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A rapid bioassay for penicillin residues in milk

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(*Received* 23 *August* 1969)

SUMMARY. A new bioassay for the detection of penicillin in milk which can be completed in $1\frac{1}{2}$ h has been developed. The test depends on measurement of pH changes in milk produced by an antibiotic sensitive organism, *Streptococcus thermophilus* BC. To measure the changes potentiometrically would require a very sensitive pH meter. Change in the enzymic coagulation time, which is extremely dependent on pH, was used in the present study to measure small changes in pH. The test is highly reliable and should be suitable as a milk selection test.

Antibiotic residues in milk are undesirable because they may be a public health hazard and also they inhibit growth of microbial cultures in the manufacture of some dairy products. The subject has been adequately reviewed (Marth & Ellickson, 1959*a*, *b*; Marth, 1961*a*, *b*; Storgårds, 1962; Albright, Tuckey & Woods, 1961).

Two courses may be followed to ensure that milk is free of antibiotic residues: (1) the prohibition or strict control of the use of antibiotics for the treatment of dairy cattle and (2) the employment of suitable tests to detect antibiotic residues in the milk, together with a suitable penalty scheme. Although the first approach has been used with some success in England, the second has been more generally adopted.

Methods for detecting antibiotic residues have been reviewed (Storgårds, 1962; Feagan, 1966). Physical methods are based on the addition of a marker to antibiotic preparations used as infusions. Bioassays, using antibiotic-sensitive bacteria, are more commonly employed at present. Those finding widespread acceptance are the disc assay methods of Kosikowski (1957) and Kosikowski & Ledford (1960) and the triphenyltetrazolium chloride (TTC) method of Neal & Calbert (1955).

The principal disadvantage of bioassays is the time required for completion—150 min for the TTC method. Moreover, a high inoculation rate of 10 % is used, thus reducing the sensitivity of the test. Discussions with Dairy Plant operators suggest that 60 min would be an acceptable time limit for routine testing.

The present paper describes a new bioassay for the detection of penicillin residues. The test may be completed within 90 min and approaches the sensitivity of the TTC test. It is highly reliable and, with a little experience, is simple to perform.

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MATERIALS AND METHODS

Principle of test. Growth of lactic acid bacteria in milk may be measured by changes in pH or titratable acidity, but very sensitive equipment is required to detect low levels of growth (Berridge, 1957). However, at around the normal pH of milk a slight decrease in pH produces a very marked decrease in the enzymatic coagulation time (ECT). Changes in ECT should, therefore, be a more sensitive indicator of growth than are changes in pH. If the culture used is sensitive to antibiotics, growth and the concomitant decrease in ECT will not occur in milk containing inhibitory levels of antibiotics. Measurement of the ECT before and after a period of incubation should therefore indicate the presence or absence of bacterial inhibitors. Such a technique has been used by Chevalier & Mocquot (1959) to assay nisin in milk, using rennet to detect the fall in pH caused by the growth of *Streptococcus lactis*.

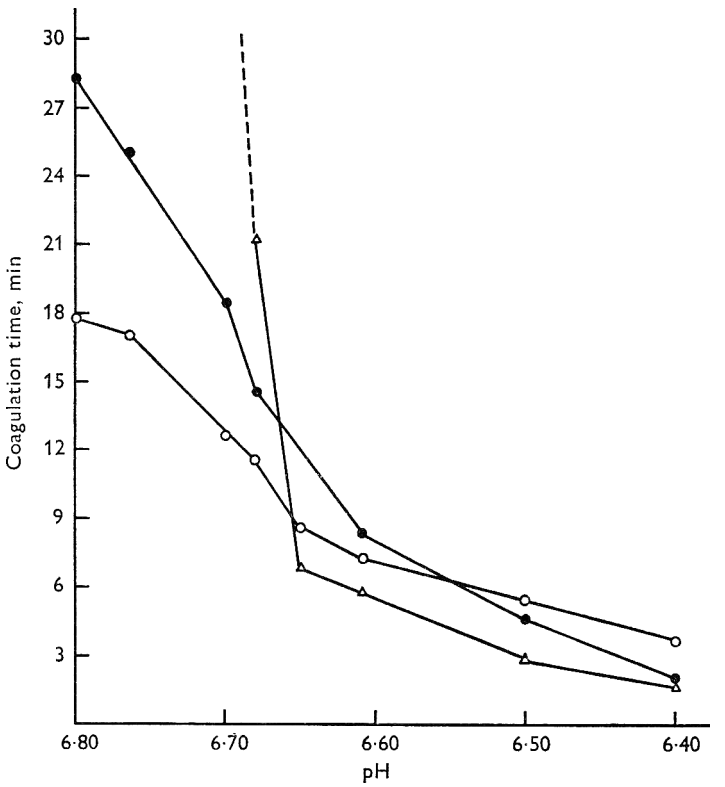

Fig. 1. The pH dependence of the milk-clotting activity at 32 °C of rennet, ○—○; bovine pepsin, ●—●; and porcine pepsin, △—△.

Milk clotting enzyme. Most proteases are capable of coagulating milk but for the present purpose the most suitable enzyme would be the one whose milk-clotting activity was most affected by changes in the pH of the substrate. In Fig. 1 is shown the dependence of the milk-clotting activity of rennet, bovine pepsin and porcine pepsin on the pH of the substrate. The milk-clotting activity of porcine pepsin was extremely pH-dependent in a narrow pH range around 6·65 but bovine pepsin

showed the greatest pH-dependence over a fairly wide pH range, and was therefore chosen as being the most suitable enzyme. The bovine pepsin used was a crude preparation prepared in this laboratory as described by Fox (1969).

A reciprocal relationship existed between the coagulation time and the quantity of pepsin used under a given set of experimental conditions. The coagulation times obtained with 3 enzyme levels at each of 4 pH values is shown in Fig. 2. It is obvious that, with increasing enzyme concentration, the difference between the ECTs of the high and the low pH samples decreased rapidly although the percentage change was virtually the same at all 3 enzyme levels. For example, at pH 6·75 and pH 6·50, the ECTs with 3 μl of enzyme/ml were 29·6 min and 4·9 min respectively (ratio 6:1), whereas at 10 μl/ml the corresponding times were 9·4 min and 1·6 min (ratio 5·9:1). We consider that the most desirable enzyme level is that which gives a clotting time of about 10 min in milk at pH 6·6.

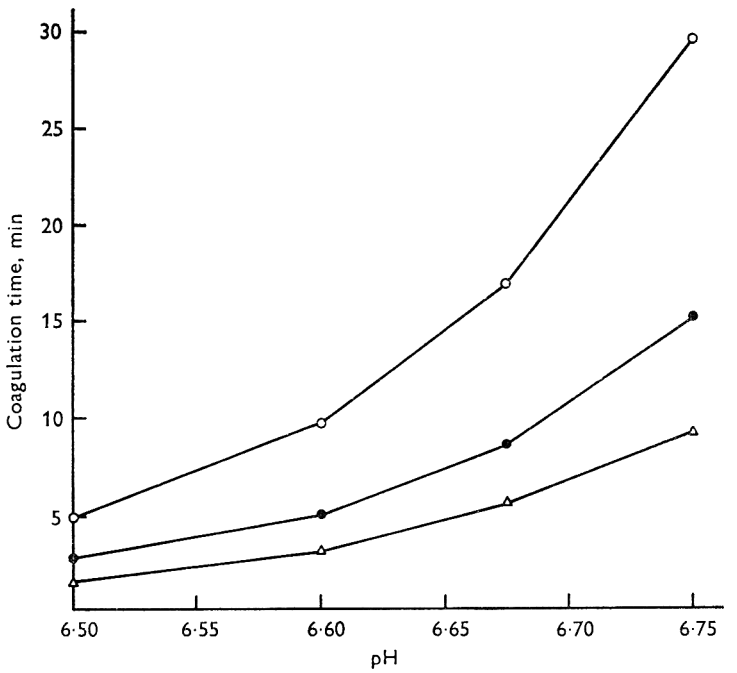

Fig. 2. The milk-clotting activity at various pH values of 3 levels of bovine pepsin: 3 μl/ml, ○—○: 6 μl/ml, ●—●: 10 μl/ml, △—△.

Measurement of coagulation time. Numerous methods have been developed for the measurement of rennet coagulation time. For the present test a simple method by which a large number of samples could be assayed simultaneously was required. An apparatus similar to that described by Sommer & Hart (1919) was used. The coagulation time was measured at 37 °C.

Test organism. Streptococcus thermophilus BC was used as bioassay organism. Working cultures were prepared by inoculating sterile skim-milk at a rate of 1–2 % and incubating at 37 °C for 16 h. Milk samples were inoculated with the working culture at a rate of 5 % unless otherwise specified.

Effect of yeast extract on growth and sensitivity of Str. thermophilus *BC*

Addition of yeast extract (YE) (Oxoid Ltd, London) to the test milk enhanced growth (Fig. 3) and reduced both the generation time and the lag phase. The effect of 0·05 % YE only is shown but levels of 0·025 and 0·1 % gave similar results. In all subsequent experiments 0·05 % YE was added to the test milk. The additions were made by adding 1 % YE to the working culture immediately before its addition to the test milk.

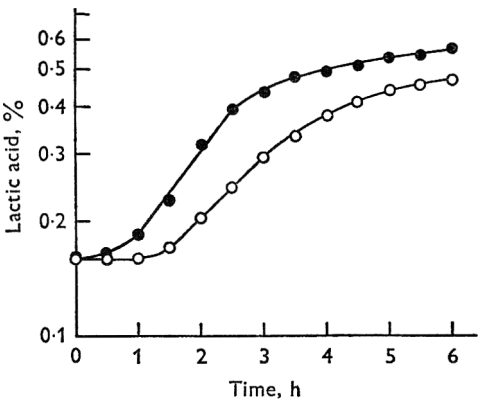

Fig. 3

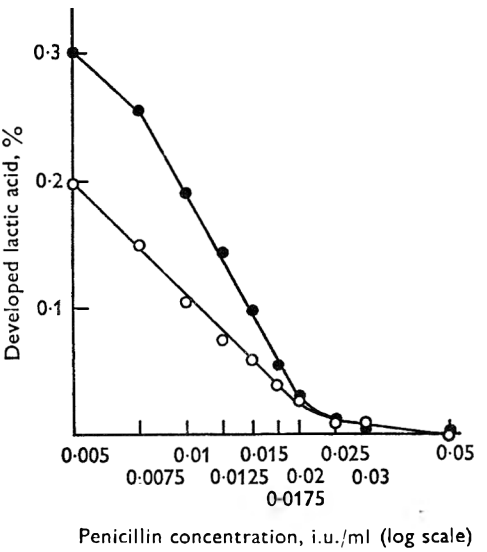

Fig. 4

Fig. 3. Effect of 0·05 % yeast extract on growth of *Streptococcus thermophilus* BC. ○—○, without yeast extract; ●—●, with yeast extract.

Fig. 4. Sensitivity of *Streptococcus thermophilus* to penicillin in the presence (●—●) or absence (○—○) of 0·05 % yeast extract. Developed lactic acid in the presence and absence of yeast extract was 0·33 and 0·22 % respectively at zero concentration of penicillin.

The sensitivity of the organism to penicillin (potassium benzyl penicillin) was slightly impaired by the presence of YE (Fig. 4). This experiment was carried out using sterile 10% non-fat milk solids. At the lowest level of antibiotic tested (0·005 i.u./ml), only slight inhibition was observed. Fifty per cent inhibition occurred at penicillin levels of 0·011 i.u./ml in presence of YE and at 0·010 i.u./ml in its absence. This slight loss in sensitivity is not important in commercial application of the test because at these levels the organism is still very sensitive. Stimulation of the organism by YE was evident even in the presence of sub-lethal amounts of penicillin ($< 0{\cdot}02$ i.u./ml).

Measurement of growth response

After the required incubation period, the test samples were chilled in an ice-water bath. The extent to which growth had occurred was assessed by: (*a*) titrating a 10-ml sample to pH 8·3 with N/9 NaOH using a Radiometer model 28 titrator; (*b*) measuring changes in pH, using a Radiometer model 25 meter; (*c*) measuring changes in enzymic coagulation time.

Milk supply

In preliminary feasibility studies, fresh whole milk or skim-milk from the Institute herd was used. The pH was adjusted to 6·7. Herd milk supplies from a local creamery, without pH adjustment, were used to evaluate the final method.

DEVELOPMENT OF THE TEST

In initial experiments penicillin was added to a portion of raw bulk milk to a final level of 0·05 i.u./ml and to a second portion was added an equivalent volume of distilled water. After inoculation the samples were incubated at 44 °C. Duplicate aliquots were withdrawn from both samples at hourly intervals and the pH values and clotting times measured. The results of a representative experiment are shown in Fig. 5.

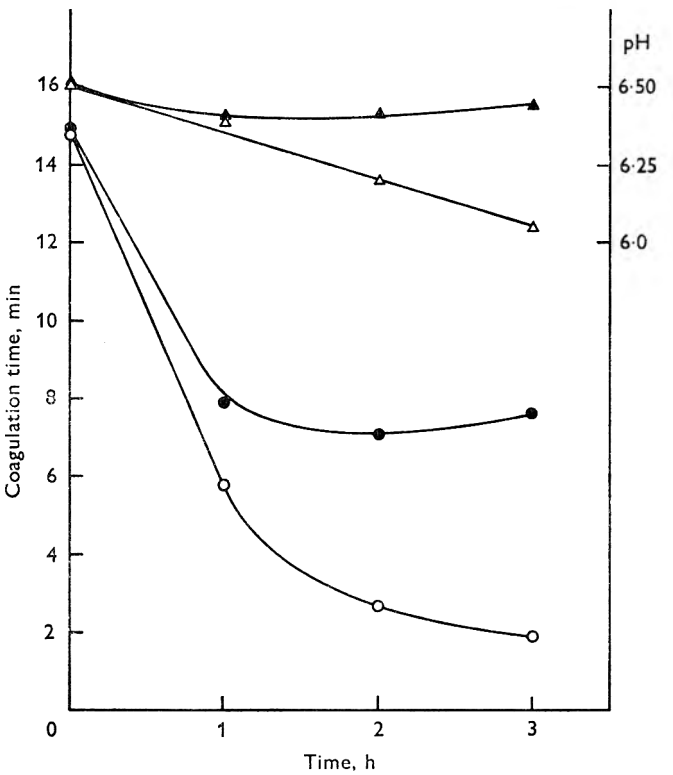

Fig. 5. The effect of incubation time on pepsin coagulation time (○) and pH (△) in presence (closed symbols) and absence (open symbols) of 0·05 i.u. penicillin/ml.

A slight decrease in the pH value and clotting time of the penicillin-containing sample usually occurred on incubation (Fig. 5) but occasionally slight increases were noted. The reason for these increases is not obvious but a similar observation was made by Phelan (1968). Assuming that an actively growing culture will produce some acid before assimilating an effective level of inhibitor, the decrease in clotting time is readily explained.

Although the decrease in the clotting time of the control (penicillin-free) sample was

very much greater than that of the penicillin-containing sample, the inconsistencies which occurred in the clotting time of the latter could lead to misinterpretation of results. A number of possible solutions to this problem were investigated. It will be seen that most of the decrease in clotting time of the penicillin-containing sample occurred shortly after the start of incubation. A preincubation period was tested but proved unsatisfactory. Various levels of starter were also investigated without success.

Table 1. *Detection of added penicillin in herd milks*

	Enzymic coagulation time, min			
	No added penicillin		Added penicillin, 0·03 i.u./ml	
Sample no.	Penase	No Penase	Penase	No Penase
1	3·45	3·43	3·00	12·00
2	2·37	2·30	2·00	7·40
3	3·50	3·39	3·00	11·35
4	2·15	2·22	2·05	6·45
5	2·30	2·11	2·12	9·00
6	3·55	3·30	2·39	15·45
7	9·05	8·15	8·00	17·15
8	5·50	5·55	6·00	10·45
9	9·30	9·41	9·35	19·45
10	6·56	6·15	6·48	14·45
11	9·15	9·00	8·10	28·15
12	4·30	4·15	4·20	8·20
13	11·42	11·24	10·50	31·11
14	10·42	10·30	10·30	23·45
15	7·21	7·22	7·19	14·15
16	10·26	9·47	10·35	21·53
17	14·05	12·47	13·11	28·25
18	9·15	8·18	9·11	18·27

A successful modification involved the addition of penicillinase (Penase, AVM Laboratories Ltd, Wrexham) to a portion of each sample (Table 1). Since enzymes capable of degrading other antibiotics have not been found, this modification limits the usefulness of the test to the detection of natural penicillins. Perhaps this is not a serious disadvantage, since *Str. thermophilus* is not as sensitive to other common antibiotics as are cheese cultures.

Final form of the test. Two 4·5 ml aliquots are taken from each milk sample. To one is added 0·2 ml of penicillinase solution (1000 units/ml) and to the other an equal amount of water. Both aliquots are inoculated with 0·25 ml of neutralized working culture containing sufficient YE to give a final concentration of 0·05 % in the test milk and incubated at 44 °C for 75 min. The ECT at 37 °C is then determined.

Interpretation of test results. If the sample contains no penicillin the test organism will grow at the same rate in both aliquots and their ECTs will be the same. On the other hand, if penicillin is present the organism will grow faster in the penicillinase-containing aliquot and a difference in the ECT of the aliquots will therefore occur.

EVALUATION OF THE METHOD

Samples from 18 bulk herd supplies were collected at a nearby creamery. All were antibiotic-negative to the TTC test. To simulate penicillin-free and contaminated samples each sample was divided into 4 aliquots, to 2 of which a solution of penicillin was added to give a final concentration in milk of 0·03 i.u./ml. To the other 2 aliquots an equivalent volume of water was added. Penicillinase was added to one of each pair of aliquots and allowed to act for 5 min. The 4 aliquots were then inoculated with neutralized culture containing YE and incubated at 44 °C for 75 min. The ECT's are summarized in Table 1.

A comparison of the last column with the other columns shows that inhibitory levels of penicillin can be readily detected by the method after 75 min incubation. In spite of wide variation in the clotting times of the milks there was no difficulty in detecting the penicillin-containing sample.

Conclusions

1. Theoretically the test should be capable of detecting all inhibitors to which *Str. thermophilus* is sensitive. However, because of inherent variations in the ECT of bulk milks and the incipient growth of the culture in the presence of inhibitory levels of antibiotics, the test was modified. As a result of the modification only penicillin residues can be detected by the test.

2. The test is very reliable for the detection of inhibitory levels of penicillin and is capable of quantitation by serial dilution. Because of the relatively short time required for completion, the test should be suitable for milk selection.

3. Further work may resolve the artifacts and make the test suitable for the detection of other inhibitors.

The authors wish to express their thanks for the excellent technical assistance given by Mr B. Wally and Mr P. Thornhill.

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With 7 plates

Influence of aggregation on the susceptibility of casein to proteolysis

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Summary. The susceptibility of the casein in milk to proteolysis was shown to be greatly influenced by its state of aggregation. In normal milk, where the casein is largely in micellar form, the α_{s1}- and β-caseins are almost inaccessible to proteolysis. On removal of the colloidal phosphate, the casein micelles disintegrate, rendering the components, especially the α_{s1}-casein, accessible to proteolysis. The role of colloidal calcium phosphate in the casein micelle is believed to be that of a non-specific aggregating agent which can be effectively replaced by calcium. Dissolved colloidal phosphate can be effectively reformed by elevation of the pH of colloidal phosphate-free (CPF) milk before equilibrium dialysis. Addition of κ-casein to CPF milk also causes aggregation of the component caseins but the micelles formed are smaller than those of normal milk.

The behaviour of micellar β-casein differs considerably from that of micellar α_{s1}-casein. The evidence suggests that part of the β-casein freely dissociates either outside or within the micelle when the temperature is reduced. The temperature dependence of the susceptibility of β-casein to proteolysis was similar in skim-milk and in solutions of sodium caseinate, and increased as the temperature was reduced. α_{s1}-Casein was quite resistant to proteolysis in normal milk but became susceptible when the micelle structure was disrupted on removal of colloidal phosphate.

It is concluded that limited proteolysis may prove a valuable technique in the study of casein micelle structure.

The proteins of cheese undergo considerable proteolysis during maturation. Although rennin is only weakly proteolytic (Berridge, 1945; Fish, 1957; Bang-Jensen, Foltmann & Rombauts, 1964), it plays an important part in this process. The products produced by rennin from casein in solution and in cheese have been investigated by Lindqvist & Storgårds (1959*a*, *b*, 1960, 1962), Cerbulis, Custer & Zittle (1958, 1960), Ledford, O'Sullivan & Nath (1966) and Ledford, Chen & Nath (1968). The influence of incubation temperature and pH on the proteolysis of casein by rennin has been recently investigated by Fox (1969).

Ledford *et al.* (1968) reported that casein in solution was more susceptible to proteolysis than was micellar casein. The present paper describes the results of a study on the influence of degree of aggregation on the susceptibility of casein to

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proteolysis. The results are of importance from 2 separate aspects: (1) they elucidate some additional factors which influence the degree of proteolysis occurring in cheese, and (2) they demonstrate the potential usefulness of limited proteolysis as a technique in the study of casein micelle structure.

EXPERIMENTAL MATERIALS AND METHODS

Milk supply.

Bulked milk from a 300-cow herd was separated at 40 °C and the skim-milk cooled immediately at 3 °C. Toluene was routinely added to all samples as a preservative.

Preparation of milks of varying colloidal phosphate content

As shown by Pyne & McGann (1960), the colloidal calcium phosphate content of milk can be readily varied, without significantly changing the other constituents of milk, by altering the pH of cold milk followed by neutralization, through dialysis against an excess of cold bulk milk. Milks containing approximately 100, 50, 25 and 0 % of their original colloidal phosphate were prepared by adjusting the pH of cold skim-milk (0–2 °C) to pH 6·6, 6·1, 5·5 and 4·8 respectively, and dialysing against a large excess of cold bulk milk, with 3 changes over a 3-day period.

Preparation of calcium-aggregated, colloidal phosphate-free (CPF) milks

Samples of CPF milk were dialysed against large excesses of bulk skim-milk containing various levels of added $CaCl_2$. All the calcium-fortified skim-milks were readjusted to pH 6·6 after the addition of $CaCl_2$. After 24 h dialysis, the pH of the milk in the dialysis was adjusted to 6·6 and dialysis continued for a further 48 h. On completion of dialysis, the pH was adjusted to 6·6 if necessary.

Preparation of synthetic milk

A portion of skim-milk at 0–2 °C was adjusted to pH 4·6 and divided into 4 aliquots. The pH of 1 aliquot was left at 4·6 while the others were adjusted to pH 7·0, 9·0 and 11·0. The 4 aliquots were then dialysed against a large excess of fresh skim-milk until pH equilibrium was established (3 days).

Simulation of the acid development during cheese manufacture

Mabbitt, Chapman & Berridge (1955) have shown that the pH changes which occur during the manufacture of Cheddar cheese can be simulated quite closely by using 1,5-gluconolactone (GAL). GAL was added to 2 sets of aliquots of skim-milk at 25 °C at levels sufficient to give concentrations of 0, 6, 15, 35 mg/ml and corresponding pH values of 6·6, 6·2, 5·8 and 5·2. Rennet (10 μl/ml of a 1:20 dilution of commercial rennet) was added to 1 set of aliquots simultaneously with the GAL, and to the other set after a 2-h holding period when equilibrium pH had been attained. The coagulated samples were centrifuged, the supernatant whey discarded and the pellet macerated and suspended in 0·1 M phosphate buffer of pH 6·6, and of volume equal to that of the removed whey.

A 1·5:5 dilution of rennet was added at 10 μl/ml to each of the samples, which were then incubated for 20 h at 32 °C.

