample of the material insoluble at pH 4·7 (para-κ-casein) was washed at pH 7 and the sialic acid determined in the insoluble portion. An equal volume of 24 % TCA was added to a portion of the fraction soluble at pH 4·7 and the precipitate collected by centrifugation. This sediment was washed with 12 % TCA, the pH adjusted to 7 and the sample dialysed to remove salts. The sialic acid in this material was determined after re-precipitating with TCA, washing and removing the TCA by dialysis. Both the para-κ-casein and the fraction soluble at pH 4·7 but insoluble in 12 % TCA contained only traces of sialic acid.

DISCUSSION

After rennin has acted on κ-casein all the sialic acid appears in the material that is soluble at pH 4·7. Moreover, the sialic acid is confined to that portion of the released material that is also soluble in 12 % TCA, i.e. the glycopeptide of Alais (1956) and Nitschmann *et al.* (1957) (Fig. 2). Since the glycopeptide contains 11·2 % nitrogen (Nitschmann & Beeby, 1960) and accounts for some 13 % of the nitrogen of κ-casein (Fig. 1), it represents approximately 17 % by weight of the κ-casein. As the molecular weight of the glycopeptide is in the vicinity of 8000 (Nitschmann *et al.* 1957; Jollés & Alais, 1960; Jollés, Alais & Jollés, 1961), κ-casein must have a molecular weight of the order of 50000.

The work of Beeby & Nitschmann (1963) indicated that κ-casein is a complex stabilized by secondary forces. If it is assumed that this complex consists of three units, each of similar molecular size, the value of 16000 for the minimum molecular weight of the protein (Waugh, 1958) can be explained in terms of the disruption of the complex under the disaggregating conditions employed during the determination of the molecular weight (low temperature, high pH). Further support for this postulate is found in the fact that para-κ-casein, which contains all the disulphide that Gillespie (pers. comm.) found in κ-casein, can be separated into two fractions, only one of which contains disulphide (Beeby, unpublished).

When rennin acts on κ-casein the rate at which sialic acid soluble at pH 4·7 appears is initially greater than the rate of appearance of sialic acid soluble in 12 % TCA

(Fig. 2). This parallels the release of soluble nitrogen (Fig. 1), and supports the contention of Beeby & Nitschmann (1963) that the glycopeptide is formed when a fraction that is first released from the κ-casein complex is subsequently split by the enzyme. However, the change with time in the ratio of sialic acid to nitrogen in the pH 4·7 filtrates suggests that the intact sialic acid-containing component carries with it part of the insoluble components of the κ-casein complex. When split to yield the glycopeptide it would not hold these components in solution and the sialic acid:nitrogen ratio would increase. This explains the low content of sialic acid in the soluble material obtained by precipitating κ-casein at pH 4·7 compared with that produced by treating κ-casein with urea (Beeby & Nitschmann, 1963). Since the sialic acid:nitrogen ratio of the TCA filtrate has a zero-time value similar to that reached by the pH 4·7 filtrate at the completion of the reaction (Fig. 3), it seems likely that the intact sialic acid-containing component is soluble to an appreciable extent in 12% TCA. As it is split by the enzyme the part that is lacking in sialic acid is precipitated by the TCA and the ratio of sialic acid to nitrogen in the filtrate increases accordingly.

If the formation of the glycopeptide involves the rupture of a single ester or peptide bond in the sialic acid-containing fraction, one would expect to find one such bond broken per molecular weight of 50000, a figure which is in agreement with the value of 55000 reported by Garnier, Mocquot & Brignon (1962) when rennin acts on κ-casein at pH 7.

Thus κ-casein, as it is isolated from whole casein, appears to be a complex of at least three components and to have a molecular weight in the region of 50000. The proportions in which the components are combined in the complex as it exists in milk, and whether the complex is present as such in the casein micelles, have yet to be determined.

The author wishes to thank Mr G. Loftus Hills and Mr R. D. Hill, both of the Division of Dairy Research, C.S.I.R.O., Melbourne, for helpful advice and criticism.

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Printed in Great Britain

The composition of Iraqi sheep's milk

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(*Received* 5 *September* 1962)

Summary. The bulked milk from a flock of Awassi sheep was analysed in two successive seasons. In the second season a wider range of analyses of samples taken from the time the first few ewes came into milk until almost the end of the production season (minimum and maximum numbers in milk 8 and 199) gave the following average values: specific gravity, 1·0366; titratable acidity, 0·217 %; pH, 6·65; fat, 6·88 %; protein, 6·18 %; lactose, 5·75 %; solids-not-fat, 12·99 %; ash, 0·928 %; CaO, 0·29 %; P_2O_5, 0·32 %; CaO in ash, 31·5 %; P_2O_5 in ash, 34·3 %.

The results are compared with the published compositional figures for sheep's and cow's milks.

There are about 10^7 sheep in Iraq with an estimated annual production (above that produced during the suckling period) of 10^8 kg of milk. Large quantities of soft and hard cheeses, butter and ghee are made, in addition to the large amounts of leben which are produced and consumed by shepherds and tribesmen. The Awassi is the most important breed and accounts for about two-thirds of the sheep population.

The present work was undertaken because few studies have been made of the composition of sheep's milk. Early work was reviewed by Godden & Puddy (1935) in their contribution on the composition of the milk of Cheviot ewes, and some other studies are mentioned by Davis & Macdonald (1953, pp. 101–2). The composition of the milk of Egyptian sheep has been studied by El-Sokkary, Sirry & Hassan (1949). Apart from the analyses reported by Godden & Puddy (1935) little information is available on the ash constituents.

EXPERIMENTAL

Samples

Samples of bulk milk were obtained in the 1960–61 and 1961–62 seasons from the Awassi flock of the Abu Ghraib Experimental Farm, Animal Husbandry Division of the Ministry of Agriculture. Lambing in this flock started in the first season on 1 November 1960 and continued for 3 months. Hand milking of the ewes started in the middle of February 1961 and ceased at the end of July. Samples of bulked milk were taken for analysis during the last 3 months of the milking season. In the second season lambing started at the end of October 1961, while milking for the purpose of this work started in December. The hand-milking season proper started on 1 February

1962 and lasted to the beginning of June. Thus sampling covered the whole of the herd's milk production period of $5\frac{1}{2}$ months.

Analytical methods

Specific gravity was measured by lactometer after cooling the milk samples to 15°C. Fat content was determined using the standard Gerber method, tests being repeated three times at least for each sample. Total solids content was determined by pipetting 1 ml of the milk into a clean, dry, weighed aluminium milk bottle cap. After quick weighing it was placed in an oven at 101°C for about $\frac{1}{2}$ h. Heating, cooling and weighing were repeated at intervals to bring the solids to a constant weight.

Twenty-five ml of the milk sample were pipetted into a porcelain basin and weighed accurately. This was then dried, ignited gently and transferred to a muffle furnace at 600°C to obtain total ash. No precautions were taken against the volatilization of alkaline chlorides. A white ash was always obtained. The ash was dissolved in 10 ml of HCl (A.R.) and distilled water, and diluted to a known volume. Part of this solution was used for the determination of phosphorus content using the colorimetric method of Rockstein & Herron (1951). An EEL colorimeter with the red filter FRI was used. The solution for the calibration graph was prepared from $KHPO_4$ (A.R.). The greater part of the ash solution was used for the determination of calcium content by the standard oxalate-permanganate method.

Additional tests were done during the second season. Titratable acidity was measured in the usual manner by titration of 10 ml of milk with N/9-NaOH solution, using phenolphthalein as indicator. Duplicate tests were made. Acidity tests and also pH determinations with a glass electrode were done immediately after receiving the samples. For the last half of the sampling period protein contents were determined. For the first five samples both the standard Kjeldahl method and a formaldehyde method were used. Quite close agreement was obtained between the results, the difference being $\pm 0{\cdot}05\%$. The formaldehyde method only was then used.

RESULTS AND DISCUSSION

Table 1 gives the results obtained for specific gravity, fat, total solids, solids-not-fat and ash for each sample in the first season, and Table 1*a* gives in addition, for the second season, the results for titratable acidity, pH and protein.

From Table 1, which represents the milk composition for the last 3 months of the milk-production season, the averages and ranges are as follows: sp.gr., 1·0338 (1·0280–1·0378); fat, 7·7% (5·3–10·0); total solids, 19·78% (18·19–24·36); solids-not-fat, 11·99% (10·09–14·36); ash, 0·886% (0·775–0·975).

From Table 1*a*, which represents the milk composition for the full lactation period, averages and ranges are as follows: sp.gr., 1·0366 (1·0305–1·0415); titratable acidity, 0·22 (0·10–0·29); pH, 6·65 (6·46–6·9); fat, 6·88% (5·7–11·8); protein, 6·184% (5·85–6·50); lactose (by difference), 5·75% (4·65–7·04); total solids, 19·870% (17·34–24·56); solids-not-fat, 12·990% (11·11–13·92); ash, 0·928% (0·864–1·018).

For the second season the averages, except for fat, were all higher than for the first incomplete season. The first season was terminated quite late, i.e. at the end of July, while the second season terminated at the normal date at the beginning of June. This and other reasons make the results of the second season the more valuable.

The figures for the second season agree well with those of El-Sokkary *et al.* (1949) for Egyptian sheep's milk and, with some exceptions, the figures quoted by Davis & Macdonald (1953). For example, the figures for ash quoted from Trillat & Forestier of 0·9–1·0 % compare closely with the figures obtained in the second season of the

Table 1. *Composition of sheep's milk—first season*

Serial no.	Date of sampling	No. of milking ewes	Sp.gr.	Fat, %	Total solids, %	Solids-not-fat %	Ash, %
1	30. iv. 61	240	1·0351	7·1	—	—	0·852
2	4. v. 61	196	1·0355	7·5	18·99	11·49	0·775
3	7. v. 61	232	1·0345	7·1	19·46	12·16	0·920
4	10. v. 61	245	1·0360	7·1	19·50	12·40	0·931
5	14. v. 61	230	1·0348	7·6	19·71	12·11	0·848
6	17. v. 61	185	1·0334	9·9	22·13	12·23	0·863
7	8. vi. 61	120	1·0341	7·4	19·23	11·83	0·976
8	11. vi. 61	125	1·0314	8·4	20·14	11·74	0·852
9	15. vi. 61	125	1·0325	8·4	19·90	11·50	0·852
10	19. vi. 61	68	1·0331	8·1	18·19	10·09	0·903
11	25. vi. 61	40	1·0328	7·5	19·03	11·53	0·971
12	2. vii. 61	46	1·0366	7·4	19·55	12·15	0·918
13	5. vii. 61	47	1·0378	6·8	19·46	12·66	0·922
14	10. vii. 61	43	1·0340	8·1	19·69	11·59	0·845
15	21. vii. 61	33	1·0310	5·3	18·42	13·12	—
16	24. vii. 61	33	1·0280	10·0	24·36	14·36	0·862

Table 1*a*. *Composition of sheep's milk—second season*

Serial no.	Date of sampling	No. of milking ewes	Titratable acidity	pH	Sp.gr.	Fat, %	Protein, %	Total solids, %	Solids-not-fat, %	Ash, %
1	20. xii. 61	8	0·22	—	1·0355	6·0	—	21·62	15·63	0·905
2	9. i. 62	10	0·25	—	1·0330	7·8	—	18·91	11·11	—
3	17. i. 62	10	0·25	—	1·0380	7·3	—	19·70	12·40	0·910
4	24. i. 62	10	0·26	—	1·0305	11·8	—	24·56	12·76	0·864
5	30. i. 62	10	0·28	—	1·0370	6·3	—	20·09	13·79	0·920
6	6. ii. 62	176	0·25	6·83	1·0415	6·8	—	20·72	13·92	0·885
7	13. ii. 62	157	0·28	6·50	1·0385	6·1	—	19·28	13·38	0·880
8	20. ii. 62	182	0·26	6·46	1·0395	5·7	—	18·57	12·87	0·867
9	27. ii. 62	174	0·29	—	1·0385	5·7	—	19·18	13·48	0·884
10	6. iii. 62	183	0·27	6·55	1·0385	6·4	—	18·63	12·23	—
11	13. iii. 62	199	0·28	6·55	1·0395	5·5	—	17·69	12·19	0·965
12	20. iii. 62	165	0·26	6·6	1·0380	4·6	5·85	17·34	12·74	0·995
13	3. iv. 62	123	0·20	6·6	1·0365	7·5	6·07	20·51	13·01	0·979
14	10. iv. 62	103	0·20	6·6	1·0360	7·4	6·15	19·44	12·04	0·921
15	17. iv. 62	50	0·17	6·7	1·0380	8·2	6·33	21·54	13·34	0·931
16	25. iv. 62	45	0·19	6·59	1·0372	6·1	6·37	19·32	13·22	0·961
17	3. v. 62	30	0·14	6·7	1·0356	7·7	6·50	20·95	13·25	0·975
18	8. v. 62	27	0·15	6·6	1·0357	7·2	6·48	21·06	13·86	0·917
19	15. v. 62	24	0·13	6·75	1·0355	7·0	6·04	18·65	11·65	0·965
20	29. v. 62	15	0·13	6·8	1·0335	7·0	6·22	19·01	12·01	0·990
21	5. vi. 62	14	0·10	6·9	1·0326	6·4	5·85	20·30	13·90	1·018

present work, although Jankowski (1953) gave a lower figure of 0·86 % in his study of the composition of Polish sheep's milk. Also protein content was, in general, higher in Iraqi sheep's milk than in the milk of most foreign breeds, but they compare closely with the findings of Besana quoted by Davis & Macdonald. It may be seen

from Tables 1 and 1*a* that the ash content increased in the second half of the milking period.

Tables 2 and 2*a* give the analyses of the ash for CaO and P_2O_5 for the two seasons.

Table 2. *Ash*, CaO *and* P_2O_5 *contents of bulk ewe's milk—first season*

Serial no.	Ash, %	CaO % in milk	CaO % in ash	P_2O_5 % in milk	P_2O_5 % in ash	Ratio CaO : P_2O_5 CaO = 1
1	0·852	0·275	32·3	—	—	—
2	0·775	0·313	40·4	0·327	42·2	1·04
3	0·920	0·316	34·3	0·337	36·6	1·06
4	0·931	0·326	35·0	0·342	36·8	1·05
5	0·848	0·298	35·1	0·304	35·8	1·02
6	0·863	0·320	37·0	0·332	38·4	1·04
7	0·976	0·290	29·7	0·325	33·6	1·12
8	0·852	0·289	33·9	0·317	37·2	1·09
9	0·852	0·295	34·6	0·318	37·3	1·08
10	0·903	0·286	31·6	0·309	34·2	1·08
11	0·971	0·285	29·3	0·310	32·0	1·09
12	0·918	0·297	32·2	0·317	34·6	1·07
13	0·922	0·294	31·8	0·324	35·2	1·10
14	0·845	0·276	32·7	0·308	36·4	1·11
16	0·862	0·271	31·4	0·309	35·8	1·13

Table 2*a*. *Ash*, CaO *and* P_2O_5 *contents of bulk ewe's milk—second season*

Serial no.	Ash, %	CaO % in milk	CaO % in ash	P_2O_5 % in milk	P_2O_5 % in ash	Ratio CaO : P_2O_5 CaO = 1
1	0·905	0·256	28·3	0·300	33·1	1·17
2	—	0·276	—	0·330	—	1·20
3	0·910	0·307	33·8	0·355	39·0	1·16
4	0·864	0·287	33·2	0·323	37·4	1·13
5	0·920	0·279	30·2	0·348	37·9	1·25
6	0·885	0·324	36·5	0·353	39·9	1·09
7	0·880	0·294	33·4	0·337	38·3	1·14
8	0·867	0·306	35·2	0·357	41·2	1·17
9	0·884	0·293	33·2	0·370	41·9	1·26
10	—	—	—	—	—	—
11	0·965	0·300	31·1	0·369	38·2	1·23
12	0·995	0·302	30·1	0·339	34·1	1·12
13	0·979	0·300	30·3	0·323	33·0	1·09
14	0·921	0·301	32·7	0·305	33·1	1·01
15	0·931	0·312	33·9	0·318	34·2	1·02
16	0·961	0·282	29·3	0·296	30·7	1·05
17	0·975	0·278	28·5	0·275	28·2	0·99
18	0·917	0·300	32·7	0·305	33·2	1·02
19	0·965	0·264	27·3	0·277	28·7	1·04
20	0·990	0·265	26·8	0·280	28·2	1·05
21	1·018	0·258	—	0·231	22·7	—

Few previous studies on ash analysis were found for comparison. Davies (1939) gave the percentages of P_2O_5 and CaO in sheep's milk as 0·293 and 0·249 respectively. These figures are low compared with the present ones, i.e. 0·32 and 0·29. Godden & Puddy (1935) studied the composition of the milk of individual ewes. They reported CaO and P_2O_5 percentages for samples taken during each of two seasons, and

averaging the results for the separate seasons gives values of 0·288 and 0·294 % for CaO and 0·418 and 0·366 % for P_2O_5. While the percentages of CaO compare closely with the present work, those of P_2O_5 are markedly higher.

Table 3. *Comparison between the* CaO *and* P_2O_5 *contents* (%, *w*/*w*) *of cow's and sheep's milk*

	Cow's milk (from Davis & Macdonald, 1953)	Sheep's milk	
		1st season (from Table 2)	2nd season (from Table 2*a*)
Mean % CaO in milk	0·190 (0·104–0·291)	0·296 (0·271–0·326)	0·290 (0·256–0·324)
Mean % P_2O_5 in milk	0·220 (0·146–0·310)	0·32 (0·304–0·342)	0·320 (0·231–0·370)
Mean % CaO in milk ash	22·37 (20·01–27·32)	33·42 (29·3–40·4)	31·47 (26·8–36·5)
Mean % P_2O_5 in milk ash	25·67 (21·57–29·33)	36·15 (32·0–42·0)	34·26 (22·7–41·9)

Comparison with cow's milk shows that ewe's milk contains considerably higher percentages of CaO and P_2O_5. Davis & Macdonald (1953, pp. 34, 36) summarized the results of several observers. Table 3 is a comparison between that summary and the composition of sheep's milk obtained in the present work. It may be seen that sheep's milk contains about 55 % more CaO and 45 % more P_2O_5 than cow's milk.

Davies (1939) gave the ratio of CaO:P_2O_5 (when CaO = 1) as between 1·15 and 1·42 for milks of different breeds of cows, and for buffalo milk as 1·02. In the present work the ratios averaged 1·077 (1·02–1·13). The CaO and P_2O_5 contents of the milk increased until the lambs were about 4 months old and then declined somewhat (Table 2*a*).

Thanks are due to the Animal Husbandry Division, Abu Ghraib, for co-operation in supplying the milk samples, and I am grateful to Mr S. Quishtaini and Mr F. Habib for technical assistance.

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Printed in Great Britain

J. Dairy Res. (1963), **30**, 87

The incidence of bacteria in cheese milk and Cheddar cheese and their association with flavour

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(*Received* 5 *September* 1962)

Summary. The numbers and types of non-starter lactic acid bacteria, lipolytic bacteria and group D streptococci in cheese milks and corresponding Cheddar cheeses have been studied and their relationship to cheese flavour discussed. Different milks, heat-treatments and starters were used, and their association with flavour investigated. The pH and fat, salt and moisture contents of the cheeses were also determined.

The non-starter lactic acid flora consisted mainly of *Lactobacillus casei*, *L. plantarum*, *L. brevis*, *L. buchnerii*, *Pediococcus* spp. and *Leuconostoc* spp. *Lactobacillus casei* was nearly always present in the milks and cheeses, predominating in most of the cheeses, particularly in those made from milk which received the most severe of the three heat-treatments tested (160°F for 17 sec). Leuconostocs were not found in any of the cheeses. Other species sometimes occurred in approximately equal numbers to *L. casei*, particularly in cheeses manufactured from the milder heat-treated milks. Cheeses made from milk receiving the highest heat-treatment contained fewest bacteria and scored lowest for flavour. The average flavour scores increased as the number of species contributing to the non-starter lactic acid flora at the time of tasting increased.

The lipolytic organisms capable of hydrolysing butterfat consisted mainly of Gram-positive cocci, predominantly *Staphylococcus saprophyticus*. The group D streptococci occurred infrequently, *Streptococcus faecium* being the most commonly occurring species.

A definite correlation was found to exist between the starters used and the flavour of the cheeses. The pH of the cheeses was associated with the flavour, but the fat, moisture and salt contents of the cheeses showed no definite effect.

The flavour of Cheddar cheese can be attributed to a complex association of chemical compounds produced during the manufacturing and ripening processes by the degradation of protein, fat and lactose. It is probable that the characteristic flavour is obtained only when the flavour components are present in certain specific proportions. However, the exact nature and balance of these constituents are not known although many compounds have been isolated. Many factors affect the development of cheese flavour but it is often difficult to assess experimentally their individual contributions because of the many variables which cannot easily be

controlled. Some of the factors involved and their role in flavour development have recently been discussed in a comprehensive review by Mabbitt (1961).

Almost all United Kingdom Cheddar cheese is manufactured from heat-treated milk, but very little information exists on the numbers and types of bacteria occurring in the heated milks and subsequently in the cheese, and on their relation to flavour. The purpose of this work was to ascertain whether any obvious correlation exists between bacteria and flavour, and to accumulate representative strains for future work. The influence on the development of flavour of the starters used, pH and the fat, salt and moisture contents of the cheeses were also studied.

METHODS

According to the milk source and place of manufacture the cheeses were examined in three groups, as follows:

(1) Cheeses made in the experimental dairy of this Institute from mixed evening and morning single herd milk.

(2) Cheeses made in the Institute experimental dairy from bulk market milk.

(3) Cheeses made in a commercial factory from bulk market milk.

Two heat treatments were used alternately for the milks from which cheeses of groups 1 and 2 were made, namely 145°F for 17 sec and 160°F for 17 sec. The holding times quoted are mean holding times, i.e. flow rate/capacity of holder. The commercial cheeses (group 3) were made from milk flash-heated to 150°F. The Institute cheeses were made using one of five pairs of single strain starters composed from three *Str. cremoris* strains (NCDO, nos. 508, 924, 1218) and three *Str. lactis* strains (NCDO, nos. 507, 509, 1007) to give a 10-week rotation of pairs. The cheeses, weighing 40 lb, were made from 40 gal of milk at fortnightly intervals during a 12-month period from April 1959 to April 1960. They were made in pairs (i.e. one group 1 and one group 2) in twin vats on the same day, using the same heat treatment and the same starters. The commercial cheeses were made at monthly intervals over a 6-month period from March to September 1960 using a mixture of 3–5 of a group of fifteen starter strains. Cheeses, selected at random from batches manufactured in vats containing from 750 to 1100 gal of milk, were either 60 lb traditional cylinders with tubular bandaging or 40 lb film-wrapped rindless blocks. They were of routine manufacture, no deliberate experimental variables being introduced. Groups 1 and 2 cheeses were ripened at 55°F and the group 3 cheeses at 50°F.

MILK AND CHEESE SAMPLING

Samples of the heat-treated milk were taken aseptically from the vats just before the addition of starter. Raw milk samples were also taken at the Institute but it was not possible to obtain representative raw milk samples at the commercial factory. Cheeses of groups 1 and 2 were sampled at 6 and 14 weeks of age, and the group 3 cheeses at 1 and 3 months. Cheeses were sampled and the samples blended and minced aseptically and homogenates prepared by the methods of Naylor & Sharpe (1958).

COLONY COUNTS AND ISOLATIONS OF BACTERIA

Total colony counts. Suitable dilutions of the milks and cheese homogenates were plated in yeast dextrose agar (YDA) (Nichols & Hoyle, 1948). The plates were incubated aerobically at 30°C for 5 days. No isolations were made.

Non-starter lactic acid bacteria. Numbers of lactobacilli, leuconostocs and pediococci were determined by plating in the selective modified Rogosa acetate agar (AcA) medium of Mabbitt & Zielinska (1956), and incubating anaerobically in an atmosphere of 90% H_2 and 10% CO_2 at 30°C for 5 days. Isolations were made from the most suitable plates by picking ten adjacent colonies into AcB (modified Rogosa acetate broth). Subsequent purification was carried out by repeatedly growing in broth and streaking on the agar medium (MRSA) of de Man, Rogosa & Sharpe (1960). Enrichment cultures of the milks were also made and examined for growth after incubation at 30°C for 1 week (10 × 1 oz screw-capped bottles/sample, each containing 1 ml milk and 10 ml AcB). Positive enrichments were confirmed by streaking on AcA.

Lipolytic bacteria. Numbers of lipolytic bacteria were determined by plating in a tributyrin medium (TA) of the following composition (w/v): Evans peptone, 1·0%; Lab-Lemco, 0·3%; Yeastrel, 0·3%; sodium chloride, 0·5%; Bacto-tryptone, 1·0%; tributyrin, 1·0%; agar, 2·0%; final pH, 6·0 ± 0·1. The plates were incubated aerobically for 5 days at 30°C, when the colonies exhibiting clear zones of lipolysis were counted. Five of these colonies were picked into yeast dextrose broth (YDB), purified and tested for their ability to hydrolyse butterfat by streaking on the butterfat agar (BFA) of Jones & Richards (1952).

Serological group D streptococci. These were enumerated by plating in the modified thallous acetate–tetrazolium–glucose agar (TlTGA) of Barnes (1956) and incubating aerobically at 45°C for 2 days. Group D streptococci were found in the cheese milks and cheeses of groups 1 and 2 so infrequently that no isolations were made, but isolations were made from the commercial cheese and milk samples, where these organisms were usually present, by picking into dextrose Lemco broth (DLB) (Nichols & Hoyle, 1948). The isolates were purified by streaking on YDA and growth in DLB.