Measurement of proteolysis. Proteolysis was measured by changes in the electrophoretic pattern of casein. As demonstrated by Fox (1969), changes in the electrophoretic pattern are a much more sensitive indicator of proteolysis than are changes in non-protein nitrogen.

The conditions under which proteolysis was performed varied widely and are more conveniently dealt with in the results section.

Gel electrophoresis

A vertical gel electrophoresis apparatus (E. C. Corporation, 220 S. St., University City, Philadelphia, Pennsylvania) was used. Electrophoresis was performed as described by Thompson, Kiddy, Johnston & Weinberg (1964). Samples were mixed with an equal volume of 7 M urea buffer and 20 μl aliquots applied to the gel.

Preparation of κ-casein. Purified κ-casein was prepared by the urea fractionation procedure of Zittle & Custer (1963). The preparation was essentially free of α_s- and β-caseins (Plate 4).

Determination of sialic acid. The analytical method of Warren (1959), with slight modifications, was used to assay for sialic acid. Due to the interference of lactose in the sialic acid assay, the TCA-insoluble rather than the TCA-soluble sialic acid was measured. After various incubation times with rennet the reaction was stopped by addition of TCA to 12%. The precipitate was removed by centrifugation, washed in 12% TCA and digested with 1 N-H_2SO_4. Subsequent steps followed Warren's procedure.

RESULTS AND DISCUSSION

Effect of colloidal calcium phosphate on the susceptibility of the casein of milk to proteolysis. Milk containing 100, 50, 25 and 0% of its original colloidal calcium phosphate level was subjected to proteolysis by rennet for 20 h, at pH 6·6 and 32 °C. The enzyme was used at a level sufficient to give a coagulation time of about 6 min in milk at pH 6·6 and 32 °C. Gel electrophoretograms of control and proteolysed samples (Plate 1) show that as the casein became more dispersed, on reduction of its colloidal calcium phosphate content, it was more susceptible to proteolysis, particularly the α_{s1}-casein. The susceptibility of CPF milk was about the same as that of sodium caseinate (Fox, 1969).

Since CPF milk differs from normal milk only in its content of colloidal calcium phosphate, it is concluded that colloidal phosphate plays a major integrating role in the structure of the casein micelle. In his model of the casein micelle, Morr (1967) proposed that the casein micelle is built up from calcium–caseinate complex units, each of which consists of a core of α_s- and β-caseins surrounded by a layer of stabilizing κ–α_s-casein complex. Colloidal calcium phosphate linked the complex units into a loose network to form micelles. Rennin and other proteolytic enzymes are able to filter through the pores of the network, split a glycopeptide from the κ-casein and thereby destabilize the whole system.

The present data do not support the views of Morr. If the various casein components were arranged stoichiometrically into subunits, the α_s- and β-caseins should be as inaccessible to enzymic attack in the subunit as they are in the intact micelle if, as is proposed by Morr (1967), the enzyme is able to percolate freely through the micelle.

This has not been found to be the case since the α_s-casein component becomes readily available for proteolysis when the micelle is disrupted.

The rate of release of sialic acid by rennet was not significantly altered by removal of colloidal phosphate (Table 1). The data suggest that, in agreement with the views of many other workers (Waugh & Noble, 1965; Payens, 1966; Rose, 1969), κ-casein is on the outside of the casein micelle, readily accessible to proteolytic enzymes. α_s- and β-caseins, on the other hand, are not normally accessible to proteolytic enzymes and become accessible only when the micelle is disrupted, e.g. by removing the cementing colloidal calcium phosphate.

Table 1. *Release of sialic acid* by rennet from milk and colloidal phosphate-free (CPF) milk*

	Normal milk		CPF milk	
Reaction time, min	A_{549nm}	Insoluble sialic acid, %	A_{549nm}	Insoluble sialic acid, %
0	1·75	—	1·85	—
3	1·43	81·8	1·37	74·1
7	1·17	66·8	1·27	68·6
11	1·02	58·3	1·06	57·3
15	0·96	54·7	0·96	51·9
20	0·88	50·3	0·68	36·8
30	0·73	41·7	0·62	33·5

* For method of assay see p. 175.

Role of colloidal phosphate in the micelle. It is suggested that colloidal calcium phosphate plays a key role in maintaining the structure of the casein micelle. Various views have been put forward as to the actual nature of the association between the colloidal phosphate and the protein moieties of the micelle. One view proposes that the colloidal calcium phosphate forms cross-links between organic serine phosphate residues of the protein (McGann & Pyne, 1960; Rose, 1965). However, ter Horst (1963) proposes that the colloidal phosphate crystallizes on the ϵ-amino groups of lysine and this idea has received some support from Rose & Colvin (1966*a*). McGann & Pyne (1960) suggest that the role of colloidal calcium phosphate in the micelle is a specific one but also entertain the possibility of its being a more or less inert filler.

To determine whether the role of colloidal phosphate in the micelle was a specific one or whether its function was replaceable, the following experiments were performed.

(*a*) If colloidal calcium phosphate acts simply as an aggregating agent its role might be replaceable by calcium alone. To investigate the aggregating ability of calcium, aliquots of CPF milk were dialysed against skim-milk to which had been added 0, 5, 10, 20 and 50 mM-$CaCl_2$/l. The electrophoretograms of these milks following proteolysis by rennet for 20 h at 32 °C are shown in Plate 2. It is apparent that the addition of 10 mM-$CaCl_2$ was sufficient to aggregate CPF milk sufficiently to render the α_s- and β-caseins inaccessible to enzymic attack. κ-Casein, however, was still accessible as is evidenced by the electrophoretograms and the formation of a clot following rennet action. Other characteristics also indicated that aggregation had occurred. Thus, CPF milk was quite translucent, being greenish-yellow in colour. On dialysis against milks of elevated calcium content, it assumed the white colour

of normal milk. Also, the casein of CPF milk precipitates from solution on direct addition of calcium chloride to a concentration of 20 mM or higher. Following dialysis against calcium-fortified milks, CPF milk assumes the calcium stability characteristics of normal milk. This finding is being further investigated. These data indicate that colloidal calcium phosphate serves as a non-specific aggregating agent and that its function can be performed by calcium ions when these are suitably added, e.g. by dialysis.

(*b*) The casein reforms aggregates, which closely resemble the micelles of normal milk, if the pH of CPF milk is readjusted to 7·0 or higher before dialysis. Samples of such milks were prepared as described above. Electrophoretograms of the proteins after proteolysis by rennet at pH 6·6 for 20 h at 32 °C are shown in Plate 3. Even though the colloidal calcium phosphate was dissolved on acidification to pH 4·6, it could apparently be effectively reformed by raising the pH before dialysis. Other properties of the milk—turbidity and calcium stability—were also re-established. The results suggest that, under the conditions employed, the association between the colloidal calcium phosphate and the protein moieties of the casein micelle is reversible and is probably not very specifically organized.

(*c*) Addition of κ-casein to CPF milk also rendered the proteins of CPF milk inaccessible to enzymic attack (Plate 4). In this experiment purified κ-casein (slot 8) was added to CPF milk to give levels in the final mixtures of 1, 2, 3, 4 and 5 times that in CPF milk. The various mixtures were then incubated with rennet at pH 6·6 for 20 h at 32 °C. Although κ-casein reversed the accessibility of CPF milk to proteolysis and also rendered it stable to Ca^{2+} ions, the turbidity, while considerably increased, was not restored to that of normal milk.

Susceptibility of the proteins of simulated cheese to proteolysis

While the removal of colloidal calcium phosphate from milk, with its concomitant dissociation of the casein micelle, renders the proteins susceptible to proteolysis, it was felt that this may not take place in cheese. Here, the decrease in pH occurs after clot formation, when the proteins may be more or less fixed. Cheddar cheese also contains most of the colloidal phosphate of milk, and although this should be in soluble form at the pH of Cheddar cheese (5·0–5·2), it may help retain the original micellar structure of the casein.

The results summarized in Plate 5 indicate that irrespective of whether the milk was clotted before or after the decrease in pH, the structure of the micelle was upset by the pH reduction and it became accessible to proteolytic attack. The conditions under which the experiment was performed, while not identical with those of the early stages of cheese ripening, are sufficiently like them to justify the suggestion that in cheese the casein is non-micellar and readily susceptible to proteolysis. Presumably the calcium phosphate of cheese is in the soluble state, simply occluded in the cheese structure.

Effect of incubation temperature on the proteolysis of micellar casein

In an earlier publication (Fox, 1969) it was demonstrated that in solution the relative susceptibility of α_s- and β-caseins to proteolysis was temperature-dependent, relatively greater proteolysis of the β-casein occurring at lower temperatures. The

influence of incubation temperature on the proteolysis of non-micellar (sodium caseinate) and micellar (skim-milk) casein is shown in Plates 6 and 7 respectively. As in our previous work, longer incubation times were employed to offset the reduced rate of reactions as the temperature was reduced.

The data in Plate 6 show the effect of incubation temperature on the proteolysis of sodium caseinate. As the temperature was decreased, β-casein became relatively more susceptible to proteolysis than α_s-casein, whereas the α_s casein still suffered extensive proteolysis at all temperatures. In contrast, the α_s-casein of normal milk was almost inaccessible to proteolysis at any of the temperatures used (Plate 7). However, its β-casein behaved similarly to that of sodium caseinate, becoming more accessible to proteolysis as the temperature was decreased.

The role of β-casein in the micelle has been the subject of considerable research. In the models of the casein micelle proposed by Waugh & co-workers (1958, 1965), a minor role is assigned to β-casein in casein micelle formation. Payens (1966), however, felt that because of its association characteristics, through which it tended to form rod-like polymers (Payens & van Markwijk, 1963), β-casein might form the nucleus of the casein micelle with α_s-casein molecules attached to it at hydrophobic regions and the whole surrounded by a layer of hydrophilic κ-casein and colloidal calcium phosphate. Rose (1968) found that supernatants obtained from milk centrifuged at different temperatures varied in the ratio of β-casein to α_s-casein. Rose had previously suggested that the loss in resolution in electronphotomicrographs of milk which had been chilled and rewarmed was due to the dissociation of β-casein from the micelle on cooling and its redeposition on the surface of the micelle on rewarming (Rose & Colvin, 1966*b*). From this evidence he proposed that β-casein was present in 2 functionally different states in the micelle (Rose, 1969); part of the β-casein (40 %) was present as a rod-like chain forming the nucleus of the micelle, much as in Payens' hypothesis, but the remainder was less tightly involved in the micelle and dissociated from it when the temperature was lowered. Downey, Murphy & Aherne (1969) adduce evidence from molecular sieve filtration data in support of Rose's view of the role of β-casein in the micelle, and have shown that about 35 % of the β-casein is tightly integrated into the micelle structure while the remaining 65 % can be readily dissociated from the micelle.

The results of the present experiments show clearly that the behaviour of micellar β-casein is very different from that of α_s-casein. In normal milk at pH 6·6 and 32 °C the α_s- and β-casein components of the micelle were quite resistant to proteolysis (Plate 1, slot 1, and Plate 7, slot 4). When the micelle was disrupted by removal of the colloidal calcium phosphate, the α_s-casein component, particularly, became susceptible to proteolysis (Plate 1, slot 4, and Plate 6, slot 4). β-Casein, which at pH 6·6 has been shown previously to be fairly resistant to proteolysis at 32 °C (Fox, 1969), became susceptible as the temperature was lowered. The increased susceptibility of β-casein was found equally with sodium caseinate and normal milk. The resistance of β-casein to proteolysis at higher temperatures was previously attributed (Fox, 1969) to the temperature-dependent aggregation characteristics of β-casein (Payens & van Markwijk, 1963; Garnier, 1966). The application of this explanation to solutions of sodium caseinate is quite plausible but this explanation of the behaviour of micellar β-casein may not be sufficiently complete. It would be quite

applicable, however, if we assume that β-casein, or at least part of it, is not rigidly integrated into the micelle structure but is free to dissociate from it when the temperature is lowered.

From the present data it is not possible to arrive at any firm conclusions about the nature of the association of β-casein with the α_s- and κ-casein components of the micelle. However, it seems clear that the roles of α_s- and β-caseins in the micelle are different. α_s-Casein appears to be quite fixed and becomes accessible to proteolysis only when the native micelle structure is disrupted. β-Casein, on the other hand, is inaccessible at higher temperatures, as in solutions of sodium caseinate. Its accessibility is not significantly affected by rupture of the native micelle structure but, as in solutions of sodium caseinate, it does become accessible to proteolytic enzymes when the temperature is reduced, under which conditions dissociation into the monomeric form occurs (Payens & van Markwijk, 1963; Garnier, 1966).

Work on the role of β-casein in the native casein micelle is continuing.

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EXPLANATION OF PLATES

Plate 1. Effect of removing colloidal calcium phosphate on the susceptibility of the proteins of milk to proteolysis by rennet at pH 6·6, 32 °C for 20 h. Slots 1, 2, 3 and 4 show the electrophoretograms of renneted milk from which 0, 25, 50 and 100 % respectively of the colloidal phosphate had been removed. C represents the pattern of the appropriate non-renneted controls.

Plate 2. Effect of increased levels of calcium on the susceptibility of colloidal phosphate-free (CPF) milk to proteolysis by rennet at pH 6·6 and 32 °C for 20 h. Slots: 1. CPF milk; no rennet. 2. CPF milk; no $CaCl_2$; rennet. 3. CPF milk; 5 mM-$CaCl_2$; rennet. 4. CPF milk; 10 mM-$CaCl_2$; rennet. 5. CPF milk; 20 mM-$CaCl_2$; rennet. 6. CPF milk; 50 mM-$CaCl_2$; rennet. 7. Skim-milk; no rennet. 8. Skim-milk; rennet.

Plate 3. Effect of increasing the pH of colloidal phosphate-free (CPF) milk before dialysis on its susceptibility to proteolysis by rennet at pH 6·6 during incubation at 32 °C for 20 h. Slots: 1. Skim-milk; control. 2. Skim-milk; rennet. 3. Sodium caseinate; rennet. 4. CPF milk; rennet. 5. CPF milk adjusted to pH 7·0; rennet. 6. CPF milk adjusted to pH 9·0; rennet. 7. CPF milk adjusted to pH 11·0; rennet. 8. CPF milk; control.

Plate 4. Influence of added κ-casein on the susceptibility of proteins of colloidal phosphate-free (CPF) milk to proteolysis at pH 6·6 and 32 °C during incubation for 20 h. Purified κ-casein was added to CPF milk to give levels of κ-casein in final mixture of 2, 3, 4 and 5 times that of CPF milk. Slots: 1. CPF milk: control. 2. CPF milk; rennet. 3. CPF milk + 2 × κ; rennet. 4. CPF milk + 3 × κ; rennet. 5. CPF + 4 × κ; rennet. 6. CPF + 5 × κ; rennet. 7. CPF + 5 × κ; control. 8. κ-casein; control.

Plate 5. Proteolysis of the casein of simulated cheese at pH 6·6, 32 °C for 20 h. Slots: 1. control, no rennet. 2. No 1,5-gluconolactone (pH 6·6). 3. 6 mg 1,5-gluconolactone/ml (pH 6·2). 4. 15 mg 1,5-gluconolactone/ml (pH 5·8). 5. 35 mg 1,5-gluconolactone/ml (pH 5·2). (Rennet added before acid development.) 6. As for 3. 7. As for 4. 8. As for 5. (Rennet added after acid development.)

Plate 6. Effect of temperature on the susceptibility of sodium caseinate to proteolysis by rennet at pH 6·6. Slots: 1. 4 °C; 6 days. 2. 10 °C; 3 days. 3. 20 °C; 2 days. 4. 32 °C; 1 day. C. Appropriate, non-renneted controls.

Plate 7. Effect of temperature on the susceptibility of the caseins of skim-milk to proteolysis by rennet at pH 6·6. Slots: 1. 4 °C; 6 days. 2. 10 °C; 3 days. 3. 20 °C; 2 days. 4. 32 °C; 1 day. C. Appropriate, non-renneted controls.

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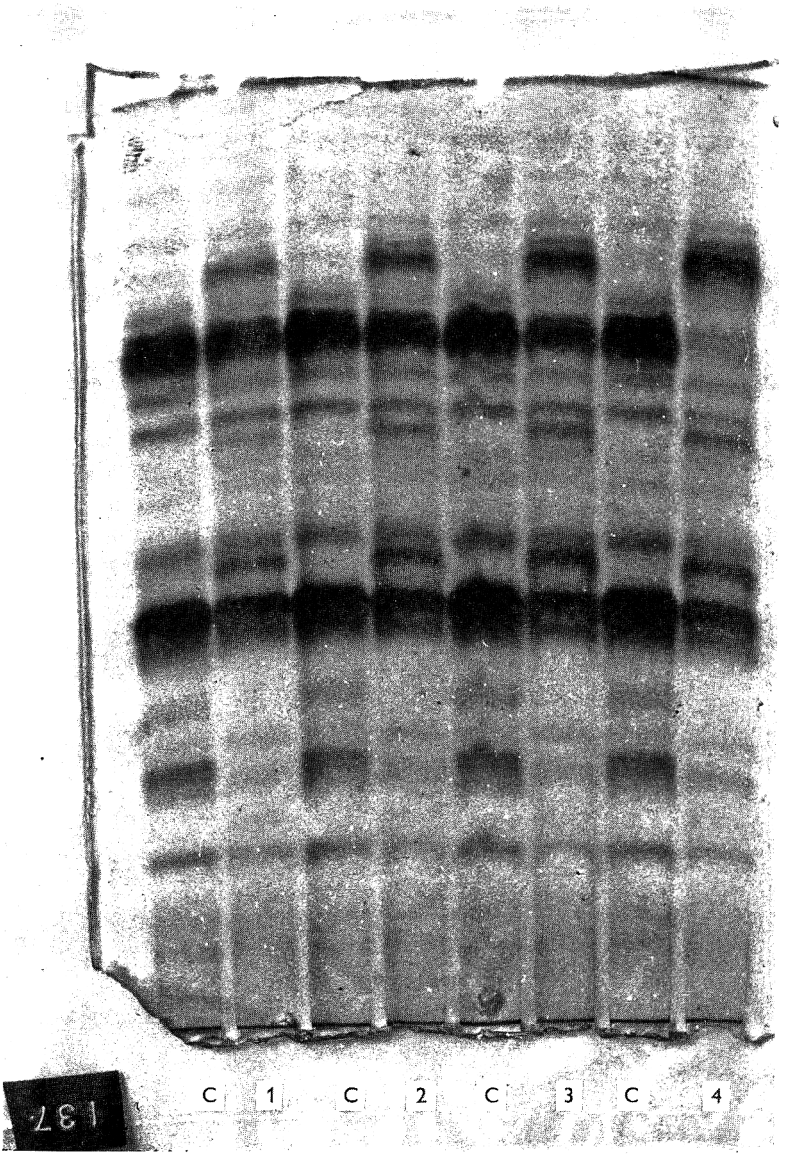

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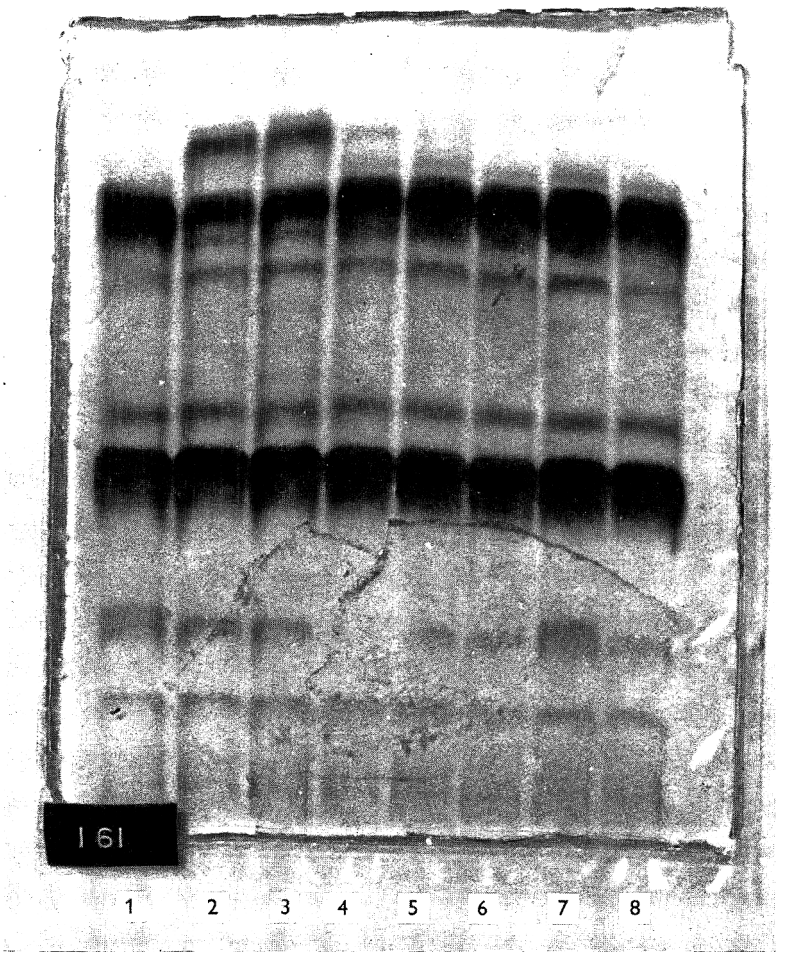

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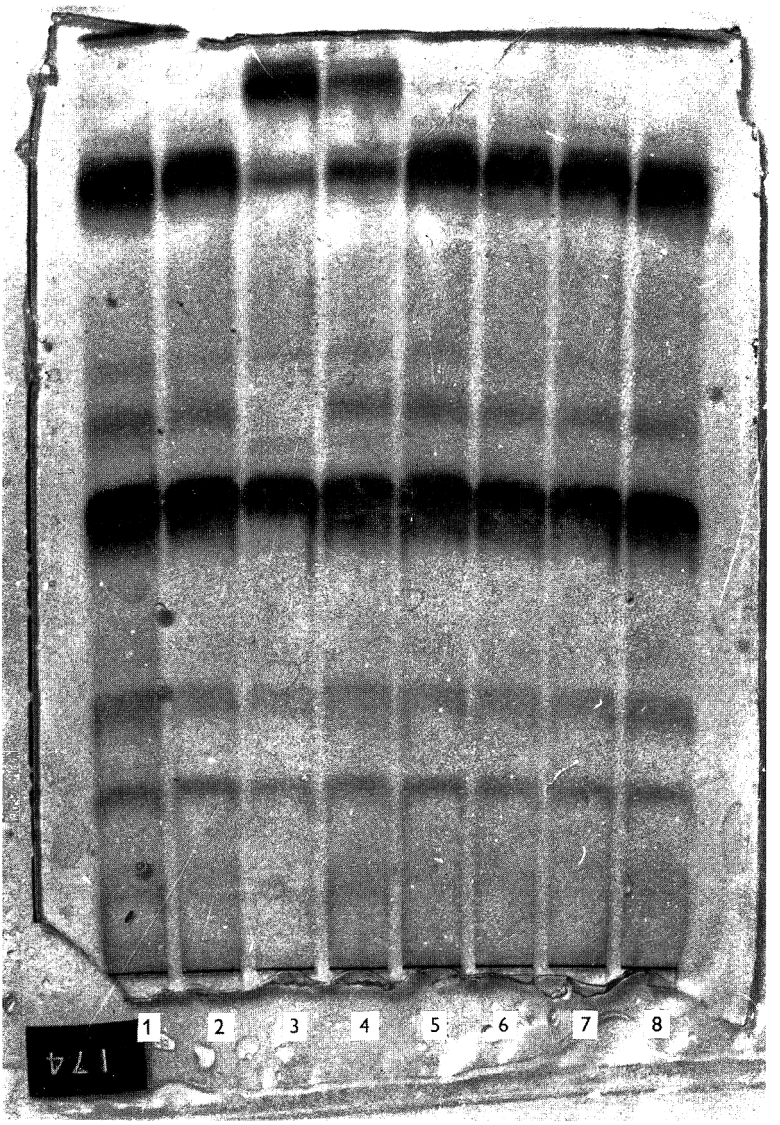

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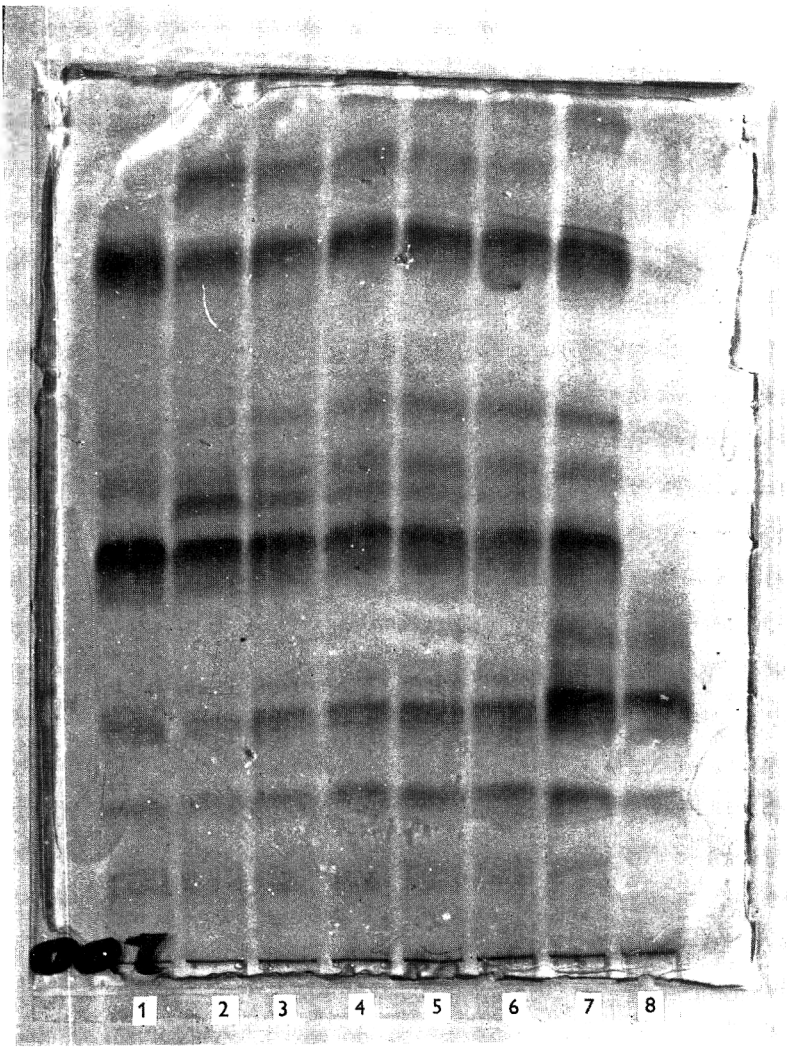

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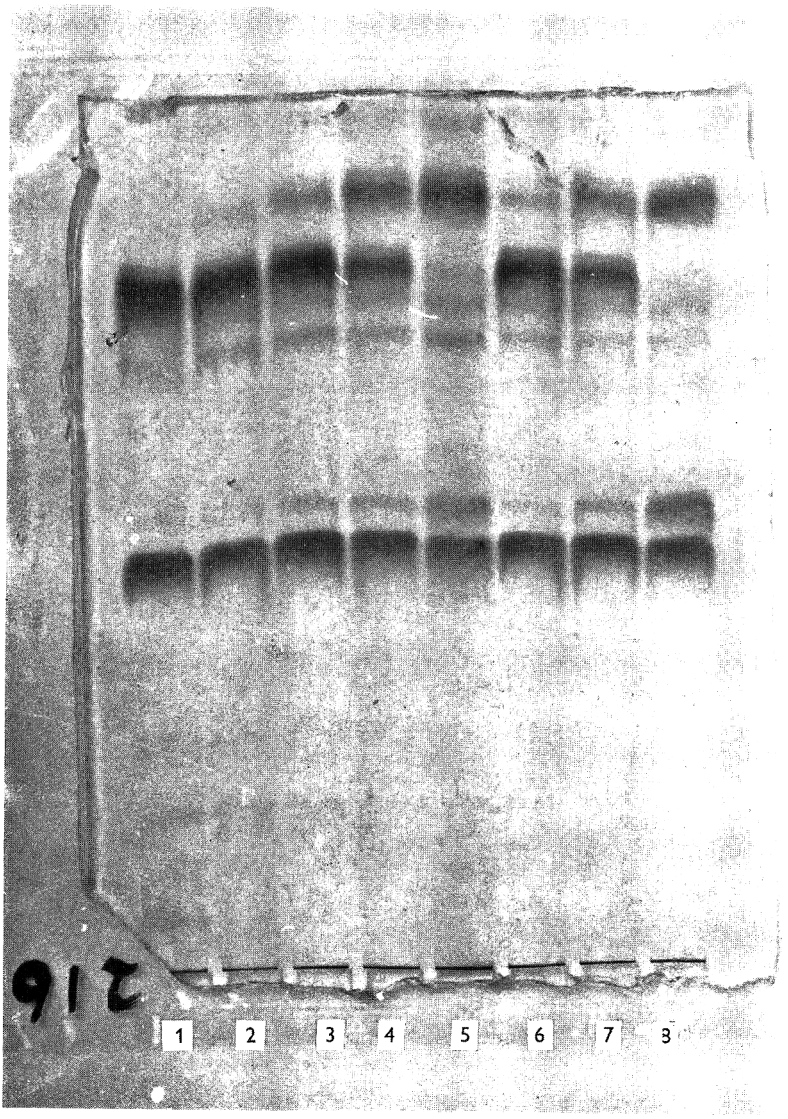
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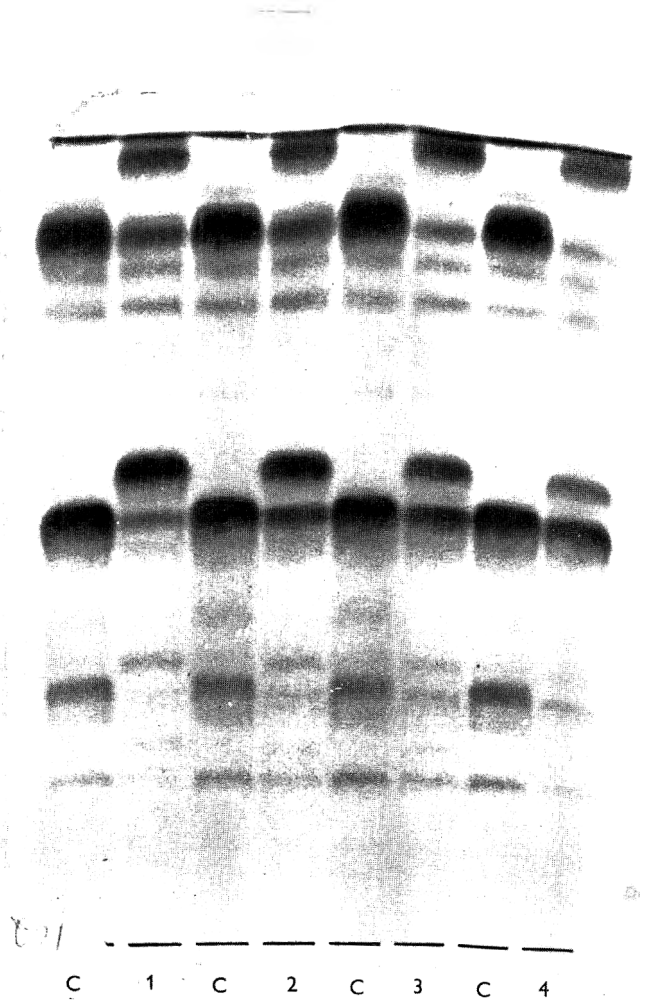
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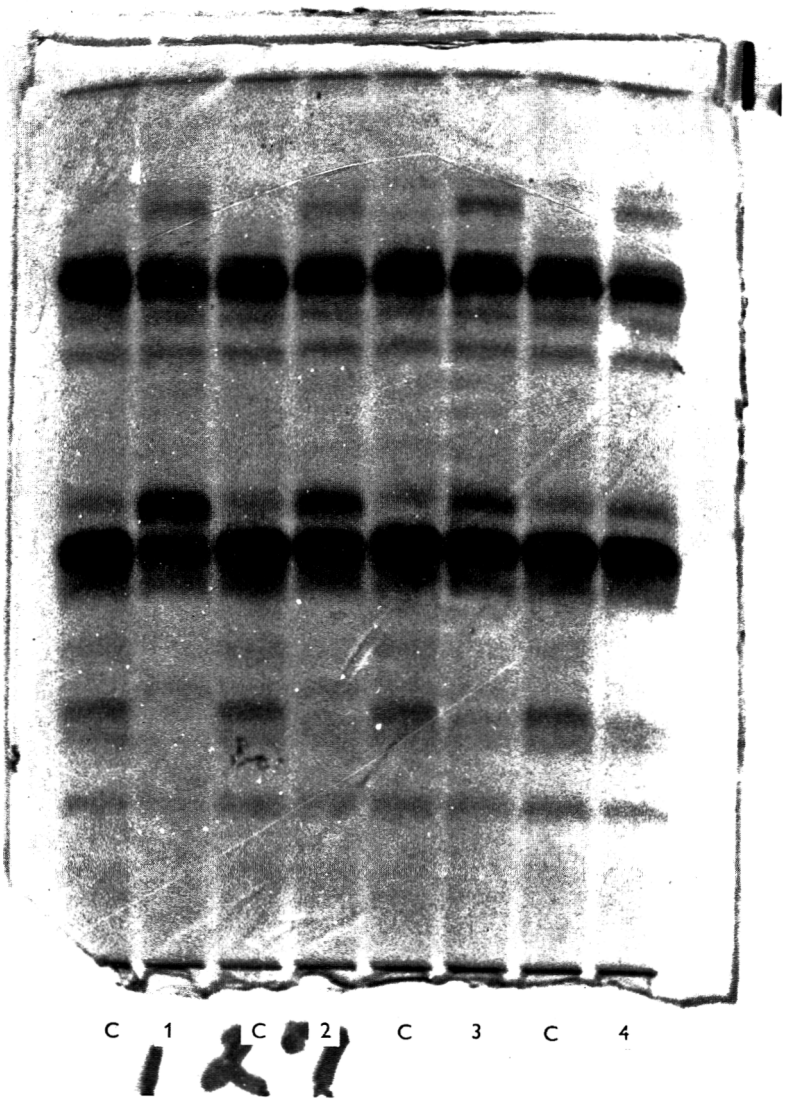

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The effect of the periodic feeding of linseed oil on the production and iodine value of milk fat

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SUMMARY. A study was made of the changes in the iodine value of milk fat from cows following the feeding of linseed oil (450 g) at intervals of 6 days. Three cows in mid-lactation and of differing age and breed were used. The experiment consisted of a series of 3 collection periods, each of 6 days' duration, with 12 days between periods. Three doses of oil were given (6 days apart) between periods 1 and 2, the last dose of oil being given immediately before the start of period 2. The mean iodine values for the 3 periods were 35·5, 40·5 and 34·7 respectively. There was a distinct pattern of change in iodine value with time after feeding the oil, a maximum of 45·5 being attained on the second day with a subsequent decline to 38·5 on the sixth day. Analysis of variance showed that these effects were significant and due to oil-feeding, whereas changes in yield and content of fat and production of milk could be accounted for by normal decline in lactation. It was concluded that the periodic feeding of a large dose of linseed oil produces a significant increase in the iodine value of milk fat and avoids the possible disadvantages of continuous fat supplementation.

The poor spreadability of refrigerated butter is largely due to the highly saturated nature of its component fatty acids. Indeed, spreadability of butter is closely correlated with its iodine value (Hill & Palmer, 1938; Dixon, 1964).

Although pasture-fed cows ingest considerable quantities of polyunsaturated fatty acids, the chylomicron fat absorbed from the gut is highly saturated (Wadsworth, 1968*a*) as a result of the extensive hydrogenation that occurs in the rumen. Hence dietary fatty acid is hydrogenated before incorporation into milk fat.

Recent studies in this laboratory on the absorption of fat by grazing cows have shown, however, that the feeding of a single, large dose of vegetable oil produces an increase in the amount and degree of unsaturation of absorbed fat and this increase is prolonged over several days (Hartmann, Harris & Lascelles, 1966; Wadsworth, 1968*b*). These observations, coupled with the fact that the mammary gland can utilize chylomicron triglyceride either directly or indirectly for the synthesis of milk fat (Lascelles, Hardwick, Linzell & Mepham, 1964) suggests that a significant softening of butter may be achieved by feeding a large single dose of oil to dairy cows.

That oil-feeding increases the degree of unsaturation of milk fat is well known, but previous experiments have usually involved daily administration of 200 g or more of

oil (see, for example, Brown, Dustman & Weakley, 1941; Parry, Sampugna & Jensen, 1964). At current prices this would not be economic. However, consistent with our recent observations on thoracic duct lymph, Aylwood, Blackwood & Smith (1937) reported that the feeding of a single dose (300 g) of linseed oil to a cow gave rise to an increase in the iodine value of the milk fat which reached a maximum of 7 units above pre-feeding levels and was prolonged for more than a week.

This prompted investigation of the possibility of obtaining a commercially significant rise in the iodine value of milk fat by dosing lactating cows with a highly unsaturated vegetable oil at less frequent intervals than have previously been used. Accordingly, a trial was instigated to assess the effect on the iodine value of milk fat of periodic administration of approximately 450 g of linseed oil to dairy cows.

EXPERIMENTAL

Animals. Three cows from the Dairy Research Unit herd were used. Details of their age, breed and stage of lactation at the commencement of the experiment, together with the results of the monthly herd recording before the experiment, are given in Table 1.

Table 1. *Details of the animals at the beginning of the experiment*

Cow	Breed	Age	No. of calvings	Date of commencement	Stage of lactation, week	Milk production, lb/day	Fat, %
1	Ayrshire	2 years 9 months	2	27. x. 67	11	29·2	2·9
2	Friesian	8 years 1 month	6	2. i. 68	15	40·4	3·9
3	Guernsey*	5 years 11 months	4	5. i. 68	10	42·6	4·3

* Cross bred.

The cows grazed a good quality pasture of ryegrass and clover. Cow 1 received no supplements whereas cows 2 and 3 received 10 and 12·5 lb of dairy pellets (15% protein) respectively each day during the first 2 sampling periods but no supplements during the last period. The cows were milked with the herd twice daily by machine.

Protocol. The experiment followed a design similar to that used by Shannon & Lascelles (1967), consisting of 3 collection periods. Oil was administered during the middle period and the other 2 periods served as controls. Time was allowed between periods for the animals to adjust to the change in diet.

Samples of milk were taken at each milking for 6 days (period 1). The cows were then dosed with 450 g of linseed oil (Meggit Pty. Ltd, Parramatta, N.S.W., Australia) at 6-day intervals. Beginning immediately after the third dose of oil, samples were collected for a further 6 days (period 2). The cows were thus on the oil-feeding régime for 12 days before the start of period 2. No more oil was given and 12 days were allowed after the completion of period 2 before commencing a final 6-day collection (period 3).

The milk from each milking was weighed. It was then thoroughly mixed and a *c.* 50 ml sample taken and stored at 4 °C in the presence of a few drops of formalin. Twice weekly the samples were warmed and stirred thoroughly, and the morning and

evening milks from each day were mixed in proportion to yield. A sample of each mixture was then taken for lipid extraction.

Analytical. Total fat in the milk was determined by the Babcock method as described by Davis & MacDonald (1953). Lipid was extracted with chloroform–methanol as described by Hartmann & Lascelles (1965). Iodine values were determined in duplicate on 20–60 mg of lipid by the Hanus method according to the Association of Official Agricultural Chemists (1965) procedure scaled down by a factor of 10.

RESULTS

Mean values for milk and fat production, fat percentage and iodine value of milk fat for each cow and each period are presented in Table 2. The iodine value rose by an average of 5 units during oil-feeding and returned to pre-feeding levels after the withdrawal of oil. By contrast, the other parameters either declined throughout the experiment (milk and fat production) or rose during the last period (fat percentage).

An analysis of variance of the results was carried out and is summarized in Table 3. The experimental design allowed the comparison of the 3 periods as though they were separate treatments. Since there was one oil-feeding and 2 control periods, partition of the 'treatments' source of variation using polynomial coefficients allowed differentiation of responses to oil-feeding (quadratic effects) from lactational trends (linear effects).

Table 2. *Milk and fat production, fat percentage and iodine value of milk fat from cows receiving* 450 *g of linseed oil at* 6*-day intervals*

(The experiment consisted of 3 collection periods, each of 6 days' duration, with 12 days between periods. Three doses of oil (6 days apart) were given between periods 1 and 2, the last dose being given immediately before the start of period 2. Values represent the means of daily determinations.)

	Period	Cow 1	Cow 2	Cow 3	Mean
Milk production, lb/day	1	26·3	41·8	42·0	36·7
	2	24·0	39·3	40·8	34·7
	3	21·5	29·9	32·1	27·8
Fat production, lb/day	1	0·87	1·47	1·55	1·30
	2	0·77	1·28	1·49	1·18
	3	0·77	1·14	1·23	1·05
Fat, %	1	3·3	3·6	3·7	3·5
	2	3·2	3·2	3·3	3·4
	3	3·6	3·8	3·8	3·8
Iodine value	1	36·0	37·0	33·5	35·5
	2	40·7	43·0	37·9	40·5
	3	36·5	36·2	31·4	34·7

There were significant treatments effects for all parameters. However, the quadratic component of the treatments terms for milk and fat production and fat percentage was not significant. While the linear component was not significant for milk or fat production, it accounted for 85 and 99·9 % respectively of the variation attributable to treatment effects. It may thus be seen that the significant changes in the yield and content of fat and production of milk were due to differences between the 2 control periods rather than to any effect of oil-feeding. Further information on this point was obtained when milk yield curves for the present and previous lactations were compared. All 3 cows were in declining lactation during the experiment. No

Table 3. *Summary of the analysis of variance of milk and fat production, fat percentage and iodine value of milk fat from* 3 *cows fed* 450 *g of linseed oil at* 6*-day intervals*

(The experiment consisted of 3 collection periods ('treatments'), each of 6 days' duration, with 12 days between periods. Three doses of oil (6 days apart) were given between periods 1 and 2, the last dose being given immediately before the start of period 2. Periods 1 and 3 served as controls.)

Source of variation	Degrees of freedom	Mean squares: Milk production	Fat production	Fat, %	Iodine value
Cows	2	1050·3***	1·937***	0·55	62·3***
Treatments	2	332·8*	0·281*	0·78*	150·9**
Linear	1	562·5	0·560	0·64*	5·4
Quadratic	1	103·1	0·001	0·93	296·3*
Treatments × cows	4	33·1*	0·039	0·06*	2·8
Linear × cows	2	39·9*	0·053	0·015*	2·0
Quadratic × cows	2	26·3	0·025	0·10	3·5
Times	5	19·1	0·021	0·102	2·3
Treatments × times	10	12·6	0·046	0·364	14·4**
Times × cows	10	7·9	0·047	0·282	5·8
Treatments × cows × times	20	11·4	0·058	0·357	3·3

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

To calculate the variance ratio for the 'treatments' term and components thereof, the mean squares for the 'treatments × cows' interaction and its corresponding components have been used as the denominator, while the mean square for 'treatments × cows × times' has been used as the denominator in the calculation of the variance ratios for the remaining sources of variation.

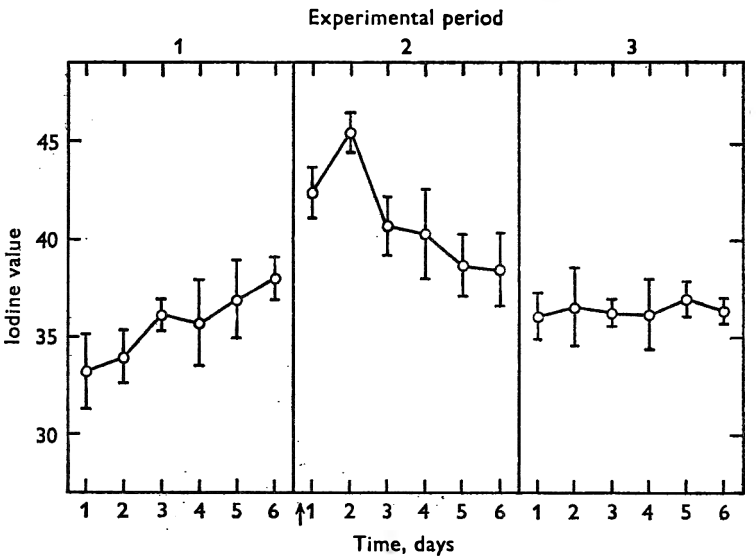

Fig. 1. Iodine value of milk fat from cows fed 450 g of linseed oil at 6-day intervals. The experiment consisted of 3 collection periods, each of 6 days' duration, with 12 days between periods. Three doses of oil (6 days apart) were given between periods 1 and 2. The last dose of oil is indicated by the arrow. Values plotted represent means ± standard errors for the results from 3 cows.

anomalies attributable to oil-feeding were observed, although the yields during periods 1 and 2 were slightly above the comparable values of the previous lactation. It is concluded that the differences between the control periods may be largely accounted for by normal decline in lactation.

On the other hand, the significant ($P < 0{\cdot}01$) treatments term for iodine value of milk fat was almost entirely accounted for by a significant ($P < 0{\cdot}05$) quadratic component (Table 3), indicating that the increase of 5 units during period 2 (Table 2) was due to the feeding of linseed oil. It is interesting that the treatments term accounted for 42 % of the total variation for iodine value whereas it contributed only 8–19 % of the total variation for milk and fat production and fat percentage.

There was also a significant ($P < 0{\cdot}01$) 'treatments × times' interaction for iodine value of milk fat (Table 3). The changes in daily iodine values are illustrated in Fig. 1, from which it is evident that the significant treatments × times term was due to a different pattern of change during the oil-feeding period compared with the control periods. Although there was an apparent change in iodine value during period 1, separate analyses of variance of the results for the 3 periods (using polynomial coefficients) showed that there were no significant changes in iodine value during periods 1 and 3 whereas there was a significant ($P < 0{\cdot}001$) linear decrease in iodine value during period 3.

DISCUSSION

The average increase of 5 units in the iodine value of milk fat evoked by oil-feeding (Table 2) is comparable to the range of seasonal variation in iodine value observed in butters from Australia and New Zealand (Mitchell, 1959; Dixon, 1964). The present observations (Fig. 1) are in agreement with previous studies on the transfer of single doses of fat into milk (Aylwood *et al.* 1937; Glascock, McWeeny & Smith, 1958; Glascock *et al.* 1966) which reported a rapid incorporation of the labelled fatty acid given orally, followed by a slow decrease in the specific activity of the milk fat over a period of several days. The principal factors responsible for these effects would appear to be (i) that large doses of unsaturated oils exceed the capacity of the rumen micro-organisms to hydrogenate lipid (Moore, Hoffman & Berry, 1945; Wadsworth, 1968*b*) and (ii) that ingested lipid is released only slowly from the rumen (cf. Lascelles, Hartmann & Harris, 1966).