IDENTIFICATION OF ISOLATES

Lactobacilli, leuconostocs and pediococci. These were identified according to the schemes of Perry & Sharpe (1960) and Rogosa & Sharpe (1959). The tests used were those of the former authors with the addition of the fermentation of arabinose, cellobiose, lactose and sucrose. Some of the strains were also tested for their ability to ferment melezitose and rhamnose.

Lipolytic bacteria. Tributyrolytic isolates which were also active against butterfat were examined for Gram-stain reaction and microscopic appearance. Approximately half of the isolates were found to be catalase-positive Gram-positive cocci and these only were classified further using the following biochemical and physiological tests based mainly on the schemes of Shaw, Stitt & Cowan (1951) and Abd-el-Malek & Gibson (1948): haemolysis; VP reaction; hydrolysis of casein, starch, gelatin and urea; reduction of nitrate; utilization of ammonia; production of coagulase, ammonia

from arginine, H_2S and pigments; fermentation of glucose, glycerol, lactose and mannitol; growth in 10, 12 and 15% salt; growth in litmus milk; growth at 15 and 45°C; and survival in milk at 60°C for 30 min.

Group D streptococci. These isolates were classified according to a scheme based on that given by Shattock (1962) using the following tests: haemolysis; growth at 45°C; sensitivity to 0·04% potassium tellurite; fermentation of arabinose, glycerol and sorbitol; liquefaction of gelatin and serological grouping.

Chemical analysis of the cheeses. The pH of the cheese samples was determined by adding 17 ml of distilled water to 12·5 g of minced cheese, filtering the suspension through muslin and measuring the pH of the filtrate electrometrically. The percentages of fat, moisture and salt in the cheeses were also determined (British Standard Institution 1955, 1952).

Cheese flavour assessment. The cheeses were scored for flavour, on a scale of 0–10, 16 weeks after manufacture, by a tasting panel of about ten members of the Institute staff. A score of 5 represented bland characterless cheeses. The range below 5 was used to indicate cheeses of poor quality, the score decreasing with quality; these cheeses were often objectionable because of undesirable flavours. Satisfactory cheeses were scored above 5, the score rising towards 10 with increasing intensity of the typical Cheddar flavour.

RESULTS

Total counts. It can be seen (Table 1) that the heat treatment of 145°F for 17 sec had little effect on the numbers of organisms present in either the single herd or bulk market milks, whereas the more severe treatment of 160°F for 17 sec resulted in a

Table 1. *Average numbers of bacteria (colony counts in yeast dextrose agar incubated for 5 days at 30°C) in milks and corresponding cheeses, and average flavour scores*

Group	No. of samples and heat treatment	Count of raw milk/ml	Count of heat-treated milk/ml	Count of cheeses (/g) at			Average flavour score
				4 weeks	6 weeks	3 months	
1. Single herd milks and Institute-made cheeses	13, 145°F for 17 sec	$3·1 \times 10^4$	$3·0 \times 10^4$	—	$4·0 \times 10^8$	$8·6 \times 10^7$	6·0
	14, 160°F for 17 sec	$9·4 \times 10^4$	$3·0 \times 10^2$	—	$8·5 \times 10^7$	$1·2 \times 10^7$	5·0
2. Bulk market milks and Institute-made cheeses	13, 145°F for 17 sec	$8·2 \times 10^5$	$2·1 \times 10^5$	—	$4·9 \times 10^8$	$1·7 \times 10^8$	5·8
	14, 160°F for 17 sec	$1·0 \times 10^6$*	$7·4 \times 10^3$*	—	$1·4 \times 10^8$	$1·9 \times 10^7$	5·0
3. Bulk market milks and commercially-made cheeses	12, 150°F, flash	—	$8·7 \times 10^4$	$2·8 \times 10^8$	—	$8·9 \times 10^7$	6·3

* Average of thirteen samples.

99% reduction. The average number of bacteria present in the heated milk at the commercial creamery was approximately equal to the numbers surviving the lower treatment in the Institute experimental dairy. The average counts of the cheeses at the first sampling were about 10^8 bacteria/g but these decreased slightly by the time of the second sampling at 3 months. It is noticeable that the two groups of cheeses with the lower average flavour score of 5·0 contained fewer bacteria than the

Table 2. *Average numbers of non-starter lactic acid bacteria (colony counts in acetate agar and enrichments incubated for 5 days at 30°C) in milks and corresponding cheeses, and average flavour scores*

Group	No. of samples and heat treatment	Count of raw milk/ml	Count of heat-treated milk/ml	Count of cheeses (/g) at 4 weeks	Count of cheeses (/g) at 6 weeks	Count of cheeses (/g) at 3 months	Average flavour score
1. Single herd milks and Institute-made cheeses	13, 145°F for 17 sec	$0{\cdot}5 \times 10^{2}$*	$0{\cdot}1 \times 10^{2}$	—	$3{\cdot}6 \times 10^{7}$	$7{\cdot}3 \times 10^{7}$	6·0
	14, 160°F for 17 sec	$0{\cdot}2 \times 10^{2}$	$< 1{\cdot}0 \times 10^{-1}$	—	$7{\cdot}0 \times 10^{6}$†	$1{\cdot}6 \times 10^{7}$†	5·0
2. Bulk market milks and Institute-made cheeses	13, 145°F for 17 sec	$3{\cdot}2 \times 10^{2}$	$0{\cdot}5 \times 10^{2}$	—	$1{\cdot}2 \times 10^{8}$	$1{\cdot}1 \times 10^{8}$	5·8
	14, 160°F for 17 sec	$5{\cdot}9 \times 10^{2}$	$2{\cdot}0 \times 10^{-1}$	—	$8{\cdot}9 \times 10^{6}$	$3{\cdot}2 \times 10^{7}$	5·0
3. Bulk market milks and commercially-made cheeses	12, 150°F, flash	—	$6{\cdot}4 \times 10^{2}$	$1{\cdot}6 \times 10^{7}$	—	$4{\cdot}2 \times 10^{7}$	6·3

* Average of twelve samples. † Include three cheeses in which no lactobacilli were detected.

Table 3. *The distribution of species of non-starter lactic acid bacteria in milk and cheese*

Type of sample	Heat treatment	No. of samples	% of samples in which the named species occurred as the whole or a substantial part of the non-starter lactic acid bacterial flora: *L. casei*	*L. plantarum*	*L. brevis*	*L. buchneri*	*Pediococcus* spp.	*Leuconostoc* spp.	Unidentified	% of samples in which non-starter lactic acid bacteria were not detected	Average flavour score
Single herd milk	145°F for 17 sec	13	69	8	46	31	0	8	38	15	—
6-week-old cheeses	145°F for 17 sec	13	85	8	15	0	15	0	0	0	—
3-month-old cheeses	145°F for 17 sec	13	92	8	23	0	0	0	0	0	6·0
Single herd milk	160°F for 17 sec	14	14	7	0	0	0	21	0	79	—
6-week-old cheeses	160°F for 17 sec	14	36	21	43	0	14	0	7	21	—
3-month-old cheeses	160°F for 17 sec	14	64	7	14	0	0	0	0	21	5·0
Bulk market milk	145°F for 17 sec	13	100	8	69	31	15	23	15	0	—
6-week-old cheeses	145°F for 17 sec	13	100	23	31	0	0	0	0	0	—
3-month-old cheeses	145°F for 17 sec	13	100	8	15	0	0	0	8	0	5·8
Bulk market milk	160°F for 17 sec	14	43	0	0	0	0	7	0	57	—
6-week-old cheeses	160°F for 17 sec	14	86	7	29	0	14	0	7	0	—
3-month-old cheeses	160°F for 17 sec	14	100	21	14	0	7	0	7	0	5·0
Bulk market milk	150°F, flash	12	83	42	0	0	33	33	42	0	—
4-week-old cheeses	150°F, flash	12	100	83	50	0	17	0	42	0	—
3-month-old cheeses	150°F, flash	12	100	67	83	0	67	0	50	0	6·3

others at both the first and second samplings, and were made from the heated milks with the lower initial numbers of bacteria.

Non-starter lactic acid bacteria. The numbers found in the raw single herd milks were lower than in the raw bulk market milks (Table 2), and were further reduced by the heat treatments, especially at 160°F when the numbers surviving were extremely low. Similar low residual average counts were obtained with the bulk market milks heated at 145 and 160°F but the market milk flash-heated to 150°F at a commercial factory still contained an average of 640 non-starter lactic acid bacteria/ml after treatment, the count being higher than in the other milks before heat treatment.

Table 4. *The influence of species of the non-starter lactic acid flora of the cheeses on the average flavour score*

(Figures in parentheses denote the number of cheeses containing the species named.)

Species	% improvement in average flavour score when the named species were present at 3 months compared with cheeses when they were not. Institute + commercial cheeses (66)
L. casei	12 (62)
L. plantarum	4 (14)
L. brevis	10 (21)
Pediococcus spp.	18 (10)
L. casei + *L. plantarum*	4 (14)
L. casei + *L. brevis*	13 (18)
L. casei + *Pediococcus* spp.	18 (10)
L. plantarum + *L. brevis*	12 (8)
L. plantarum + *Pediococcus* spp.	18 (5)
L. brevis + *Pediococcus* spp.	18 (9)

The average numbers in all the cheese groups exceeded 10^7/g at 3 months, showing only a slight increase over the numbers present at the first sampling. The groups of cheeses with the lowest average flavour scores (5·0) contained the lowest number of non-starter lactic acid bacteria at both samplings, but the group of commercial cheeses which scored highest for flavour did not correspondingly contain the highest number of these organisms.

The distribution of species of the non-starter lactic acid bacteria is shown in Table 3. The milks heated at 145°F for 17 sec contained more species than similar milks heated at 160°F for 17 sec. This is emphasized by the fact that non-starter lactic acid bacteria were not detected in 79 % of the single herd milks and 57 % of the bulk market milks given the higher heat treatment. *L. casei* usually occurred more frequently than any other species in the heated milks irrespective of the source of the milk or the heat treatment used.

The flora of the Institute cheeses was also dominated by *L. casei*, *L. brevis* and *L. plantarum* occurring much less frequently. Commercial cheeses made from milk flash-heated to 150°F presented a different picture, however. Although every cheese contained *L. casei*, strains of *L. plantarum*, *L. brevis* and pediococci were also present in equivalent numbers in a large proportion of these cheeses. *Leuconostoc* spp. were

found in samples representing each type of milk but were never found in any of the cheese samples, and it would appear that they are little involved in the development of the typical Cheddar cheese flavour.

L. casei was numerically the most important organism, occurring much more frequently than any other species. The occurrence of this organism in the milk almost inevitably resulted in its presence in large numbers in the cheese, and even when milk containing no *L. casei* was used it usually entered the vat during cheesemaking to yield large numbers in the cheese at 6 weeks or earlier. The results also indicate that in the absence of *L. casei* in the milk, another species might multiply to predominate in the cheese, but if *L. casei* is also present it usually outgrows all other species. Sometimes one or more species were found in equal numbers with *L. casei* in cheeses made from milk containing greater numbers of lactobacilli, i.e. milk given the milder heat treatments, but rarely did they outgrow *L. casei* in the cheese. The numerical preponderance of *L. casei* in the cheeses did not, however, necessarily imply that it was the most important species associated with good Cheddar cheese flavour development. Table 4 shows the percentage flavour improvement obtained when a particular species or combination of species was present in the cheeses tested compared with those cheeses in which the species or combination of species was absent. [The results do not indicate the influence of other species which might also be present with the species or group of species being considered.] The greatest improvement (18 %) occurred when pediococci were present, and this improvement was unaffected by the presence of *L. casei*, *L. plantarum* or *L. brevis*. Cheeses containing *L. plantarum* showed the smallest improvement in flavour (4 %) although cheeses also containing *L. brevis* or pediococci showed greater improvement. Cheeses containing *L. casei* or *L. brevis* showed approximately equal improvements. A similar pattern of results was obtained when the Institute cheeses alone were considered, except that the percentage improvements were usually less and cheeses containing *L. plantarum* had a lower average flavour score than those cheeses in which this organism was absent. It is noticeable that no species or pair of species when present resulted in a lowering of the combined average flavour scores of the Institute and commercial cheeses. This was due to the comparatively high average flavour scores of the commercial cheeses which usually contained *L. casei*, *L. brevis*, *L. plantarum* and pediococci at 3 months.

The results above show that the non-starter lactic acid flora of the cheeses consisted of various combinations of *L. casei*, *L. plantarum*, *L. brevis* and *Pediococcus* spp., and Table 5 indicates that the average flavour score improved with increasing numbers of species. The severity of heat treatment of the milk influenced the types of bacteria present in the cheeses in addition to affecting numbers.

Lipolytic bacteria. The average counts of tributyrolytic bacteria (Table 6) were lower in the milks processed at the Institute and the respective cheeses, whereas the highest average count occurred in the factory-processed milks and cheeses. The groups of cheeses with the greater number of lipolytic bacteria also had the higher average flavour score, and vice versa. Numbers of tributyrolytic bacteria in the cheeses were more or less constant between the first and second samplings. This might indicate a state of equilibrium but it is more likely that at the low storage temperature the population remained static or was decreasing slowly.

In the course of this study, 304 strains of tributyrolytic bacteria were isolated from

milk and 618 strains from cheese. Details given in Table 7 show that only 12 % of the milk isolates and 13 % of the cheese isolates were able to hydrolyse butterfat, the largest proportion being found in milk heated at 160 °F for 17 sec and the associated cheeses, particularly the single-herd milks and cheeses. It was considered that the lipolytic bacteria most likely to influence cheese flavour were those organisms capable of splitting butterfat and accordingly only these were studied further. Of these isolates 38 % were Gram-positive bacilli (aerobic spore-formers), 7 % were Gram-

Table 5. *The association of the number of species of non-starter lactic acid bacteria, and the average flavour score*

(Figures in parentheses indicate the number of cheeses contributing to the average flavour score.)

No. of species of *L. casei*, *L. plantarum*, *L. brevis* and *Pediococcus* spp. detected in cheeses at 3 months	Average flavour score
0	4·96 (3)
1	5·50 (40)
2	5·60 (13)
3	6·10 (7)
4	6·58 (5)

Table 6. *Average numbers of tributyrolytic bacteria*

(Colony counts in tributyrin agar incubated for 5 days at 30 °C in milks and corresponding cheeses, and average flavour scores.)

Group	No. of samples and heat treatment	Count of raw milk/ml	Count of heat-treated milk/ml	Count of cheeses (/g) at 4 weeks	Count of cheeses (/g) at 6 weeks	Count of cheeses (/g) at 3 months	Average flavour scores
1. Single herd milks and Institute-made cheeses	13, 145 °F for 17 sec	$3·3 \times 10^4$	$3·7 \times 10^2$	—	$3·0 \times 10^4$	$1·8 \times 10^4$	6·0
	14, 160 °F for 17 sec	$6·4 \times 10^4$*	$5·9 \times 10^2$	—	$5·2 \times 10^2$	$5·9 \times 10^2$	5·0
2. Bulk market milks and Institute-made cheeses	13, 145 °F for 17 sec	$2·4 \times 10^5$†	$1·2 \times 10^4$	—	$5·0 \times 10^4$	$6·2 \times 10^4$	5·8
	14, 160 °F for 17 sec	$8·1 \times 10^5$†	$2·0 \times 10^3$	—	$6·9 \times 10^3$	$2·0 \times 10^3$	5·0
3. Bulk market milks and commercially-made cheeses	12, 150 °F, flash	—	$4·7 \times 10^4$	$4·8 \times 10^5$	—	$4·8 \times 10^5$	6·3

* Average of thirteen samples. † Average of twelve samples.

Table 7. *A simple grouping of butterfat-splitting bacteria isolated from milk and cheese*

Type of sample	No. of samples	No. of tributyrolytic isolates	% of tributyrolytic isolates able to hydrolyse butterfat	% of butterfat-splitting isolates grouped microscopically as: Gram+ bacilli	Gram+ cocci	Gram− bacilli	Yeasts
1. Single herd milk, 145 °F for 17 sec	13	54	9	60	40	—	—
2. Single herd milk, 160 °F for 17 sec	14	60	33	30	55	15	—
3. Bulk market milk, 145 °F for 17 sec	13	60	3	50	50	—	—
4. Bulk market milk, 160 °F for 17 sec	14	70	9	67	33	—	—
5. Bulk market milk, 150 °F, flash	12	60	5	—	—	100	—
6. Cheeses made from 1	26	120	13	44	56	—	—
7. Cheeses made from 2	28	102	30	37	63	—	—
8. Cheeses made from 3	26	130	4	20	80	—	—
9. Cheeses made from 4	28	126	17	32	59	9	—
10. Cheeses made from 5	24	140	5	57	29	—	14

negative bacilli and one isolate was a yeast; no further identifications were carried out with these strains. Most of the butterfat-splitting isolates were Gram-positive cocci (54 %) which were classified further as described above. Table 8 shows that 10 % of these strains were coagulase-positive belonging to group 1A of Abd-el-Malek & Gibson (1948) or the *Staph. aureus* group of Shaw *et al.* (1951). Most of the remainder were *Staph. saprophyticus* or were divided between Abd-el-Malek & Gibson's groups 1B and 1D in the ratio of approximately 2:1 respectively. Three strains isolated from milk were unidentified, not fitting into any of the groups or species listed in either scheme. Most of the strains were salt-tolerant (15 % NaCl) and also produced H_2S when grown in YDB. The metabolic activity of these organisms in cheese during ripening is doubtful, but if there is any activity either directly or by the release of enzymes during lysis of cells, then the properties of lipolysis and H_2S production might be expected to have some influence on the development of flavour.

Table 8. *Classification of Gram-positive catalase-positive cocci isolated from milk and Cheddar cheese*

		Classification according to Abd-el-Malek & Gibson (1948)					Classification according to Shaw, Stitt & Cowan (1951)			
No. of strains	Source	Group 1 A	Group 1 B	Group 1 C	Group 1 D	Unidenti-fied	*Staph. aureus*	*Staph. sapro-phyticus*	*Staph. lactis*	Unidenti-fied
16*	Milk	1	11	—	1	3	1	12	—	3
43†	Cheese	5	26	1	11	—	5	36	2	—

* 12 strains grew in Lemco broth containing 15 % NaCl and 13 strains produced H_2S.
† 41 strains grew in Lemco broth containing 15 % NaCl and also produced H_2S.

Table 9. *The occurrence of group D streptococci in milks and corresponding cheeses*

Type of sample	Total no. of samples	No. of samples containing group D streptococci	Average counts of group D streptococci (colony counts on TITGA /ml or /g)	Average flavour scores
1. Single herd milk, raw	24	3	20·0	—
2. Single herd milk, 145 °F for 17 sec	12	2	1·6	—
3. Single herd milk, 160 °F for 17 sec	14	0	< 1·0	—
4. Bulk market milk, raw	24	8	57·5	—
5. Bulk market milk, 145 °F for 17 sec	12	2	89·3	—
6. Bulk market milk, 160 °F for 17 sec	14	1	< 1·0	—
7. Bulk market milk, 150 °F, flash	12	11	2176·0	—
8. Cheeses made from 2 — 6 weeks	13	2	23·0	6·0
8. Cheeses made from 2 — 3 months	12	1	250	
9. Cheeses made from 3 — 6 weeks	13	2	3·8	5·0
9. Cheeses made from 3 — 3 months	14	1	1543·0	
10. Cheeses made from 5 — 6 weeks	13	3	2856·0	5·8
10. Cheeses made from 5 — 3 months	12	3	20354·0	
11. Cheeses made from 6 — 6 weeks	13	3	183	5·0
11. Cheeses made from 6 — 3 months	14	4	131	
12. Cheeses made from 7 — 4 weeks	12	10	8496	6·3
12. Cheeses made from 7 — 3 months	12	9	3732	

Group D streptococci. These organisms occurred infrequently in the Institute milks and cheeses but were nearly always present in the commercial milks and cheeses as shown in Table 9. The few Institute cheeses containing group D streptococci, sometimes in fairly high numbers, did not necessarily score higher for flavour than the average score for the type of cheeses to which they belonged. When these organisms were detected in a particular cheese, they were rarely found at both samplings but in only one or other of the samples. Group D streptococci occurred, however, in nearly all the milk and cheese samples from the commercial factory and for this reason, and because these cheeses scored higher for flavour than the Institute cheeses, representative strains were isolated and studied further to determine the incidence of different species. The results (Table 10) show that the most commonly occurring

Table 10. *Classification of serological group D streptococci isolated from commercial bulk milk and corresponding cheeses*

		Classified according to Shattock (1962)					
No. of strains	Source	*Str. faecalis*	*Str. faecalis* var. *zymogenes*	*Str. faecalis* var. *liquefaciens*	*Str. faecium*	*Str. bovis*	Unclassified
21	Milk	2	2	2	13*	1	1
28	Cheese	—	—	—	25	1†	2

* Two strains failed to react with group D antisera. † One strain failed to react with group D antisera.

species in the milks and cheeses was *Str. faecium*. *Str. faecalis* and its variants were found occasionally in the cheese milk but not in any of the cheeses. One strain of *Str. bovis* was isolated from a heated cheese milk and a similar strain was later isolated from the cheese made from this milk. Three strains from unrelated milk and cheese samples were unclassified. Although *Str. faecium* appeared to occur most frequently in the cheeses, there was little association with the development of Cheddar cheese flavour. However, the commercial cheeses in which group D streptococci were nearly always detected scored higher for flavour than any other group of cheeses.

The effect of starter on cheese flavour. Cheeses were made in the Institute dairy using pairs of single strain starters in a 10-week rotation. The average flavour scores awarded to the groups of cheeses made using the different starter pairs are shown in Table 11. The results indicate that the best starter pair was *Str. cremoris* 1218 and *Str. lactis* 509. The pair used to make the cheeses having the lowest average flavour score was *Str. cremoris* 508 and *Str. lactis* 507. The two worst pairs each contained *Str. cremoris* 508 and this would suggest the unsuitability of this strain as a cheese starter under these conditions of manufacture. The average pH values of the cheeses made using the five starter pairs were similar, suggesting that the relatively large flavour differences were due to factors other than the quantity of acid produced. However, the rate of acid production might have had some effect.

It was not possible to compare the starters used in the commercial factory.

Chemical analysis of the cheeses. Examination of the average flavour scores and pH values at 3 months of the sixty-six cheeses studied in this investigation showed that the flavour tended to improve as the acidity of the cheese decreased within the pH range 4·95–5·35. Statistical analysis indicated that the coefficient of correlation

between flavours scores and pH readings could be expressed as $r = 0{\cdot}298$ (64 D.F.), $0{\cdot}02 > P > 0{\cdot}01$. Cheeses in which an increase in pH value occurred between the first and second samplings had a higher average flavour score (5·93) than those cheeses showing no change or a decrease in pH during that period (5·29).

The relationships between flavour and percentage salt, moisture and fat contents at 3 months are given in Table 12. There is no evidence of any correlation between the fat, salt or moisture content of Cheddar cheese at 3 months and cheese flavour, within the ranges considered.

Table 11. *The relationship between the average flavour scores of Institute Cheddar cheeses and the starters used*

Starter pairs	Average flavour score of cheeses made from milk heated at 145 °F for 17 sec	Average flavour score of cheeses made from milk heated at 160 °F for 17 sec	Average flavour score of all cheeses	Average pH of all cheeses at 3 months
Str. cremoris 924, *Str. lactis* 1007	5·68	5·2	5·44	5·17
Str. cremoris 1218, *Str. lactis* 509	6·72	5·92	6·46	5·17
Str. cremoris 508, *Str. lactis* 507	5·52	4·23	4·75	5·15
Str. cremoris 508, *Str. cremoris* 924	4·75	5·11	4·97	5·08
Str. lactis 507, *Str. lactis* 1007	6·22	5·16	5·59	5·16

Table 12. *The relationship between the percentages of salt, moisture and fat in Cheddar cheese and flavour*

(The figures in parentheses denote the number of cheeses contributing to the average flavour score.)

Range of salt content at 3 months	Average flavour score	Range of moisture content at 3 months	Average flavour score	Range of fat content at 3 months	Average flavour score
1·15–1·25	6·55 (2)	32·5–33·4	6·6 (3)	31·6–33·5	5·96 (19)
1·26–1·35	6·60 (1)	33·5–34·4	5·77 (8)	33·6–35·5	5·39 (24)
1·36–1·45	5·30 (3)	34·5–35·4	5·20 (11)	35·6–37·5	5·82 (19)
1·46–1·55	4·43 (3)	35·5–36·4	5·47 (21)	37·6–39·5	6·45 (2)
1·56–1·65	5·75 (11)	36·5–37·4	5·98 (15)	—	—
1·66–1·75	5·60 (23)	37·5–38·4	6·22 (4)	—	—
1·76–1·85	5·70 (14)	38·5–39·4	6·75 (2)	—	—
1·86–1·95	6·48 (7)	—	—	—	—

DISCUSSION

The bacteriological results described above give some support to the arguments in favour of a positive influence of microorganisms on cheese flavour, cheeses containing the greater number of bacteria scoring higher for flavour. The numbers and types of bacteria in the cheese milk, and ultimately in the cheese, are controlled largely by the severity of the heat treatment to which the milk is subjected, although adventitious contamination during cheesemaking must also contribute to the cheese flora. The poorer flavour of cheeses made from the more severely heated milks cannot be dissociated from chemical and physical changes. If it is considered desirable, for public health reasons, to subject cheese milk to a minimum heat-treatment capable of destroying pathogenic bacteria (such as the 161 °F for 15 sec treatment required in

the U.K. Milk Regulations (1960) governing the pasteurization of milk), it might be necessary to add selected strains of non-pathogenic bacteria to the cheese milk after heat-treatment to obtain a superior-flavoured Cheddar cheese.

The lipolytic bacteria and serological group D streptococci did not increase in numbers during ripening and, in fact, because mechanical concentration of bacteria in the curd particles normally occurs during cheesemaking, the numbers probably decreased during the first few weeks of ripening. It can reasonably be assumed, therefore, that because these bacteria were present in similar numbers at the first and second samplings they must be scattered singly throughout the cheese and are either static or, if dividing, as many new cells are being formed as old cells are dying. The comparatively low numbers of these bacteria in the cheese make the latter hypothesis unlikely and it is probable that isolated bacteria exist in a dormant or near-dormant state. It is difficult to see how such bacteria could influence flavour unless they were present originally in considerably higher numbers and subsequently contributed to the flavour by the action of enzymes released by autolysis of the cells during the ripening period. If this occurred, however, it is difficult to explain why the bacteria did not continue to decrease in numbers during the period between the two samplings. Conditions in the cheese, such as low pH, high salt content and low temperature, do not favour the multiplication and associated metabolic activity of these bacteria.

The situation is very different with the lactobacilli and pediococci, however, which are actively multiplying in the cheese with the formation of colonies. It is feasible that these organisms have some effect on flavour, and the results suggest that certain species may contribute more towards flavour than others. It has already been shown that many strains of lactobacilli produce H_2S under conditions of low pH, anaerobiosis and low sugar concentration (Sharpe & Franklin, 1962), and they may also do this in cheese. They may influence the flavour in other ways although they appear to have little or no activity against butterfat and casein. Their role might be associated with the growth or metabolic by-products of other bacteria.

The starter streptococci, which are very active during the early stages of cheesemaking and attain large numbers in the cheese, probably have a marked effect on cheese flavour during this period, and also later because of the continued influence of the by-products of their earlier activity and to the release of enzymes into the cheese during cell lysis. The results, in fact, showed fairly large differences in average flavour scores when different pairs of single strain starters were used. The quantity and quality of the enzymes and metabolic by-products produced would be expected not only to influence the flavour directly but also to affect the growth and metabolic activity of other bacteria in the cheese, particularly the non-starter lactic acid flora. Acid production in the cheese by the starter streptococci is obviously of importance and the results of pH measurements of the cheeses confirm this. Although acid development is essential to good cheesemaking, too much acidity can adversely affect the flavour as exemplified by the lowering of flavour scores as the pH decreases in the range 4·95–5·35.

Kristoffersen & Gould (1960) demonstrated that satisfactory flavour development in Cheddar cheese is related to both proteolytic and lipolytic activities during ripening, and that the two processes must proceed at definite, interdependent rates before optimum flavour quality results. This study has confirmed that bacterial activity does

influence cheese flavour, its development being correlated with the numbers and types of bacteria present. The influence may be indirect, for example, by the release of bacterial enzymes into the cheese milk as a result of the destruction of bacteria during heat-treatment of the milk and the subsequent activity of these enzymes in the cheese, or by the release of proteolytic and lipolytic enzymes in the cheese due to autolysis of microorganisms during ripening. Alternatively the influence may be a direct result of the metabolic activity of certain actively multiplying strains in the cheese.

This work has shown that certain strains and species improved the flavour more than others, but as good cheese flavour is usually thought to be due to a correct blend of a complex mixture of components, it is also possible that a correct blend of bacteria in the cheese might be necessary to achieve the best flavour. Towards the end of this investigation it was demonstrated, using a selective medium technique (Gyllenberg, Eklund, Antila & Vartiovaara, 1960), that Gram-negative bacilli were sometimes present in considerable numbers (10^4–10^6/g) in the commercial cheeses, and the possible influence of these organisms must be considered. Further assessment of the role of bacteria is likely to depend on associative growth studies of different strains and species, and the manufacture of cheese under carefully controlled bacteriological conditions so that the effect of adding selected strains and mixtures of strains can be evaluated.

We wish to thank the Milk Marketing Board of England and Wales for their co-operation and for providing laboratory facilities at one of their creameries; Miss H. R. Chapman and Miss A. J. W. Harrison for making the Institute cheeses; Mr A. W. Wagstaff for carrying out the chemical analyses of the cheeses and numerous other colleagues at the Institute for their help and co-operation during this work.

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Printed in Great Britain

The preparation of κ-casein

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(*Received* 25 *September* 1962)

Summary. A method is described for the preparation of κ-casein and for its purification by chromatography on DEAE cellulose. The conditions of preparation have been chosen so as to avoid drastic alteration of the κ-casein and permit the isolation in relatively pure form of one-half to two-thirds of the amount theoretically available.

Since the discovery by Waugh & von Hippel (1956) of a rennin-sensitive, Ca-stable fraction in casein which they named κ-casein, there has been considerable interest in the properties of this fraction and a number of methods has been suggested for its preparation (e.g. McKenzie & Wake, 1961; Tsugo & Yamauchi, 1960; Swaisgood & Brunner, 1962; Cheeseman, 1962; Long, van Winkle & Gould, 1958). These methods generally rely to some extent upon the process suggested by Waugh & von Hippel (1956) for the separation of the κ fraction from the α–κ complex, but use protracted or relatively inefficient means for the subsequent purification of the crude κ-casein. The work of Beeby & Nitschmann (1963) suggests that κ-casein itself is not a single protein but is a complex, and that the soluble material split from κ-casein by rennin may also be removed by urea. This makes it desirable to develop a method for preparing κ-casein that does not involve the use of reagents such as urea, or TCA-urea, and that results in a greater yield of final product. The method described in this paper is based upon that of Waugh and von Hippel for the separation of the crude κ-casein, but uses a final chromatographic separation in conditions which minimize the break-up of the κ-casein complex.

EXPERIMENTAL

Materials and methods

All chemicals were commercial products of reagent grade. Water was de-ionized by means of an Elgastat de-ionizing column. Fresh raw bulk milk was used; it and the protein solutions prepared from it were preserved during preparation by thymol. Crystalline rennin was used and the DEAE cellulose was Whatman DE50. Fractions were collected on a L.K.B. fraction collector equipped with a UV absorptiometer recording transmission at 253 mμ.

Nitrogen was determined by semi-micro Kjeldahl distillation, and sialic acid by the method of Warren (1959). Disulphide was determined by the method of Allison & Cecil (1958) using phenyl-mercury acetate in lieu of phenyl-mercury hydroxide as the titrant.

The buffers for eluting protein from the DEAE cellulose columns were: A, 0·05 N-acetic acid made to pH 6·25 with NaOH; and B, 0·10N-acetic acid adjusted to pH 6·25 with NaOH, to which was added $CaCl_2$ to a final strength of 0·5M and the pH adjusted to 4·5 with 1·0N-HCl.

Electrophoresis was performed in an L.K.B. apparatus, using a liquid density gradient which was approximately linear from 0 to 45 % in glycerol and in which the protein gradient was initially exponential. Runs were usually of 40–44 h duration with an applied voltage of 500 V in phosphate buffer of ionic strength 0·05 and pH 9·5.

The preparation of crude κ-casein

Three litres of raw milk were warmed to 35°C and centrifuged in two lots of 1500 ml in a Servall RC2 centrifuge (GSA Rotor, 9000 rev/min). After 15 min at 35°C, the temperature control was adjusted to 3°C and the run continued for a further 15 min. The frozen fat layer was removed, any sediment discarded and the two lots of fat-free skim-milk were combined. The skim-milk was warmed to 30°C and the pH adjusted to 4·5 by the slow addition with stirring of 1·0N-HCl. The precipitate was filtered and washed on Nylon cloth in a large Buchner funnel. The precipitate was then dispersed in water by means of a Waring Blendor, then redissolved in 3 l of water by the slow addition with stirring of 1·0N-NaOH, the pH being kept below 8. When solution was complete, the casein was again precipitated, and redissolved as described above, except that the final volume of the solution was 1400 ml. This solution containing 5–6 % protein was cooled to 3°C and 100 ml of similarly cooled 4M-$CaCl_2$ solution added slowly with stirring, the pH of the solution being kept between 6·7 and 7·2. The suspension was kept cool for approximately 1 h, then warmed to 35°C and spun for 15 min at this temperature in the Servall centrifuge (GSA Rotor, 5000 rev/min). The supernatant was removed and dialysed overnight against 16 l of water at 3°C. The dialysed solution was then concentrated in a low-temperature vacuum evaporator to a volume of about 250 ml, the evaporator being operated so that the temperature of the solution did not exceed 40°C. (Alternatively the dialysed solution was adjusted to pH 4·7, and the precipitate of crude κ-casein was redissolved in a volume of 250 ml at pH 7–7·5 by the slow addition of 1·0N-NaOH.) The solution was then cooled to 3°C and 20 ml of 4M-$CaCl_2$ added, the pH being kept between 6·7 and 7·2. The solution was then warmed to 35°C and spun at 60000 ***g*** for 45 min in the Spinco Model L ultracentrifuge (Rotor, 21, 21000 rev/min). The precipitate and any fatty layer were discarded and the clear supernatant was dialysed for 3–4 h against 5 l of water, with stirring, then overnight against 16 l of buffer A at 3°C. The dialysed solution was then either used directly for chromatographic purification, or freeze-dried for subsequent use. A yield of 9–12 g of protein material was obtainable at this stage.

Chromatographic purification of crude κ-casein

The crude κ-casein was purified by chromatography on a column of DEAE cellulose prepared in the following way. Fifteen grams of DEAE cellulose was agitated in 500 ml of 0·1N-NaOH, allowed to settle for ½ h, and the excess liquid containing fines decanted. The slurry was poured into a glass column of diameter about 4 cm with a layer of ⅛ inch of glass wool packed into the bottom of the column. The

cellulose was packed in the column under an air pressure of not more than 4–5 lb/in^2. It was then washed with 300 ml of water followed by 300 ml of 0·05N-acetic acid until the pH of the effluent was 3. It was finally washed with 250–300 ml of buffer A until the pH of the effluent was 6. The column was then cooled in ice water for at least 15 min before use, and was kept in ice water throughout the run.

A volume of the dialysed crude κ-casein solution containing 800–1200 mg of protein was diluted with buffer A to a volume of about 40 ml, cooled to 3 °C and applied to the column. It was washed into the column with 2 × 10 ml lots of cooled buffer A,

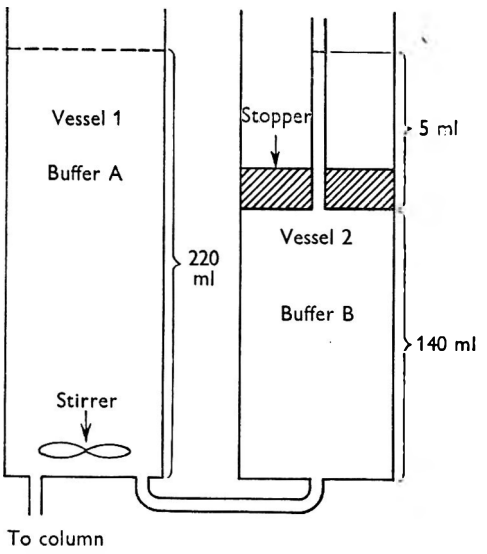

Fig. 1

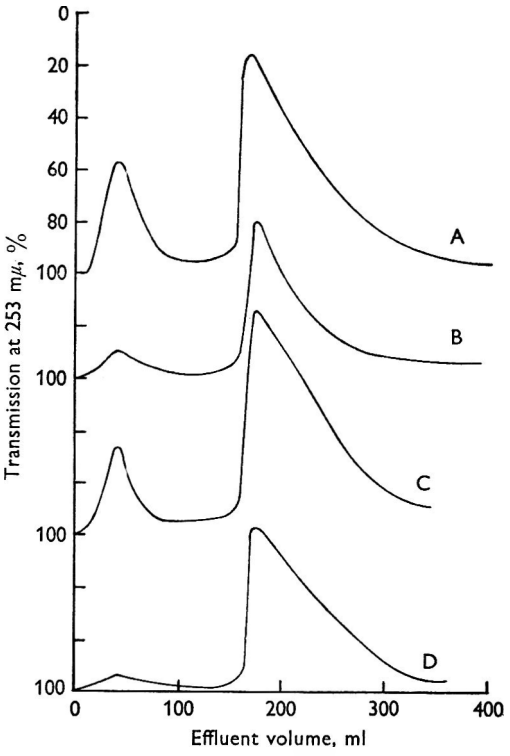

Fig. 2

Fig. 1. Arrangement of vessels for generating gradient for column chromatography.

Fig. 2. Chromatograms of κ-casein on DEAE cellulose. A, 700 mg crude κ-casein, final centrifugation, 35000*g*; B, 390 mg purified κ-casein, main peak of A, rechromatographed; C, 930 mg crude κ-casein, final centrifugation 60000*g*; D, 480 mg purified κ-casein, main peak of C, rechromatographed.

and elution commenced at a flow rate of 2–3 ml/min. The flow rate was not critical but too slow a rate was avoided because of the possibility of β-casein precipitating in the absorptiometer cell as the solution warmed in passing through. Elution was mainly performed in two stages, using mixtures of buffers A and B.

The first stage of the gradient was made up of 5 ml of buffer B mixed with 80 ml of buffers A in the arrangement shown in Fig. 1; in the second stage 140 ml of buffer B was mixed with an equal volume of the buffer in vessel 1 to give a steeper linear gradient in $CaCl_2$ concentration. The gradient in the first stage is described by the

relation [Ca] = $\frac{1}{176}$ ln 220/[220 − (16V/17)] for 0 < V < 85, where V is the volume of flow in ml; for the second stage 85 < V < 365, [Ca] = 0·03 + (V − 85)/280 × 0·47. The elution of β-casein occurred first and was found to be fairly complete after about 80 ml. The elution of κ-casein increased sharply after about 140 ml, typical chromatograms being shown in Fig. 2. Just before the sharp increase in the elution of κ-casein occurred, the molarity of Ca in the eluate was almost 0·01 M. After a further 120–140 ml most of the κ-casein had been eluted and the molarity of Ca was 0·25 while the pH had dropped to 4·8–5·2. The material used for further work was contained in the fractions of the κ-casein peak having absorptions greater than 15 %. These fractions were pooled, dialysed against two changes of a large excess of 0·01 M-NaCl at 3 °C, and freeze-dried. The yield of dry protein was usually about two-thirds of the total protein placed on the column.

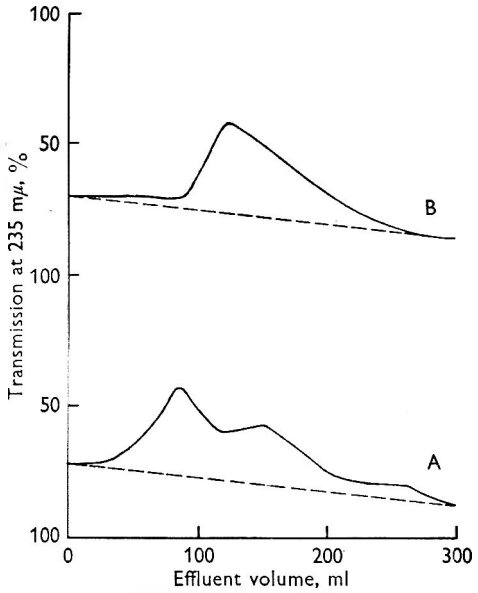

Fig. 3. Density gradient electropherograms of κ-casein. A, crude κ-casein; B, purified κ-casein. Amount of protein placed in gradient, 65 mg; duration of run A 44 h, run B 41 h; applied voltage, 500; buffer, Na_2HPO_4, pH 9·5; ionic strength 0·05. Dotted lines represent transmission of buffer.

Elution of protein was not complete at the end of the second stage of the gradient. Elution was therefore hastened by washing with 250 ml of 0·1 N-acetic acid, then 100 ml of distilled water followed by 250 ml of 0·1 N-NaOH. Alternatively, the elution with 0·1 N-acetic acid could be followed by elution with 1·0 N-acetic acid. Elution of protein was then complete. The material which could be eluted in each stage is shown in Table 1. The sum of the amounts of material shown as recovered in each stage does not equal the total amount placed on the column, mainly because the material from the tail of the κ-casein peak was not collected. However, Kjeldahl nitrogen determinations performed on all fractions eluted showed complete recovery of material.

When the material comprising the main κ-casein peak was collected as described,

dialysed against starting buffer, freeze-dried and rechromatographed, a main κ-casein peak occurred in the same position as in the original chromatogram (Fig. 2). The β-casein was present in small quantity; as determined by Kjeldahl nitrogen the proportion of β-casein was only 1 % of the purified protein, in comparison with 5–8 % in the crude κ-casein (Table 1, and Fig. 2D). The efficiency of the chromatographic removal of the β-casein was also tested by electrophoresis in a liquid density gradient, under conditions in which β-casein could be distinguished from κ-casein. The electropherograms which are given in Fig. 3 show that the β-casein in the purified material is undetectable, although it is clearly present in the crude κ-casein.

Composition of the fractions eluted

The identity of the different caseins was established in the following ways. β-Casein is soluble in the presence of Ca^{2+} in the cold, but is insoluble or gives a turbid suspension at 25 °C. κ-Casein is soluble in the presence of Ca^{2+}, and contains sialic acid and cystine, neither of which is present in the other casein fractions. Para-κ-casein contains cystine, but is lacking in sialic acid.

The fraction identified as β-casein contained no detectable disulphide and little sialic acid (Table 1). The material from the κ-casein peak contained both cystine and sialic acid, was sensitive to rennin, and was soluble in the presence of Ca^{2+}. For example, the cystine content was about one mole per 45000–48000 g of κ-casein,

Table 1. *Composition of crude and chromatographically fractionated κ-casein*

Preparation no.	Crude material		β peak		κ peak		HAc peak		NaOH peak		Yield of purified κ casein, %
	mg	% NANA	mg	% NANA	mg	% NANA	mg	% NANA	mg	% NANA	
1	760	—	ND	0·1	445	1·4	25	1·8	52·5	0·2	59
2	705	—	40	0·1	430	1·3	30	1·4	59	0·2	61
3	1·280	1·25	77	0·35	940	1·4	40	1·35	51	0·5	73
4	700	1·8	53	0·25	430	1·95	48	1·6	20	1·34	62
4R	390	1·9	—	—	250	1·8	—	—	—	—	64
5	930	1·5	—	—	480	—	—	—	—	—	52
5R	480	—	5	—	250	1·6	—	—	—	—	52
6	1·180	1·15	37	—	705	1·5	—	—	—	—	60
7	1·100	1·1	—	—	600	1·8	—	—	—	—	55

Preps. 4R, 5R represents rechromatographed preps. 4 and 5 respectively.

Preps. 6 and 7 were prepared from the same milk. For no. 6 the crude κ-casein solution was concentrated by acid precipitation, while no. 7 was concentrated by evaporation at low temperature.

NANA = *N*-acetyl neuraminic acid.

compared with one mole per 52000 g in the crude material (Prep. 1, Table 1). The material eluted with 0·1 N-acetic acid tended to have a sialic acid content similar to that of the main peak (Table 1). The remaining fraction eluted with 0·1 N-NaOH contained cystine and little sialic acid. It was aggregated at pH 7, but could be dissolved in 6 M urea at pH 11, and was therefore similar to para-κ-casein. The relative proportions of these fractions for several preparations are shown in Table 1.

The proportion of sialic acid in the κ-casein was different, depending upon the method of preparation. For the preparation in which concentration of the crude

κ-casein was effected by acid precipitation of the κ-casein and redissolving the precipitate, the sialic acid content was smaller (1·2–1·5 %) than that of κ-casein which had been prepared at this stage by concentration in vacuo (1·8–2·0 %, Table 1). This effect was checked by direct comparison of the sialic acid content of κ-caseins prepared by each of these methods from the same raw milk (Table 1). This result shows that concentration by means of low-temperature evaporation is to be preferred.

DISCUSSION

The method of preparation of the crude κ-casein given in the first part of this paper is not original, but there are steps in the process which are worthy of comment. For example, the method of removing fat from the raw milk was more effective than use of continuous cream separators. Complete removal is important, as fat present in later stages of the preparation tends to become firmly associated with the κ-casein. Again, the removal of $CaCl_2$ after the precipitation of the α and β caseins is effected by dialysis, since the use of oxalate to remove the Ca^{2+} can result in considerable loss of yield (Long *et al.* 1958). The second treatment of the crude κ-casein solution with 0·25 M-$CaCl_2$ is required to reduce the β-casein content to a minimum, because both the purity of the final product and the amount of protein that can be placed on the chromatographic column are adversely affected by increased content of β-casein in the crude κ-casein preparation. For the same reason, the crude κ-casein is centrifuged at 60000 ***g*** so that removal of β-casein may be as complete as possible (cf. A and C, Fig. 2).

As was pointed out earlier, κ-casein is a complex (Beeby & Nitschmann, 1963) and that part of the complex which is released by rennin may also be set free by reagents such as urea. In solutions of κ-casein, this part of the complex may be considered to be in equilibrium between at least two states—those of dissolved monomer and of κ-casein complex—since the sialic acid containing part is selectively lost on acid precipitation of the complex. When part of this fraction is lost the remainder tends to be distributed uniformly throughout the κ-casein since on chromatography the bulk of the κ-casein elutes with a fairly constant proportion of sialic acid. This is so whether the sialic acid content is relatively high or low, suggesting that it is possible to form stable aggregates having different proportions of the sialic-containing fraction and that there is no strong tendency to a preferred combining ratio between the parts of the κ-casein complex. This behaviour presents problems of peculiar difficulty in the preparation of the intact κ-casein complex, and shows the necessity for avoiding the use of reagents such as urea and TCA–urea, or of conditions of high pH and low temperature in which disassociation of the complex may occur. The use of such reagents or conditions is likely to result in a reduced yield because of the formation of para-κ-casein, and in the reduction of the sialic acid content of the final product.

This view is supported by the rather large variations in the proportion of sialic acid in κ-casein prepared by various methods, ranging from 0·79 % (Cheeseman, 1962) to 2·2 % (Cayen, Henneberry & Baker, 1962), and by the fact that chromatography of κ-casein at high pH and in urea leads to the formation of two fractions, one of which could only be eluted by NaOH (Dumas, 1961).

For the foregoing reasons, the chromatographic purification of the crude κ-casein

was performed not in alkaline but in mildly acid conditions, in which disaggregation of the κ-complex is less likely. Precipitation of the casein at the iso-electric point (pH 4·7) was prevented by adjusting the gradient of elution so that the eluant contained more than 0·25M-$CaCl_2$ as its pH approached 4·7. Whole casein, and κ-casein, do not precipitate from solution at pH 4·7 in the presence of 0·5M-$CaCl_2$; this was therefore chosen as the displacement reagent. Since displacement of the κ-casein was effected more readily in acid conditions, a pH gradient as well as a salt gradient was used; under these conditions, β- and κ-casein can be effectively separated, without the need for large volumes of eluant. At the same time, there is little tendency for the κ-casein complex to dissociate, as is shown by the fact that both the cystine and sialic acid contents increased to an equal extent during the purification. This is not so when chromatography is effected in conditions of high pH in the presence of urea (Dumas, 1961).

At present the composition of the κ-casein complex as it exists in milk is not known with certainty, and a similar uncertainty exists regarding the effect of the treatments used to prepare the crude κ-casein in altering this composition. In making a study of the reactions of the κ-casein complex, it is desirable that the method of preparation preserve the complex in as intact a state as possible. The chromatographic separation described in this paper represents a step in that direction.

It is a pleasure to acknowledge the technical assistance of Miss R. R. Hansen, and the helpful discussions with Mr R. Beeby, both of the Division of Dairy Research.

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Printed in Great Britain

Reviews of the progress of dairy science

Section F. Milk-borne disease

(*Received* 16 *October* 1962)

INTRODUCTION

In his last biennial review of milk-borne diseases, Dr John Smith (1961) referred to the changing relative importance of the various milk-borne infections. Some previously important conditions (e.g. streptococcal infection from milk) were now rare and others, previously considered of little importance, were now attributed greater significance. Bovine tuberculosis, once a scourge, had been controlled or eradicated in most western countries, and infection of the human subject with bovine bacilli was uncommon and only seen when there were breakdowns in animal control or milk pasteurization. There is also the changing situation in regard to micro-organismal diseases transmitted by milk, in regard to the survivability of viruses in heat-treated milk and the possible role of more recently recognized viruses in human disease. Hazards other than micro-organismal, in the form of radionuclides, toxic chemicals and antibodies, and even the role of milk constituents in arterial disease, are now subjects of importance. These newer problems have already developed an extensive literature.

An attempt is made here to sail a course through this well-charted but nevertheless vast ocean of data; it is hoped that it will be a course reasonably acceptable to most readers, and that those accustomed to appreciation of Dr John Smith's elegant reviews will bear with the present author.

MILK CONSUMPTION

A total of 5065×10^6 gal of milk and milk products were consumed in Britain in 1961, of which 1583×10^6 gal was in liquid milk form (Milk Marketing Board, 1962).