Extrapolation of these results to practical situations must be approached with care for a number of reasons. Firstly, it has been found that growing steers fed liberal allowances of oil daily over prolonged periods show no increase in the unsaturation of the body fat (Thomas, Culbertson & Beard, 1934; Willey, Riggs, Colby, Butler & Reiser, 1952). This suggests that the rumen microflora may adapt to long-term feeding of unsaturated oils, resulting in increased efficiency of hydrogenation. No data appear to be available on the long-term effects of oil-feeding on the iodine value of milk fat. However, oil has been fed daily at high levels for periods up to 6 weeks with no evidence of decline in iodine value (Allen, 1935; Brown *et al.* 1941; Parry *et al.* 1964). Furthermore, seasonal variation in iodine value is probably due to dietary factors (McDowall & McGillivray, 1963) which does not support the idea of adaptation to highly unsaturated diets. Nevertheless, this possibility cannot be excluded.

Secondly, the effect of fat supplementation on the gross composition and production of milk by dairy cows is not well understood, despite the voluminous literature on the subject (see, for example, Van Soest, 1963). Indeed, Storry, Rook & Hall (1967) concluded from their review of the literature that the effect of feeding tallow and vegetable oils was variable depending on the experimental conditions employed.

However, there is evidence to support the view that, at relatively low levels of lipid intake, increases in dietary fat exert a positive effect on the secretion of milk and milk fat, whereas at high levels of fat supplementation the fat content of milk is depressed and the yield of milk and fat may be decreased (Orth & Kaufmann, 1957; Larsen, Klausen & Frederiksen, 1966; Storry, Hall & Johnson, 1968; Dr A. K. Lascelles, private communication). In the present experiment, the feeding of linseed oil had no effect on the yield and content of fat or production of milk (Tables 2 and 3) in agreement with the observations of Hartmann *et al.* (1966), who fed similar amounts of safflower oil. It would appear, therefore, that the infrequent feeding of large doses of oil avoids the possible detrimental effects of continuous supplementation with fat at high levels.

Finally, the effect of stage of lactation on response to oil-feeding is not clear, but since the contribution of blood lipid to milk fat synthesis appears to vary with stage of lactation (Decaen & Adda, 1966; Stull, Brown, Valdez & Tucker, 1966) this may be an important factor. In this connexion it should be pointed out that the 3 cows in this experiment were all in a similar phase of lactation (Table 1).

The advice and encouragement given by Professor A. K. Lascelles during this work is gratefully acknowledged. Thanks are also due to Miss C. Salt for technical assistance and to Messrs G. H. Gray and K. McKean for care of the animals. This work was supported by the Australian Dairy Produce Board and University Research Grants.

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The effect of the modification of arginine side chains in casein on the coagulation of rennin-altered casein

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Summary. The modification of arginine residues in casein by treatment with glyoxal at pH 8·6 resulted in an inhibition of the coagulation of rennin-treated casein. The effective residues are on the κ-casein fraction and inhibition of coagulation was complete when about 1·5 residues of arginine/mole of κ-casein had been altered. It is suggested that the arginine forms part of a positively charged region that also includes lysine and histidine residues, and that this region is important in the coagulation of the casein.

Recent work on the modification of lysine residues in casein (Hill & Craker, 1968) showed that the rennin-induced coagulation of casein could be inhibited by modification of a small number of the lysine residues on the κ-casein fraction. The lysine was modified by treatment with 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride at pH 8, and it was found that the effectiveness of the treatment depended greatly upon the proportion of disaggregating agent, such as acetone or isopropanol, which was present in the reaction mixture. In Fig. 1 data from Hill & Craker (1968) have been replotted to illustrate this effect. It is clear that the treatment became much more effective when the proportion of disaggregating agent reached about 9%; at this stage treatment with as little as 1·3–3 moles of dansyl chloride/10^5 g of whole casein may cause almost complete inhibition of coagulation. As the important lysine residues are on the κ-casein and there is about one mole of κ-casein/10^5 g whole casein, the dansyl chloride must react almost exclusively with these critical residues when inhibition of coagulation is caused by such small amounts of dansyl chloride. From these results it can be inferred, firstly, that the critical residues are normally not readily accessible but become so in disaggregating conditions and, secondly, that once rendered accessible they are much more reactive than are the other lysine residues.

In considering reasons for the enhanced reactivity of these lysine residues, it seemed that a probable cause could be the presence nearby of a functional group with a strongly bound positive charge, such as arginine. This would lower the pK of a neighbouring ϵ-amino group, and would enhance its reactivity towards reagents such as dansyl chloride which react with the unprotonated form of the lysine. If such a structure existed, modification of arginine side chains would be expected to have an effect on the coagulation of the casein. The present paper presents the results of experiments designed to test this hypothesis. They show that modification of

arginine side chains on the κ-casein does inhibit coagulation, and taken in conjunction with other results (Jollès, Alais & Jollès, 1968) point to the existence in κ-casein of a positively charged region which plays an important part in coagulation.

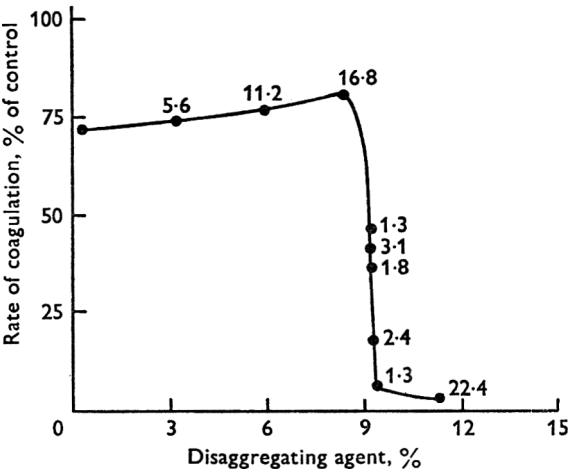

Fig. 1. The effect of the proportion of disaggregating agent present during reaction with dansyl chloride on the inhibition by this reagent of the rennin-induced coagulation of casein (based on data of Hill & Craker, 1968). Figures beside the points indicate moles of dansyl chloride/10^5 g of casein used in the treatment (pH 8, 20 °C). Disaggregating agent, isopropanol or acetone.

EXPERIMENTAL

Caseins

α_s- and κ-caseins were prepared by the method of Zittle & Custer (1963), while whole casein was prepared from fresh skim-milk by precipitation at pH 4·7, and washing and redissolving at pH 7. This process was repeated and the redissolved casein was centrifuged at 60000 *g* for 30 min to remove traces of fat: 100-ml portions of bulk solution were kept frozen, and thawed for use as required.

Arginine modification

Arginine side chains in the caseins were modified by the glyoxal method of Nakaya, Horinishi & Shibata (1967), except that the treatment was made milder by reducing glyoxal concentrations from 0·06 M to 0·01–0·02 M, by reducing the pH of the buffer (2% sodium bicarbonate) from 9·2 to 8·6 and by limiting the time of treatment at room temperature to 30–40 min instead of up to 24 h.

After the treatment the proteins, of concentration about 1·5%, were separated from reagents by gel chromatography on Sephadex G 25 in 0·05 M-sodium acetate buffer of pH 6·2. Control solutions were treated similarly except for the use of glyoxal. The extent of modification of the arginine residues was determined by assaying the arginine in the intact proteins by the method of Sakaguchi (Macpherson, 1946). Control and treated proteins and arginine standards were included in these tests. Although the glyoxal treatment is stated by Nakaya *et al.* (1967) to be specific for arginine, Bowes & Cater (1968) reported that reaction occurred with lysine or hydroxylysine residues in collagen after treatment with 0·06 M-glyoxal for 24 h. In

the much milder conditions of the present work, there was no measurable reaction with lysine residues as there was no significant difference between the lysine content (46–7 residues/10^5 g casein as estimated by formol titration at pH 8) of control caseins and caseins in which the modification was sufficient to cause complete inhibition of coagulation.

Rennin was prepared by salt precipitation and ion exchange chromatography as described by Hill & Laing (1967). Rates of coagulation of rennin-treated samples were determined at pH 6·5 and 30 °C in solutions containing 0·05 M-sodium acetate and 0·015 M-calcium chloride. The amounts of rennin used were sufficient to cause coagulation of the controls in 2–8 min.

Protein concentrations were estimated either from Kjeldahl nitrogen determinations (using a semi-micro distillation method) or from the optical density at 280 nm of suitably diluted solutions adjusted to pH 11·5. The relation between optical density and protein concentration was obtained from standard solutions of the different proteins. Quadrufos, a linear polyphosphate, was the product of Rumford Chemical Co., Rumford, Rhode Is., New York.

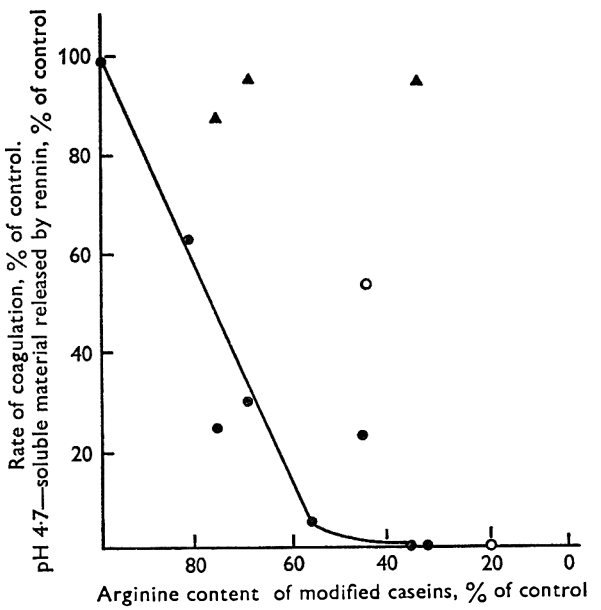

Fig. 2. The effect of modifying arginine side chains on the rate of coagulation of casein by rennin. Coagulation tested at 30 °C in 0·05 M-sodium acetate, 0·015 M-calcium chloride (pH 6·5). ●, Casein treated with glyoxal at pH 8·6 in 2% sodium bicarbonate; ○, casein treated with glyoxal at pH 8·6 in 2% sodium bicarbonate containing 10%, v/v, isopropanol; ▲, material soluble at pH 4·7, released by rennin.

RESULTS

Effect of modifying whole casein

From Fig. 2 it can be seen that there is an approximately linear relation between the extent of modification of arginine side chains and the reduction in the rate of coagulation. Inhibition of coagulation was complete when the arginine content of the modified casein, as determined by the Sakaguchi test, was about 50% of that of the control. Fig. 2 also shows that the release by rennin of pH 4·7 soluble material was

not affected in the modified caseins. The effects of the treatment must therefore have been due to an interference with the polymerization of the casein, and not to inhibition of the primary enzymic phase of the rennin action. More evidence on this point was provided by experiments in which the arginine on para-casein was modified, when it was found that the coagulation of the para-casein in the presence of Ca^{2+} was inhibited similarly to that of the modified caseins of Fig. 2.

There was a marked difference between the effects of disaggregating agent in these experiments and in those in which lysine residues were modified (Fig. 1). The use of 10% isopropanol in the glyoxal treatment increased the extent of modification of arginine, but did not increase the efficiency of the treatment in inhibiting coagulation (Fig. 2). This indicates that the arginine residues which are important to the coagulation are accessible, unlike the corresponding lysine ones. It also provides further evidence that the glyoxal treatment was not affecting lysine residues.

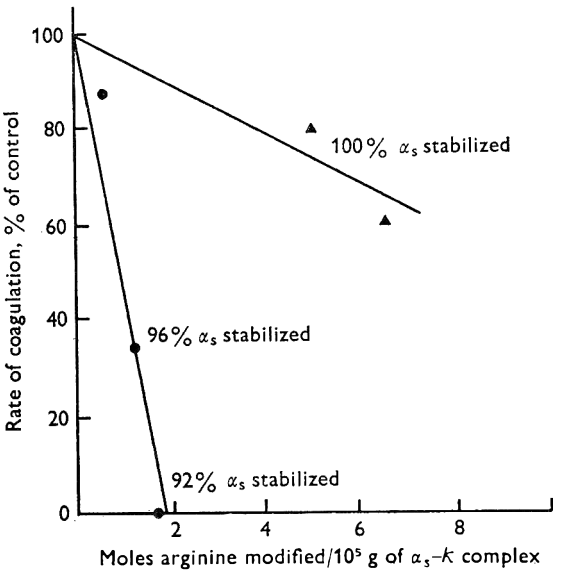

Fig. 3. The effect of modifying arginine in α_s- or κ-casein on the rate of coagulation of the α_s-κ-casein complex by rennin. Ratio of κ-casein to α_s-casein, 1:3·5–4; coagulation test as in Fig. 2; total protein approx. 0·35%. ●, κ-Casein modified; ▲, α_s-casein modified.

Effect of modifying α_s- or κ-casein

To determine whether the foregoing effects were connected with the modification of arginine on a particular casein fraction, κ- and α_s-caseins were modified by the glyoxal treatment and the extent of modification determined by the Sakaguchi test. Modified κ- and unmodified α_s-caseins were used in another series. The ratio of κ- to α_s-casein was 1:3·5–4, and rates of coagulation of the rennin-treated samples were determined as previously described and compared with those of control samples, prepared from unmodified α_s- and κ-caseins. The effectiveness of the stabilization of the α_s-caseins by the κ-caseins was determined by using solutions of these samples which had been kept for 30 min in the conditions used for the rennin tests, namely, pH 6·5, 30 °C, 0·05 M-NaAc, 0·15 M-$CaCl_2$. The protein content of these solutions was

then determined before and after a centrifuge treatment at 2000 *g* for 10 min, and the proportion of α_s-casein kept in solution by the κ-casein was calculated on the assumption that any precipitate was α_s-casein.

In Fig. 3, the relative rates of coagulation of these samples have been plotted as a function of the number of arginine residues modified per 10^5 g of α_s-κ complex. This presentation differentiates between general effects of the modification such as a change of the net charge on the complex, and specific effects which may be caused by the alteration of residues that take part directly in the coagulation. From Fig. 3 it is clear that modification of arginine residues on α_s-casein had relatively little effect. On the other hand, modification of the κ-casein arginines had a potent effect, as coagulation no longer occurred when 1·8 moles of arginine/10^5 g casein were modified. Assuming a molecular weight of 19000 for κ-casein (Mackinlay & Wake, 1964) this corresponds to about 1·5 arginine residues modified/mole of κ-casein. At this relatively low level of modification there was little effect on the ability of the κ-casein to stabilize α_s-casein (Fig. 3), and the modified α_s-caseins were fully stabilized in the conditions of the test.

The modification of the κ-casein also affected the self-aggregation of rennin-treated κ-caseins. Sufficient rennin was added to cause strong turbidity in the control κ-casein solution (10 mg/ml 0·05 M-sodium acetate, pH 6·5, 25 °C) in several minutes. Solutions of κ-casein with one modified arginine residue/mole developed a relatively slight turbidity after 1 h, while solutions containing κ-caseins with 1·5 and 2 such residues remained almost completely clear. It is of interest to note that a similar inhibition of the self-aggregation of κ-casein (which occurs in the absence of Ca^{2+}) was caused by 5×10^{-3} M-Quadrufos.

DISCUSSION

The interpretation of the results of experiments in which functional groups of caseins are modified is always subject to some uncertainty, because of the possibility of unsought effects due to changes of conformation and of charge. In the present work, the use of mild conditions of treatment and the fact that these treatments had little effect on the stabilizing power of the κ-casein toward α_s-casein make it unlikely that significant changes in conformation occurred. In addition, it is clear from the experiments with modified α_s-caseins that change of charge cannot be regarded as an adequate explanation for the highly specific effects caused by modifying κ-casein (Fig. 3). The results, in fact, are in accord with the suggestion that arginine residues on the κ-casein, as well as the lysine residues, play an important part in the coagulation of rennin-altered casein. The further suggestion that at least one of the lysine residues is specially reactive because of the charge from a nearby arginine side chain is supported by recently published work of Jollès *et al.* (1968). These workers reported the presence near the rennin-sensitive bond in κ-casein of the sequence lys-his-pro-pro-his-lys, while studies in our laboratory (Beeby, 1969, private communication) have shown the presence of arginine, as well as histidine and lysine, in this region. Although direct evidence is as yet lacking regarding the location in the α-casein molecule of the residues involved in coagulation, it is worth-while to point out that this segment of the protein contains a relatively high proportion of positively charged functional groups. Furthermore, they are situated where they could reasonably be

expected to be masked by the macropeptide. After the release of the masking peptide by the action of rennin, this region could play its part in the coagulation by interacting with a negatively charged region of suitable configuration on another protein molecule. Areas in which there is a concentration of negatively charged groups do exist in α_s-casein, as several workers have shown (Williams & Sanger, 1959; Osterburg, 1961; Reeves & Latour, 1958). Interactions of this type between clusters of oppositely charged groups would be relatively stable, compared with the interaction between any pair of groups. On the other hand, modification of any of the functional groups in the interacting clusters could be expected to weaken the interaction as a whole. This conforms with the observations that modification of arginine, or lysine (Hill & Craker, 1968) or histidine (Hill & Laing, 1965) causes an inhibition of the coagulation. The efficiency of linear polyphosphates such as Quadrufos in preventing the coagulation of rennin-treated κ-casein may also be explained by their ability to form multiple ionic linkages with such a positively charged region. In this connexion, Melnychyn & Wolcott (1967) have shown that Quadrufos does form a complex with κ-casein sufficiently stable to survive the conditions of gel electrophoresis.

It is clear from the evidence in the present paper that the modification of arginine side chains in casein causes an inhibition of the coagulation of rennin-altered casein. The important arginine residues are located in the κ-casein, and inhibition of coagulation is complete when about 1·5 arginine residues/mole of κ-casein have been altered. It is suggested that the arginine forms part of a positively charged region that also contains lysine and histidine side chains, and that this region is involved in the coagulation of the casein. It is commonly known that this coagulation depends upon the formation of linkages mediated by calcium and also upon hydrophobic bonds; it is the purpose of this paper to point out some additional and equally important factors.

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The effects of intravenous infusions of insulin and of sodium succinate on milk secretion in the goat

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Summary. The effects of intravenous infusion of insulin and of sodium succinate on milk yield and composition and on the yields of milk constituents was investigated in the goat. Depression of plasma glucose concentration to less than 40 mg/100 ml in response to infusion of insulin was associated with a decrease in milk volume and lactose yield, and increases in the contents of fat and casein and in the yield of fat. The increase in fat yield was the result of an increased output of C_{12}–C_{18} fatty acids; the output of C_4–C_{10} acids was slightly reduced. Infusion of succinate caused a reduction in the yields of milk and of lactose, protein and fat. There was no consistent change in milk composition.

The origin of the effects is discussed.

Hypoglycaemia induced by injection or intravenous infusion of insulin is associated in the lactating cow and goat with a decrease in the yields of milk and of milk lactose but the yield of protein is maintained and that of fat slightly increased (Kronfeld, Mayer, Robertson & Raggi, 1963; Rook, Storry & Wheelock, 1965; Linzell, 1967). In a preliminary study in the cow (Rook *et al.* 1965), intravenous infusion of succinate, which has little effect on plasma glucose concentration but may interfere with the oxidation of acetate, was also found to decrease milk yield but the yields of lactose, protein and fat were all depressed. A comparison has therefore been made of the effects of intravenous infusion of insulin and of succinate on milk secretion in the goat.

EXPERIMENTAL

Animals and diets

Two lactating goats (Sally and Colworth) of the White Saanen breed were used. They were offered a diet of hay, dried grass and a proprietary concentrate mixture rationed according to the yield of milk at the beginning of an experiment.

Experimental procedure

Two experiments were done with each animal; in one the effect of intravenous infusion of insulin, and in the other that of succinate, was investigated. In all experiments there was a preliminary control period of 48 h. This was followed by an

experimental period in which a continuous infusion of insulin or of sodium succinate into the jugular vein was given through an indwelling polythene cannula. The insulin (B.P. grade, Burroughs Wellcome & Co. Ltd) in isotonic saline was given over a period of several days, initially at a rate of 5 i.u./12 h but increasing to a level at which the yield of milk was markedly depressed. There was then a final control period of 96 h. The sodium succinate (sodium succinate hexahydrate, AR) was given at the rate of 40–50 g/12 h for a period of 24 h. In Sally this was followed by a final control period of 48 h, but in Colworth there was an intermediate control period of 24 h followed by an infusion of succinate for an additional 24 h and then a final control period of 48 h.

Throughout the experimental and control periods, the animals were milked at regular, 12-h intervals, the weights of milk recorded and samples taken for chemical analysis. Shortly after milking a sample of blood was taken into a hypodermic syringe containing heparin, from an indwelling polythene cannula in the jugular vein opposite to that used for infusion. The plasma was separated and stored at −20 °C.

Methods of analysis

Milk samples were analysed for water, lactose, fat and total and non-casein nitrogen by methods described previously (Rook & Line, 1961). Sodium and potassium were determined by a flame photometric method, using standards containing a background mineral composition similar to that present in the aqueous phase of milk. A methanol–chloroform (1:2) extract of milk fat was prepared and the methyl esters were analysed by gas-liquid chromatography (Storry, Rook & Hall, 1967). Blood plasma was analysed for glucose by the glucose oxidase method (Huggett & Nixon, 1957).

RESULTS

Intravenous infusion of insulin

Similar results were obtained for the 2 animals and the details for Colworth of the effects on plasma glucose concentration and on the yields of milk and on the contents and yields of the major milk constituents are given in Fig. 1. Plasma glucose concentration declined progressively with increasing rate of insulin infusion from an initial value of 60–70 mg/100 ml to a minimum of 20 mg/100 ml. On cessation of the insulin infusion the glucose concentration rose to 108 mg/100 ml and then returned to about the pre-treatment value. Initially there was no effect of reduction in glucose concentration on milk secretion but at a concentration of 30 mg/100 ml both milk volume and lactose yield were markedly depressed and to about the same extent, and at a glucose concentration of 20 mg/100 ml the values were less than half the pre-treatment values.

The yield of casein was unaffected but there was significant ($P < 0{\cdot}05$) increase in fat yield to about 125% of the pre-treatment value. The associated change in the composition of milk fat is given in Table 1, and indicates that the increased yields of fat arose from an increased secretion of C_{12}–C_{18} acids. The output of C_4–C_{10} acids was slightly reduced.

The reduction in milk volume was associated with a slight fall in lactose content

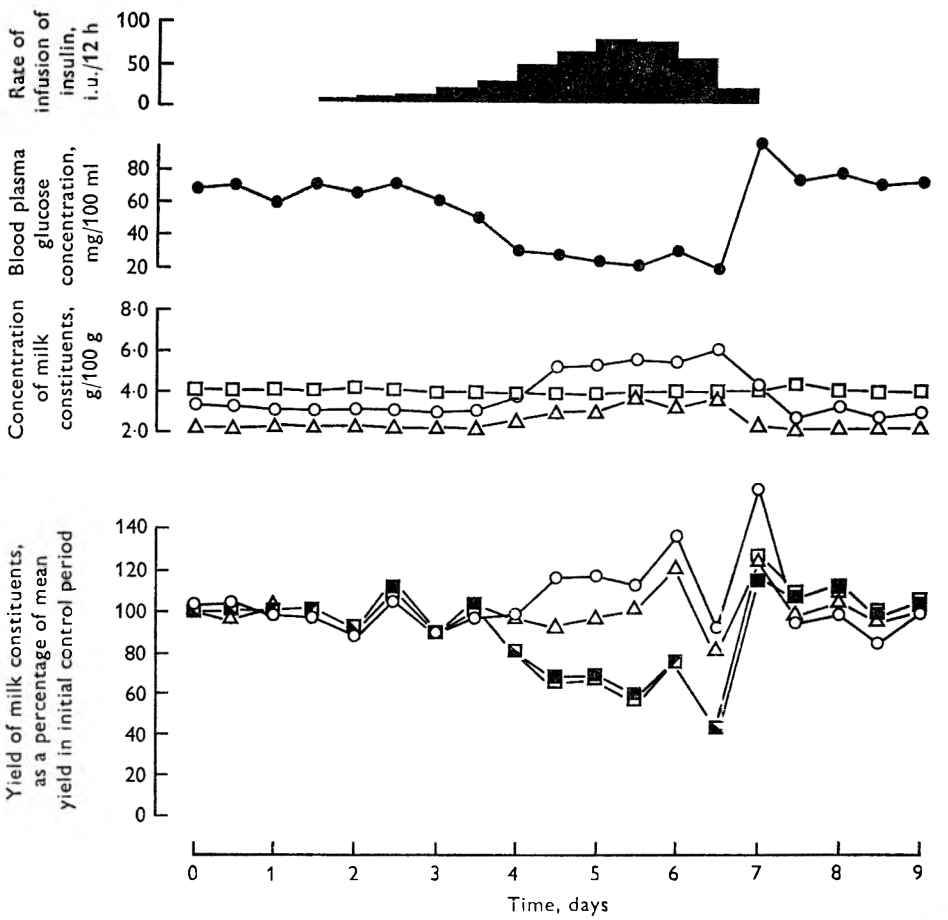

Fig. 1. Effect in Colworth of intravenous infusion of insulin on blood plasma glucose concentration and on the yield and content in milk of fat, ○; casein, △; lactose, □, and water, ■.

Table 1. *The effects of intravenous infusion of insulin or of succinate on the fatty acid composition (molar percentage) of milk fat in the goat*

(Values are means, with S.E., for Colworth.)

	Insulin infusion		Succinate infusion	
Fatty acid	Control	Experimental	Control	Experimental
C_4–C_{10}	28·6 ± 1·0	24·1 ± 0·6**	27·8 ± 0·7	28·7 ± 1·7
C_{12}	6·7 ± 0·3	7·1 ± 0·3	7·6 ± 0·3	8·2 ± 0·3
C_{14}	14·5 ± 0·5	14·7 ± 0·4	15·4 ± 0·4	14·5 ± 0·7
C_{16}	23·4 ± 0·8	24·7 ± 0·5	24·6 ± 0·6	22·9 ± 0·8
$C_{18:0}$	4·9 ± 0·3	5·9 ± 0·3*	4·9 ± 0·3	5·3 ± 0·5
$C_{18:1}$	18·9 ± 0·7	21·4 ± 0·4**	17·6 ± 0·7	19·2 ± 0·6
$C_{18:2}$	3·1 ± 0·5	2·2 ± 0·3	2·1 ± 0·3	1·9 ± 0·3

* $P < 0·05$. ** $P < 0·01$.

Table 2. *The effects of intravenous infusion of sodium succinate on milk yield and composition and yield of milk constituents in the goat*

Animal	Sally				Colworth							
	Pre-control	Infusion		Post-control	Pre-control	Infusion		Intermediate control		Infusion		Post-control
12 h period	1–4	5	6	7–10	1–4	5	6	7	8	9	10	11–14
Rate of infusion of succinate,* g/12 h	—	19·6	16·6	—	—	17·0	22·4	—	—	19·0	22·4	—
Rate of milk secretion, g/12 h	847 ± 38	587	670	816 ± 45	818 ± 8	656	614	807	809	698	657	841 ± 3
Fat, %	3·26 ± 0·09	4·30	3·39	3·48 ± 0·28	3·31 ± 0·14	3·50	3·25	3·85	3·25	3·40	3·00	3·09 ± 0·05
Casein, %	2·54 ± 0·03	2·92	2·92	2·83 ± 0·05	2·16 ± 0·04	2·30	2·20	2·17	2·30	2·30	2·28	2·27 ± 0·05
Non-casein proteins, %	0·84 ± 0·06	1·06	1·20	0·93 ± 0·05	0·59 ± 0·02	0·63	0·66	0·73	0·65	0·59	0·62	0·60 ± 0·03
Lactose, %	3·51 ± 0·02	3·52	3·66	3·65 ± 0·02	4·35 ± 0·04	4·62	4·62	4·27	4·21	4·32	4·34	4·05 ± 0·02
Yield of fat, g/12 h	27·7 ± 1·8	25·2	22·8	28·0 ± 1·9	27·1 ± 1·7	22·9	19·5	31·1	26·3	23·7	19·7	26·0 ± 0·5
Yield of casein, g/12 h	21·9 ± 1·0	17·2	19·7	22·3 ± 1·3	17·6 ± 0·3	15·1	13·5	17·5	18·6	16·1	15·0	19·1 ± 0·4
Yield of lactose, g/12 h	29·8 ± 1·3	20·7	24·5	29·7 ± 1·6	35·6 ± 1·2	30·3	28·4	34·5	34·0	30·2	28·5	34·1 ± 0·2

* Expressed as succinic acid.

but the contents of casein and especially of fat were increased. There was no effect on sodium content but a significant ($P < 0{\cdot}05$) inverse relationship between lactose and potassium contents was observed:

$$\text{lactose (g/100 ml milk)} = 7{\cdot}845 - 0{\cdot}0182\ (\pm 0{\cdot}0061)\ \text{K (mg/100 ml)}$$

Intravenous infusion of sodium succinate (Table 2)

Infusion of succinate was invariably associated with a significant ($P < 0{\cdot}05$) reduction in the volume of milk secreted. There were simultaneous decreases in the yields of fat, casein and lactose and, to a lesser extent, in non-casein proteins. Changes in milk composition were irregular and, for Colworth, lactose content decreased over the period of the experiment. No significant change was observed in fat composition (Table 1) or in the content of sodium or potassium.

Plasma glucose concentration was unaltered or slightly increased.

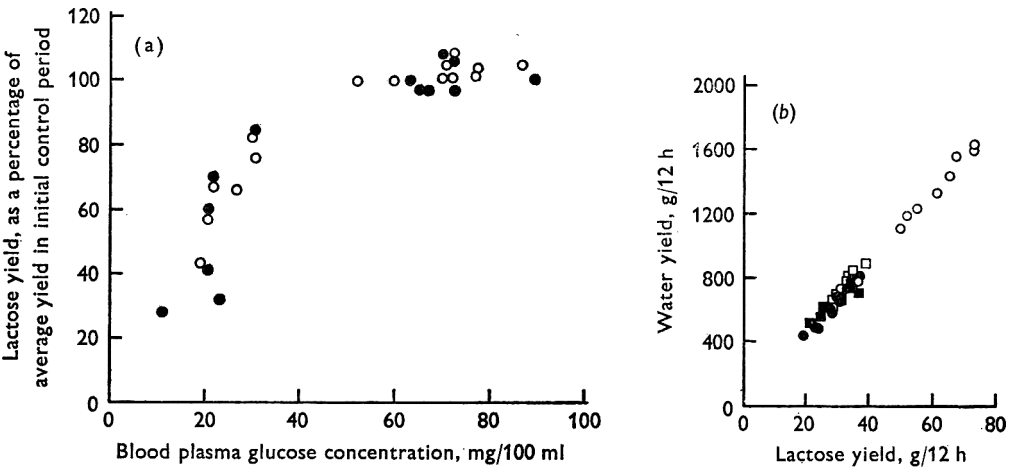

Fig. 2. The relationship between (*a*) lactose yield and blood plasma glucose concentration (●—Sally, expt 1; ○—Colworth, expt 1) and (*b*) lactose yield and the output of water in milk (○—Sally, expt 1; ■—Sally, expt 2; □—Colworth, expt 1; ●—Colworth, expt 2).

DISCUSSION

The results confirm earlier observations that a depression in plasma glucose concentration in response to the intravenous infusion of insulin selectively depresses secretion of lactose and that there is an associated reduction in the volume of milk secreted. The relationships between plasma glucose concentration and lactose yield and between the outputs of lactose and of water in milk are shown in Fig. 2. As observed previously (Rook *et al.* 1965), a reduction in milk yield also followed the intravenous infusion of succinate, but the secretion of all the major constituents was affected and there was no associated decrease in plasma glucose concentration.

Glucose is the major precursor of milk lactose in the ruminant (Linzell, 1968) and the relationship between plasma glucose concentration and lactose yield demonstrated during the intravenous infusion of insulin appears to reflect a precursor–product relationship, lactose synthesis reaching a maximum at a glucose concentration

of about 40 mg/100 ml. As milk is iso-osmotic with blood (Wheelock, Rook & Dodd, 1965) and as lactose is the major contributor to osmotic activity of the materials synthesized within the gland, there are associated changes in the output of milk water. In addition to its role as a precursor of lactose, glucose also makes a major contribution to the materials oxidized within the gland (Linzell, 1968) but there was no evidence that a depression in plasma glucose concentration interfered with the synthesis and secretion of fat or proteins. Synthesis of these materials would be expected to be sensitive to energy made available through oxidation within the mammary gland.

A possible explanation for the effect of infusion of succinate on the synthesis and secretion of fat, protein and lactose is that there is an interference with the oxidation of acetate by the tricarboxylic acid cycle (Krebs, 1961; Krebs, Salvin & Johnson, 1938). In support of such a possibility, increases in the yields of fat, protein and lactose have been observed in response to the intraruminal infusion of acetic acid (Rook & Balch, 1961), which is associated with an increase in the concentration of plasma acetate. These observations suggest, therefore, that the availability of adenosine triphosphate for the synthesis of milk constituents is dependent more on the supply of acetate than of glucose. Wood, Peeters, Verbeke, Lauryssens & Jacobson (1965) have demonstrated that the major route for the oxidation of glucose in the lactating ruminant mammary gland is the pentose phosphate pathway, important for the provision of reduced nicotinamide adenine dinucleotide phosphate required in the synthesis of fatty acids. Within the ruminant mammary gland there may be some limitation on the metabolism of glucose to acetate by the glycolytic pathway.

During the infusion of insulin, the secretion in milk fat of C_{12}–C_{18} fatty acids was increased. These acids are derived partly or wholly from fatty acids of the plasma triglycerides. A possible explanation for the increased secretion is that the profound fall in glucose concentration promotes a release of adrenaline, causing a mobilization of free fatty acids from fat depots and consequent increases in the conversion of fatty acids to triglycerides in the liver, in the circulating level of triglycerides in the plasma and in the uptake of triglyceride fatty acids by the mammary gland.

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Extraction of the 260 nm-absorbing material from Group N streptococci as a method for estimating cell growth

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Summary. A method is described for estimating the growth of Group N streptococci in milk and other media by measuring the 260 nm-absorbing material extracted from the cells with 0·1 M-NaOH at 100 °C. A good correlation between the absorbance at 260 nm of the hot alkali extracts and the dry weight of the cells was obtained for cultures of *Streptococcus lactis* during the logarithmic and early stationary phases of growth.

A simple chemical method based on the extraction and measurement of 260 nm-absorbing material (purines and pyrimidines) has been used to measure growth in tissue cultures (McIntire & Smith, 1958). In the present investigation correlation with dry weight of the 260 nm-absorbing material extracted from Group N streptococci, harvested from a casein coagulum, indicated that it could be used as a satisfactory measure of the growth of these organisms in milk.

EXPERIMENTAL AND RESULTS

Growth media. Bacterial cells (*Streptococcus lactis* C10) were grown in skim-milk or in a broth medium. Fresh skim-milk was autoclaved at 115 °C for 10 min within 1 h of collection and stored at 0·5 °C until required. The broth medium contained per l: tryptone (Difco), 30 g; yeast extract (Difco), 10 g; lactose, 5 g; KH_2PO_4, 5 g; 'Lab Lemco' meat extract (Oxoid), 2 g. The medium was adjusted to pH 6·5 and sterilized by autoclaving for 20 min at 115 °C.

Separation of bacterial cells from coagulated milk cultures. Bacterial cells were separated from coagulated milk cultures by dissolving the casein in cold sodium hydroxide solution. Five ml portions of the coagulated milk culture in test tubes were chilled in ice-water. Excess of ice-cold 1 M-NaOH was added to disperse the coagulum and to dissolve the casein. The cells were removed by centrifugation and washed twice with 10 ml quantities of ice-cold distilled water. Cells grown in a broth medium were centrifuged and washed free of the growth medium without the prior addition of cold sodium hydroxide solution.

Extraction of nucleic acids with sodium hydroxide at 100 °*C.* The bacterial cells were suspended in 5 ml of 0·1 M-NaOH and heated at 100 °C for 30 min. After removal of the cell debris by centrifugation the UV spectrum of the supernatant was measured.

This showed an absorbance peak at 260 nm, characteristic of nucleic acid, as shown in Fig. 1, curve *a*.

As shown in Table 1, a heating period of 10 min at 100 °C gave absorbance values for the extraction of nucleic acid with 0·1 M-NaOH which did not differ markedly from those obtained after heating for 30 min. As heating for 30 min did not change the UV spectrum of the supernatant, heating for this period was adopted as standard procedure.

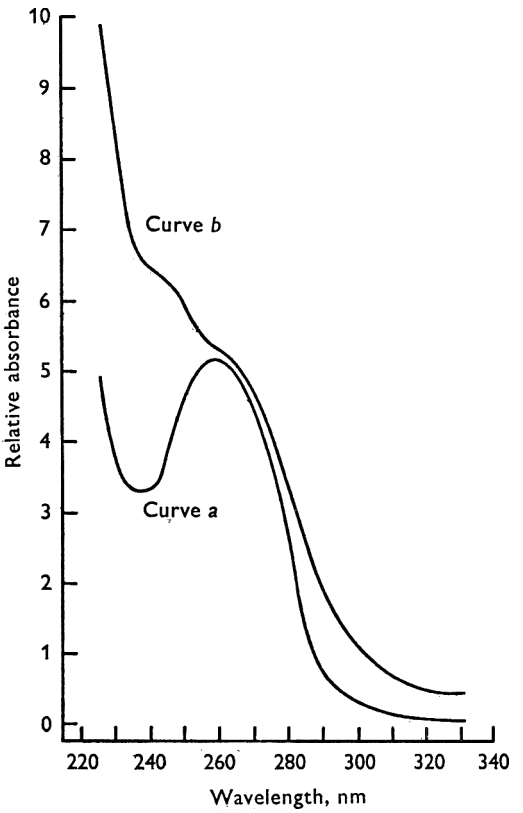

Fig. 1. UV spectra of sodium hydroxide extracts of *Streptococcus lactis* C10. Washed cells were extracted with 0·1 M-NaOH (curve *a*) and 1 M-NaOH (curve *b*) at 100 °C for 30 min.

Table 1. *The effect of varying the heating time on the extraction of* 260 *nm-absorbing material from* Streptococcus lactis *C*10 *with* 0·1 *M-sodium hydroxide*

Time heated at 100 °C, min	Absorbance at 260 nm
5	2·055
10	2·160
15	2·100
20	2·175
25	2·190
30	2·190

Extraction of nucleic acid material was maximal when the cells were suspended in either 0·5 M- or 1 M-NaOH and heated at 100 °C for 30 min, as is shown in Table 2. The amount of nucleic acid material extracted with 0·1 M-NaOH was 96% of that

Table 2. *Extraction of* 260 *nm-absorbing material from* Streptococcus lactis *C* 10 *incubated with varying concentrations of sodium hydroxide at* 100 °*C for* 30 *min*

Extracting medium	Absorbance at 260 nm
Water	1·155
0·1 M-NaOH	3·045
0·5 M-NaOH	3·171
1 M-NaOH	3·171

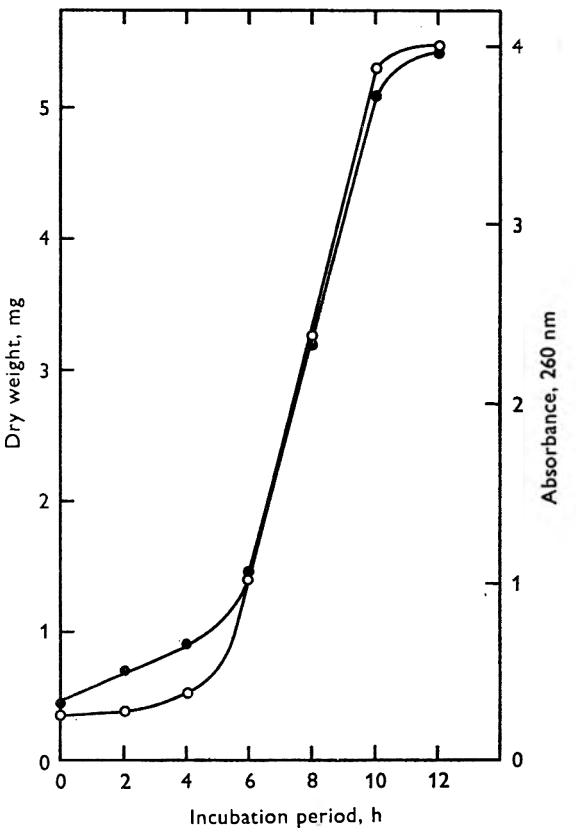

Fig. 2. Comparison between absorbance of 0·1 M-NaOH extracts of cells at 260 nm (○) and dry weight (●) of cells during growth of *Streptococcus lactis* C 10.

extracted with 0·5–1 M-NaOH. Extraction of the bacterial cells with 1 M-NaOH at 100 °C resulted in almost complete digestion of the cells, and only a very small quantity of fine precipitate was obtained on prolonged centrifugation. However, the UV spectrum of the supernatant from this extract did not show the characteristic absorbance peak at 260 nm (Fig. 1, curve *b*), the absorbance being higher at 225–255 nm.

Relationship between absorbance at 260 *nm and dry weight of cells during growth of* Str. lactis *C* 10. To examine whether the absorbance at 260 nm of the extract obtained by treating cells with 0·1 M-NaOH was related to the dry weight of the cells at different stages of growth, the following procedure was followed. Sterile skim-milk, centrifuged aseptically at 35000 *g* to remove cellular material, was inoculated with

Str. lactis C10. After thorough mixing, 5 ml portions were taken and incubated for the required time at 30 °C. The cells were recovered from the coagulated milk cultures as described above, and separate samples were taken for drying to constant weight at 110 °C and for extraction with 0·1 M-NaOH at 100 °C for 30 min. A good correlation between the results obtained by the 2 methods was observed for the logarithmic and early stationary phases of growth, as is shown in Fig. 2.

DISCUSSION

Mitchell & Moyle (1951) examined the nucleic acid content of the cell envelope of *Streptococcus pyogenes* by dissolving the cell residue in 1 M-NaOH at 35 °C for 15 h. They estimated RNA by absorbance at 260 nm after acidification of the extract to remove the DNA fraction. The UV spectrum did not, however, show a characteristic peak at 260 nm. In the present investigation a spectrum similar to that obtained by Mitchell & Moyle was obtained when cells of *Str. lactis* C10 were extracted with 1 M-NaOH. The high absorbance at wavelengths below 260 nm may be indicative of the extraction of protein. This was avoided by using 0·1 M-NaOH as the extracting medium.

The growth of Group N streptococci in milk cannot be measured by simple turbidimetry because of the opacity of the milk and the formation of an acid coagulum. Small clots are often formed during growth of these organisms in milk which precipitate with the cells on centrifugation. Estimation of growth from measurement of pH or acid production is only valid when the organisms are grown anaerobically. Depending on the oxygen tension in the medium, the amount of lactic acid produced per unit increase in cell numbers may be as low as 50 % of that produced anaerobically (Jago, 1957).

The present method has been shown empirically to be satisfactory for estimating Group N streptococci during their logarithmic or early stationary phases of growth in milk (and in other media) as indicated by the correlation between the absorbance of the hot alkali extracts at 260 nm and the dry weight of the cells. The simplicity of the method not only allows large numbers of cultures to be handled at the one time but also allows them to be stored at low temperatures (−20 °C) for any length of time without loss of 260 nm-absorbing material. The only condition affecting the latter procedure is that the cultures should be separated from the medium before cold storage ready for immediate extraction with 0·1 M-NaOH.

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Measurement of the quantity of lactose passing into mammary venous plasma and lymph in goats and in a cow

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Summary. Lactose in the plasma of 12 lactating goats and one cow with healthy udders has been measured by a sensitive and specific enzymic assay. Mean values (± S.E.) for arterial and venous concentrations were 52 ± 11 μM and 70 ± 11 μM respectively. Simultaneous measurement of mammary blood flow, milk yield and milk lactose concentration showed that 2·70 ± 0·25 % (mean ± S.E.) of the total lactose production passed into the plasma. This rose to 42·7 % in a goat when milking was suspended for 46 h. No lactose was found in the plasma of male and non-lactating female goats, but it was detected 4 days before parturition in a nulliparous goat.

It has been known for many years that lactose occurs in the urine during the puerperium in several species, its mammary origin being indicated by its disappearance after removal of these glands (Moore & Parker, 1900; Marshall & Kirkness, 1907). Measurements of urine lactose concentrations in lactating cows showed 0–468 μM for young animals, and up to 2050 μM for older ones, including some that had had clinical mastitis (Wheelock & Rook, 1966). The concentration in both plasma and urine rose considerably when milking was suspended during lactation (Wheelock & Rook, 1966). Lactose has also been determined in the mammary venous plasma (155 μM) and lymph (366 μM) of cows (Heyndrickx & Peeters, 1960); however, the lymph was collected *post mortem*, under which conditions mammary cell contents are known to be released into it (Linzell, 1960*a*).

In the course of other work we detected lactose in the plasma of lactating goats and became interested in obtaining quantitative data on this loss. Simultaneous measurements of mammary plasma flow and milk lactose yield have enabled us to calculate what fraction of the total lactose is lost to the plasma. A further reason for examining this species is that, unlike the cow, goats do not suffer from chronic subclinical mastitis; in our herd mastitis is uncommon, but usually severe and often fatal.

METHODS AND MATERIALS

Blood and lymph samples were collected from 13 conscious goats (Saanen and Welsh) and a Jersey cow, and from 3 perfused goat mammary glands. Mammary venous blood was taken from the milk vein with the precaution that the external

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pudic vein was manually occluded during sampling (Linzell, 1960*b*, 1966*a*). General systemic blood was taken from a carotid loop in 6 goats and the cow, and in the remainder of the goats from the right atrium (Linzell, 1966*b*). Lymph was collected from a conscious goat via a PVC cannula inserted the previous day, under local anaesthesia, into the main lymph duct draining one half of the udder (Linzell, 1960*a*). Mammary blood flow was measured by the method of Linzell (1966*a*). In the perfusion experiments, samples were taken from the blood entering and leaving the glands, and from the lymphatics afferent and efferent to the main lymph node.

Evidence for the absence of mastitis. None of the animals had apparently had mastitis and 4 of the goats were in their first lactation. In this herd, all animals are milked twice daily after washing the udder with chlorhexidine, and the milkers are instructed to examine the fore-milk for clots. Periodic determination of the milk leucocyte count, as assessed by the California mastitis test (Blackburn, 1965), and the catalase test (Willits & Babel, 1965) has been in the range accepted as normal in cows, and occasional culture of fore-milk on blood agar has revealed no infection. Routine clinical and histological examination of the udders of all goats killed in other experiments (12–20/year) has not detected unsuspected mastitis. When mastitis has been recognized in our herd it has always caused a fall in yield of the gland affected. In the animals used in these experiments the milk yields of each gland, recorded separately twice daily throughout lactation, have agreed within 11 %.

Analytical. Lactose was determined in duplicate by a specific enzymic method (Reithel, 1963). Milk samples were diluted with water and assayed directly. Plasma and lymph samples (2 ml) were first incubated with glucose oxidase (4 μg Sigma Type V) under O_2/CO_2 (95/5, v/v) for 2 h at 37 °C to destroy the glucose present. The recovery of lactose added to male goat plasma (100 μM) treated in this way was 95 %. The precision of the method estimated from 29 duplicates was 1·5 μM (S.D.). The lactose from a larger sample of mammary venous plasma (41 ml), which had been deproteinized with perchloric acid, was further characterized by its adsorption onto charcoal and desorption by 20 %. v/v, aqueous ethanol (Whistler & Durso, 1950). Lactose was also measured in milk by the chemical method of Hyvärinen & Nikkilä (1962) adapted for the Autoanalyser (Technicon, Chauncey, N.Y.). The chemical and enzymic methods agreed within 10 %. Glucose was measured by the hexokinase method (Slein. 1963).