Harris & Seldon (1962) say that this was 4·88 pints per head per week, only fractional changes in this figure having occurred since 1953–54; but the figure had risen since 1933–34, when it was about 3 pints per week. According to these authors, milk consumption began to fall after the war, because of rising prices and competition from other foods and drinks, and the National Milk Publicity Council began an advertising

campaign to increase the sales of milk and cream. Milk had prestige as a health food, but faced competition, a catering tendency to use dried milk, and a public impression that milk was expensive and fattening. Publicity in the first year cost £300000, representing 0·1 % of retail turnover of $£3 \times 10^8$, or one-hundredth of a penny per pint. £428000 was spent in 1955–56, and consumption stabilized at 0·66 pints per head per day. By 1958 milk sales rose by 14 million gal or about 1 %. By 1961 a sales increase of 23 million gallons against 1951–52 figures had been recorded. Changes in milk drinking habits were noted, e.g. milk drinking almost doubled among the age-group 16–30, more was consumed outside the home, it was drunk on its own. The authors note variations in milk consumption in different parts of the country, listed in Table 1; relatively low consumption in some northern and Welsh areas is believed to be a relic from depression years, when many families lived on skim-milk for economy.

Table 1. *Analysis by region of milk and cream consumption per person per week*

	Milk, pints	Cream, pints
Southern and south-eastern	5·51	0·02
London	5·34	0·02
Midland	5·18	0·01
North Midland	5·04	0·02
Eastern	4·99	0·02
South-western	4·72	0·03
North-western	4·65	0·02
Wales	4·48	0·02
Scotland	4·38	0·01
Northern	4·37	0·01
East and West Ridings of Yorkshire	4·16	0·01

Finally, Harris & Seldon conclude, in relation to advertising: 'With a reservation about the pidgin English in "drinka pinta milka day", this is one of the most intelligently planned advertising campaigns we have examined. Moreover, it is being accompanied by efforts to overhaul the industry's marketing methods in accordance with changing social habits and the counter-attractions of competitors.' They note that the word 'pinta' is to be included in the next edition of Chambers's Dictionary as 'a drink of milk'.

In the U.S.A., Faulkner, Taylor & Schlafman (1962) report that in step with the population increase of about 50 million in 20 years, the total production of milk rose from the 1940 total of 109×10^9 lb, of which 43×10^9 lb (39 %) was fluid milk and cream, to a total of 122×10^9 lb, of which 59×10^9 lb (48 %) was fluid. [10·3 lb = 1 imperial gallon].

Heat treatments and standards

The Milk Marketing Board Report shows that 95 % of liquid milk in England and Wales is pasteurized. In Scotland, the Report of the Chief Medical Officer to the Department of Health (1962) indicates that the pasteurization level is lower, at 79 %; thus the opportunity for milk-borne disease appears to be greater there than in England and Wales.

The detail of the remaining percentage of raw milk in England and Wales available

to transmit milk-borne disease is of interest. Strauss (1960) stated that producer-retailers in 1960 were almost the sole source of milk for liquid consumption fresh from the farm and not subjected to heat treatment, and further (E. Strauss, pers. comm.) that the main markets for T.T. farm-bottled milk were rural areas and suburban areas of large towns.

As Kaplan, Abdussalam & Bijlenga (1962) point out, no authentic outbreak of disease has been attributed to properly pasteurized, bottled and delivered milk, but the infallibility of the pasteurization process is not absolute, as the conditions necessary for its adequate performance are not always fulfilled.

McCallum (1960) described a bacteriological survey, made between 1956 and 1959, of the flora of Glasgow milk as it reached the consumer. She concluded that the resazurin test is capable of detecting only the 'worst' milk. There was complete correlation between coliform test and the psychrophile count for both certified and pasteurized milk. Though the psychrophile count is considered by some to be a more accurate indicator than the coliform test of the bacteriological standard of milk, it is cumbersome to perform, has a long incubation period, and is subject to all the errors of the 37 °C count. The coliform test, on the other hand, detects milks which fail the psychrophile count and is easier to perform. The methylene-blue test was found to be more reliable than the resazurin test and, if a dye test were favoured, might take its place. The results obtained in this survey showed that consumer milk in Glasgow was not of as high a standard as it might be. For certified milk, insufficiently rapid cooling, and for pasteurized milk, badly cleaned bottles, were thought to be the most frequent reasons why samples failed the coliform test. In certified milk a number of samples contained coagulase-positive, penicillin-resistant staphylococci type 42D. Although phage-type 42D is not often a cause of disease in man, it was considered undesirable that consumer milk should contain large numbers of such organisms.

For Scotland as a whole (Scottish Health Statistics, 1960), the following proportions of different grades of milk failed to satisfy the prescribed bacteriological tests: 21·63 % certified, 17·38 % T.T., 3·7 % pasteurized, and 3·08 % T.T. pasteurized.

Mackenzie (1960) concluded that from a purely laboratory standpoint, five tests should be used for a milk control programme: on raw milk, the direct microscopic count supplemented by the resazurin reduction test, which is of value in the detection of milk in which well-marked clumping of bacteria has taken place; and on pasteurized products the 'big three'—phosphatase test, coliform M.P.N., and standard plate counts. To these must be added special tests indicated by circumstances and active field inspection services.

In the fourth edition of Chalmers's *Bacteria in Relation to the Milk Supply* (1962), additions have been made to the chapter on routine examination of milk samples, including recent research on tests for the keeping quality of milk. There is a separate chapter on investigations of the phosphatase test and in-bottle pasteurization. The quaternary ammonium compounds are described, and the coli-aerogenes group of organisms is given greater attention.

This is a comprehensive book and while it is intended mainly as a guide for the commercial dairy bacteriologist, it should also be of value to medical officers of health, public health inspectors, and others concerned with the control of milk supplies.

Milk standards

The United States Department of Health, Education, and Welfare have in 1960 issued a bulletin on 'Procedures for Testing Pasteurization Equipment'. This revises earlier publications on this subject and provides a ready reference for the milk sanitation trainee on methods of conducting the important tests on pasteurization plant equipment. This manual covers pasteurization plants and equipment, tests on vat-type pasteurization, the short-time high-temperature process and vacuum pasteurizer equipment.

A report on milk powder by the Ministry of Agriculture (1961) pointed out that there were no bacterial standards for milk powder in this country, but the milk powder industry had an agreed code of practice designed to keep hygienic standards high. Later the Food Standards Committee Report on Dried Milk (Ministry of Agriculture, 1962) set out a schedule of standards of constitution, but this did not cover bacteriological content.

Delivery methods

In regard to delivery to the customer, the relative merits of bottle and carton are not yet widely appreciated. Dixon (1961) has outlined the advantages of milk cartons. 'Tetra Paks' are used in fifty-three countries. In Sweden they are used for 70 % of total milk distribution and for 100 % in some cities. In London, the Express Dairy Company delivers all half-pints of milk in these cartons; and in Essex, Lord Rayleigh's Farms Inc. carry out a retail distribution of some 10000 gal daily in them, at the same price as bottled milk. Although the glass bottle still has its adherents, as shown by Brooks (1962), the problem is one of public awareness. In spite of the obvious advantages of cartons in comparisons with bottles, it is not yet generally agreed that the two methods of packing are similar in cost.

Burton (1960) says that it is becoming recognized that it is not possible to sterilize, by use of the conventional bottle-washing system alone, the inside surfaces of milk bottles to a standard suitable for aseptic filling processes, and that it seems necessary for the washing to be followed by a separate sterilizing process. The relatively new 'uperization' process (an ultra-high-temperature treatment) offers germ-free milk with an automatic sterile filling process (Alpura Ltd., Berne). It is claimed that uperization results in total destruction of all bacteria whilst retaining the characteristics of raw milk, e.g. vitamin content.

The increasing development of the milk vending machine is a feature of current interest. The Milk Marketing Board states that at the end of June 1962 about 6000 milk vending machines were in use. These machines must dispense designated milk, but about half of them apparently purvey raw milk (T.T.)—fifteen of the twenty-nine in Bristol do so (F. J. Redstone, pers. comm.). Current legislation excludes 'catering sales' from the requirement to hold a licence for these machines, and milk vending machines in this category could escape sampling by the licensing authority. Raw milk from machines provides a small but distinct and random opportunity for the ingestion of raw milk containing disease organisms (Bothwell, 1960; Howard, 1962).

PUBLICATIONS BY THE WORLD HEALTH ORGANIZATION AND THE FOOD AND AGRICULTURE ORGANIZATION

There have been a number of publications from these organizations of the United Nations which are relevant to the problems of milk-borne disease. The Joint FAO/WHO Expert Committee on Milk Hygiene (1960) in its second report dealt with the hygiene of liquid milk, hygiene control of the preparation of milk products, water supply in relation to milk hygiene, and the special problems of milk hygiene in warm countries. It reported that staphylococcal infection derived from bovine mastitis has taken the place in many countries of mastitis caused by streptococci. The indiscriminate use of antibiotics by intramammary infusion in the treatment of mastitis has resulted in the production of antibiotic-resistant staphylococci, with resultant epidemiologic and therapeutic problems when dissemination occurs in the human population. Rapid cooling of all milk to 10 °C or lower would prevent the formation of staphylococcal enterotoxin. The use of inferior quality milk for cheesemaking is condemned, as it often leads to retention in the cheese of staphylococci and enterotoxin.

This Committee refers to the possible development of 'markers' to detect low levels of antibiotics in milk at farms, collecting stations and dairy plants, the most promising results being obtained with dye preparations and chlorophyll. The report deals also with insecticides and radionuclides in milk. Urgent studies, it says, are required on the problem of insecticides in milk and on the monitoring of milk for radionuclides. It quotes the Joint WHO/FAO Expert Committee on Methods of Radiochemical Analysis (1959) as follows:

> Monitoring of milk is important, because this food item is a major route by means of which environmental contamination reaches the human population in many countries. In addition, levels of certain radionuclides in milk are a reliable reflexion of the contamination of the cow's diet consumed within the past few days. In general, sampling procedures should be governed by local and regional conditions and the objectives of the analyses. Sampling can be done at collection centres or processing plants, so that bulk supplies or individual units can be sampled systematically. Depending upon the objectives, pooling and reduction of sample size can be easily accomplished. Depending upon the source of contamination, it may be desirable to measure one or more of the following radionuclides in milk; ^{89}Sr, ^{90}Sr, ^{137}Cs, ^{131}I, and perhaps ^{140}Ba and ^{226}Ra. When ^{131}I or ^{140}Ba are to be measured for checking recent or variable contamination, it is essential to complete the assay as soon as possible and to record the times of sample collection and assay, because of the short half-lives of these nuclides.

The Report deals at some length with diseases transmitted through milk products, and it is emphasized that ageing in itself does not ensure freedom from pathogens, which can persist in cheese for many months. A useful code of principles governing milk and milk products is given as an appendix.

The Committee also refers to the more recently recognized tick-borne encephalitis and its transmission by either tick bite or raw milk, its excretion in cow's milk having been demonstrated experimentally. Heating to 65–70 °C for 20 min is necessary to destroy this virus, 60 °C for 20 min being insufficient. Butter can maintain the virus for 2 months at 4 °C, and curds for about 2 weeks. Prevention of the disease therefore can be accomplished by heating the milk to temperatures and for periods of time above levels previously indicated. Since these time-temperature levels are different

from those commonly used in pasteurization procedures, the Committee strongly recommends further research on the point.

A WHO Monograph on Milk Hygiene has been compiled by Kaplan *et al.* (1962). This is an extremely comprehensive study of the subject, with approximately 350 references, and it should be a basic source of data in this field. These authors list the viral, rickettsial, bacterial, clostridial, protozoal, helminth and other diseases transmitted to man through milk. An important part of the study deals with heat destruction of organisms, and they say:

> It is often stated that no authentic outbreak of disease has been attributed to properly pasteurized milk. This claim is valid if the term 'properly pasteurized' is taken to indicate that three conditions have been fulfilled: (1) the milk was adequately cooled and kept cool before pasteurization so as to prevent the formation of heat-resistant staphylococcal enterotoxin; (2) the pasteurizing equipment was functioning adequately; and (3) precautions were taken to prevent post-pasteurization contamination. Unfortunately it often happens that one or more of these prerequisites is lacking and this is responsible for much milk-borne disease. Two points may be noted however. Firstly, modern microbiological studies have shown that temperature inactivation is a much more complicated phenomenon than was formerly believed; future studies, especially with micro-organisms of borderline susceptibility to some pasteurization temperatures now used (Q fever, tuberculosis), should take this into account (see, for example, Daoust, Read & Litsky, 1961). Secondly, the survival of pathogenic viral agents requires careful study with the latest virological techniques, developed only in the last few years. Apart from very few investigations made some years ago, no adequate studies have been reported on the survival of pathogenic viruses in milk. On the basis of practical experience over many years however there seems no reason to doubt that the pasteurization procedures now used, especially those involving the higher temperature range, are adequate for protection, in spite of certain theoretical possibilities indicated in laboratory experiments.

These authors also deal with more recently recognized viruses likely to produce milk-borne disease, e.g. the enteroviruses and also certain strains of *Escherichia coli*. Infectious hepatitis must, they say, be considered as one of the most serious viral diseases of which milk may be an important vehicle of transmission; they refer to the observations of Poskanzer & Beadenkopf (1961) on the inability of chlorination to destroy the virus under certain circumstances. They refer to the urgent need to ensure national notification of milk-borne diseases, which they list.

In addition to the micro-organismal conditions, antibiotics, milk allergy, toxic chemicals, preservatives, disinfectants, radionuclides, plant toxins, flavours and drugs excreted in milk, and other causes of unwholesome milk are covered. An example of the completeness of this work is the interesting reference to the epidemics of glaucoma associated with dropsy in India and traced to contamination of cooling oils with the seed-oil of prickly yellow poppy (*Argemone mexicana*) which contains the alkaloids sanguinarine and dihydrosanguinarine. It is noted that recently Hakim, Mijovic & Walker (1961) have suggested the possibility of sanguinarine being excreted in the milk of cows feeding on fumaria weeds (e.g. *Fumaris officinalis*) in poor pastures, these authors believing that the wide geographical distribution of these weeds may be of significance in relation to the widely prevalent endemic glaucoma of man—an observation of the kind which might have profound significance for the epidemiology and ecology of human disease.

The Food and Agriculture Organization of the United Nations (1962) have published a study of Animal Disease and Human Health. Written for non-specialist

as well as specialist readers, this publication describes the main zoonoses and the main threats to animal health. The estimated world animal population, as given in tabular form, is shown in Table 2.

Table 2

Domestic animals	Estimated number in millions	Percentage of world population
Sheep	967·0	33·0
Cattle	890·0	32·0
Pigs	485·5	15·0
Goats	340·8	12·3
Equines:		
Horses	70·2	2·3
Mules	15·8	0·6
Asses	33·5	1·3
Buffaloes	94·5	3·2
Camels	10·3	0·3
	2907·6	100·0
Chickens:		
Broilers (meat)	2400·0	72·0
Hens (laying)	600·0	18·0
Turkeys	150·0	4·0
Ducks and geese	200·0	6·0
	3350·0	100·0

The FAO has also published a monograph by McDiarmid (1960) on 'Diseases of Free-living Wild Animals'. There is now much evidence that wild life must seriously be considered in schemes for the control and eradication of diseases in livestock and this comprehensive monograph gives an excellent summary of the information available on the subject. This study will be a standard reference in regard to the interrelationship of human epidemiology and animal epizootiology. The extensive bibliography is divided into sections at the end of each chapter.

Perhaps the most basic necessity for attempting to deal with diseases is that the information about them should be complete. In fields other than milk-borne diseases, much has been written in recent years about the inadequacy of the data available about disease and health services—an inadequacy of such proportions that conclusions about the nature of the problems or how they are being dealt with can hardly be drawn. There are deficiencies in the material that is available when it is officially asked for, but what is worse are those cases where the primary information is not even officially asked for.

The World Health Organization (1959), in a survey of existing legislation on notification of communicable diseases, cites four reasons for notification, namely: (1) it gives information as to incidence—rates, age and sex, time, place; (2) it is essential for immediate local and general control; (3) it ensures provision of appropriate care, including hospital care if necessary; (4) it provides a comprehensive list of cases requiring further study and research, without which no progress is possible.

The conclusion of this WHO survey reads as follows:

> Of the factors for success in the conquest of communicable disease—one of the compelling problems of our time—the regular notification of cases to the health services by medical

practitioners is undoubtedly one of the most important; for without such information the health authorities are unable to take the necessary measures. This collaboration between medical practitioners and the health services must be based on suitable regulations. This survey of the legislation of about fifty countries brings out the existence of important differences in their notification requirements as to both the number of notifiable diseases, which may vary between ten and seventy, and the notification procedure. The terms used to designate the scheduled diseases are often vague; and this imprecision, together with the additional burden on medical practitioners in some countries to notify more than one authority, may result in discouraging them from collaborating with health authorities to the fullest extent. Various attempts to improve notification regulations have been made both on the national and international level. Incompleteness of notification and differences which exist between countries in the methods of notification impede accurate comparative studies that are valid on the international level from being made; yet such studies are important both for the countries themselves and for the success of the world-wide campaigns undertaken by the WHO.

In the list of infectious diseases notifiable in various countries, given in this survey, there are variations in regard to the milk-borne diseases; for example, England and Wales constitute a small area in which brucellosis, the major milk-borne disease in that area, remains unnotifiable. The lesser milk-borne diseases like salmonellosis and tuberculosis are notifiable. It is clearly time for adoption of an international schedule of notifiable diseases, so that prevention will no longer be hindered simply by lack of knowledge of their existence.

THE RELATIVE IMPORTANCE OF THE MILK-BORNE DISEASES

Leech, Davis, Macrae & Withers (1960) compiled a report based on a national survey of disease, wastage and husbandry in British dairy herds in 1957–58. They give the figures shown in Table 3. Acute mastitis and Johne's disease were the two

Table 3. *National estimates of total depreciation in market value caused by four disease conditions in* 1957–58

Disease	Estimated total depreciation £
Mastitis	540000
Johne's disease	626000
Injuries and accidents	520000
Trouble at calving	316000

diseases causing the greatest total loss through depreciation in market value. The report continues as follows:

> The outstanding feature of the diseases of dairy herds was the importance of diseases peculiar to the female, particularly mastitis and those closely associated with parturition. Of the remainder, the most important specific diseases appear to have been Johne's disease, grass tetany, and foul-in-the-foot. There were marked differences between breeds, sizes of herd, and different parts of the country, in the incidence of several important diseases.
>
> The following features of the incidence of certain disease conditions, deserve further and more precise investigation: 1. The implication of the seasonal curve of abortions that the main reservoir of brucellosis infection may be the apparently normal cow excreting infection after a full-term parturition; 2. The relatively high incidence of stillbirths at autumn and winter parturitions, particularly of heifers, compared with spring and summer. The feeding of in-calf heifers and dry cows might merit investigation; 3. The regional differences in the incidence of acetonaemia and grass tetany, which were strikingly similar for the two diseases.

Smith (1960) found *Mycobacterium johnei* in the milk of one out of twenty cows suffering from clinical Johne's disease and this supports the view of Doyle (1954) that its excretion in the milk is infrequent. Its absence from fifty-two herd samples from ten farms where Johne's disease was a serious problem indicated that in affected herds contamination of the milk is not heavy or common. Although the disease is a serious problem in animals, it is not transmissible to human subjects.

Steele (1961), in a review of animal diseases transmissible to man, says that brucellosis is probably the most widespread of zoonoses, with estimated human infection of hundreds of thousands. In the U.S.A. in 1947 the infection rate in cattle had been estimated at 20–30%, in swine 5–10%, while more than 10000 cases of human brucellosis were diagnosed. In 1958, after a national campaign to eradicate brucellosis, less than 800 human cases occurred and most of these were among persons in occupational contact with animals and animal products. By the end of 1960, twenty-six States showed less than 1% of cattle with positive serum agglutination. During 1961 brucellosis in swine and goats was receiving more attention with a view to elimination. Sheep brucellosis was never a disease entity of any consequence in the U.S.A., although it is a major animal and human health problem in the U.S.S.R., according to this author. In Southern Europe, Africa and Asia goats are the main source of infection for human brucellosis.

Brucellosis must therefore take first place in any assessment of relative significance of milk-borne disease in countries where bovine TB is controlled. Though mastitis is present in up to 30% of animals, only 25% of this is staphylococcal and when excreted in the milk even this pathogen will not produce toxin if the milk is cooled immediately. Brucellosis is transmissible from the freshest milk and causes chronic disease as well as death in animals and men, while staphylococcal infection is an enteritic toxaemia, brief in duration and, so far as is known, without chronicity or fatality. Furthermore, even if it were possible to eliminate staphylococcal mastitis completely from animals, there would still remain in man himself a reservoir of strains possibly more virulent than those in the cow. Though Johne's disease is also a significant animal problem it is not transmissible to man so far as is known and its secretion in milk is very uncommon.

BRUCELLOSIS

General

The World Health Organization (1962) recorded the incidence of human brucellosis, in all countries where the disease is notified, between April 1961 and March 1962. The highest numbers of cases were in Italy (5663) and Spain (3718), with an incidence ranging from twenty to 600 cases in other European countries. There were no cases in Denmark and Sweden, where the disease has been eradicated. No cases are reported in England and Wales, but this is because brucellosis is not notifiable to local health authorities there; in fact, some 90–100 cases per annum are known to occur, from the Public Health Laboratory Service returns, but this is a laboratory diagnostic return and not a legal notification system carrying the right to investigate.

In the U.S.A. in 1961–62 there were 530 cases, in Canada 113, and in Peru 178. In Asia the main known incidence was in Iran (1166 cases). In Australia there were

forty-five cases and in New Zealand forty-one. Algeria had forty-five cases; there were only about twenty reported in all other parts of Africa.

Steele (1962) reported that the annual incidence in the U.S.A. decreased from 6321 cases in 1947 to 580 in 1961. The decline had been less rapid in recent years, partly because swine brucellosis had remained a problem and had become the principal source of occupational human disease. The estimated rate of infection in swine was 1–2%, with about 5% of herds affected. Pigs are raised on about 1847000 farms in the U.S.A. and some 850000 persons are said to be exposed in handling them; about 131000 herds are infected, exposing this number of farm families. Distribution of human brucellosis is highest in the mid-west, particularly Iowa, Kansas and Illinois, followed by other mid-west areas, and southern states including Texas. Most cases in the south are related to persons handling infected animals or ingestion of contaminated milk products, while in the north and west packing-house workers and butchers are mainly affected, although milk is involved in some cases.

Also in the U.S.A., Schirger, Nichols, Martin, Wellman & Weed (1960) gave an account of experiences with 224 patients, more than half of whom were occupationally connected with animal husbandry. In patients with bacteremic brucellosis, agglutination titres were 1/200 or more. Most of them had clinical manifestations of severe systemic infection and they were treated satisfactorily with tetracycline and streptomycin. A few patients with localized infections had recurrent constitutional symptoms and in these cases agglutination titres often were low or absent; surgical drainage or excision of localized infections was of value in diagnosis and treatment. *Brucella suis* was the strain most commonly isolated from localized lesions.

The *Canadian Journal of Public Health* (1960) discussed a programme for brucellosis control in Canada, which had commenced in 1957–58. By March 1960 over 3×10^6 cattle (about 27% of the cattle population) were under supervision, i.e. they were either in the 134 areas where testing was under way or in the 107 areas which had been certified as having a level of infection not exceeding 1% of the cattle population and 5% of the herd population for 3 years. Over 37000 reactor animals had been identified and slaughtered. Results indicated that bovine brucellosis could be eradicated under Canadian conditions.

The World Health Organization (1961*a*) stated that brucellosis was common among sheep and goats in the U.S.S.R., where these animals were the source of 85–90% of human contact cases with clinical symptoms. Human disease transmitted through milk and milk products had been reduced considerably by hygienic processing methods. Between 1952 and 1958 vaccination had reduced human disease by nearly 60%, and over a 5-year period the frequency of cases among exposed persons gave an average of 0·5% for vaccinated persons and 12·3% for unvaccinated.

A report from the FAO/WHO Brucellosis Center in Japan (1961) stated that brucellosis there had been observed mostly among dairy cows, the type of infecting organisms being different between imported Jerseys (*Br. abortus* type I predominant) and domestic Holsteins (*Br. abortus* type II). It was considered highly probable that man and domestic animals other than dairy cows show non-specific reaction to the agglutination test—a point on which there is not much published work.

In France, Jacotot (1961) considered that bovine brucellosis was a threat to the agricultural economy, extracting a toll two or three times that which tuberculosis

took before the introduction of an eradication scheme. Brucellosis was a growing danger to the population; it could very easily cause characteristic clinical disease and in many other cases caused an inapparent state of infection which aggravated other concomitant infections.

A village epidemic of brucellosis in Israel, due to *Br. melitensis* from infected sheep and goats, was investigated by Rozansky, Weber, Lehman & Bali (1961). Of 309 inhabitants, eighty-four were infected and seventy-nine clinically ill with brucellosis. Of forty-six families only nine escaped infection. The population at risk was comprised mainly of herdsmen and animal handlers. All age groups were susceptible, but morbidity increased with age, correlated with increased risk of exposure in older age-groups. Severity of illness varied considerably but there were no fatalities and all cases responded to antibiotic therapy. *Br. melitensis* was isolated from twenty-eight out of thirty-six patients subjected to blood culture, and specific agglutinins at an acceptable level were demonstrated in all but three of those who were clinically ill. The number of persons without evidence of clinical brucellosis who had significant brucella agglutinins was very small. Persistence of specific agglutinins up to 2 years after recovery from clinical illness was recorded.