RESULTS AND DISCUSSION

Lactose was found in the plasma of all animals with functioning mammary glands that were examined (12 goats, 1 cow and a rat). However, none was detected in the systemic plasma of 2 castrated male goats, nor of 2 non-lactating female goats (one ovariectomized). These findings are consistent with the mammary gland being the sole source of plasma lactose.

Plasma lactose at parturition. In a nulliparous goat the systemic plasma lactose concentrations at 4, 2 and 0·5 days before parturition were 4, 6 and 33 μM respectively. In 2 primiparous goats on the day of parturition the systemic plasma concentrations were 31 and 165 μM, and the mammary venous plasma concentrations were 41 and 212 μM respectively. In a parturient rat the systemic plasma concentration was found to be 36 μM.

Plasma lactose during lactation. In 9 of the goats and in the cow, during established lactation, arterial plasma lactose averaged 52 μM, with S.E. = $\pm$ 11 μM and range 21–137. There was a consistently positive arteriovenous difference of mean value $19 \pm 2{\cdot}5$ μM (range 9–31). Although there was no correlation between the arteriovenous difference and either the number of pregnancies or stage of lactation, a possible correlation did exist between the arterial concentration of lactose and the number of previous lactations (up to 7) ($r = 0{\cdot}57$; $0{\cdot}05 < P < 0{\cdot}1$), indicating that the disposal of this sugar may be impaired in older animals. In man, renal plasma flow and glomerular filtration rate both decline with age (Shock, 1958).

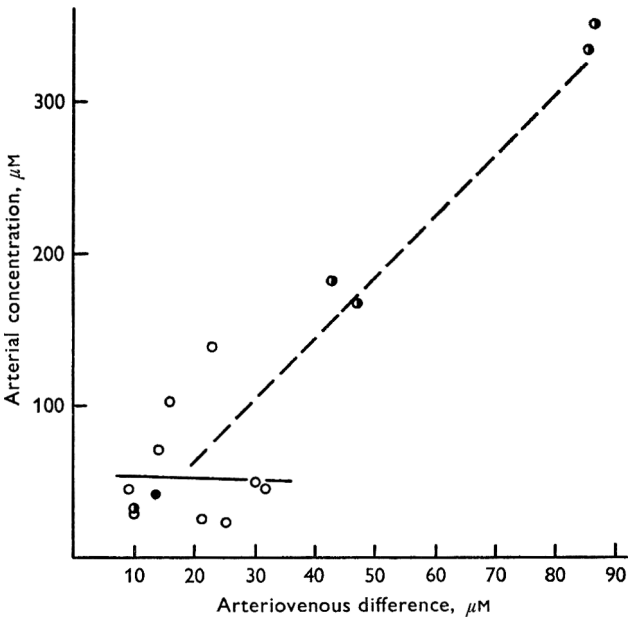

Fig. 1. The relation between plasma arterial concentration and the arteriovenous difference of lactose in goats (○) and a cow (●). Under normal twice-daily milking there is no correlation (solid line), but when data for parturient goats and a goat with a distended udder (◑) are included the correlation coefficient is significant. ($r = 0{\cdot}92$; $P = 0{\cdot}001$.)

Arterial lactose concentration during normal lactation must also reflect the mechanism of removal from the circulation, since there was no correlation with its rate of input of lactose by the mammary gland (Fig. 1). However, large variations which occur at parturition or on the cessation of milking correlate well with the increased arteriovenous difference (Fig. 1).

From data on simultaneously determined mammary plasma flow, arteriovenous plasma lactose and milk lactose output, we could calculate what proportion of lactose was lost to the plasma. Nine estimations made in 5 goats and a cow gave a mean of 2·7, with S.E. = $\pm 0{\cdot}25$ and range 1·8–3·8. Since the percent loss of lactose correlated with its arteriovenous difference (Fig. 2; $r = 0{\cdot}94$; $P = 0{\cdot}001$) but not with the lactose yield, it reflects a variation in permeability rather than of synthetic activity of the udder.

Plasma lactose after cessation of milking. The effect of udder distention produced

by omitting 3 milkings in a goat is shown in Table 1. Noticeable features are: (1) mammary blood flow rose slightly; (2) mammary lactose production fell more than total milk production and mammary glucose uptake; (3) at maximum distention about 40 % of the lactose produced by the udder was passing into the plasma, in excellent agreement with the figure of 50 % passing into urine under similar circumstances in cows (Wheelock & Rook, 1966); (4) the loss of lactose into the plasma did not cease immediately after milking, indicating that udder distention itself was not the cause of lactose release, but rather that this had altered the udder permeability. In another lactating goat arteriovenous differences of 10 and 13·5 μM were measured before and after morning milking.

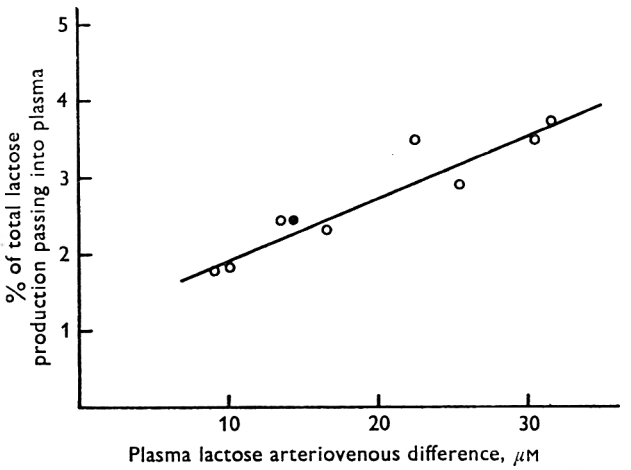

Fig. 2. The relation between the percentage of the total lactose production which passes into plasma and the mammary plasma lactose arteriovenous difference. ○, Goats; ●, cow.

Lactose in lymph. Lactose was present in lymph from 4 lactating goat mammary glands. In a conscious animal the concentration equalled that in arterial plasma (45 μM), but in a perfused gland the concentration was higher than in mammary venous plasma (50 μM) both before and after passage through the lymph node (53 and 63 μM respectively). In 2 other perfused glands lymph concentrations were 143 μM. In all cases the proportion of lactose that is lost via the lymph was negligible because in the conscious animal the rate of mammary blood flow was about 600 times that of lymph (see also Linzell, 1960*a*).

Wheelock & Rook (1966) concluded that there is 'a continuous slight loss of lactose into the blood from the actively secreting mammary gland', but that 'when the usual system of milking twice a day is practised the total loss of lactose into the urine is negligible in comparison with the amount secreted by the mammary gland'. We agree with their conclusion that this loss is a physiological effect not necessarily indicative of mild mastitis. Our data confirm the loss of lactose into the plasma throughout normal lactation, but suggest that it is not insignificant. At times when milk is not being removed from the teats—just prior to parturition, and on cessation of milking—the loss of lactose into plasma assumes great importance. Further work is needed to decide the physiological significance of the normal loss of lactose into

Table 1. *The effect of udder distention on the passage of lactose into blood in a lactating goat*

(During the cessation of regular milking the rate of milk secretion was measured by the rate of increase in udder volume (Linzell, 1966*c*).)

Stage of lactation, days	Time after milking, h	Plasma glucose, mM		Plasma lactose, μM		Udder plasma flow, ml/min	Milk yield, ml/min	Milk lactose, mM	Lactose output μM/min		% of lactose transferred to plasma
		Arterial	Arterio-venous difference	Arterial	Arterio-venous difference				Plasma	Milk	
42	5	—	—	137	23	448	2·47	117	10·3	289	3·5
147	3	3·67	1·72	102	16	290	1·7	117	4·65	199	2·3
148	27	3·50	1·38	181	43	343	1·39	76	14·75	106	12·2
149	46	3·55	0·84	333	86	326	0·8	58	28·1	46·5	37·7
149	0·25	4·52	1·20	350	87	288	0·6	56	25·1	33·6	42·7

the plasma to see whether it escapes from the basal portion of the secretory cell or is resorbed from secreted milk in the alveoli and ducts, and to determine if plasma lactose is all cleared by the kidney or whether some is taken up and re-utilized by the liver in order to conserve carbohydrate.

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Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization

I. An experimental ultra-high-temperature sterilizer and its characteristics

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Summary. An experimental ultra-high-temperature milk sterilizer, of 1140 l/h capacity and capable of operation as a plate-type indirect heating plant or as a steam-into-milk direct heating plant, has been installed for comparisons of the milk produced by the direct and indirect processes. The sterilizer and its ancillary equipment are described, together with methods of plant sterilization and milk processing. Time–temperature profiles of the plant are given for both modes of operation.

Methods for the ultra-high-temperature (UHT) sterilization of milk fall into 2 general groups, the direct and the indirect. In the direct method, milk is heated to the final sterilizing temperature by mixing it with steam: the condensate produced is then removed during the cooling stage when the hot milk is injected into a vacuum chamber for expansion cooling. With the indirect method, heat is transferred from the heating medium, which may be steam or pressurized hot water, to the milk through a conducting wall so that the heating and heated fluids never come into contact.

Plants employing these alternative methods have specific practical advantages and disadvantages. However, it has also been widely claimed that the milk produced by direct UHT processes is of better quality than that produced by indirect processes in that there is less change in the organoleptic and nutritional properties of the milk. There are theoretical reasons for supposing that the direct process might have these advantages, because the high rates of heat transfer obtainable by mixing with steam and by evaporative cooling allow higher milk processing temperatures for shorter effective processing times. These higher temperatures and shorter times in turn increase the sporicidal effect of the process relative to the chemical change produced (Burton, 1965).

It has never been determined conclusively to what extent, if at all, this theoretical benefit is realized in practice. True comparisons between milks processed on different plants have not been possible, even when the plants have been installed in the same country. Analytical methods have differed, and organoleptic comparisons are not valid unless they are made by the same taste panel. Even where a single group of workers has used the same methods for the examination of milk from different UHT

sterilizers (Lembke, Frahm & Wegener, 1968), it has not been possible to impose standard operating conditions on commercial plant, and a common supply of raw milk has been impracticable.

Zadow (1969) has compared some of the chemical effects of processing using a laboratory scale sterilizing plant capable of use either as a direct or as an indirect heater, and Rossikhina, Mastakov & Seleznev (1969) have used a similar plant to obtain results on vitamin destruction during processing. In neither case does it appear to have been recognized that equal holding times will not give equal severities of heat treatment by the 2 processing methods because the time–temperature profiles are not the same. Nor does the same milk appear to have been used for the comparative tests.

To overcome some of the difficulties in making a true comparison of milk processed by the direct and indirect methods, an experimental 250 gal/h (1140 l/h) UHT sterilizer has been installed. This plant, designed and built by the APV Co. Ltd, of Crawley, is such that the rearrangement of some of the pipework converts it from a steam-into-milk direct heating system to a plate-type indirect heating plant. It is proposed to use this plant for comparative experiments with a common bulk of milk, employing the same chemical and biochemical analytical techniques, and with the operating conditions chosen so that the bacteriological efficiency of the plant, i.e. the proportion of resistant spores that would be destroyed by the heat-treatment process, is the same for both of its modes of operation.

The purpose of this paper is to describe the experimental plant and its services, and to give some of its operating characteristics.

DESCRIPTION OF THE EXPERIMENTAL PLANT AND ITS OPERATION

General

The plant comprises 7 plate heat exchanger sections, P–V, in a common frame (Figs 1, 2), with a deaerating vessel, steam injection head and expansion vessel, aseptic homogenizer, pumps and controls.

The heat exchanger sections are of the single pass type on the milk side. Sections R and S are regenerator sections. Section T is a low-temperature heating section using hot water at a controlled temperature. Section U has a dual purpose: with the indirect system it is the final heater, supplied with steam at a pressure determined by the temperature control system, but with the direct system it acts as a condenser for the vapours from the expansion chamber. Section V is a 'crash cooler', used only with the indirect system to give rapid cooling from the full sterilizing temperature. Section Q is the final milk cooling section which controls the milk outlet temperature. Section P is used in both systems to cool the water circulated during plant sterilization to below boiling point before it is returned to the balance tank.

The deaerator vessel 7 is an experimental unit built for this sterilizer, designed to give effective deaeration under vacuum without flash boiling of the milk. Milk is sprayed into the top of the vessel as a thin circular horizontal sheet, which takes an umbrella shape under the influence of gravity. The milk does not reach the surface of the vessel until the continuous sheet has disintegrated. With a milk temperature

of 82 °C (180 °F) and a vacuum corresponding to 87 °C (188 °F) (12 inHg vacuum) the oxygen content can be reduced from about 9 ppm to 1 ppm.

The steam injection head 18 (Fig. 2) is a standard Uperiser unit having a proportion of the steam orifices closed by an internal sleeve to make it suitable for a milk flow rate of 250 gal/h. It is mounted vertically with the milk flow upwards.

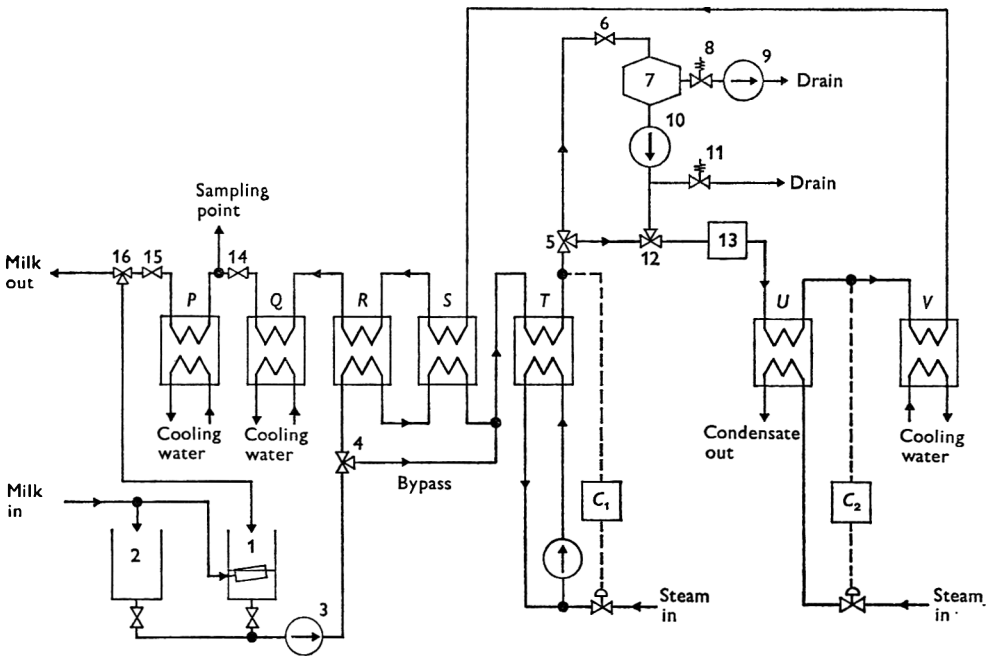

Fig. 1. Flow diagram for indirect-heating experimental plant: 1, float controlled balance tank; 2, tank for experimental milk batches; 3, centrifugal pump; 4, 3-way cock controlling regeneration; 5, 3-way cock controlling deaeration; 6, valve controlling flow into deaerator; 7, deaerator; 8, spring-loaded vacuum controller; 9, vacuum pump; 10, centrifugal pump; 11, pneumatically balanced bleed valve; 12, 3-way cock controlling deaeration; 13, homogenizer; 14, back pressure valve for normal operation; 15, back pressure valve for plant sterilization; 16, 3-way cock controlling milk discharge. *P*, Cooler for recirculating water during plant sterilization; *Q*, final milk cooler; *R*, regenerator; *S*, regenerator; *T*, hot water heating section; *U*, final steam heating section; *V*, crash cooler; C_1, hot water temperature controller; C_2, final milk temperature controller.

The homogenizer 13 is an aseptic Manton–Gaulin 2-stage unit which can be included in the circuit before or after the sterilizing sections by suitable connexion of the pipework. The homogenizing pressure is limited to 200 atm (3000 lb/in.^{2}).

The high pressure pump 17 is a 2-stage helical rotor pump (Mono Pumps Ltd, London, E.C.1) fitted with a hydraulic variable speed gearbox.

The plant recorder-controllers C_1, C_2 and C_3 are conventional pneumatic 2-term types (Taylor Instrument Co., Stevenage, Herts). C_1 controls and records the milk temperature at the outlet of the hot water section T, and C_2 controls and records the final milk sterilizing temperature, both operating from sensing elements in the milk line. C_3 is a ratio controller which is used only in the direct heating system as will be described later.

Water for the crash cooler V and the sterilizing cooler P is obtained from an

ion-exchange water softener (The Permutit Co. Ltd, Isleworth, Middx). Unsoftened mains water is used in the milk cooling section Q and in the condenser of the direct heating system.

The steam supply arrangements are governed by the need to produce steam of suitable quality for mixing with milk. Water for the hot water heating section T is heated with steam from the normal services at a pressure of about 1·3 atm (20 lb/in.^{2}).

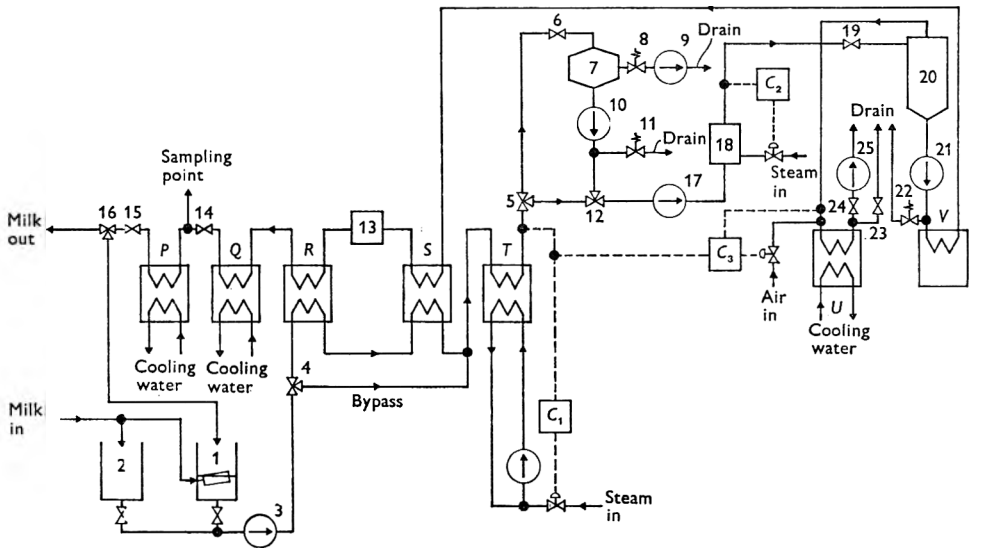

Fig. 2. Flow diagram for direct-heating experimental plant: 17, high pressure pump; 18, steam injector; 19, expansion valve; 20, expansion chamber; 21, aseptic centrifugal pump; 22, pneumatically balanced bleed valve; 23, back pressure valve for plant sterilization; 24, isolating valve for vacuum pump; 25, vacuum pump. U, Condenser; V, crash cooler, not in use; C_3, ratio controller.

Steam for the final sterilizing stage, whether direct or indirect, is obtained from an oil-fired packaged boiler (700 lb steam/h, Spencer-Bonecourt-Clarkson Ltd, London, W.C.1). This is fed with potable water softened as mentioned above, and the feed water is given the minimum amount of additional treatment for boiler operation. Clean condensate is returned to the feed tank.

The steam supply arrangements for direct heating of milk are generally as recommended by the National Association of Dairy Equipment Manufacturers (NADEM, 1963). Steam passes from the plant control valves through a length of uninsulated pipe for desuperheating, and then to a centrifugal separator. From there it goes to a ceramic filter of 60 μm pore size (Aerox Ltd, Stroud, Glos). Between this filter and the injection head, the steam is carried in stainless steel and a steam sampling point is fitted.

The packaged boiler operates as an on-off system controlled by the generated steam pressure. In normal operation, the steam pressure on load cycles between approximately 9 atm (135 lb/in.^{2}) and 10·7 atm (160 lb/in.^{2}). With indirect processing the control system can absorb this variation without any effect on the processing

temperatures. However, with direct processing these pressure fluctuations cause variations in the sterilizing temperature which cannot be removed by normal controller adjustments. An additional pneumatically operated pressure controller has therefore been connected to a diaphragm-operated valve which bleeds steam to atmosphere. This keeps the steam pressure constant by causing the boiler to operate continually. For experimental purposes this is an effective, although uneconomic, way of avoiding fluctuations in the process temperature.

Operation as an indirect-heating plant

The arrangement of the experimental plant as an indirect heater is shown in Fig. 1. Two balance tanks 1 and 2 are arranged in parallel. No. 1 is the float-controlled balance tank used in normal operation and for recirculation during cleaning and sterilization of the plant; no. 2 is used to contain experimental batches of milk, e.g. inoculated milk for the bacteriological experiments which will be described in a later paper.

Milk is pumped with a centrifugal pump 3 through the regenerator sections *R* and *S* which can be by-passed by means of the cock 4. In normal operation milk leaves the regenerator sections at about 50 °C (122 °F). The hot water section *T* heats the milk further to 82 °C (180 °F) under the control of controller C_1. The milk then passes to the deaeration vessel 7, where air is removed under vacuum, and is pumped, 10, to the homogenizer 13. The level of the milk in the deaeration vessel and outlet pipe is maintained constant by the pneumatically balanced pressure relief valve 11. If the level rises so as to increase the inlet pressure to pump 10 the outlet pressure of the pump rises correspondingly and overcomes the air loading of valve 11. This opens, allowing excess milk to drain to waste, and so restores the original milk level. The rate of milk flow into the deaerator is controlled by valve 6 so that a minimum amount of milk for effective level control is discharged through valve 11. The deaeration vessel can be by-passed by means of cocks 5 and 12. The milk is homogenized (13) at a total pressure of 200 atm (3000 lb/in.^{2}). After homogenization the milk is heated to the final sterilization temperature in the steam heating section *U* under the control of controller C_2.

After a short holding period (3·6 s mean) the milk is cooled rapidly to about 88 °C (190 °F) in the crash cooler *V*, and more slowly in the regeneration sections *S* and *R* to about 51 °C (124 °F). The final milk cooling section (*Q*) reduces the milk temperature to about 25 °C (77 °F). A graduated valve 14 at the outlet of the final milk cooling section enables the back pressure to be maintained high enough (4 atm ≡ 60 lb/in.^{2}) to prevent boiling of the milk or separation of dissolved gases at the highest processing temperatures.

An aseptic sampling point is fitted after the back pressure valve 14. Milk in excess of that taken through the sampling point is passed through heat exchanger section *P*, which is only effective as a cooler during plant sterilization, and then to a 3-way cock 16 which allows either recirculation or the discharge of the plant output to waste or to a storage tank. A graduated valve 15 provides back pressure during plant sterilization.

Plant sterilization and milk processing (*indirect process*). The plant is sterilized by recirculating pressurized hot water so that all parts of the plant from the steam heating

section U to the sterilizing cooling section P are at a temperature above 130 °C (266 °F) for at least 45 min.

Water at a temperature of about 75 °C (167 °F) from the float-controlled balance tank 1 is pumped by pump 3, by-passing the regenerator sections R and S by means of cock 4, directly to the hot water section T, where its temperature is raised to 82 °C (180 °F) under the control of controller C_1. The water then passes to the deaerating vessel 7, and through pump 10 to the homogenizer 13. When deaeration is not required, the vessel is by-passed by means of cocks 5 and 12. The homogenizer pumps the water through the heating section U, where its temperature is raised to 140 °C (284 °F) under the control of controller C_2, and then without any cooling through the crash cooling section V, the regenerator sections S and R, and the final milk cooler section Q to the sterilizing cooler section P. In this last section the temperature of the water is reduced to about 75 °C (167 °F) before it is returned to the float-controlled tank through cock 16. The graduated valve 15 is set to maintain a back pressure of about 4 atm (60 lb/in.^{2}) during sterilization.

The sampling point is sterilized by allowing a small bleed of hot water to pass through it during plant sterilization.

To change the plant from sterilization to normal operation, softened mains cooling water is supplied to the crash cooling section and, after a short delay, the regenerator sections are brought into circuit by the operation of cock 4. Back pressure control is taken over by adjusting valve 14 while valve 15 is opened, and cooling water is removed from the sterilizing cooler section.

The required milk processing temperature is set by controller C_2, and cooling water is supplied to the final cooling section. When the plant conditions have become stable, the circulating water is diverted to waste through cock 16 and milk is admitted to the float-controlled balance tank. When undiluted milk appears at the plant outlet, it is diverted to a storage tank.

Operation as a direct-heating plant

The arrangement of the plant as a direct heater is shown in Fig. 2. From the balance tanks through the regenerator sections, hot water section and deaeration vessel the circuit is identical with that for the indirect-heating plant. The milk passes from the deaerator (or hot water section if the deaerator is not in use) to a high pressure pump 17 which pumps the milk through the steam injector 18. The steam supply is controlled by controller C_2 to give the required operating temperature.

After a short holding period (3·0 s mean) the milk passes through the expansion valve 19 into the expansion chamber 20. Stable operation of the steam injector depends in part on an adequate milk pressure in the injector. The valve 19 is adjusted to give a pressure of about 5·4 atm (80 lb/in.^{2}) at the beginning of operation, and the corresponding steam pressure is about 6·4 atm (96 lb/in.^{2}). These pressures increase as processing continues, partly because of obstruction of the expansion valve by deposits and partly because of obstruction of the steam inlet orifices. The setting of valve 19 is not changed during an operating run.

The expansion chamber is held at a constant pressure below atmospheric by condensing the vapours in the condensing section U and removing the condensate and

incondensable gases with a vacuum pump 25. The pressure in the expansion vessel and hence the temperature of the milk after expansion cooling is controlled by the ratio controller C_3 as described later.

Milk at a temperature of about 84 °C (183 °F) leaves the expansion chamber and is pumped by the aseptic centrifugal pump 21 through the crash cooler, which is not supplied with cooling water, to the regenerator section *S*. The level of the milk is maintained constant in the vertical outlet tube below the expansion chamber by the pneumatically balanced pressure relief valve 22. This operates in the same way as valve 11 which is associated with the level control of the deaeration vessel 7, as described above.

After being cooled to a temperature of about 52 °C (126 °F) in the regenerator section *S*, the milk passes to the homogenizer 13, where it is homogenized at a total pressure of 200 atm (3000 lb/in.^{2}). The milk then leaves the plant through the regenerator section *R*, milk cooling section *Q* and sterilizing cooler section *P* as described for the indirect-heating plant.

Plant sterilization and milk processing (*direct process*). The plant is sterilized by recirculating pressurized hot water so that all parts of the plant from the steam injector 18 to the sterilizing cooler section *P* are at a temperature above 130 °C (266 °F) for at least 45 min.

The system, as far as the steam injector, is as described for the indirect system. The water is heated to 140 °C (284 °F) in the steam injector under the control of controller C_2.

The water then passes to the expansion vessel 20, which is allowed to fill with water. A small amount overflows through the condenser section *U*, sterilizing the vapour passages, and is bled to waste through valve 23 with the vacuum pump isolated by valve 24. The condenser is not supplied with water at this stage. Most of the water is pumped from the expansion vessel by pump 21 through sections *V* and *S* to the homogenizer 13. A small volume also passes to waste through valve 22, which is supplied with a higher air pressure than during normal operation to withstand the higher liquid pressure. The homogenizer pumps the water through the regenerator section *R* and the final milk cooling section *Q*, which is not supplied with cooling water. In the sterilizing cooler section *P* the temperature of the circulating water is reduced to about 75 °C (167 °F) before being returned to the balance tank 1 through cock 16.

Back pressure is maintained in the system during plant sterilization by the settings of valves 15 and 23, and is about 8 atm (120 lb/in.^{2}) from the injector 18 to the expansion vessel 20, 2·7 atm (40 lb/in.^{2}) in the expansion vessel and condenser, and 4 atm (60 lb/in.^{2}) from the aseptic pump 21 to the graduated valve 15.

The sampling point is sterilized as in the indirectly heated system.

When plant sterilization is complete, cooling water is first supplied to the condenser *U* and valve 23 is opened to allow the pressure in the expansion vessel to fall to atmospheric. The vacuum pump 25 is started, valve 24 opened and valve 23 closed: the control of the vacuum in the expansion vessel is then taken over by controller C_3. The air pressure to valve 22 is reduced to a preset value to maintain the correct level in the expansion vessel. The regenerator sections are brought into circuit by operating cock 4, cooling water is removed from the sterilizing cooler and is

introduced to the final milk cooler. The control of the operating back pressure is transferred from valve 15 to valve 14.

When the required operating temperature has been reached under the control of controller C_2, the circulating water is replaced by milk as with the indirect system.

Setting of the ratio controller. For the total solids content of the milk to be unaffected by the direct-heating process, the temperatures of the milk before steam injection and after expansion cooling must differ by a small constant amount (Hallström, 1966). In this experimental plant, the temperature before steam injection is held constant by controller C_1, which controls the water temperature in heater T. The differential temperature therefore depends only on the temperature after expansion cooling, which is varied by controlling the vacuum in the expansion vessel 20 by means of an air bleed into the vapour line to the condenser V. The air bleed is regulated by a diaphragm valve, controlled by controller C_3 so that the 2 temperatures sensed by C_3 have the required constant difference.

The difference in temperatures required to give the correct milk composition depends on the heat content of the steam at the temperature and pressure of steam injection, on the heat content of the vapour at the temperature and pressure of evaporative cooling, and on heat losses from the system and steam dryness fractions. The heat contents can be obtained from the operating conditions, but the heat losses and the degrees of dryness of the steam and the expansion vapours can only be estimated. Therefore, the true difference in temperature required in operation for any individual plant can only be determined empirically.

To determine the correct setting of controller C_3 for these experiments, the float tank 1 was closed with a liquid-tight lid fitted with a vertical transparent calibrated tube. The plant was filled with water until a level was visible in the tube. The water was circulated through the plant and heated to the normal operating temperature, and the change in volume over a period of time was measured for a series of values of the differential temperature as determined by the setting of controller C_3. The differential temperature to give no change in volume was found by interpolation to be 2·1 degC, so that for a water temperature before steam injection of 82·2 °C (180 °F) the required temperature after expansion cooling was 84·3 °C (183·8 °F). That this was the temperature differential was confirmed by processing a batch of milk and determining total solids contents for samples taken before and after processing. Volume measurement was found to be a much more accurate control of dilution or concentration of the milk than was its direct analysis.

TIME–TEMPERATURE PROFILES

The advantages that have been claimed for milk produced by the direct-heating process arise from the different time–temperature conditions imposed by the direct and indirect systems. So that any differences in the milks produced by the 2 systems could be interpreted more satisfactorily, time–temperature curves for the experimental sterilizer operating as an indirect and as a direct heater were constructed.

Temperatures at different points in the plant were measured either with mercury-in-glass thermometers mounted in stainless steel pockets in the milk lines, or with copper-constantan thermocouples mounted in the milk lines or fitted between pairs

of plates in the heat exchanger sections. The mounting of the thermocouples and the method of measurement were generally as described by Burton (1958). Thermometers and thermocouples were calibrated against an NPL-certified mercury-in-glass thermometer in an oil bath.

Mean flow times for the indirect-heating system were calculated from the volume of each part of the plant and a milk throughput of 250 gal/h.

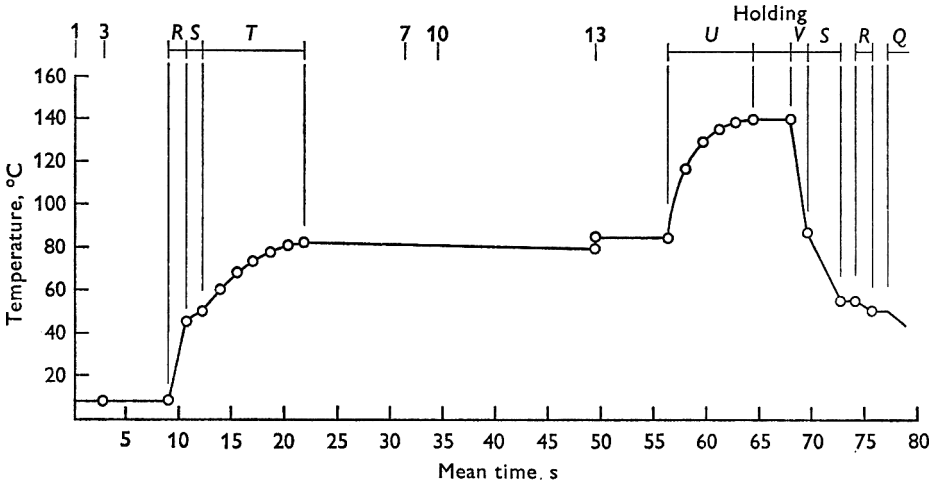

Fig. 3. Time–temperature profile for indirect-heating experimental plant.

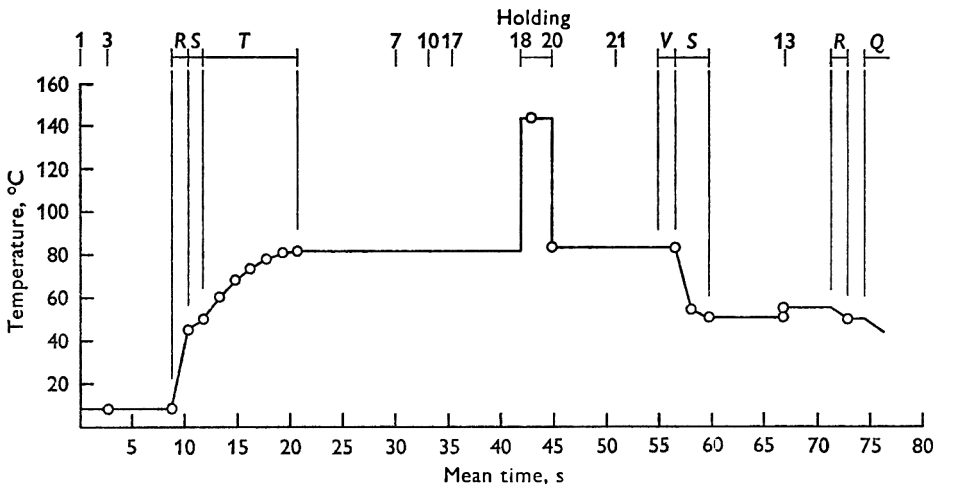

Fig. 4. Time-temperature profile for direct-heating experimental plant.

For the direct-heating system, it was assumed that the temperature rose instantaneously in the steam injector and fell instantaneously in the expansion vessel. The milk flow rates used for the calculation of residence times in other parts of the plant varied in different parts of the system. At points after the relief valve 22, the flow rate was taken as 250 gal/h as determined by the homogenizer. The flow rate from the balance tank to the injector was set at 260 gal/h by adjustment of the speed

of the high pressure pump 17. The amount of condensate added in the steam injector was calculated from the operating temperatures to be 31 gal/h. The flow rate through the holding tube was therefore taken as 291 gal/h. Approximately 10 gal/h were lost to waste through valve 22 in controlling the milk level in the expansion vessel.

A measured temperature rise of 5 degC (9 degF) was caused by homogenization: this agrees exactly with that calculated from the homogenizing pressure of 200 atm (3000 lb/in.^{2}).

The complete time–temperature curves so obtained are shown in Fig. 3 for indirect heating and in Fig. 4 for direct heating. Only the parts of the curves above about 130 °C (265 °F) are important in determining the bacteriological performance of the plant, and only those parts above about 80 °C (175 °F) are important in causing chemical changes. This must be remembered in considering the relevance of these curves to the bacteriological and chemical results to be given in later papers.

We wish to thank the members of the staff of the APV Co. Ltd for their close cooperation during the design and construction of this plant, and throughout the experimental work.

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Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization

II. The sporicidal efficiency of an experimental plant for direct and indirect processing

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SUMMARY. The variation of sporicidal efficiency with processing temperature was determined for an experimental ultra-high-temperature (UHT) milk sterilizer operating alternatively as an indirect or as a direct heater. Whole milk was inoculated with large numbers of spores of *Bacillus subtilis* 786 and *Bacillus stearothermophilus* TH 24, and the proportion of spores surviving the sterilizing process was calculated from dilution and colony counts on the untreated and treated milk. The results for *B. subtilis* spores were unreliable, and the dilution count results for *B. stearothermophilus* spores were influenced by the inhibitory effect of the UHT processed milk. The results for the colony counts of *B. stearothermophilus* spores were preferred as a basis for the comparison of the direct and indirect processes. Over the range of processing temperatures 137–145 °C it was found that the sterilizing temperature had to be 3–4 degC higher with direct heating than with indirect heating to give equal spore destructions.

To be valid, comparisons of the quality of the milks produced by direct and indirect heating ultra-high-temperature (UHT) processes require standardization of the heat treatments. In the present studies, it was decided that processes having equal sporicidal efficiencies should be compared. Experiments were therefore performed with the experimental plant described previously (Burton & Perkin, 1970) to determine the variation of sporicidal efficiency with processing temperature for both the direct and indirect modes of operation. From these results, pairs of temperatures giving equal kills of resistant spores could be selected for future work.

EXPERIMENTAL METHODS

Test organisms. The sporicidal efficiency of the UHT plant was determined for spores of *Bacillus subtilis* 786 (NCDO 1069), a heat-resistant mesophile, and for spores of *Bacillus stearothermophilus* TH 24 (NCDO 1096), a very heat-resistant thermophile. These strains have been extensively used for similar work (Williams,

* Present address: Glaxo Research Ltd, Greenford, Middlesex.

Franklin, Chapman & Clegg, 1957; Franklin, Williams, Chapman & Clegg, 1958; Franklin, Williams & Clegg, 1958; Franklin, Williams, Burton, Chapman & Clegg, 1959).

Spores were grown on bacillus spore agar (BSA) as described by Williams *et al.* (1957) for *B. subtilis* and by Franklin *et al.* (1959) for *B. stearothermophilus*. In both cases the concentration of Mn^{2+} in the growth medium was 1 ppm. After harvesting, the spores were resuspended in distilled water and stored in a refrigerator at approximately 4 °C until used.

Sampling arrangements. A sterilizable sampling outlet was fitted at the exit from the plant. Its construction is shown in Fig. 1(*a*).

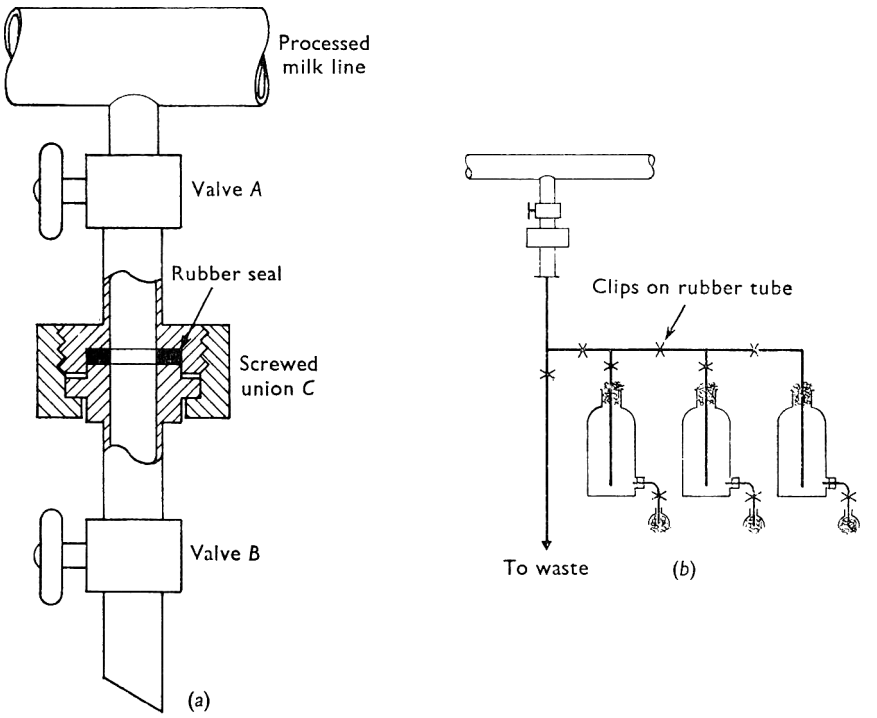

Fig. 1. Construction of sampling outlet and arrangement of sampling jars.

The processed milk samples were taken via the sampling outlet into specially fitted 10-l aspirator jars. It was convenient to test up to 3 treatment temperatures in each experimental run, and accordingly 3 of these jars were joined to a common manifold, which in turn was joined to a matching connector component which replaced the bottom part of the sampling outlet. A tube to waste was also included in the sampling assembly so that the sampling outlet and supply tube could be flushed with the milk being sampled without the jars being filled. This arrangement is shown diagrammatically in Fig. 1(*b*).

The manifold consisted of stainless steel tubes connected with lengths of rubber tubing. The inlet tubes to the jars passed through non-absorbent cotton wool plugs closing the top jar opening, and extended to the bottom of the jars so that milk entered below the level of liquid already present and frothing was minimized. A

hooded tube was fitted to the bottom outlet of each jar for aseptic dispensing of the sample into smaller volumes. The flow of milk was controlled by clips on the rubber tubing at the points shown.

To ensure sterility of the sampling system, a double autoclaving procedure was used. The jars and manifold were first autoclaved as disconnected components in steam at 130 °C for 30 min. To each jar was then immediately added direct from a steamer 1 l hot nutrient broth of pH 7·0 ± 0·1, containing per l: 10 g Evans peptone, 10 g Lab-Lemco and 5 g NaCl. The manifold and jars containing broth were assembled while still hot, and were re-autoclaved at 130 °C for 30 min. The assembly was incubated for several days at room temperature before use to check on sterility.

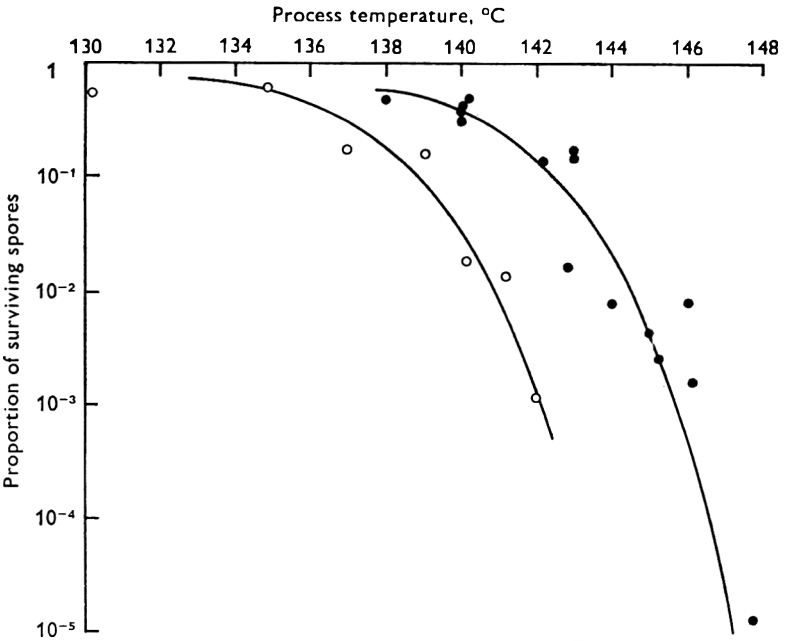

Fig. 2. Proportion of *Bacillus stearothermophilus* spores (colony counts) surviving UHT processing at different temperatures. ○, Indirect heating; ●, direct heating.

Plant operation. The plant was sterilized by the circulation of water as described by Burton & Perkin (1970). To sterilize the sampling outlet with the plant, the inner valve *A* of the sampling outlet was opened fully, and the outer valve *B* was opened sufficiently to give a slight flow of water through it. When plant sterilization was complete, valves *A* and *B* were both closed. The screwed union *C* between them was then broken, and the lower part including valve *B* removed. This was replaced by the matching connector attached to the sterile sampling jar assembly and autoclaved with it. Aseptic precautions were taken and the connecting pieces were flamed during these manipulations.

The plant was set to operate under the required temperature conditions and was then changed to milk processing as described by Burton & Perkin (1970). The experimental tank 2 (Figs 1 and 2, Burton & Perkin, 1970), in parallel with the inlet float

tank, was filled with approximately 180 l raw milk, and sufficient of both spore suspensions was added to give a count of *c.* 10^5–10^6 *B. stearothermophilus* spores/ml and *c.* 10^6–10^7 *B. subtilis* spores/ml. The inoculated milk was mixed thoroughly and a control sample taken into a sterile bottle immediately before the milk was processed.

When the plant conditions were stable at the required temperature, the milk supply to the float tank was cut off. When the float tank was almost empty, the inoculated milk was allowed to enter the plant from tank 2. The time required for milk to pass through the plant and reach the sampling point was known by calculation from the volume of the plant and the milk flow rate. After rather more than this time had elapsed, valve *A* on the sampling outlet was opened and the valve and supply tube to the sampling assembly were flushed by milk passing to waste. By opening the appropriate clips on the rubber tubing and closing the flow to waste, the first of the 3 aspirator jars was filled to the 10 l level with treated milk. Valve *A* was then closed and the filled jar isolated from the remainder of the assembly by closing the clip on the inlet tube to the jar.

When all the inoculated milk from the experimental tank had passed through the plant, normal milk was allowed to enter the float tank again and the plant operating conditions were altered as required and allowed to re-stabilize.

A further 180 l of inoculated milk was prepared in tank 2, and after a sample for control counts had been taken a second experiment was carried out as described above.

Normally this was repeated 3 times, so that each aspirator jar contained the milk produced under one set of constant operating conditions (see p. 220). When more than one treatment temperature was used the highest temperature was used first and the lowest last, to reduce errors in the results which might occur if the sampling assembly supply tubes contained surviving spores from previous treatments at lower temperatures.

Determination of spore counts. The numbers of spores present in the inoculated raw milk samples, after steaming at 100 °C for 30 min to heat-activate the spores, were determined by colony and dilution counts. Both counting methods, without steaming, were also used to enumerate *B. stearothermophilus* spores in the processed milks, but dilution counts only were used for the *B. subtilis* spores in the processed samples because of the low number of survivors.

Colony counts were made in BSA (containing 1·5% agar) incubated 2 days at 55 °C for *B. stearothermophilus* and in starch milk agar (SMA) (Grinsted & Clegg, 1955) incubated 2 days at 37 °C for *B. subtilis*. Dilutions before plating, where necessary, were made in $\frac{1}{4}$-strength Ringer's solution.

Dilution counts were made by incubating appropriate quantities of the milks (e.g. 1000, 100, 10, 1·0, 0·1, 0·01 ml, etc.) for 7 days at 37 °C for *B. subtilis* and at 55 °C for *B. stearothermophilus*. Five replicates were incubated at each level, samples of $\leqslant$ 1 ml being made up to 10 ml with sterile nutrient broth. Positive growth was determined by inspection for marked physical change and by microscopic examination. Doubtful positives were confirmed by streaking on BSA or SMA plates. The most probable number of spores present was determined from the pattern of positive samples by the use of Hoskins' (1934) probability tables.