In South Africa, Schrire (1962) reviewed the history, incidence and distribution of human brucellosis. He reported the results of a survey of 116 brucellosis patients and also a serologic survey of 142 Africans. Between 1920 and 1959, 513 cases of human brucellosis were notified. The distribution of the 116 survey cases showed a high proportion from the eastern Transvaal and S.W. Africa, indicating the endemic nature of the disease in those areas, where cattle and goats are the probable reservoir animals. Of these 116 cases, forty-six were at occupational risk, comprising veterinary surgeons, stock inspectors, cattle farmers, butchers and laboratory workers handling infected animals or material. The sex incidence was 77 % male. A number of cultures from different parts of the country had been identified and it is evident that both *Br. melitensis* and *Br. abortus* have been isolated from human sources in several regions.

Other reports of brucellosis in South Africa have been published by Lewis (1959), Kirsten (1961), and van Drimmelen (1961). Kirsten believes that brucellosis can be responsible for spontaneous human abortion and he refers to work done to show that about 20 % of placentas from a series of spontaneous abortions in an endemic area showed positive agglutination tests. He refers to handling infective material as a cause of infection and in these cases it could occur via the gastro-intestinal tract. After investigating the histories of women whose husbands had the disease, he believes transmission by the venereal route is possible and he points out that in animals this is a recognized route of infection. Van Drimmelen says that four epidemiologically distinct forms of the disease exist in South Africa: *Br. melitensis* in goats and sheep; *Br. abortus* in cattle; *Br. abortusovis*, common in small stock, but self-limiting and not known to be transmitted to man; and *Br. ovigenitalium*, causing infectious infertility of rams and also not pathogenic for man or other animals. The two last names are Van Drimmelen's suggestions for Brucella organisms with intermediate characteristics between *Br. abortus* and *Br. melitensis*.

Alton (1960) has reported on the frequent occurrence of dissociated strains of *Br. melitensis* in Maltese goat milk—less pathogenic and markedly less agglutinogenic

for rabbits than smooth strains. An antigen not present in smooth strains was demonstrated by agglutinin absorption methods.

Diagnosis

Although a positive blood culture is a definitive diagnosis, it has to be accepted that this procedure has only yielded a low percentage of successes in most people's hands, e.g. less than 5 % in series in the U.K. where *Br. abortus* is the predominant organism, though as high as 50 % or so when *Br. melitensis* is the pathogen. In the remaining 95 % of *Br. abortus* cases, reliance has to be placed on the tube agglutination test. Here interpretation is crucial. Wilson (1955) and Dalrymple-Champneys (1960*b*) put 1/80 as the diagnostic titre in the presence of other indications—i.e. clinical indications. Spink (1960) puts it at 1/100 (using American antigen). Rising titres are significant; high titres occur, however, with no apparent or known illness, and very low ones in clinically definite cases.

Wallis (1960) has written of the problem of diagnosing psychiatric conditions where the true diagnosis is brucellosis, and Evans (1961) has again drawn attention to the unreliability of diagnostic tests in chronic brucellosis and the 'intolerable situation of patients suffering from chronic brucellosis who live for years under the shadow of a diagnosis of neurasthenia, which vaguely implies malingering'.

The problems of diagnosis and interpretation of the epidemiologic significance of raised titres in brucellosis are not yet solved, and categoric statements about the significance of this or that titre require careful assessment. WHO/FAO antigen and other national antigens are sometimes used in different laboratories in the same country and the results of tests with these antigens are not comparable.

A number of papers describe culture methods. Brinley Morgan (1960) found that a basal medium containing 0·5 % (v/v) Tween 40 was able to support the growth of 80 % of the *Br. abortus* Wilson Type II strains studied. Other strains of *Br. abortus*, *Br. suis*, *Br. melitensis* and intermediate types grew on the basal medium either with or without the addition of Tween 40. The Tween agar medium containing the antibiotics bacitracin, polymyxin, and actidione (with or without ethyl violet) as well as the Albimi-antibiotic medium, would not support the growth of *Br. abortus* type II strains.

Richardson (1959) has utilized monolayer tissue culture to study the multiplication of *Br. abortus* in cells from various organs of the cow. *Br. abortus* was shown to invade and multiply rapidly within bovine cells from foetal skin and kidney as well as adult uterine mucosa, testes, spleen, bone marrow and lung.

Calderone & Pickett (1961) described the use of 5-day embryonated eggs, Albimi's brucella agar, and egg yolk, which were compared as to their suitability for supporting growth of brucellae from blood of rabbits experimentally infected with these organisms. Tubed egg yolk was found to be a superior medium for the growth of brucellae from small inocula.

Fogel & Lewis (1960) reported successful culture of *Br. melitensis* from the liver tissue following percutaneous needle biopsy in a case where repeated blood culture was negative. In their case it was important to establish, for medico-legal reasons, the precise identity of the aetiologic organism.

Hauschild & Pivnick (1961) report that continuous culture of *Br. abortus* S. 19 can

be carried out for 3 weeks with populations up to 2×10^{11} viable cells/ml, without establishing non-smooth variations; but before continuous culture can be used for vaccine production several additional problems have to be considered and continuously-grown S. 19 must be tested on animals. Revick, Walker & Pivnick (1916) reported a case of oral infection with S. 19 vaccine in a laboratory worker.

Olitzki (1959) found that *Br. abortus*, *Br. melitensis* and *Br. suis* possess at least six soluble antigens, which could be demonstrated by the use of the agar gel precipitation technique. These antigens differed in their relative concentration in bacterial extracts of different origins and in their ability to produce antibody titres in immune sera. No antigen specific for a single species was demonstrated.

Clinical aspects

One of the measures of the importance of brucellosis as a human pathogen lies in the multiplicity of lesions and the chronicity of the disease. In this connexion, Schirger, Dearing & Waugh (1959) described a case of brucellosis of 17 years' duration, in which the diagnosis was substantiated by culture methods. Asymptomatic splenomegaly detected on routine roentgenograms of the thorax, results of blood studies suggestive of hypersplenism, and elevated brucella agglutinin titres provided the initial clues to the diagnosis.

Kelly, Martin, Schirger & Weed (1960) observed that brucellosis of the bones and joints appeared to be primarily a disease affecting men engaged in occupations relating to animal husbandry. Among twenty-two patients at the Mayo Clinic with culturally-proved skeletal brucellosis, the agglutination titres of serum were often low or absent. *Br. suis* and *Br. abortus* were the strains most commonly isolated from skeletal lesions. Osteomyelitis was the hardest of all the lesions to treat successfully; they found that treatment with a combination of tetracycline and streptomycin gave satisfactory results.

Again, Aguilar & Elvidge (1961) considered that the radiologic and pathologic appearance of advanced brucellar spondylitis suggested that destructive invasiveness begins in the intervertebral disk rather than as an extension from the bony matrix; they described a case which demonstrated primary involvement of the disk based on histopathologic evidence of destruction of the nucleus pulposus, microscopic invasion of the cartilaginous plate and absence of changes in the bone. The special affinity for the intervertebral disk, a notochordal derivative, they thought might be explained by the predilection of the brucella organism for embryonic tissues, and that brucellosis might be added to the few entities that cause particular involvement of the intervertebral disk.

Peery & Belter (1960) have reviewed forty-four cases of fatal brucellosis in the literature, with particular reference to heart disease. These cases were all culturally proved and they excluded all other cases from their consideration. Endocarditis was by far the commonest manifestation (80 % of cases). It occurred in 95 % of *Br. abortus* infections, 69 % of *Br. melitensis*, and 43 % of *Br. suis* cases.

Konwaler, Carpenter & Ohno (1960) studied cardiac pathology in thirty-seven guinea-pigs, at intervals of from 39 to 383 days after a single inoculation with either *Br. suis* or *Br. abortus* S. 19. The experimental evidence indicated that

brucella infection causes microscopic lesions in the heart of the guinea-pig and that the heart is not as resistant to the infection as might be inferred from gross examination.

Griffith & Norris (1961), in an analysis based upon the study of 144 proved cases of brucellosis and the study of 275 autopsied cases of calcific aortic stenosis, were unable to determine any aetiologic relationship.

Tapie, Monnier, Frejevu, Mole & André (1960) describe a case of brucellotic perinephritis. Circulatory collapse had to be dealt with by noradrenaline and corticosteroids. Biopsy confirmed the diagnosis and a pure culture of *Br. melitensis* was obtained.

Phages

At a World Health Organization round-table discussion on brucella phages (1961*b*) representatives of European countries and the U.S.A., under the chairmanship of A. W. Stableforth, summarized the current information on phages. They intimated that a review of all phage publications would be produced. They thought the brucella phages might not be as useful as the salmonella phages for classification and epidemiology, since they did not show enough diversity in their host range and serology.

Brucellosis in hares and dogs

Brucellosis was found in hares in Czechoslovakia (*Czech. J. Socialist agric. Sci.* 1961), in all three regions, nineteen districts and thirty-eight localities, and was usually connected with pigs infected with *Br. suis* and in some cases with infected cattle. Domestic animals were generally considered free of the condition, though in some instances transfer of infection from hares to domestic animals was thought to occur. All strains recovered were typical *Br. suis* and eradication of brucellosis from hares was thought to be necessary.

Schnurrenberger (1961) reported brucellosis in a 4-year-old male bloodhound; listlessness, anorexia, swelling of scrotum and testes, and puffy swelling of the right tibial-tarsal joint developed after exercise; the swelling of the hock joint and scrotum were reminiscent of orchitis and hygroma in bovine brucellosis. Agglutinins were positive and the condition apparently cleared in 4 days.

In connexion with the infection of dogs, there is no evidence that they are vectors or intermediate hosts for brucellae. Any role they may have is simply as mechanical carriers of, for example, conception products or aborted foetuses.

McDiarmid, in 1960, summarized the state of knowledge up to that date on the carriage of brucellosis by wild animals.

Vaccination against brucellosis

Hoptman (1959) has made an extensive review of the literature on brucellosis from the U.S.S.R. (150 references) and has referred to the early work of Vershilova on vaccination, originating in 1946. This latter author (Vershilova, 1961) has recently described extensive action in Russia to eliminate brucellosis among farm animals and to protect human beings by vaccination with live vaccine. Although there has been no decrease in animal infection in a number of districts, vaccination reduced human infection by nearly 60 % between 1952 and 1958. The vaccine consisted of a bovine

strain of brucella, *Br. abortus* 19-BA, a strain from a dissociated colony of a culture of *Br. abortus* 19 that proved more effective than strains of *Br. melitensis* and *Br. suis*. Cross immunity exists and epidemiology seems to show that brucellosis of cattle or pig immunizes against sheep brucellosis. The dose by subcutaneous administration should contain $4–6 \times 10^8$ viable brucellae. Observation over 5 years of frequency of cases in persons exposed to infection gave an average of 0·5 % for vaccinated and 12·3 % for unvaccinated; while in brucellosis areas 200000 persons were vaccinated and the incidence was 3·3 times less than among unvaccinated. Cutaneous administration seemed to give as good results as subcutaneous. Revaccination is done a year later to establish solid immunity.

Olitzki (1960) in Israel, vaccinated 223 persons with a streptomycin-dependent strain of *Br. abortus* 19. Severe post-vaccinal reactions were absent, though very slight rises in temperature and slight local swelling were found to deter people from vaccination. Peroral administration of the vaccine did not provoke post-vaccinal reaction. Given orally, at least 10^8 cells of *Br. melitensis* Rev. I (the strain used) were required to develop a rise in agglutinin titre.

Elberg (1961) reported that injection of the Rev. I strain of *Br. melitensis* into *Cynomologus phillipinensis* raised resistance to infection by 1 to 3 powers of 10 compared with non-immunized animals; bacteraemia was rarely observed; no alteration of virulence on passage subcutaneously or intravenously occurred in the goat or guinea-pig.

The use of *Br. melitensis* vaccines in control of animal infection has been reviewed by the World Health Organization Veterinary Public Health Unit (1961*c*).

Therapy

Spink (1960) has examined the current status of therapy for brucellosis in the human subject. He states that agglutination titres of 1/100 or over are consistent with active disease, and that it has been established in experimental brucellosis and in man that antibiotics will suppress the infection, reduce complications, and shorten the course of the disease. The tetracycline drugs constitute the treatment of choice and are recommended in doses of 500 mg every 6 h for a minimum of 21 days, orally. If the drug is well tolerated, it can be repeated if a relapse occurs. In the more severe cases, 1–2 g of streptomycin daily for 14 days can be administered intramuscularly. Relapse may take place two or three times before recovery and the treatment may be repeated each time; but Spink thinks it doubtful if more than three courses of antibiotic therapy are beneficial, except in the rare patient in whom suppuration is present. He comments that at present neither vaccine nor antibiotic therapy can be recommended for acute or chronic illness unless the diagnosis of brucellosis can be supported by dependable bacteriological or serological data. Subjective complaints, with or without a positive brucella skin test, do not constitute sufficient evidence for a diagnosis of active brucellosis.

In experimental brucellosis of mice, Spink & Bradley (1960) indicated that intermittent therapy of two periods of 1 month each resulted in about the same degree of suppression as that obtained with prolonged continuous therapy.

Britain

It is generally accepted that *Br. abortus* is the predominant type of infecting organism in Britain, a reasonable assumption since this is the organism grown from positive human blood cultures, except in the case of *Br. suis* in one instance (Williams, Entwhistle, Masters & Woods, 1957) of a child in Ireland. *Br. melitensis* has not been reported from a human case in this country. Blood cultures are positive in 1–4 % of cases in series of human brucellosis in this country, so that the assessment of infecting organism is based on a very small sample of the total.

In milk and products of conception there have been reports of an increase in the proportion of *Br. melitensis* and its variants. Stableforth (1960*a*, *b*) and W. J. Brinley Morgan (pers. comm.) report a continuing rise in the percentage of *Br. melitensis* and melitensis-like organisms in material and milk submitted, from thirty-eight herds infected in 1940–50 to a total of 112 herds infected in 1951–56, while strains from milk and genital sources isolated in Britain in 1958–59 (415 strains) showed 8·7 % *Br. melitensis* in milk and 11·4 % from genital sources.

An analysis of brucella strains typed between October 1956 and December 1960 at the Brucella Reference Laboratory (J. M. Payne, pers. comm.) shows that there has been a considerable rise in the isolations of *Br. melitensis*, and a reduction in the dye-sensitive strains by about half. In that period of about 4 years, only eight strains of brucella from human sources were typed. In the same period the number of human brucellosis cases reported to the Public Health Laboratory Service would, on an average of ninety per annum, total nearly 400, so that apparently in only about 2 % of human cases is the organism available for typing. If more blood cultures were positive and the organism typed, it is possible that *Br. melitensis* might be isolated.

Dalrymple-Champneys (1960*a*) reviewed the global situation in regard to brucellosis and referred to the epidemiologic potential of enzootics in swine traced to hares infected with brucella organisms, infection in hares having been reported from France, Germany, Denmark and Switzerland. He wondered if the attempts to find wild carriers had been thorough enough. The emergence of *Br. melitensis* variants in Britain might present a problem. He said it was estimated that brucellosis in cattle cost the U.S.A. about £3½ million in 1947, and that the figure for Switzerland was probably £1½ million at the time of writing.

A *Lancet* editorial (1961) considered that brucellosis should be the next disease to be eradicated, following the extinction of bovine tuberculosis. Though all milk-borne disease could be abolished tomorrow if all milk was pasteurized, and even though more than 90 % of it is now, the writer thought it asking a lot to demand that every pint produced in the depth of the country be collected, pasteurized, and returned before a drop could be drunk by the producer and his family. Probably less than 500 developed undulant fever each year out of half a million people who drink raw milk. Universal pasteurization would be unlikely to reduce the incidence of infection by as much as half. (In fact, overall recent PHLS figures indicate that about 70 % might be eliminated by pasteurization: author.) To free man of undulant fever, no measures short of eradicating brucella infection in cattle would suffice.

Bothwell (1960) has reviewed the epidemiology of brucellosis in Britain. He referred to the eighteen fatalities between 1950 and 1958. Hepatitis occurred in five

cases (two with cirrhosis), two had endocarditis, and one pulmonary embolism. Of seventeen with known occupation, six patients had some connexion with farming. Table 4 shows percentages of milk pasteurized and a summary of liquid milk sales to final consumers for June 1959.

Table 4

Percentages of milk supply pasteurized in 1959

	%
England and Wales	94
Scotland	78
Northern Ireland	93
Eire: Dublin	87
Other urban areas	75
Rural (46 % of population)	nil

Summary of liquid milk sales to final consumers, June 1959

(Quantities in millions of gallons.)

	Total	Heat-treated		Untreated
		Bottled	Bulk	
Producer retailers	5·0	0·8	—	4·2
Depots	0·9	0·7	0·2	—
Processing dairymen	73·9	70·4	3·0	0·5
Non-processing dairymen	33·2	30·2	1·4	1·6
Totals	113·0	102·1	4·6	6·3

He points out that the routine distribution of T.T. milk (some of which is raw) tends to occur more in some sections of the community than in others and tends to be associated with higher income levels. The main markets for T.T. farm bottled milk are rural areas and suburban areas of large towns. Table 5 gives detailed information concerning social classes and income groups, obtained during a 1958 survey and described in the National Milk Publicity Council's booklet, *How Housewives Buy Milk.*

Attention is drawn to the inadequacy of current legislative provision for the control of brucellosis, particularly the need for general notification of brucellosis in human subjects and the need to unify the legal aspects of brucellosis in one piece of legislation.

Table 5. *T.T. milk received by households from the milkman*

	Number having milk delivered of any type	T.T. milk excluding Channel Is., %
Class:		
Well-to-do and middle-class	100	40
Lower middle-class	532	32
Working-class and poor	1125	23
Income:		
Under £520 (head of household)	816	22
£520–£780	464	29
£781–£1196	154	36
£1197 and over	61	56
No information	262	23
Total	1757	26

Serology and skin tests

Brodigan, McDiarmid, Mann & Skone (1961), in their study from the Isle of Wight, found that 5 % of 132 psychiatric in-patients had titres of 1/80 or more, and 3 % of 1395 blood donors and pregnant women had titres of 1/80 or more (using PHLS antigen). These figures are much higher than those reported by Bartram, Bothwell, Jebb, McDiarmid & Preston (1962) in Oxfordshire, namely, 0·23 % in sera from 3431 pregnant women at or over 1/100 (PHLS antigen), and 0·41 % or 8690 blood-donor sera at or over 1/10 (FAO antigen), and it was concluded that this was most likely due to more exposure to infected raw milk in the Isle of Wight than in the Oxfordshire area, the 1959–60 proportions of pasteurized milk being 77·3 % and 90–95 % respectively. In the latter paper (Bartram *et al.* 1962) the authors concluded that the presence of *Br. abortus* agglutinins detected by the tube test was not associated with human abortion in thirty-five pregnant women studied, but they suggested that the problem should be studied in a series using blocking antibody tests. They also suggested that the follow-up of positive serum agglutinins could lead to finding an infected herd of animals, and did so on five occasions in the series.

By comparison, Moreno & Castaneda (1961), in Mexico, found 0·83 % suspicious brucellosis vectors (thirty-two cases) in a group of 3819 blood-bank donors over 16 months, implying, they suggested, a serious risk to the potential recipient. They used the surface fixation reaction with brucella antigen. In Japan, Uede *et al.* (1960), using FAO antigen, found 1·5 % at 1/20 in milk industry personnel, 2·5 % at 1/20 in slaughterhouse workers, and none at 1/20 in healthy adults. Shibata, Suzuki, Isayama & Shimizu (1961) stated that instability of brucella titres in animal sera can be due to incubation periods and temperatures, repeated freezing and thawing, exposure to sunlight, and shaking—all of which seem to decrease the titre.

Brodigan *et al.* (1961), in the Isle of Wight, also carried out skin tests, which indicated infection in about 17 % of the adult population. Of 685 children, 5 % gave positive skin tests. Raw milk was drunk at home by 36 % of children attending urban schools and by 67 % of children in rural schools. The incidence of positive skin tests was highest (17 %) among eighty-five children who attended rural schools and drank raw milk at home. On the basis of single examinations, thirty-three out of 520 registered dairy herds on the island were found to be producing milk infected by *Br. abortus*. These authors also obtained data on the incidence of agglutinins and skin sensitivity among adults occupationally exposed to infection by contact with animals, as well as among others known to be drinking raw milk from infected herds. They concluded that infection is widespread among dairy herds on the island, and that consumption of infected milk is associated with a high rate of infection among adults and children; much of this is subclinical, but cases of overt disease occur.

Occupation

Bothwell (1961) points out that the percentage of persons in series of cases of human brucellosis in Britain who are in occupations which might be considered as carrying contact risks (i.e. farming, dairying, or veterinary work) ranges from 18·0 % (Dalrymple-Champneys, 1960*b*), 18·5 % in N.E. Scotland cases (Smith, 1951), 22·2 % in the PHLS Series (G. S. Wilson, pers. comm.) and 22·9 % in an Oxfordshire series

(Bothwell, 1960), to 34·0% in an Irish series (Northern Ireland Department of Health, pers. comm.). In the 1960–61 PHLS cases (G. S. Wilson, pers. comm.) the percentage of persons known to be occupationally exposed was not more than 25%, and Wilson says this suggests that the great majority get infected in other ways. Milk is the most likely source for these 75% of cases. Wilson points out that even persons occupationally at risk may have been infected via milk.

However, in rural areas the percentage of cases of human brucellosis in persons occupationally exposed may be as high as 50%, as found by Bothwell (1961) in series of cases in rural areas around Oxford. These indicated the endemicity of the disease and the need to plot cases over years rather than months, the futility of a local notification order for say 3 years being apparent. These localized areas of human cases were associated with infected herds; clearly, by following up the initial case and sampling herds in the area, most if not all of the subsequent human cases could have been eliminated.

In other parts of the world, the occupationally exposed fraction of series of cases appears to be much higher than in Britain. Thus in Israel Rozansky *et al.* (1961) reported a clear predominance of contact, but in this series apparently animal husbandry was the almost exclusive occupation of the community and the infecting organism was *Br. melitensis*. Again, the current *Br. suis* problem in the U.S.A. derives from meat-handler risks and thus the occupational proportion of cases is high. Though in Britain there is this apparent main infection source in milk, it is important that the 50% of occupational cases in rural areas should be a guide to the necessity for attention (by eradication) to the animal disease, rather than reliance on pasteurization of milk as a prophylactic.

Proportion of children in human brucellosis cases

Usually children account for very small proportions of human series—2–10% of totals. Bothwell (1962) has reviewed British and American work on brucellosis in childhood and has given an account of a series of seventeen childhood cases in England. Only one of these had a positive blood culture; remittent or irregular pyrexia was seen in fourteen and the undulant variety in two; thirteen cases appeared to be due to drinking raw milk and in four instances this was tuberculin-tested. These cases accounted for about 30% of a series of cases of all ages in Oxfordshire and it was considered that special paediatric interest and improving recognition were factors in this; the series was small, however.

Essame (1962) has described a farming outbreak of brucellosis involving eight people in two families (adults and children); this is an unusual occurrence which, together with other observations, suggests that brucellosis in family groups might be more frequently observed.

Immunity in brucellosis is closely linked to the mononuclear cell reaction (Elberg, Schneider & Fong, 1957; Elberg & Meyer, 1958).

Bekierkunst & Sulitzeanu (1958) showed that mice can be protected against brucella infection by BCG vaccine. Such a situation has interesting epidemiologic implications, viz. that the generally diminishing tuberculin sensitivity of the child population might be associated with reduced resistance to brucellosis, and thus that smaller

doses of brucella in milk might be expected to produce clinical disease more often than in the past. It should also mean that intensive BCG vaccination of 13–14-year-olds in school would reduce the incidence of brucellosis, at any rate for ten years, since this is the protection apparently conferred by BCG against tuberculosis. Again, tuberculin skin positivity should be negatively correlated with brucellin skin positivity, but so far as we know this has not yet been demonstrated or attempted. Angle, Algie, Baumgartner & Lunsford (1938) did note the *absence* of *positive* correlation between positive brucellergen and positive tuberculin reactions.

Infection in milk

In the recent survey in England and Wales by the Ministry of Agriculture, provisional results appear to indicate that herd infection is about 22 % (varying from 11 to 30 % from region to region), and that overall about 1 % of cows are secreting infected milk (i.e. about 32000 cows). The Public Health Laboratory Service (1961) and earlier studies in the Oxford area (McDiarmid, 1960) have indicated that 4–5 % of churn milk supplies are infected.

Milking machines

Kerr, Pearson & Rankin (1958) drew attention to the role of milking machines in the transfer of infection in animals. In the report for 1960 on 'Animal Health Services in Great Britain' (1962), an experiment to examine this point was reported. Field observations had suggested that such transmission does not occur commonly. Eight milking cows, all of which had been vaccinated with S. 19 vaccine during calfhood, were used. Two of them were experimentally infected with *Br. abortus* S. 544 into each of two quarters. Infection having been established, each cow was placed in a separate loose-box and subsequently machine-milked always in the same order and procedure. With the first four cows, one infected and three non-infected, the teat clusters were transferred directly from one cow to another. For the other four cows (one infected) the clusters were dipped into a dairy disinfectant (Deosan) between cows. Quarter milk samples were collected twice weekly, and vaginal mucus and blood samples were collected weekly from all eight cows. No evidence had been forthcoming to suggest that any spread of infection during the 10-week period covered at the time of the report had occurred.

Legal aspects

Bothwell, McDiarmid, Bartram, Mackenzie-Wintle & Williamson (1962) of the Oxford Brucellosis Group have reviewed the brucellosis situation in man and in animals in the U.K., together with the legislation and arrangements for control. They conclude that control of brucellosis requires national action, and recommend that the term 'undulant fever' be officially interred as misleading and obsolete, and that 'brucellosis' be used instead, to bring this country into line with WHO practice. They outline the necessity for notification of disease as an essential to control, indicating that countries which have eliminated brucellosis have invariably used this as a preliminary necessity. They examine the numerous fallacies surrounding the question of legal notification in this country. They point to the loopholes and anomalies in

existing legislation on the control of infected milk from infected herds, and the ineffectiveness or absence of legislation dealing with the control of infection in animals.

They suggest that the control of this disease could be a three-stage process: (1) Make legislation for routinely dealing with brucellosis comprehensive, in the form of 'Brucellosis Regulations' made under the various Acts relating to human and animal disease; the authors make detailed recommendations under this heading. (2) Establish pilot eradication areas; they suggest areas around Compton and in the Isle of Wight; in these areas compensation on slaughter would be introduced. (3) A general eradication scheme which would be simplified by attending to items (1) and (2) first, with S. 19 vaccination made compulsory. They believe that these suggestions if implemented would put the elimination of brucellosis on a sound basis without dislocating the animal husbandry industry. In an appendix they give details of existing legislation on brucellosis. This report has been submitted to the Ministry of Agriculture and to the Ministry of Health.

TUBERCULOSIS

The World Health Organization (1960) Expert Committee on Tuberculosis considered that bovine tuberculosis is probably not of great importance at present, because in most parts of the world milk is routinely boiled before consumption, and in other areas pasteurization of milk and control of bovine tuberculosis infection have been put into extensive practice. Where tuberculosis in cattle is still a problem, however, it is also a menace to man, and complete eradication of human tuberculosis in these countries could not be achieved without attention being paid to the reduction or preferably eradication of tuberculosis in cattle.

The Report of the Animal Health Services in Great Britain for 1960 (1962) described the successful completion within 10 years of the area eradication plan for bovine tuberculosis in Britain. In 1937 Parliament promoted the Attested Herds Scheme, but with the Second World War intervening the concentration of effort to this end did not occur until 1950–60. Tuberculin testing continued for some considerable time as a few pockets of infection were bound to remain. Of Irish cattle imported in 1960, 1·2% were reactors, but with the whole of Britain included in Eradication and Attested Areas on 1 March 1960, cattle imported from Ireland from that date, unless accompanied by attestation certificates, were sent for immediate slaughter.

In Eire, the Veterinary Annual (1961) referred to a four-stage bovine tuberculosis eradication scheme, which was in full swing by mid-1959. By 1961, all northern counties along the border with Northern Ireland were at stage 2, i.e. clearance, the disease had been eradicated from the seven western counties, and about one-quarter of all cattle in the country had achieved attested status.

In the U.S.A., Ranney (1960*a*) reported that the State-Federal co-operative tuberculosis (cattle) eradication scheme was still a long way short of the ultimate goal. Reactors were found in twenty-five States in the previous year, following reports of lesions found at meat inspection. The same author (1960*b*) referred to the reversal of the downward trend in tuberculosis infection rate in cattle in the U.S.A. in 1955. In 1959 the incidence rose to twenty-three per 10000 slaughtered animals, though there was a slight decrease in reactors in 1960. Multiple factors were considered

responsible for this trend, mainly shortage of veterinarians in the war years, shortage of trained help in agriculture, complacency, failure to maintain minimum requirements for country reaccreditation, growing livestock population, failure to emphasize proper cleansing, disinfecting and quarantine; also, increased care in testing and more careful tracing of contacts had caused an increase in the number of cases found.

Winter (1960), reporting on the Co-operative State Federation Animal Disease Eradication Programme in Michigan, said that in 10 years from 1930 the percentage of reactors fell from 0·5 to 0·1 %. By 1954, rising reactor rates stimulated a review of the situation and in 1958–59 Michigan reported more than 30 % of the nation's reactors. A cutback in testing was responsible for unidentified pockets and centres for disease spread, while the use of meat inspection reports rather than necropsy reports was a factor. Re-education of the public and veterinary profession on a resurgent disease was necessary.

In New Zealand, according to Jamieson (1960), the infection rate was reduced from 7·8 % in 1950 to 1·1 % in 1960.

In Turkey, Karasu (1959) reported high incidence of tuberculosis in humans and low in cattle; 8 % of the cattle, however, yielded positive results, 0·8 % of milk samples and 17·6 % of butter samples containing the organisms. Of 2260 strains of tubercle bacilli from humans, 6 % were bovine; this type was associated with 5 % of pulmonary tuberculosis in man and 22 % of extra-pulmonary tuberculosis.

In Hungary, Szabo & Kertay (1959) reported that of 248 strains of tubercle bacilli from humans, 35 % were bovine and this type was found in one-third of cases of urogenital tuberculosis.

In Japan, in experimental infection of calves with human tubercle bacilli, Shibata *et al.* (1960) found no lesions, though the organisms were re-isolated from five of the eight tested.

In Britain, Marks (1961), in a Public Health Laboratory Service report on drug resistance in untreated pulmonary tuberculosis in England and Wales in 1960, found that the incidence of bovine tubercle bacilli was 0·5 % (six strains) in 1773 strains contributed to the survey from patients aged 15 or more. These six strains were sensitive to streptomycin and ionazid, but three strains were resistant to PAS and all were virulent for rabbits, and gave dysgonic, smooth, poorly-pigmented growth at 37 °C and a trace at 25 °C.

As there is no planned system of collection of tubercle organisms from the country as a whole, it is not possible to say what the current incidence is in Britain.

In the Animal Health Services report (1962), already referred to, it is reported that the Central Veterinary Laboratory of the Ministry of Agriculture examined 850 specimens for evidence of tuberculosis in 1960, and found 149 confirmed cattle lesions, twelve being avian. Tubercle bacilli, of which sixteen were bovine, three human, and one avian, were also isolated from twenty out of 465 cattle which had no visible lesions on post-mortem examination in the field. One herd had apparently been infected with bovine-type bacilli from a human source, and transfer of human-type infection from attendants to cattle was also recorded. Progress with eradication of bovine tuberculosis was shown in seventy suspected milk samples of which only two contained tubercle bacilli, both of which were avian.

Indirect evidence of exposure to tuberculosis is available in the tuberculin positivity of skin tests in school children examined prior to BCG vaccination. Thus, tuberculin testing of 8839 school children in several areas in England with bovine and human PPD (Weybridge) has been carried out by Stewart, Embleton & van Zwanenberg (1961), using the Heaf multiple-puncture technique. In two districts known to have had a supply of milk infected with tubercle bacilli and in which there had since been a high incidence of non-respiratory tuberculosis, more reactors were found to bovine than to human PPD and the bovine reactions were larger than those due to human PPD. In two towns with a clean milk supply and a low incidence of non-respiratory tuberculosis, there were more reactors to human PPD and these were larger than those to the bovine PPD. Comparative tuberculin testing of the human suspect with bovine and human PPD may, they suggest, be used to differentiate human and bovine sources of mycobacterial infections. A qualitative difference between these two PPD preparations was shown as well as a quantitative difference. These authors point out that in the case of children over the age of 9 in one town, they had been exposed to known outbreaks in 1950 and in 1953–54 of milk-borne tuberculous infections, while those over 6 had been exposed to the 1953–54 outbreak; they believe that these milk-borne infections were responsible for the high incidence of infection, the higher incidence of bovine PPD reactors than human PPD reactors, and the high incidence of non-pulmonary tuberculosis in that town.

In Eire, M. P. Flynn (pers. comm.), rechecking eighty-four low-grade reactors with comparative Heaf tests, using human tuberculin and avian tuberculin, retested twenty-four who reacted only to avian tuberculin or more strongly to it than to the human, and reactions were equal or inconclusive in five further children. This author made a detailed analysis of the results of tuberculin tests on unselected school children (2029 rural and 703 urban). More rural-dwelling children gave a stronger reaction to avian tubercle, and all had contact with fowls. Handling of fowls and eggs appears to be particularly associated with avian infection of rural children, infection occurring through the mouth or by contaminated hands. Morbidity in these children, if any, was slight. Avian tuberculosis was widespread in fowl flocks in the country.

In Britain there have been several reports of tubercle infection arising from a breakdown in the tuberculin-testing procedure, with resultant infection of human subjects by milk. George & Payne (1961) reported three cases of tuberculous cervical adenitis in children in a village school in Yorkshire, two in September and one in November, 1959; all were 9 years old and all had positive Heaf tests. A positive biologic test was obtained from the school milk at the end of October and confirmed in November from a sample of village milk. Thirty-one of 128 cattle in the herd were found to be reactors; an old cow which had been removed to the knacker's yard in early October was thought by the veterinary surgeon to have been the source. Another breakdown in an attested herd was reported by the same authors. In Berwickshire, discovery of a tuberculous lymph gland in a slaughtered bullock led to investigation of the herd and forty-three reactors were found in 118 animals; forty-three store cattle sold from the herd were found to be reactors also. The extreme rapidity with which the breakdown proceeded once it had occurred is emphasized. George & Payne drew attention to 'the danger of relaxing, at the present time, measures designed to ensure the freedom of milk from tubercle bacilli'. They urged

extension of pasteurization to protect against the risk not only of tuberculosis but also of other milk-borne diseases such as undulant fever.

Wynne-Griffith (1960) reported an instance of a boy aged 6 years found to be positive, having converted from negative the previous year; two others in the family, both under school age, were then tested and one (aged 4) was positive and the other (14 months) negative. Chest X-rays were negative. The family lived on a farm and two milking cows there had been found to be reactors at a routine TB test 3 months before; fifteen out of twenty-five cattle were later slaughtered. It was concluded that the two children had been infected by the cattle, particularly as the youngest, who was negative, had never had milk from this herd.

In Oxfordshire, Black & Sutherland (1961) reported two incidents of tuberculous infection by milk from attested herds. In one herd, six reactor cows were found at routine test, and at retest later nineteen further reactors were found, so that in due course the whole milking herd was slaughtered. Most of the milk had been pasteurized, but three families who took raw milk from the herd produced five, four, and three positive reactors, one of whom had erythema nodosum; chest X-rays were negative; all the reactors were put on chemotherapy prophylactically. In the second incident, a cow from an attested herd had been slaughtered in London and found to be tuberculous, in December 1959. When tested in June 1959, the Friesian herd had been negative, including the slaughtered animal, but in September 1959 this cow had mastitis in one quarter of the udder. A test made then showed thirty-five positive and six inconclusive reactors; these animals were slaughtered and glandular involvement found in eighteen. About 20 gal of raw milk had been sold daily from this herd in the local village and all consumers up to 21 years were therefore tested; of 160 tested, sixty-six with no history of previous positive reaction or BCG were found to be positive reactors; of twenty-three with previous BCG vaccination ten had strongly positive reactions, probably indicating recent superimposed infection.

It is clear that relaxation in the schedules of testing may be followed by rapid disaster for herds and those who drink the untreated milk. It cannot be expected that all breakdowns in the tuberculin testing net will be immediately detected.

Comparative tuberculin testing offers a way to discover routinely what continuing role milk-borne infection might play in tuberculin conversion, since such testing could be readily incorporated in the pre-BCG skin testing of school children. It is to be hoped that standardized PPD materials will be produced and that work to standardize the interpretation of comparative tuberculin tests can be carried out.

STAPHYLOCOCCOSIS

Blackburn (1960) states that mastitis caused by staphylococci and by streptococci other than *Streptococcus agalactiae* has now assumed a greater role and these types of mastitis do not respond so well to antibiotic therapy. Much of the mastitis is subclinical and therefore largely ignored. Recent Reports of the Animal Health Services in Great Britain (1959, 1960) give results obtained from clinical cases of mastitis in cows, which show that of all organisms obtained staphylococci constituted 24·1 % in 1958, 24·0 % of 5086 cases in 1959, and 21·8 % of 3335 cases in 1960. It has been pointed out that there are twenty or more kinds of infection causing mastitis (Murphy,

1956) and, although any of these can be important in individual herds, 99 % of all mastitis is accounted for by infection with *Str. agalactiae*, other streptococci, staphylococci and bacilli.

White, Rattray & Davidson (1962) obtained 502 coagulase-positive staphylococci from cows known to be carriers (normal and mastitic cows), and attempted to type these isolates by a slide-agglutination technique with absorbed sera prepared from sera against bovine strains of the organism: 94·8 % were typed into three groups. Strains isolated from clinical cases of mastitis fell into the same three groups as those isolated from bovine udders without clinical symptoms. Results were consistent with the presence of a common antigen or antigens and a strain-specific antigen or antigens. Twelve coagulase-negative staphylococci from the bovine udder and the thirteen human international types of coagulase-positive staphylococci failed to react with the cross-absorbed sera used.

Malik & Singh (1960) tested for phage type (using ten standard phages) a total of sixty-three coagulase-positive strains of staphylococci, comprising forty-four from milk samples, three from caprine mastitis, one from an ovine abscess, and fifteen from human sources, and also seventeen coagulase-negative strains. Approximately 86 % of the bovine strains were phage-typed and fell into four phage types; all of these strains were lysed by phage 42F. Apart from two human ones, no other strains were susceptible to the phages used.

Minamimoto, Shibata & Ishii (1959) estimated that in Japan about 30 % of cows suffered from mastitis. Some of the strains which they isolated from infected cows were typable with bacteriophages in respect to group I and coincided with the so-called pathogenic staphylococcus in properties.

According to Ozer & Demirer (1959), tests on ten strains of mastitis staphylococci indicated a 99·9 % destruction with either 30 min at 65 °C, 45 sec at 72 °C, or 1 min at 85 °C.

Staphylococcal food poisoning occurs when toxin has accumulated. Inadequate cooling of raw milk or inadequate heat treatment followed by poor cooling will allow growth of staphylococci. Growth of organisms can occur in inadequately heat-treated or cooled milk.

Thus, George & Olson (1960) have investigated the growth of *Staphylococcus aureus* in condensed skim-milk at low and moderate temperatures. Maximum populations were generally attained after 16 days at 50 °F, 4 days at 60 °F, 52 h at 70 °F and 24 h at 80 °F.

Staphylococcal persistence was shown by Foltz, Mickelsen, Martin & Hunter (1960), who found staphylococci in 10 % of chocolate drink samples and 42·85 % of 'low fat' samples, out of a total of 207 samples of seven pasteurized dairy products. Coagulase-positive staphylococci were found in 3·38 % of the samples, representing four products.

The growth of staphylococci in cheese, butter and cream has also been the subject of numerous papers. Thatcher, Comtois, Ross & Erdman (1959) presented data to show that staphylococci with phage patterns of strains commonly associated respectively with cattle and with human infections are widely distributed, often in large numbers, in Canadian cheese made from unpasteurized milk. Mammary infection of cattle is considered to be contributory. Enterotoxin was demonstrated in

eight of 149 specimens tested from individual vats of cheese from a single factory, selected because its product was highly contaminated with staphylococci. The toxic cheese contained up to $1{\cdot}5 \times 10^6$ staphylococci/g. No staphylococci were found among 236 specimens of imported cheese, for the most part made from pasteurized milk or heat-processed after manufacture.

Thatcher & Ross (1960) carried out laboratory experiments to demonstrate the development of staphylococci in milk and in cheese made from milk, subjected to faults of inadequate cooling, and contamination with antibiotics and with staphylococci; their experiments simulated extreme faults. Potential health hazards arising from consumption of cheese made from unpasteurized milk gave enhanced risks of staphylococcal food-poisoning due to the following factors, with effect either singly or collectively: (*a*) inadequate cooling of milk contaminated with staphylococci; (*b*) antibiotic residues in milk used for making cheese; (*c*) strains of staphylococci resistant to antibiotics present in the milk. Three instances of the infection of cattle with the 'hospital epidemic strain', 80/81, are cited by these authors, who considered that if the trend towards an increase in the proportion of staphylococci in milk with these phage patterns continued, then faulty cheese manufacture might introduce into the community a large population of these organisms.

Walker, Harmon & Stine (1960) reported on the survival of staphylococci in Colby cheese. They used (i) milk inoculated with 13000 to 500000 *Staph. aureus* organisms/g of milk, and (ii) milk from cows with subclinical staphylococcal mastitis. Organisms were subsequently enumerated on plate count agar and Staphylococcus Medium 110. Percentages of coagulase-positive colonies in (i) were 89, 84, 87, 51, 57, 20, 16 and 15 at approximately 1, 15, 30, 45, 60, 75, 90 and 120 days respectively; and in (ii) were 75, 83, 22, 9, 0 and 5 at approximately 1, 15, 30, 45, 60, and 75 days respectively.

Allen & Stovall (1960) traced an outbreak of food-poisoning in Wisconsin in 1958 to staphylococci in Colby cheese.

Roughley & McLeod (1961) investigated the survival of staphylococci in cheese made from pasteurized milk which was inoculated with broth cultures of five strains of *Staph. aureus*. The *Staph. aureus* count increased gradually during the cheese-manufacturing process up to hooping. During curing the counts generally showed a marked decline, and no *Staph. aureus* could be isolated from any of the samples after 70 days. The authors point out, however, that the absence of staphylococci does not guarantee the absence of toxins.

In this connexion, Post, Bliss & O'Keefe (1961) suggested that the absence of cases of food-poisoning following consumption of a pasteurized cream and pastry product containing five strains of *Staph. aureus* was due to the restricted development of toxins by competitive growth of other organisms, and that the product had been rejected by consumers because of its soggy and unappetizing appearance when kept under conditions favourable to the growth of *Staph. aureus*.

In Russia, Nefed'eva, Petrovich & Petrushina (1961) reported on the results of the phage-typing of staphylococci isolated from the nose, mouth and hands, equipment, and various foods including butter and kefir. Of the thirty-six butter samples, eleven contained staphylococci, of which six belonged to the third lytic group. Staphylococci lysed by 3–4 phages of the third lytic group were found to be the chief causative agents in thirty cases of staphylococcal poisoning investigated.

Mickelsen, Foltz, Martin & Hunter (1961) found that ninety-five out of 125 samples of cheese, representing twenty different varieties, contained staphylococci; thirty-three of these contained *Staph. epidermis*, eighty-eight *Staph. aureus*, and twenty-five contained both. Six samples of Cheddar cheese and one sample each of Blue, Bondost and Brick cheese contained coagulase-positive *Staph. aureus*; phage-typing indicating that seven of these samples contained organisms belonging to lytic group III.

Donnelly, Black & Lewis (1962) consider that Staphylococcus Medium 110 is the most efficient means of isolating staphylococci from samples of Cheddar cheese. Of thirteen samples examined from a food poisoning outbreak, eleven contained coagulase-positive staphylococci; while 19% of 343 retail samples of Cheddar cheese obtained in 1959–61 contained coagulase-positive staphylococci. Three out of nine selected cultures from cheese involved in the food-poisoning outbreak were positive for enterotoxin.

Obiger (1961) studied the pathogenicity of 372 strains of staphylococci isolated from various sources, including can milk, individual cow samples and quarter samples from healthy and infected udders. Tests were carried out on mice and guinea-pigs (by intraperitoneal or intrapleural injection of fresh cultures, by feeding culture filtrates heated for 30 min at 100°C, or by intraperitoneal injection of heated or unheated toxins) and on kittens (fed unheated culture filtrates or given intraperitoneal injection of heated or unheated culture filtrates). Inconsistent results were obtained, indicating that animal tests are not a reliable means of demonstrating pathogenicity, probably due to individuality of test animals and variability in toxin production.

Sharpe, Neave & Reiter (1962) described methods of isolation and some characteristics of coagulase-positive and coagulase-negative staphylococci associated with dairying. Excessive multiplication during cheesemaking of the coagulase-positive staphylococci present in raw or insufficiently heat-treated milk may result in large numbers in the cheese, and also sometimes in the presence of enterotoxin. A high pH during manufacture due to suppression of the starter by penicillin or bacteriophage allows such multiplication. As the enterotoxin may remain active after the staphylococci have died out, the number of these organisms is not an indication of the presence or absence of toxin. A comparison by these authors of selective media for isolating coagulase-positive staphylococci from cheese favoured a tellurite glycine egg yolk medium (Baird Parker, 1962). They described cheesemaking experiments, and observed that lipolytic coagulase-negative staphylococci in cheese may contribute towards the flavour, whilst penicillinase-producing strains may be used to inactivate penicillin present in milk used for cheese and yogurt making.

Outbreaks of staphylococcal food-poisoning

The Public Health Laboratory Service (1960) reported three staphylococcal outbreaks in 1959. Two were associated with raw milk and both of these occurred in July, one in a Hampshire school and one in a Welsh hotel, both being due to *Staph. aureus* phage-type 42D. In one the source of infection was not found; in the other it was a cow with mastitis due to the same phage-type of *Staph. aureus*. The third outbreak was associated with cream and occurred in a hotel at Christmas. The

cream had been stored in an open container, sometimes kept in the refrigerator but often in the warm kitchen. Over 100 persons who ate it at different times became ill 1–6 h afterwards. *Staph. aureus* was isolated from the cream but the strain was not phage-typed and none of the patients was examined. In 1960 there were two outbreaks (PHLS, 1961), which were associated with milk and due to *Staph. aureus*. In one of these over 100 persons were affected, in the other thirteen were ill; in neither was the source of infection clearly demonstrated. In 1961, the PHLS (1962) reported two outbreaks associated with milk and due to *Staph. aureus*; one was among campers, twenty-six of whom became ill, whose milk supply came from a cow with mastitis on a local farm; in the second outbreak all three members of a family had symptoms after drinking milk from their own cow. There were also three outbreaks associated with cheese in 1961; in one, the vehicle of infection was Australian Cheddar cheese supplied by one firm to four canteens, causing symptoms in about seventy persons; the two other outbreaks, involving at least 120 cases, were in hospitals, and associated with New Zealand Cheddar cheese from which *Staph. aureus* was isolated.

Steede & Iredale (1962) reported an outbreak in February 1960 of staphylococcal food-poisoning from raw milk involving twelve service personnel and one civilian, all of whom developed vomiting and diarrhoea after drinking raw T.T. milk; ineffective cooling arrangements were thought to be responsible. *Staph. aureus* was isolated from nine of thirty-two cows in the herd and was phage-type 29/52/6/7/42E/47/54/73/75. One cow had clinical mastitis but the milk from that animal was not in use at the time. A subsequent investigation showed that *Staph. aureus* was found commonly in churn milk from local farms; fifteen of fifty-five samples were positive in one farm and eighteen of thirty-nine in another. There was some evidence that when the mean temperature of the churn was over 60 °F the incidence of *Staph. aureus* was higher.

Munch-Petersen (1960) has made a comprehensive review of the problem of food-borne epidemics of staphylococcal food-poisoning in Australia, with special reference to dairy products; he gives an extensive bibliography and it would appear that the incidence of staphylococcal infection in man is increasing, as is the incidence of bovine mastitis. Hodge (1960) has reported on problems of outbreaks of staphylococcal food poisoning in the U.S.A.

In a review of laboratory and epidemiological aspects of food-borne diseases, Galton & Steele (1961) say that bovine mastitis of staphylococcal aetiology is a world problem and appears to have become much more prevalent in the past few years. Widespread antibiotic mastitis treatment favours the development of antibiotic-resistant strains of staphylococci. Although the actual interrelationship of human and animal strains of staphylococci has not been established with certainty, important information is being accumulated to indicate that both animal to human and human to animal transmission occurs. In a comparative study of the properties of 263 coagulase-positive staphylococci from butter or cheese and from human clinical sites, Thatcher & Simon (1956) found that the isolates from dairy products were predominantly phage-type 42D, but this type was rarely encountered among the isolates from clinical sites. However, this so-called bovine form has been recovered from severe cases of human enteritis that developed as a sequel to antibiotic therapy, and it has also been established as capable of causing food poisoning in man. The applica-

tion of phage-typing to the epidemiological study of staphylococci isolated from bovine milk was studied by Williams Smith (1948). He found that 93 % were typable and that 42D was the most common type. However, phage-typing did not distinguish strains isolated from cows with mastitis from those found in milk from apparently normal udders; he found also that two phage types may occur in the milk from one animal and therefore considered that phage-typing may be limited as an epidemiologic tool in the study of bovine mastitis. Galton & Steele go on to say that the first evidence of phage-type 80/81 staphylococci in milk and in dairy employees was obtained by Wallace, Quisenberry & Deharne (1960), who, during a survey of staphylococcus phage-types in milk, isolated type 80/81 from bulk milk and from the milk from three cows in the herd; further investigation revealed staphylococcal infection in one dairy employee and his family caused by the same phage type. There is recent epidemiological evidence to indicate further that the animal to human chain of transmission of staphylococci is reversible. Zinn, Anderson & Skaggs (1961) studied persistent furunculosis in a herd of dairy cows. The initially infected herd on this farm had been disposed of, since all attempts at treatment had failed, and a replacement herd purchased. The second herd became infected shortly after arrival at the farm and was studied by these authors for about a year. Phage-type 80/81 staphylococci were isolated repeatedly from furuncles on the cows' udders, the milk, the milking machine inflations and bulk milk. Two dairy attendants were found to be nasal carriers of phage-type 80/81 and this same type was isolated from skin lesions on these individuals. One of the attendants was undergoing clinical treatment at about the same time that the disease appeared in the initial herd. Both cattle and human isolates were resistant to penicillin, dihydrostreptomycin and chlortetracycline.

Dauer (1961) has given a 10-year résumé of food- and water-borne disease outbreaks in the United States for the period 1951–60. In 1960, there were five outbreaks and forty-eight cases due to milk and milk products. Fluid milk was the vehicle of infection in only one of the five outbreaks reported, the other four being attributed to various milk products. In the instance involving fluid milk, five cases of staphylococcal food poisoning were traced to unpasteurized milk from a fresh cow. Many coagulase-positive staphylococci were isolated from a specimen of the cow's milk. Another outbreak involving five cases followed consumption of ice cream made with unrefrigerated raw milk that had been obtained from one cow. There was no evidence of mastitis in the cow.

Krasnitskaya (1960) has given details of staphylococcal food-poisoning in Russia, stating that in recent years staphylococci have been responsible for 20–30 % of cases of bacterial food poisoning there. In 1958, 46 % of all cases of food-poisoning resulted from consumption of dairy products, sour milk products being chiefly responsible.

Control

Sharpe *et al.* (1962) quote van Heyningen (1950), who stated that progress in research of staphylococcal food-poisoning is stifled almost to the point of extinction by the difficulty of testing for toxin. They refer also to a paper by Bergdoll (1962) relating to the purification of staphylococcal toxin and they point out that this has to be tested either on animals (cats and monkeys) or on human volunteers. Until

laboratories can test for toxin, the evidence for the incrimination of suspected food is circumstantial only.

These authors, in their comprehensive review of this subject, point out that, while it is true that we do not know the conditions which promote the production of enterotoxin and that little progress can be made until reliable and simple tests are available for detecting and measuring it, the solution for protecting the consumer against toxic milk, cream or cheese is simply provided by refrigeration and pasteurization. The elimination of staphylococci, which are often resistant to antibiotic treatment, is difficult, but the use of strictly hygienic milking methods promises a decrease in the incidence of infection of animals.

In this connexion, the report on the Animal Health Services in Great Britain for 1960 (1962) contains details of control measures applied against mastitis in the laboratory herd. After milking each cow, the udder has been washed with a sterile cloth soaked in 1:5000 chlorhexidine and the teat-cup cluster and milk tube have been flushed out with running cold water. Initially, each infected quarter was treated with an antibiotic until a bacteriological cure was obtained. The report indicated that, although it was too soon to conclude that the earlier mastitis-producing strains had been completely eliminated, it seemed that the control measures used were sufficient to suppress the staphylococci to an extremely low level. The incidence of staphylococcal mastitis was reduced from twenty-two cases (thirteen cows) in 1959 to five cases (five cows) in the first 11 months of 1960, at least one and probably two of these resulting from recurrence of previous infection.

SALMONELLOSIS

Huckstep (1962) has written a monograph on 'Typhoid Fever and other Salmonella Infections', and includes experience gained in dealing with 1300 cases of typhoid, many of them in Kenya. There are chapters on prophylaxis, and one on other salmonella infections.

Taylor (1960) in a general review of the whole problem of salmonellosis in Britain, stated that milk and milk products rarely cause human salmonella infection and attributed this to the high level of pasteurization (95 % of milk), the fact that most cheese is now made from pasteurized milk, and that cream is now commonly pasteurized. Infection of the human subject can, however, still occur following errors in pasteurization.

Typhoid outbreaks have been reported from Eire and from Canada.

McCarthy & Dunlevy (1960) describe a milk-borne outbreak of typhoid in Arklow (population 5203), Ireland. Twenty cases occurred within a month (eight male, twelve female); nineteen regularly drank milk from the same supplier while the other one drank it on a visit. This dairyman supplied about fifty families. Fourteen of the twenty cases were aged 15 or under, as were five other unconfirmed cases. No deaths and no carriers resulted. A dairyman had left the source dairy a fortnight before the first case occurred; he was ill, with coryza, cough, and pains in chest and abdomen; he was found to have a Vi agglutination 1/20 + +, though Widal and faeces were repeatedly negative. He was regarded as a typhoid carrier and the source of the outbreak.

Dumas, Foley & Breton (1961) described an outbreak of 197 cases of typhoid fever

in March 1959 in Montmagny (population 6000), Canada. All but nine of the patients lived in Montmagny; of these nine, five had visited the homes where contaminated milk was consumed and four were secondary cases infected by a student who contracted the illness in Montmagny and returned to his home before diagnosis—his four siblings developed typhoid fever.

The municipal waterworks and the municipal sewerage system were not incriminated. Some 600 quarts of raw milk were sold daily in the town, distributed by four principal dairymen, two living in the town and two in the country. These milk suppliers had always maintained adequate hygiene standards and no case of typhoid fever had occurred in the Montmagny town or rural outskirts during the previous 2 years, but a few cases were reported annually in people who lived on the banks of the river and who obtained their water from this polluted stream; two of the dairymen of Montmagny lived on the bank of this river. The outbreak started when eight cases of typhoid fever were admitted to hospital on serological diagnosis. All the families of these cases were buying raw milk from the same dairyman. This dairyman's well had dried up over a month previously and river water had been used as a source of supply and for cleaning dairy equipment. The water was not boiled; all members of the dairyman's family drank it. It was chlorinated for washing dairy utensils, but these were rinsed with polluted water to remove dust from them; they were not sterilized. A son of the dairyman had been ill for 2 weeks but had continued to work in the dairy with all the other members of the family, and eight out of the ten members later developed typhoid fever, the father and one son alone being spared although they were great consumers of raw milk. Between 1 March and 12 April, 179 cases were reported; the other cases were reported at the rate of one or two a week from 12 April to 14 June.

Further cases, apparently unconnected with the outbreak, had drunk raw milk from a restaurant where the operator was not supposed to sell raw milk. Four typhoid cases occurred in two neighbouring families although they lived downstream from the dairyman implicated. They obtained water from the river and produced their own milk, having no relations with the dairyman concerned. The aetiological agent in these cases was a phage-type E–1, which was that isolated from all the other patients. One hundred and twenty-three of the 197 cases in the outbreak were under the age of 20 years. The cases were fairly mild. No deaths occurred. There were no intestinal complications, perforations or haemorrhages; myocarditis was diagnosed in a few cases who recovered without sequelae. Oral chlormycetin was exclusively the treatment.

Hudemann (1959) examined 140 samples of foods of both plant and animal origin for typhoid-Vi and salmonella phages. Of six milk product samples, two were found to contain typhoid-Vi phages and three salmonella phages. But the technique was not considered suitable for routine use.

The Public Health Laboratory Service (1960) reported two outbreaks in 1959 of food poisoning due to *Salmonella typhimurium*, associated with milk. One occurred at a dairy farm, with twenty-six cases, confined to the farmer and employees and their families; the milk was pasteurized except for that consumed by these families. Though there was good epidemiologic evidence to incriminate the milk, salmonellae were not isolated from samples examined nor from cow faeces, so the source was not discovered. The second outbreak originated among calves which fell sick while being

reared on a farm, and the infection spread to raw milk, possibly by the hands of a milker who was also concerned in calf rearing; at least two young children who drank the milk had symptoms and one adult was a symptomless excretor.

An outbreak due to *Salm. typhimurium* in 1960 was also reported (Public Health Laboratory Service, 1961), in which about 300 customers of a dairy selling unpasteurized tuberculin-tested milk were at risk, and at least sixty cases occurred among them and among persons on the farm and workers in the bottling plant. *Salm. typhimurium* was isolated from anal swabs from two of the thirty-six cows in the herd and from a sample of milk from one of them.

Parry (1962) has reported a milk-borne outbreak due to *Salm. typhimurium*. In July 1961, sixteen cases of gastroenteritis due to *Salm. typhimurium* phage-type 1, var. 5, were traced to unpasteurized tuberculin-tested milk from one dairy in Liverpool. All sixteen cases were notified between 19 July and 5 August; two were hospitalized. *Salm. typhimurium* was isolated from the stools of thirteen patients, and five symptomless excretors of this phage-type of salmonella were found among forty-seven home contacts. The majority (60 %) of the salmonella excretors were under 15 and half of them were under 5. The infected milk supply came from a herd of thirty-six tuberculin-tested cows, supplying milk to 740 households. Diarrhoea had occurred in one of the cows, which died on 25 July. The milk infected amounted to only about 18 gal out of a total of 105 gal distributed daily. About ninety families, numbering 220 people, were possibly at risk out of a total of 740 families (2220 people). The author thought it possible that the original source of the infection was bovine, but that it was most likely that infection was transmitted by faecal contamination of the milk.

The Public Health Laboratory Service (1962) reported five outbreaks of food poisoning due to salmonellae in 1961; one of these was that reported by Parry, reviewed in the previous paragraph; the second, also due to *Salm. typhimurium*, produced at least sixty cases, the organism being isolated from the farmer and herdsman and from the milk of two cows in the herd; in a third outbreak due to *Salm. typhimurium*, the organism was isolated from about twenty persons, who drank farm-bottled T.T. milk, and from two cows in the herd. In an outbreak due to *Salm. enteritidis* var. *jena*, cases occurred in several families, and the organism was isolated from patients and from the faeces of a sick cow on the farm supplying the milk. In a larger outbreak, comprising 123 cases and excretors, the source of infection was traced to a cow with symptomless mastitis due to *Salm. heidelberg*; animal feeding stuffs were thought to be the most probable origin. In addition to these five outbreaks associated with milk, there was one outbreak in which the vehicle of infection was cheese; *Salm. bovis-morbificans* was isolated from cheese brought home from France by a family.

In India, a family outbreak of food poisoning due to *Salm. weltevreden* was reported by Mathur (1959), involving six people in attacks of gastroenteritis and fever for 5–9 days. The source of infection was milk which had been boiled at a farm, then transferred to another container and consumed twelve hours later without further heating, presumably having been contaminated after the original bottling.

Dauer (1961) reports that ten cases of *Salm. montevideo* infection followed the eating of home-made ice cream made with raw milk and a cracked egg found in a

hen's nest. Five cases of gastroenteritis of undetermined aetiology occurred in a family group who ate cottage cheese purchased from a store. The probable source of infection was not found.

Q FEVER

In the past 10 years, Marmion and his colleagues have dealt with various aspects of Q fever in Britain (Marmion, Stewart, Richmond, Barber & Stoker, 1954; Marmion, Stoker, Walker & Carpenter, 1956; Marmion & Harvey, 1956; Marmion & Stoker, 1956; Marmion, 1959; Stoker, Brown, Kett, Collings & Marmion, 1955). But in the period under review there have not been many papers on this disease published in Britain. The majority of the papers on Q fever stem from the U.S.A., but the disease is being detected wherever it is looked for.

Luoto (1960) in the U.S.A. reported on the nation-wide occurrence of Q fever infections in cattle and the fact that the disease was already recognized as a public health problem in some areas. At least 300 human cases had been found in southern California, and 350 cases associated with sheep in northern California, during epidemiologic studies in 1948 to 1949. Further cases occurred annually. For many years cases originating from cattle or sheep had been reported in Texas and in Idaho, where epidemics were studied in 1947 and in 1958. In Iowa, ten proven cases and evidence of infection in eighty-five individuals were found, but only 3% of dairy herds were infected. Human infection had occurred in eighteen of the thirty-five States with known infected cows, and occasional human cases in four newly 'infected' states—Maryland, Pennsylvania, New York and New Jersey. The true incidence of human infection or disease was unknown, because many cases in the U.S.A. were unrecognized. Even during the 1959 epidemic in Idaho, most of the ninety-three confirmed cases were diagnosed by 10% of the local physicians, many of whom had diagnosed cases in previous years.

Luoto & Pickens (1961) have given a résumé of recent research seeking to define the Q fever position. They refer to previously established factors, namely, that bovine infections are widespread and increasing in the U.S.A. and will result in extensive human contagion and disease, and that the nation-wide occurrence of Q fever among dairy cattle indicates numerous reservoirs and sources of infection. Prevalence rates in man remained to be determined in most states and, until adequate knowledge concerning the human disease became available, the public health significance of this omnipresent animal infection would remain obscure and a matter for concern. In Montana, dairy cattle appeared to serve as an important (if not the sole) source and reservoir. Sale and removal of positive cows was repeatedly incriminated in the introduction of Q fever to previously negative herds and areas. Once the organism is introduced by incoming cattle, it spreads and permeates the environment, possibly through air- or water-borne routes, via wild-life vectors, or by other means. These authors discussed the modes of human infection and pointed out that more than the mere presence of the agent in the environment is necessary for human involvement. They say that ingestion of infected milk does not lead to cases in many areas and that man is not highly susceptible via the gastro-intestinal tract. A neutralizing factor is present in infected milk and this may render the organisms less virulent, but still antigenic, for man. Strain differences among *Coxiella burnetii* might be responsible

for lack of human disease in some areas. Protection from infected milk requires, they suggest, new standards for pasteurization of milk—elevation of temperature to 145°F for 30 min or 161°F for 15 sec being required. They conclude that additional knowledge is needed for satisfactory definition of the public health problem.

Stoenner *et al.* (1961) studied the incidence of antibodies against *Co. burnetii* and of clinical symptoms associated with Q fever in two areas of Idaho, amongst families associated with 119 infected herds and families associated with 141 Q fever-free herds. In the former group 26 % of 333 persons were sero-positive and in the latter group 14 % of 394. Although dairy cattle were a potential source of human infection, a significant amount of clinical illness was not associated with these infections.

Tjalma & Braun (1960) say that Q fever has little or no veterinary importance but definite public health significance, and thus may be more difficult to control than a zoonotic disease having both veterinary and human clinical importance. Tjalma (1958) had stated that prior to 1957 Q fever had not been reported in man or animals in Iowa, except that one case of human Q fever occurring in 1955 had been investigated.

A serologic survey of Q fever in 36921 human sera has been made by Braun (1962) in the U.S.A. Of these sera, 19189 were diagnostic specimens from febrile patients and 17732 were premarital specimens. An over-all reactor rate of 0·3 % positive was found. Follow-up of suspect infections, using clinical and bacteriologic data, resulted in identification and diagnosis of Q fever in twenty-two males. Contact with infected dairy cattle had occurred in three farmers; occupational exposure in three abattoir employees and one milk-plant employee; while the remaining fifteen had no discernible source. Dairy cattle serologic studies showed increased prevalence, from 0·7 % in 1956–57 to 1·6 % in 1960, the herd-positive rate increasing from 3·25 to 9·6 %.

The American Veterinary Medical Association Council on Public Health and Regulatory Veterinary Medicine (1961) reviewed the clinical and diagnostic aspects of Q fever and its epidemiologic features, and mentioned the high susceptibility of dairy cattle and sheep, though cattle are completely asymptomatic. In control measures they include vaccination of man with inactivated vaccines prepared from *Co. burnetii*, pasteurization of milk, and also search for occupational exposure, contact with live stock, the consumption of raw milk, and association with other infected environments.

Ferris, Hanson & Brandly (1961), in Illinois, tested *Co. burnetii* Q fever antibody by capillary agglutination (CA) test, with the following results: of 8575 dairy cattle 7 % were positive and 16 % of the herds; of 4733 swine 0·3 % were positive and 2·4 % of the herds; only two of 862 sheep were positive. The authors said that Q fever in man had not been adequately investigated in Illinois. About 23 % of thirty-seven veterinarians in 1958, and 10 % of 100 veterinarians in 1959, were positive to CA and CF tests. Absence of human cases might be due to failure of physicians to diagnose, and inapparent infection was shown to exist.

In Canada, Fish & Labzoffsky (1960) collected composite raw milk samples from 200 dairy herds in Western Ontario and subjected them to the capillary agglutination test. Samples from fourteen herds gave positive reaction. Individual milk samples from the 334 animals in these fourteen herds and blood samples from those with positive reaction for Q fever were examined by capillary agglutination tube method and complement-fixation test. Sixty animals (17·9 %) produced a reaction in milk

and blood; all fourteen herds contained reactors, the incidence being from one to eight animals within herds.

J. A. McKiel (pers. comm.) also investigated Q fever in Ontario, with results as follows: of 4567 herd milks sampled, 103 were positive in the CA test for *Co. burnetii* antibodies, giving a herd reactor rate of 2·3 %. The highest reactor rate in any of the test areas was 14·1 % and in this area the average reactor rate within herds was 32 %. CF titres of 1/64 or higher in guinea pigs resulted from inoculation of five of six herd milks, nine of fourteen individual cow milks, and one of three bovine placental specimens tested. A strain (CNP) of *Co. burnetii* was isolated in embryonated eggs from guinea pigs inoculated with a seropositive bovine milk. Coxiellae were seen in smears of spleens of milk-inoculated guinea pigs and of yolk sacs of CNP-infected fertile eggs. Suspensions of these yolk sacs in guinea-pigs resulted in production of CF antibodies in high titre to *Co. burnetii*. Tissues of one rat and one swallow out of 256 small mammals and birds tested produced antibody for *Co. burnetii* in guinea-pigs. In a serologic study of the occurrence of Q fever antibodies in human sera, only one of 2453 sera (0·04 %) reacted in the capillary tube test. In a complement fixation test on 1775 human sera, eight sera (0·5 %) had antibody titres of 1/8 to 1/32, and two sera (0·1 %) had titres of 1/16 to 1/32.

In Ibadan, Collard & Udeozo (1959) tested 204 sera from blood donors and found eleven (5·4 %) had complement-fixing antibody to *Co. burnetii* at a serum titre of 1/20 or greater. Of 119 sera from pregnant women, only one (0·8 %) had complement-fixing antibody. This preponderant male incidence was compared with similar reports from California. These authors also tested cattle, goat, sheep and chicken sera, which showed a high incidence of positive sera, mostly at low titre, infection being more prevalent among goats (44 %) and chickens (36 %). They suggest that main sources of human infection in Ibadan are through inhalation of infected dust particles and through contact with goats and fowls, these forming a potent animal reservoir.

In Belgium, Jadin, Thomas & Leonard (1959) reported investigations into the incidence of *Co. burnetii* agglutinations in cattle, and carried out micro-agglutinations on 169 specimens of cow serum, which did not show the presence of *Br. abortus*. Of these, 107 were positive (63·3 %). Of the sera in the area, 30 % were positive for *Br. abortus* and 44·3 % for Q fever.

In Germany, Trüeb, Boese & Posch (1960) reported an epidemic of Q fever affecting 515 persons in the lower Rhine district in 1958, which occurred in three waves; the primary source of infection was believed to have been a cattle auction where four cows excreted *Co. burnetii* in their milk.

A recent British paper is by Marmion (1962) who has reviewed the course and clinical features of seven proven and two suspected cases of Q fever endocarditis (seven men and two women aged between 30 and 60). The aortic valve was affected in five cases, the mitral in two, and both in one case. A definite or equivocal attack of rheumatic fever occurred before the onset of endocarditis in six patients, and a clinically recognizable attack of Q fever in seven. Subacute endocarditis lasted from 5 months to 5 years before fatal termination, and clinically resembled subacute bacterial endocarditis in many but not all respects. Blood cultures taken during the phase of endocarditis were nearly always bacteriologically sterile, but *Co. burnetii* was isolated from the blood of four cases, and Q fever complement-fixing antibody

was present at high titre in all. In seven cases examined after death, *Co. burnetii* was isolated from vegetations on the heart valves and coxiella-like organisms seen in sections. The author drew attention to the diagnostic value of complement-fixation tests with the phase 1 antigens of *Co. burnetii* in detecting chronic, active infection.

TICK-BORNE VIRUS INFECTION

The epidemiology of tick-borne biphasic milk fever has been further elucidated by workers in Russia and Central Europe.

Drozdov (1959*a*), in Russia, reported epidemiologic investigations of outbreaks of biphasic milk fever, of which the virus appears similar in immunological characteristics to the tick-borne encephalitis viruses. Milk was frequently implicated and in one outbreak four strains of virus were isolated from 203 samples of goat's milk; the virus could not be isolated from cow's milk, but antibodies to biphasic milk fever virus were detected in a high proportion of cows, indicating that cows, acting as hosts to infected ticks (the main reservoir of infection), are not generally infected to a sufficient degree to produce milk contamination, although this is considered likely to occur following infection with massive doses.

Blaskovic & Gresikova (1959), however, demonstrated that milk of farm animals may transmit tick-borne encephalitis virus to man. Goats and cows artificially infected with the virus excreted it in milk during 2–8 days after; in blood it usually appeared more quickly and disappeared earlier. The virus remained viable in dairy products and sour milk for varying periods of time.

Gresikova, Havranek & Gorner (1960) studied the heat resistance of tick-borne encephalitis in milk at temperatures ranging from 55 to 85 °C, at intervals of 5 deg, for 10 sec. The virus was inactivated in milk during pasteurization at both 72 and 85 °C for 10 sec.

Pogodina (1959) presented case reports of families owning goats and cows; they considered that latent immunization against tick-borne encephalitis occurs via the alimentary canal when fresh milk from infected goats is consumed, and that infection from this source does not always produce clinical symptoms; also that the possibility of infection via cow's milk was doubtful.

Drozdov (1959*b*) isolated virus strains from the blood of a patient infected with biphasic milk fever in Russia, and also from the milk of goats and *Ixodes ricinus* ticks. Results indicated that all strains were identical. Symptoms of encephalitis similar to those observed after intracerebral infection of tick-borne encephalitis virus and Omsk haemorrhagic fever virus were produced in white mice and a kid by intracerebral injection of the isolated virus.

Drozdov (1959*c*) also reported that strains of a filterable virus isolated from the blood of a patient with biphasic milk fever, the blood of goats, and *I. ricinus* ticks, were identical antigenically and immunologically. Later (1960*a*) he reported that experiments with patients' sera established a high antigenic and immunological similarity between viruses causing biphasic milk fever, tick-borne encephalitis, louping-ill, and Omsk haemorrhagic fever.

Gresikova-Kohutova (1959*a*) reported studies on survival of the virus of tick-borne encephalitis in dairy products at refrigerator temperature. The titre remained

almost unchanged in cow's milk for 2 weeks and in cream and butter for 2 months; in quarg it fell as the acidity increased, the virus being absent after 2 months.

The same author (1959*b*) centrifuged a Hypr strain of tick-borne encephalitis virus in 20 % mouse brain suspension and diluted the supernatant with saline, saline + 10 % normal inactivated rabbit serum, or milk. The virus was inactivated at 50 °C for 20 min in saline and at 65 °C for 20 min in milk. In general, milk afforded better protection over the temperature range tested (40–70 °C for 20 min). Pasteurization of milk by the holder method caused a marked decrease in titre of the virus but did not inactivate it completely.

This author further reported (1959*c*) that the optimum environmental pH for the tick-borne encephalitis virus isolated in Czechoslovakia was found to be pH 7·6–8·2. The Hypr (M40) virus however was able to survive at a pH range of 2·55–10·45 over a 24 h period at 4 °C; it survived a pH of 4–5 for 24 h in sour milk.

The role of the *I. ricinus* tick was experimentally demonstrated by Gresikova & Rehacek (1959), who infected two ewes with louping-ill virus by means of infected *I. ricinus* ticks, and one of them excreted the virus in milk on the 7th and 8th day after infection. Two sheep infected with a human strain of Czechoslovakian tick-borne encephalitis excreted the virus in milk between the 3rd and 7th day after infection. One cow excreted encephalitis virus in milk on the 3rd and 4th days, and the virus was detected in blood on the 2nd and 6th days after infection.

Further, Drozdov (1960*b*), in experiments with five pairs of goats, used four different strains of virus of the tick-borne encephalitis group, which were injected twice subcutaneously or intramuscularly, and blood and milk samples examined from the 3rd day after injection and subsequently at 1–2 day intervals. All the viruses were found to be capable of entering the milk and blood under the conditions of this experiment. Louping-ill virus was shown to possess highest pathogenicity for goats.

ONCOGENIC VIRUS

Gross (1961) in his monograph on oncogenic viruses, refers to the development of leukaemia in successive generations of cattle. Information about possible familial incidence of leukaemia in cattle is scanty, as only relatively few animals are allowed to live out their life span and be observed for possible development of disease. Cattle have a relatively long life-span and records are not available to survey the incidence of disease through several successive generations, whereas in the mouse ten or even twenty successive generations of animals can be observed by a single human observer in an experiment—this would be impossible in cattle. Nevertheless, there is evidence that leukaemia may also develop in several members of the same family of cattle—in some instances in two or three successive generations. Gross cites papers by Schaper (1938), who noted twenty-three cases of leukaemia developing in a cow and its offspring; by Lockau (1933) and others, who made similar reports; and by Czymoch (1937), who observed eight cases of leukaemic cows with leukaemic offspring, including two leukaemic calves from the same cow, and also that sixteen out of twenty leukaemic cows bred in a particular district were the offspring of one and the same bull, which eventually also died from leukaemia. Both Czymoch and Schaper observed many cases of leukaemia in herds of cattle in which a particular bull was

used for breeding purposes, the occurrence of the disease ceasing on removal of the bull.

Gross pointed out that it is difficult to evaluate the incidence of leukaemia in cattle, but at slaughter (when they are relatively young) 1 in 9000 cattle has the disease. He says that the possibility should be considered that in cattle, as in chickens and mice, leukaemia may be caused by an agent transmitted in certain families from one generation to another and that such an agent may be responsible for the development of this disease in more than one member of the same family.

It is unknown whether these oncogenic viruses are present in cow's milk, whether they have any role in human disease, or what the survival rate might be in raw milk or heat-treated milk.

In another study relating to oncogenic virus, Huebner, Rowe, Hartley & Lane (1962) demonstrated the presence of polyoma virus in mice infecting grain and silage stores on dairy and other farms, in Montgomery and Frederick Counties, Maryland, U.S.A., a grain- and livestock-producing area north of Washington, D.C., which also supplies a large part of the milk for that city. They reported also on continuing surveys for polyoma infection in mouse populations on one of the five positive farms and in three feed mills located in a small town in the same area. They used a haemagglutination-inhibition (HI) test to demonstrate antibodies to polyoma. They point out that *Mus musculus* have world-wide distribution and are grass seed and grain feeders. As much as 25 % of grain stocks may be destroyed by them, and the mice per 100 m^3 of rick increases from forty-seven in November–December to over 1100 in May–June. As many as 2000 living and dead mice may be taken from the upper portion of a single rick. They quote figures given by Southern & Laurie (1946) which showed that over 95 % of all grain ricks in England were infested with house mice.

Grain storage areas infested with breeding colonies of mice are important foci of mouse virus infections, with consequent opportunity for widespread dissemination—the authors say 'at least to mice' and hypothesize that the basic natural cycle of polyoma and other viruses exists in agricultural ecologies. Further studies may, they think, reveal previously unknown viral zoonoses, as well as explanations for the survival of known mouse viruses in natural and laboratory colonies.

The urine is a more prolific outlet than faeces for polyoma virus; over a million median infectious doses can often be demonstrated in 1 ml of urine from mice infected as new-borns. These authors estimate that in a 9-month period about 150 l of urine may be deposited in the upper layers of a grain rick. Polyoma virus is very hardy, surviving 37 °C undiminished for long periods, and accumulation of virus can be considerable, even on undamaged grain. Other common and comparatively resistant viruses, e.g. mouse adenovirus, K virus, hepatitis, reoviruses, lymphocytic choriomeningitis virus, and others might be expected to occur on such grain.

Low level haemagglutination-inhibition responses were obtained from 10 % of persons sampled in houses with heavy mouse infestations but the authors did not think this very significant. Cows on the farm were also negative, though apparently positive for reovirus.

No mention is made of testing preheated milk for virus, but clearly the presence of polyoma and other tumour-producing viruses—albeit at present of mice—in situations where access to milk supplies could occur, must be of concern, and many questions

of potential survival of polyoma virus and other animal viruses in milk and their heat lability in milk arise.

The Medical World News of 12 May 1961 contained a report that for the first time a pox-type virus had been shown as the specific cause of a simian tumour, and in a series of experiments in five human volunteers it was shown that the virus can produce benign tumours in man, these being due to Yaba virus.

At the very least it can be said that much more needs to be known about the possible presence of oncogenic virus in milk supplied for human consumption.

PESTICIDES AND ANTIBIOTICS IN RELATION TO ANIMALS AND MILK SUPPLIES

The Research Study Group on Toxic Chemicals in Agriculture and Food Storage (Ministry of Agriculture, 1961) reported that it had received no evidence of harm to consumers of crops or food treated with pesticides and that official recommendations for their safe use were so framed that any residues in food should be far below any dose liable to harm a consumer. Nevertheless, they recommended further selective survey work to determine pesticide residues in food, home-produced and imported, to serve as a check on whether such recommendations were being properly observed.

In the U.S.A., Clifford, Bassen & Mills (1959) reported a 1958 survey by the Food and Drug Administration, in which 936 samples of raw milk from forty-eight dairies in sixteen metropolitan areas in all sections of the U.S. were analysed for residues of chlorinated organic pesticides by a paper chromatographic method. Investigations to determine the source of pesticide residues were limited to eight producers whose milk contained 'substantial' residues. Of these, the source of contamination was definitely accounted for in only three cases. In the milk from one producer, the feeding of DDT-contaminated corn silage (1·2–12 ppm. DDT) was responsible for high residues, 4·8 and 6·33 ppm. DDT. In the other two cases, contamination was traced to careless spraying of barns with concentrated DDT solution by a commercial exterminator.

Papworth (1961), in the *Veterinary Annual*, has reviewed the subject of the toxicity of pesticides in relation to animals, but he did not adduce evidence for toxic effects on men. He refers to papers describing toxic effects from bitch's milk (Burgisser, 1960) and residues of dieldrin in cow's milk; but cows did not excrete malathion in the milk even when fed levels of 8 ppm. for 3 weeks (Smith *et al.* 1960).

Sanders (1962) points out that total losses to farm crops from pests of all types may be in the region of £1×10^8 in the U.K., and that chemical pesticides can play an important part in reducing these losses, as they have done in increasing the yield of arable crops on British farms by 30–40 % in the last 20 years. Although no specific cases of poisoning by toxic chemicals or residues had been reported, it was harder to prove that no human being had suffered any sort of ill-effects from such residues. Laboratory animals had been fed through a life-time with doses of the new pesticides far in excess of those likely to be encountered in human food and as far as it goes the evidence seems to show that responses to equivalent doses by men and laboratory animals are similar. On the other hand, insecticidal seed dressings can be a menace to wild birds and to mammals eating the birds, but restrictions on the use of such dressings were agreed in 1961 and now these risks are believed to be slight.

The Joint Committee on Antibiotics in Animal Feeding (1962) noted in their report that the Scientific Sub-Committee accepted that the permitted feeding of antibiotics since 1953 had been of economic benefit; in young pigs and poultry it significantly lowered the cost of production, through either improved rates of growth or improved efficiency of feed conversion, or both; these effects did not occur in germ-free chicks and it would seem that the effects in farm animals are often due to control of sub-clinical infection. They state that there is no evidence that feeding antibiotics at permitted levels has any harmful effects on animals and after slaughter there are no more than traces of antibiotics in carcases or animal products. They consider the only remaining potential hazard to animals or human health that can be foreseen arises from the effect of antibiotics on bacterial populations.

Frazer (1962), in an address to the conference of the Royal Institute of Public Health and Hygiene, has recently reinforced these views in relation to the possibility that, if the particular antibiotic is one used clinically, its indiscriminate use in animals producing milk or meat might lead to the development of resistant organisms on a large scale, and a further danger might arise from the possibility that the injected antibiotic might so far alter the bowel-flora as to result in gastro-intestinal distributors and other difficulties. He also said that milk or meat from animals so treated retained varying quantities of the antibiotic, in an amount sufficient to cause a reaction in sensitive individuals.

MILK FATS IN RELATION TO HEART DISEASE

Davis (1961*a*) has reviewed the evidence relating to diet and coronary artery disease with reference to the role of essential fatty acids, and particularly to fats derived from milk and dairy products. He emphasizes the difference between normal intake and excessive intake and concludes, *inter alia*, that the amount of properly controlled experimental evidence on the relationship between cardiovascular disease and fat intake and type is practically negligible, and that drastic recommendations, such as the avoidance of all dairy produce, are unwise, because the solids-not-fat fraction of dairy products is probably the most valuable constituent in our diet. Meat fat might be reduced first, and secondly butter and margarine, but no reduction should be made in whole milk or cheese. In another communication, the same author (1961*b*) indicates that if the teaching of certain schools is extended into the future we shall have healthy middle-aged men thinking they can avoid disease by sipping maize-oil in front of a television set or at a banquet, and by taking tablets or capsules while travelling everywhere by car.

In regard to excess intake of milk and milk fats, Briggs, Rubenberg, O'Neal, Thomas & Hartroft (1960) reported a study of the incidence of myocardial infarcts among three groups of autopsied patients who were matched for age, sex, race, and place and period of death: (1) patients with peptic ulcers who had been treated with a Sippy diet or milk products; (2) patients with peptic ulcers who were not known to have been so treated; (3) non-ulcer patients matched with the other two groups. This study was carried out as a sequel to reports that the incidence of myocardial infarcts is higher among persons with chronic peptic ulcers than others. Dietary difference might account for this, and milk products are suspect because they are commonly

used by these patients. Butter fat in particular is suspect because of its effect on blood coagulation and clot lysis, because it is a major constituent of diets that produce coronary thrombosis and myocardial infarcts in experimental animals, and because of its effect on blood cholesterol levels of man under certain conditions. Ten hospitals in the U.S.A. and five in the U.K. provided the clinical and autopsy data. In each of groups (1) and (2) there were ninety-seven patients in the U.S.A. and ninety-five in the U.K.; the control groups (3) numbered 194 and 190 respectively. In the U.S.A. the incidence of myocardial infarct was identical in ulcer patients without Sippy diet and in non-ulcer controls (15 %); incidence in the Sippy-treated ulcer group was significantly higher at 36 %, while the elimination of twenty-one patients on diet for less than a year left seventy-six patients with an incidence of 42 %. In the U.K., incidence of infarcts in patients on milk diet was 18 %, significantly higher than in the non-Sippy ulcer group (3 %) and the controls (8 %).

After allowing for other factors, it was tempting to think that the high incidence of myocardial infarcts among Sippy-treated patients was a result of the butterfat content of their diet; but the authors point out that of course mere association does not constitute proof and further study is needed before definitive conclusions are drawn.

An Interdepartmental Committee (1960) on Milk Composition in the United Kingdom concluded that the S.N.F. fraction of milk should be maintained, as certain population groups have inadequate intakes of protein and calcium. Unless these are increased, through consumption of more milk, dairy products or other foods, an increase in the S.N.F. content of milk would be desirable, but this could only be done gradually. This does not apply to the fatty fraction of milk, as the diet as a whole contains more than adequate amounts of vitamins A and D. The Committee stressed the need for more research into the relationship between milk fat and arterial degeneration, but thought no reduction was called for in the fat content of milk; if it were necessary to reduce the amount of milk fat consumed, butter would be considered first. The fat of milk has an indirect value, because people prefer whole milk and there has been no increase in consumption of skim-milk in this country, as in some parts of the world; thus maintenance of milk fat at its present level ensures that milk remains an esteemed article of diet.

RADIONUCLIDES IN MILK

The hazards to man of nuclear and allied radiations are now of world-wide concern and the two British reports of the Medical Research Council (1956, 1960) are detailed expositions of all the ramifications of the subject. It is, of course, in relation to their great *potential* somatic and genetic damage that the radionuclides are the subject of concern and not in relation to a delineated existing disease problem.

Appleyard (1961) points out that the acute radiation syndrome is well known and well described in man. The median lethal total body dose for man is probably not far from 500 rad. At doses above this not much can be done. At doses well below, recovery is straightforward and apparently complete with little external aid, and for short exposures over a wide range of doses there are also extensive data. It is in the range of doses well below the median lethal, say 5–100 rad total body dose, that

another weakness is apparent; there are no really satisfactory biological indications of exposure; most of the ordinary peripheral blood examinations, e.g. differential white cell count, are rather remote from the radiation effect and rather easily upset by other factors. In Hiroshima and Nagasaki people exposed to radiation have been followed up and there is no doubt about the subsequent increased incidence of leukaemia within the following 10 years. An increase in malignancies other than leukaemia and further delayed in time has also been found in these studies. In general, however, there is a major gap in our understanding of the genetic effects of radiation on people.

Newcombe & James (1959) state that about 4 % of all individuals born will suffer at some time during their lives from serious defects in which heredity plays an obvious and major part, and it is now believed that something between a quarter and the whole of this 'load' of hereditary defects is directly maintained by the rate of naturally occurring new mutations in each generation. The new mutations presumably make up for the defective individuals who die young or who fail to reproduce. The frequency of the conditions which are mutation-maintained will increase with any rise in the level of ionizing radiation. Present levels of exposure resulting from medical X-rays average about 3 roentgens per generation per person and might eventually increase hereditary effects by as much as 10 %. Genetic effect of fall-out from nuclear weapon testing is probably about one-thirtieth of this, but while this appears small as a percenage, it seems large if expressed as absolute numbers of seriously affected individuals per generation in the world population. These authors considered the uncertainties in calculations—one of the chief stemming from ignorance concerning the respective roles of selection and mutation in maintaining our present load of hereditary defects. They discussed a method by which we might obtain the necessary insight into the operation of these two factors to influence the genetic component of population health and well-being.

Newcombe (1960), who has made a comprehensive study of this subject, considers that population genetic studies must be based on the family as the important unit and, within the family, on a chronology of the events of marriage, birth, death and illness or handicap. Conventional surveys are not adequate for the purpose but much of the required information already exists in the routine records of vital events and illnesses relating to whole populations. The ordinary methods of vital statistics are incapable of extracting such family data from the routine records on a massive scale, but development studies carried out in Canada indicate that this extraction can be achieved with the aid of electronic computers.

The need for accurate data in the field of general radiation exposure of populations is a special illustration of the whole problem of ensuring accurate data about all diseases, as well as those that are milk-borne.

An Expert Committee on Radioactive Materials in Food and Agriculture was set up by the FAO to review the behaviour of radioactive substances in terrestrial and aquatic food chains, including levels in food and agricultural materials, and practices which affect radioactive contamination of human diets; to indicate improvements in surveys of radioactive substances in food and agricultural material; and to make recommendations regarding research on food chains to further understanding and reduction of radioactive contamination of human diet. The report of this Committee

(FAO, 1960) noted that the quantity of ^{131}I which reaches milk depends on the extent to which the deposit is intercepted on plants which cattle consume, which will vary greatly according to the nature of the herbage and the extent of removal of ^{131}I from leaves by rain. An average of 5–10 % of ingested ^{131}I appears in cow's milk and 20–30 % in that of goats and sheep.

In 1961 the FAO published a summary of world information on dietary levels of ^{90}Sr and ^{137}Cs. An approximate correlation between latitude and ^{90}Sr in milk was found, values for U.K., U.S.A., Russia, Germany and France being similar and for Norway and Canada slightly higher, but the few values for Sweden and Finland were lower. Values were low for the Mediterranean area, India, Japan, South Africa, and Australia, and very low for Central America. A general rise in level occurred between 1957 and 1959 in the northern hemisphere, correlated with heavy fall-out. In 1960 a considerable decrease in ^{137}Cs was observed, because of the cessation of weapons testing.

McNeill & Trojan (1960) related the ^{137}Cs content of Toronto milk in 1959 to the feeding arrangements of the animals—in barns on winter feed up to April, then out to pasture in May and exposed to fall-out contamination as a result of bomb tests in late 1958. Two distinct rates of decrease of the Cs/K ratio supported the assumption that most of the Cs entering cattle comes from foliage directly rather than via the grass root-system. Peaks of Sr/Cs ratio and Cs/K ratio were reached in May–June 1959.

Miettinen, Paakkola, Nasanen, Vuorinen & Merten (1961) state that milk and milk products are the main source (85 %) of caesium in the Finnish diet. An analysis of grass and milk in Finland in 1959 for ^{90}Sr and ^{137}Cs showed similar results to that of milk of other countries in corresponding climatic conditions.

Madshus & Baarli (1960) reported that in Norway the value of the total fall-out was higher in the spring and summer of 1958 than in the preceding year, ranging up to 119 mc/km^2 in April. The ^{137}Cs content of dried milk showed seasonal variations, with a remarkable increase in spring and summer and a decrease in late autumn and winter, in 1958 and in 1959.

Ellis, Howells, Russell & Templeton (1960) studied deposition of ^{90}Sr on farm land and its transfer to milk after the reactor accident at Windscale in 1957, and after fall-out from weapons tests. Deposition averaged 160 $\mu\mu$c/m^2 on farms within two square miles of Windscale and fell rapidly with distance from the works. At farms farther from the works most of the ^{90}Sr deposition came from weapons test fall-out. The highest level of ^{90}Sr in milk was 0·2 $\mu\mu$c/g of Ca, which was lower than the permissible amount after such incidents (2 $\mu\mu$c/g of Ca) recommended by the Medical Research Council. Relatively more ^{90}Sr was transferred to milk from weapons test fall-out deposition than from material emitted from Windscale.

An editorial in the *British Medical Journal* (1961) reviewed the question of radioactive iodine in milk. Every nuclear explosion generates ^{131}I in the fission products of uranium or plutonium in bombs. It was pointed out that explosions at several thousand feet resulted in a fraction of the fall-out being distributed promptly and in lethal doses over a relatively small area of the earth's surface, while most of the radioactive material is propelled into the stratosphere, from which it descends gradually over a period of a year or two. A third fraction of the fission products is trapped in the

lower atmosphere and deposited in a few weeks, covering the hemisphere in which the explosion occurred, and this is the source of iodine about which concern is expressed. This isotope is not found in the stratospheric fall-out because its short half-life allows it to decay during the months it remains in the upper air. In 1958, measurable amounts of ^{131}I were detected in the air after heavy programmes of nuclear weapon testing in U.S.A. and U.S.S.R., while in October 1961 tests had again released large amounts of ^{131}I which had found its way into milk. A Medical Research Council report (1961) advised that an acceptable radiation dose would not be exceeded in any age-group unless the average concentration of ^{131}I in milk of 130 $\mu\mu c/l$ was exceeded over a period of a year. The *British Medical Journal* considered that a stricter standard would be necessary when a nation's whole milk supply was threatened. During October 1961 the average iodine concentration was about 130 $\mu\mu c/l$ (higher in Scotland but lower in most parts of England). Children are vulnerable to ^{131}I, particularly at 6 months, as up to this age the thyroid gland remains constant in size though the milk intake increases roughly in proportion to the infant's body weight. The *British Medical Journal* saw no reason for advising dried milk for children at that time.

The Agricultural Research Council Radiobiological Laboratory (1961*a*) reported on the quantity of ^{90}Sr in milk produced in the U.K. in 1959–60. During the late spring and early summer of 1960, cattle were grazing for the first time on herbage which had grown after the rate of fall-out had been relatively low for several months; on average, milk produced then contained 48 % of the ^{90}Sr observed a year previously. The mean for the 12 months ending June 1960 was 10·3 $\mu\mu c$ of $^{90}Sr/l$ or 8·4 $\mu\mu c/g$ of Ca; this was about 15 % less than the figure at the end of 1959. Investigations continued in 'special areas' where high rainfall and local agricultural conditions lead to higher levels in milk than elsewhere, and where the effective delay period in transfer of ^{90}Sr from rain to milk is longer than elsewhere.

In a later report (1961*b*) the A.R.C. Radiobiological Laboratory gave the estimated mean ratio of $^{90}Sr/Ca$ in the average diet in the U.K. during 1960 as 6·4 $\mu\mu c/g$, less than three-quarters of the value for 1959; while the mean concentration of ^{90}Sr in milk was 7·8 $\mu\mu c/l$ or 6·4 $\mu\mu c/g$ of Ca, about two-thirds of that for 1959. The decrease reflected reduced rate of fall-out with suspension of weapon testing.

A more recent report from this laboratory (1962) contains the results of measurements of ^{131}I in milk between December 1961 and April 1962, and of ^{90}Sr in milk during the latter half of 1961. The levels of ^{131}I in milk were negligible between December 1961 and April 1962. During the 32 weeks following nuclear weapon tests in autumn 1961, the average amount of ^{131}I entering milk in the U.K. was 17 % of that which would give rise to the annual average of 130 $\mu\mu c/l$ which the M.R.C. stated would not cause the acceptable radiation dose to be exceeded for any age-group; between major regions the values ranged from 11 to 24 %.

Measurements of ^{90}Sr in milk showed that values in the summer of 1961 were similar to those of a year before, but between October and December the ratio was on average about 20 % higher than the same period in 1960, attributed to tests in autumn 1961. However, the mean ratio for 1961 was about 10 % less than that for 1960. But the highest levels of ^{90}Sr in milk resulting from these tests was not expected before mid-1962.

The average mixed diet of U.K. population during 1961 was estimated to contain 6·2 $\mu\mu$c of ^{90}Sr/g of Ca (similar to the value for 1960). Working levels giving a margin of safety below maximum permissible levels were derived from M.R.C. recommendations: for individuals in general population, 400 $\mu\mu$c of ^{90}Sr/g of Ca; average for whole population, 130 $\mu\mu$c of ^{90}Sr/g of Ca. During 1961, levels of ^{90}Sr in milk and in total diet were less than one-twentieth of the working level for the whole population; in localized areas the levels in milk were a little above one-tenth of the working level for individuals.

Garner (1959) concluded from available data that if a cow's milk is fit for human consumption there is no danger to the cow itself from ^{131}I or ^{90}Sr.

The fall-out from nuclear tests has stimulated some countries to analyse food and water to discover their content of radioactive material and to estimate the total intake into the body of the longer-lived isotopes, ^{90}Sr and ^{137}Cs. Such research has required a large number of radiochemical analyses, since analysis of one source only (e.g. water) sheds very little light on the whole picture. Estimates have been checked by analysing human bones obtained at post-mortem examination for ^{90}Sr (WHO 1961*d*).

Bryant & Loutit (1961) made an assessment of the accuracy and precision of the methods for the determination of ^{90}Sr, stable Sr and Ca in human bone. The degree of contamination of an individual with ^{90}Sr depends largely upon his diet, the contamination of which has been determined mainly by the rate of fall-out. These authors show that the degree of contamination of bone (*a*) differs in infants according to domicile, being higher in Wales and probably lower than average in London and the home counties; (*b*) differs between bones of an individual adult, depending on rate of turnover of mineral in the respective bones; and (*c*) is relatively constant between bones of growing children.

Scott Russell (1962) stated that although Sr and Ca do not move at identical rates through biological systems, their behaviour is sufficiently similar for the fate of ^{90}Sr to be largely inferred from a knowledge of the metabolism of Ca. Although about 65 % of ^{90}Sr consumed by the population comes from milk and milk products, this does not necessarily mean that reduction in intake of milk might lessen the hazard. Strontium and calcium are transferred from diet to bone in a relatively constant ratio, calcium dilutes the ^{90}Sr in the skeleton, and retention is determined by the ratio of ^{90}Sr:Ca in total diet rather than its content of ^{90}Sr alone. The ratio of ^{90}Sr:Ca in milk is relatively similar to that in other foods; thus variations in the quantity of milk consumed have little effect on the ratio of ^{90}Sr to calcium in the total diet and the exposure level will not be affected.

Differences in the amount of deposition of fall-out in various regions depend upon variations in rainfall. Rainfall in most of the U.K. is between 25 and 75 inches a year and the variation in total deposition of ^{90}Sr varies by a factor of 3. Examination of ^{90}Sr content of milk and of potatoes in different regions shows that the extent of contamination varies with the amount of rainfall.

The Health and Safety Laboratory of the U.S. Atomic Energy Commission (1962), in their quarterly report of 1 April, presented current data from the fall-out programme and the U.S. Naval Research Laboratory. Radionuclide levels in fall-out, water, and milk were several times higher during 1957–59 than either before or after. Inter-

pretative reports and notes deal with ^{90}Sr levels in diet, U.S. wheat, and tap water, and also included was a report on errors in ^{90}Sr measurements made during 1961. A bibliography of recent literature pertinent to fall-out studies was given.

Removing radionuclides from milk

About 85 % of radioactive strontium and radioactive caesium in cow's milk can be removed by treatment with cation exchange resins saturated with non-radioactive cations, e.g. Ca, without apparently lowering the quality of the milk (Migicovsky, 1959). ^{131}I has been removed from milk up to the extent of 98 % by treatment with an anion exchange resin in the chloride form. Preliminary laboratory experiments have also been conducted on the removal of ^{137}Ca from meat by treatment with sodium chloride.

Singer & Armstrong (1960) have shown that up to 75 % of the ^{85}Sr content of milk could be removed by passing it through a column of pulverized protein-free or fat-free bone treated with 4N-$CaCl_2$ solution or 8N-KOH solution. After such use pulverized bone could be repeatedly regenerated.

Radionuclides in water used in dairying

The 4th report of the Ministry of Housing and Local Government (1962) on radio-strontium and radiocaesium in drinking water in the U.K. gave results of measurements on samples taken in 1961. Assuming an average ingestion of 1 l/day, the estimated average daily ingestion of ^{90}Sr in drinking water in 1961 was 0·25 $\mu\mu c$ per person. This amount is small compared with the average daily intake in the diet, which for 1961 was estimated at 6·71 $\mu\mu c$ per person. The daily ingestion of ^{137}Cs in drinking water in 1961 was estimated to be 0·13 $\mu\mu c$ per person.

These figures are of value when related to the use of contaminated water in dairy operations.

MISCELLANEOUS

Wende & Dienst (1961) reported a localized area in Georgia with a high incidence of attenuated infection with *T. gondii* in a herd of cattle and swine. The high incidence (51 %) of toxoplasmosis in the occupants of two cottages was related to association with the infected animals but not related to consumption of infected meat or milk supply, which was obtained from an outside dairy.

Nevot, Lafont & Lafont (1960) investigated the destruction of shigella in milk by pasteurization, and found that *Shigella dysenteriae*, *Sh. flexneri* and *Sh. sonnei* were destroyed by pasteurization when correctly carried out.

Elliker (1961), discussing time and temperature combinations for inactivation of pathogenic microorganisms, gives times for destruction of the pathogenic micro-organisms in milk and milk products. He refers to *Listeria monocytogenes*, an organism that infects man and twenty-six other mammals and birds, and causes an increase in monocytes, necrotic or granulomatous foci in various organisms and conjunctivo-keratitis. Pasteurization in the laboratory at 149 °F for 35 sec, 167 °F for 10 sec and at 185 °F in a commercial flash drum pasteurizer destroyed a mixed suspension of fifty-four strains of the organism. Significance in raw milk supplies

was not indicated. Bruhn, Møller-Madsen, Pedersen & Jensen (1960) determined temperatures required for destruction of a number of organisms with 16 sec exposure in milk. *St. pyogenes* was destroyed at 141·6°F, *Staph. aureus* at 151·5°F, *Salm. typhimurium* at 151·5°F, *Br. abortus* at 153·5°F, and *Str. faecalis* at 163·8°F. All but the last were destroyed at 153·4°F for 16 sec. However, they considered questionable whether or not this organism should be classed as a true pathogen. A comparison of temperatures required for destruction of *Escherichia coli* and various pathogens by the come-up time method of pasteurization was made, and higher temperatures were required for *E. coli* than for the pathogens tested.

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