The spore counts for the treated milks were corrected to allow for dilution by the 1 l of broth present in each 10 l aspirator jar before sampling.

RESULTS AND DISCUSSION

The control and treated milk spore counts are summarized for all the experiments in Table 1 for *B. stearothermophilus* and in Table 2 for *B. subtilis*. Whenever repeat experiments were made with the same processing temperatures, as for example with *B. stearothermophilus* spores in runs 3 and 7, the reproducibility of the results was good.

The proportions of surviving spores are given as a function of processing temperature in Fig. 2 (*B. stearothermophilus*, colony counts), Fig. 3 (*B. stearothermophilus*, dilution counts) and Fig. 4 (*B. subtilis*, dilution counts).

Table 1. *The effect of UHT processing on the destruction of* Bacillus stearothermophilus (*TH* 24) *spores*

Expt no.	Processing system	Temp., °C	Spores/ml before processing: Colony count	Spores/ml before processing: Dilution count	Spores/ml after processing: Colony count	Spores/ml after processing: Dilution count
1*a*	Direct	135·9	$7{\cdot}1 \times 10^{5}$	$3{\cdot}0 \times 10^{5}$	$> 1{\cdot}0 \times 10^{5}$	$> 3{\cdot}52 \times 10^{-1}$
1*b*	Direct	133·7	$6{\cdot}35 \times 10^{5}$	$2{\cdot}9 \times 10^{5}$	$9{\cdot}85 \times 10^{4}$	$> 1{\cdot}76 \times 10^{2}$
1*c*	Direct	131·7	$3{\cdot}0 \times 10^{5}$	$4{\cdot}5 \times 10^{5}$	$4{\cdot}24 \times 10^{5}$	$> 1{\cdot}76 \times 10^{2}$
2*a*	Direct	142·2	$2{\cdot}5 \times 10^{5}$	$1{\cdot}7 \times 10^{5}$	$3{\cdot}6 \times 10^{4}$	$> 3{\cdot}5 \times 10^{2}$
2*b*	Direct	140·0	$2{\cdot}0 \times 10^{5}$	$5{\cdot}15 \times 10^{5}$	$6{\cdot}0 \times 10^{4}$	$> 3{\cdot}5 \times 10^{3}$
2*c*	Direct	138·0	$2{\cdot}1 \times 10^{5}$	$1{\cdot}7 \times 10^{5}$	$1{\cdot}0 \times 10^{5}$	$3{\cdot}7 \times 10^{4}$
3*a*	Direct	140·2	$6{\cdot}8 \times 10^{4}$	$7{\cdot}9 \times 10^{4}$	$3{\cdot}1 \times 10^{4}$	$8{\cdot}7 \times 10^{3}$
3*b*	Direct	140·0	$1{\cdot}03 \times 10^{5}$	$7{\cdot}0 \times 10^{4}$	$4{\cdot}0 \times 10^{4}$	$1{\cdot}2 \times 10^{4}$
3*c*	Direct	140·0	$8{\cdot}3 \times 10^{4}$	$7{\cdot}0 \times 10^{4}$	$3{\cdot}3 \times 10^{4}$	$8{\cdot}7 \times 10^{3}$
4	Direct	146·0	$9{\cdot}6 \times 10^{4}$	$2{\cdot}2 \times 10^{5}$	$6{\cdot}99 \times 10^{2}$	$2{\cdot}0 \times 10^{-4}$
5*a*	Direct	147·8	$9{\cdot}9 \times 10^{5}$	$7{\cdot}85 \times 10^{4}$	$1{\cdot}32 \times 10^{1}$	$4{\cdot}4 \times 10^{-2}$
5*b*	Direct	145·0	$1{\cdot}16 \times 10^{6}$	$2{\cdot}65 \times 10^{4}$	$4{\cdot}78 \times 10^{3}$	$4{\cdot}4 \times 10^{1}$
5*c*	Direct	142·8	$9{\cdot}1 \times 10^{5}$	$2{\cdot}35 \times 10^{5}$	$1{\cdot}46 \times 10^{4}$	$2{\cdot}86 \times 10^{2}$
6*a*	Direct	146·1	$7{\cdot}35 \times 10^{5}$	$3{\cdot}4 \times 10^{5}$	$1{\cdot}18 \times 10^{3}$	$4{\cdot}4 \times 10^{-1}$
6*b*	Direct	145·2	$8{\cdot}90 \times 10^{5}$	$1{\cdot}1 \times 10^{5}$	$2{\cdot}2 \times 10^{3}$	$4{\cdot}4 \times 10^{-1}$
6*c*	Direct	144·0	$7{\cdot}05 \times 10^{5}$	$1{\cdot}1 \times 10^{5}$	$5{\cdot}23 \times 10^{3}$	$2{\cdot}42 \times 10^{1}$
7*a*	Direct	143·0	$1{\cdot}4 \times 10^{5}$	Not tested	$2{\cdot}04 \times 10^{4}$	Not tested
7*b*	Direct	143·0	$1{\cdot}4 \times 10^{5}$	Not tested	$2{\cdot}2 \times 10^{4}$	Not tested
8*a*	Indirect	140·2	$6{\cdot}0 \times 10^{5}$	$2{\cdot}45 \times 10^{5}$	$1{\cdot}15 \times 10^{4}$	$1{\cdot}74 \times 10^{2}$
8*b*	Indirect	134·9	$1{\cdot}18 \times 10^{6}$	$2{\cdot}4 \times 10^{5}$	$7{\cdot}2 \times 10^{4}$	$2{\cdot}02 \times 10^{4}$
8*c*	Indirect	130·2	$3{\cdot}3 \times 10^{5}$	$1{\cdot}1 \times 10^{5}$	$1{\cdot}75 \times 10^{5}$	$5{\cdot}28 \times 10^{4}$
9*a*	Indirect	142·0	$6{\cdot}35 \times 10^{5}$	$3{\cdot}5 \times 10^{5}$	$7{\cdot}59 \times 10^{2}$	$1{\cdot}02 \times 10^{-1}$
9*b*	Indirect	137·0	$4{\cdot}35 \times 10^{5}$	$1{\cdot}3 \times 10^{5}$	$7{\cdot}7 \times 10^{4}$	$1{\cdot}11 \times 10^{4}$
10*a*	Indirect	141·2	$1{\cdot}17 \times 10^{5}$	$3{\cdot}5 \times 10^{5}$	$1{\cdot}54 \times 10^{3}$	$2{\cdot}2 \times 10^{1}$
10*b*	Indirect	139·1	$1{\cdot}52 \times 10^{5}$	$2{\cdot}4 \times 10^{5}$	$2{\cdot}5 \times 10^{4}$	$1{\cdot}05 \times 10^{4}$

The results for the colony counts of *B. stearothermophilus* showed good reproducibility and the expected variation of lethal effect with processing temperature. The results closely followed the curves, which would be expected from the assumption of a Q_{10} for spore destruction of 29. The results for indirect heating showed a greater lethal effect than with direct heating for the same processing temperature: this was to be expected because the holding time with indirect heating was longer than with direct heating (3·6 s versus 3·0 s; Burton & Perkin, 1970) while the effect of the final heating section increased further the effective holding time with indirect heating. The lethal effect for a given processing temperature was less than was found in previous experiments on a different plate-type indirect-heating plant (Franklin *et al.* 1959). This was because the plant studied in the present experiments was of more

modern design, with increased heat transfer rates in the steam heating section. This allowed shorter flow times in this section (8 s in the present plant (Burton & Perkin, 1970) as against 17 s in the earlier plant (Burton, 1958)), and hence there was a smaller contribution from the steam heating section to the total lethal effect.

Table 2. *The effect of UHT processing on the destruction of* Bacillus subtilis (786) *spores*

Expt no.	Processing system	Temp., °C	Spores/ml before processing: Colony count	Spores/ml before processing: Dilution count	Spores/ml after processing: Dilution count
1*a*	Direct	135·9	$5{\cdot}35 \times 10^{6}$	$1{\cdot}45 \times 10^{7}$	$> 1{\cdot}76 \times 10^{-1}$
1*b*	Direct	133·7	$3{\cdot}8 \times 10^{6}$	$1{\cdot}35 \times 10^{7}$	$> 1{\cdot}76 \times 10^{-1}$
1*c*	Direct	131·7	$2{\cdot}45 \times 10^{6}$	$1{\cdot}65 \times 10^{7}$	$> 1{\cdot}76 \times 10^{3}$
2*a*	Direct	142·2	$3{\cdot}9 \times 10^{6}$	$5{\cdot}15 \times 10^{6}$	$9{\cdot}2 \times 10^{-2}$
2*b*	Direct	140·0	$4{\cdot}1 \times 10^{6}$	$1{\cdot}45 \times 10^{7}$	$1{\cdot}98 \times 10^{1}$
2*c*	Direct	138·0	$4{\cdot}4 \times 10^{6}$	$8{\cdot}9 \times 10^{6}$	$5{\cdot}67 \times 10^{1}$
4	Direct	146·0	$7{\cdot}65 \times 10^{6}$	$1{\cdot}3 \times 10^{7}$	$4{\cdot}4 \times 10^{-4}$
5*a*	Direct	147·8	$8{\cdot}15 \times 10^{6}$	$1{\cdot}01 \times 10^{7}$	$5{\cdot}0 \times 10^{-1}$
5*b*	Direct	145·0	$1{\cdot}19 \times 10^{7}$	$5{\cdot}15 \times 10^{6}$	$2{\cdot}2 \times 10^{-1}$
5*c*	Direct	142·8	$5{\cdot}4 \times 10^{6}$	$1{\cdot}65 \times 10^{7}$	$1{\cdot}98 \times 10^{-1}$
6*a*	Direct	146·1	Not tested	$8{\cdot}6 \times 10^{6}$	$2{\cdot}2 \times 10^{-1}$
6*b*	Direct	145·2	Not tested	$7{\cdot}4 \times 10^{6}$	$4{\cdot}95 \times 10^{-1}$
6c	Direct	144·0	Not tested	$8{\cdot}6 \times 10^{6}$	$1{\cdot}98 \times 10^{-1}$
8*a*	Indirect	140·2	$7{\cdot}45 \times 10^{6}$	$1{\cdot}16 \times 10^{7}$	$1{\cdot}6 \times 10^{1}$
8*b*	Indirect	134·9	$5{\cdot}2 \times 10^{6}$	$2{\cdot}4 \times 10^{6}$	$9{\cdot}1 \times 10^{-4}$
8*c*	Indirect	130·2	$7{\cdot}2 \times 10^{6}$	$1{\cdot}45 \times 10^{7}$	$1{\cdot}0 \times 10^{6}$
9*b*	Indirect	137·0	$8{\cdot}4 \times 10^{6}$	$5{\cdot}4 \times 10^{7}$	$1{\cdot}01 \times 10^{-1}$
9*c*	Indirect	132·0	$8{\cdot}7 \times 10^{6}$	$2{\cdot}8 \times 10^{7}$	$3{\cdot}85 \times 10^{4}$

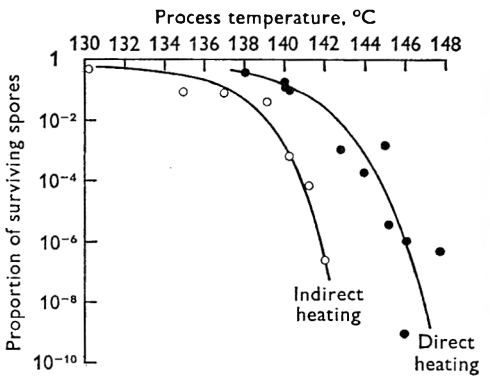

Fig. 3

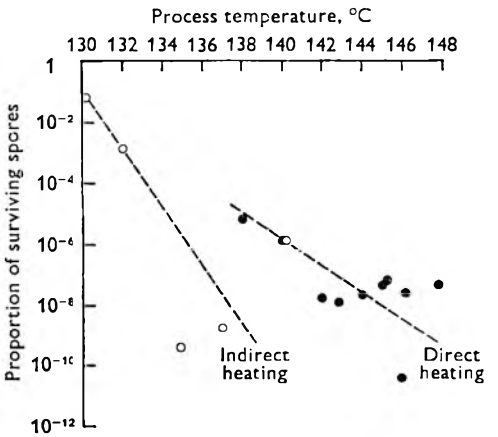

Fig. 4

Fig. 3. Proportion of *Bacillus stearothermophilus* spores (dilution counts) surviving UHT processing at different temperatures. ○, Indirect heating; ●, direct heating.

Fig. 4. Proportion of *Bacillus subtilis* spores (dilution counts) surviving UHT processing at different temperatures. ○, Indirect heating; ●, direct heating.

The results for the dilution counts of *B. stearothermophilus* spores (Fig. 3) were also satisfactory, and showed a similar difference between the effects of direct and indirect heating. However, the dilution counts showed greater apparent lethal effects

for the same processing conditions than did the colony counts. The difference was small when there was a high proportion of survivors, but it increased to about 4 log cycles at the lowest levels of survivors, i.e. at the highest temperatures. This is the result of the inhibitory action of the UHT processed milk on the germination and growth of the heated spores, an effect that has been noted previously to lead to falsely high values for sterilizing effect when dilution counts are used (Franklin *et al.* 1959; Franklin, 1970). The inhibitory effect is increasingly diluted out at high proportions of survivors by the addition of broth to the very small quantities of milk required for incubation.

Table 3. *Pairs of processing temperatures required to give the same sporicidal effect against* Bacillus stearothermophilus (*TH* 24) *spores with indirect and direct heating*

Processing temp., °C		
	Direct system	
Indirect system	From colony counts	From dilution counts
137·0	140·6	140·0
138·0	141·6	140·8
139·0	142·6	141·7
140·0	143·5	143·1
141·0	144·5	144·6
142·0	145·5	146·4

The results for the dilution counts of *B. subtilis* spores were inconsistent. No conclusions could be drawn from these results other than that the indirect heating system gave a greater lethal effect than the direct system for the same processing temperature, as was to be expected. Survivor counts were again significantly depressed due to the inhibition of spore germination and growth by the UHT milk, a situation made worse for *B. subtilis* because of its lower heat resistance and the consequent lower proportions of surviving spores compared with *B. stearothermophilus*. This prevented the use of colony counts and also removed the possibility of broth addition to dilute out the inhibitory effect. In most of the experimental runs with *B. subtilis*, numerous skips were evident in the dilution counts and it is obvious that these results are unreliable. It is possible that the inhibitory effect against *B. subtilis* spores varies with the milk in which they are suspended and the severity of the heat treatment that the milk and spores have received.

For a comparison of the sporicidal performances of the experimental plant with direct and indirect heating, only the results with *B. stearothermophilus* spores could be used, and of these the colony count results were preferable because of their greater reliability. Table 3 shows the pairs of processing temperatures which gave the same sporicidal effect. In general, the direct heating system required a processing temperature 3–4 degC higher than the indirect heating system to give equivalent results, and this difference was maintained over the whole of the practical range of processing temperatures. In the proposed comparative tests on the UHT milks produced by the 2 systems, therefore, processing temperatures differing by 3–4 degC will be used.

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Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization

III. A note on the results for spore destruction obtained with an experimental ultra-high-temperature milk sterilizer

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Summary. The bacteriological results of Franklin, Underwood, Perkin & Burton (1970) are analysed to show that colony counts of *Bacillus stearothermophilus* spores in UHT-treated milk are influenced by the inhibitory action of the milk, so that the sporicidal effects of the UHT process as calculated from the results are too high. Only the elimination of the inhibitory factor will allow true sporicidal effects to be determined.

The purpose of the bacteriological experiments of Franklin *et al.* (1970) was to find the operating temperatures required to give equal sporicidal effects by direct and indirect processing with an experimental UHT plant. The results gave this information without any ambiguity. However, when they are examined more closely some other conclusions follow, which strictly are outside the scope of the comparison of direct and indirect heating but which have an important bearing on the interpretation of the sporicidal effects apparently obtained.

It is known that, because of an inhibitory factor present in milk, dilution counts can give much lower apparent numbers of survivors of *Bacillus stearothermophilus* spores than colony counts when UHT milk sterilizing processes are compared (Franklin, Williams, Burton, Chapman & Clegg, 1959; Franklin, 1970; Franklin *et al.* 1970). It has been assumed from this that dilution counts are unreliable and unsuitable for determining the sterilizing efficiency of a UHT plant (Burton, 1969), but that colony counts are sufficiently accurate to be used with some confidence. However, an examination of the results of Franklin *et al.* (1970) suggests that this confidence may be misplaced, and that it may be difficult or even impossible to obtain a true value for the kill of spores where inhibition is present.

DISCUSSION

The curves relating the proportion of surviving *B. stearothermophilus* spores to processing temperature, given in Fig. 2 of Franklin *et al.* (1970), refer to constant processing times, although the effective holding time is somewhat longer for indirect heating than for direct heating. The Q_{10} for spore destruction over the temperature

range studied can therefore be calculated as the ratio of the logarithms of the proportions of surviving organisms at 10 degC intervals of processing temperature (Burton, 1958). Both the curves indicate a Q_{10} of 29, which is unusually high for the thermal kill of *B. stearothermophilus* spores: laboratory results on spores of the same strain gave a Q_{10} of 11·5 (Franklin *et al.* 1959; Burton *et al.* 1959).

Extrapolation of the laboratory results of Franklin *et al.* (1959), using this lower Q_{10} of 11·5, still leads to an overestimate of the effect of a direct-heating UHT process, in which heating to and cooling from the sterilizing temperature is virtually instantaneous. For example, using the nomenclature of Burton (1958), from the laboratory results at 120 °C (Burton *et al.* 1959):

$$K_{120} = 0{\cdot}260 \text{ min}^{-1}$$

If the Q_{10} is 11·5, then:

$$K_{140} = 0{\cdot}260 \times 11{\cdot}5^2 \text{ min}^{-1} = 34{\cdot}3 \text{ min}^{-1} = 0{\cdot}57 \text{ s}^{-1}$$

The experimental UHT plant has a mean holding time of 3·0 s with direct heating (Burton & Perkin, 1970). The logarithm of the proportion of surviving spores of *B. stearothermophilus* at a processing temperature of 140 °C should therefore be:

$$L_{140} = -K_{140}t = -0{\cdot}57 \times 3{\cdot}0 = -1{\cdot}7$$

In fact, as may be seen from Fig. 2 of Franklin *et al.* (1970), L_{140} was found to be only about $-0{\cdot}4$. The Q_{10} value of 11·5 found from the laboratory results therefore cannot be used to extrapolate to temperatures as high as 140 °C.

The Q_{10} required to give accurate extrapolation can be calculated from the experimentally determined proportion of survivors ($L_{140} = -0{\cdot}4$). If the mean holding time is 3·0 s, then

$$K_{140} = 0{\cdot}4/3{\cdot}0 = 0{\cdot}133 \text{ s}^{-1} = 8{\cdot}0 \text{ min}^{-1}$$

But

$$K_{120} = 0{\cdot}260 \text{ min}^{-1}$$

Therefore

$$K_{140}/K_{120} = Q_{20} = Q_{10}^2 = 8{\cdot}0/0{\cdot}260$$

$$Q_{10} = 5{\cdot}5$$

This value of Q_{10} has been derived from the results of direct heating alone, since this gives practically instantaneous heating and cooling to and from the sterilizing temperature, and so simplifies the calculations. However, by using the time-temperature curves given by Burton & Perkin (1970) and the method of calculation set out by Burton (1958) the relative bacteriological results for direct and indirect processing can be checked.

Using the Q_{10} value of 5·5 derived above, and the published time–temperature curves, it is found that the thermal kill of *B. stearothermophilus* spores with indirect heating should be very nearly the same as with direct heating at a temperature 3·5 degC higher. The logarithms of the proportions of surviving spores are then in the ratio 1·09 (indirect):1·00 (direct). The required difference in temperatures for this result is independent of the absolute level of survivors.

The difference in temperature of 3·5 degC was that found experimentally for equivalent bacteriological effect. This, therefore, supports the hypothesis that the effective Q_{10} for spore destruction at high processing temperatures is about 5·5, and consequently lower than is found at lower temperatures. Evidence for such a

reduction in the Q_{10} at higher temperatures has been given for *Bacillus cereus* spores by Miller & Kandler (1967).

It is necessary to explain the discrepancy between the Q_{10} value of 5·5 obtained by the extrapolation of laboratory results at 120 °C to plant results at 140 °C and also by the comparison of direct and indirect processing, and that of 29 obtained from the shapes of the thermal death–temperature curves for direct and indirect processing. The important difference appears to be that the low values of Q_{10} are obtained from comparisons of the same proportion of surviving spores, but with different processing temperatures: the high values are deduced from results covering the whole range of proportions of surviving spores covered in the experiments. Such a discrepancy between the Q_{10} values would be found if the colony count results were subject to an inhibitory effect.

The amount of inhibitory effect would vary with the number of dilutions between the initial sample of milk and spore suspension actually plated. If the numbers of surviving spores in the initial sample were low, no dilution would occur before plating, and inhibition would be likely to occur. If the numbers of survivors were increasingly large, the numbers of dilutions required before plating would progressively increase, and so the inhibitory factor initially present would be increasingly diluted out. Therefore, low numbers of surviving organisms would be underestimated, and high numbers would be estimated correctly. This would cause a falsely high value of Q_{10} to be derived from the shape of a temperature–survivor curve if the curve covered a wide range of numbers of surviving organisms, as in the results under discussion.

The inhibition would not affect the derivation of the low Q_{10} value from the extrapolation of the laboratory results, since the proportion of survivors at a processing temperature of 140 °C is sufficiently high for the inhibitory factor to have been diluted out by the number of dilutions required during counting. Nor would it affect the confirmation of this value from the comparison of the curves for direct and indirect processing: in this case the comparison is always between processes giving the same number of survivors, so that the required dilution for counting is the same in each, and any effect of the inhibitory factor has been partially or completely diluted out to the same extent in each. The effect of inhibition shows itself only in a shape of the derived thermal death–temperature curve which overestimates the thermal death at relatively high temperatures.

On the assumptions that high proportions of surviving *B. stearothermophilus* spores were correctly measured in the experiments of Franklin *et al.* (1970) because the inhibitory factor had been diluted out, and that the correct Q_{10} for the thermal death of the spores was 5·5, the true curve relating the proportion of surviving spores to processing temperature for direct heating is shown in Fig. 1 (Curve *A*). The curve obtained experimentally and having a Q_{10} of 29 is also shown (Curve *B*). According to the hypothesis put forward, the difference between the 2 curves represents the inhibitory effect of UHT milk carried over into the plating medium.

Franklin (1958) showed that UHT milk in bacillus spore agar (BSA) as plating medium reduced the counts obtained, but that a 5 % concentration of UHT milk in BSA was needed to reduce the counts 20-fold. H. M. Underwood (private communication) has also found inhibition in colony counts of spores suspended in UHT milk.

The degree of inhibition varied with the amount of carry-over of milk into the BSA plating medium, and so depended on the dilution of the suspension before plating. For conditions corresponding to the lowest proportion of survivors in Curve *B*, Fig. 1, the inhibition amounted to a factor of about 100. For the dilutions used at other parts of Curve *B*, no inhibition could be detected.

Busta (1966) carried out comprehensive experiments in which *B. stearothermophilus* spores were suspended in milk which was then heat-treated at temperatures up to 128 °C. He found an inhibition factor of about 10^4 for conditions corresponding to the lowest proportion of survivors in Curve *B*. This degree of inhibition is greater than that found by Franklin (1958) and H. M. Underwood (private communication), and is more than sufficient to account for the difference between Curve *A* and Curve *B*. Busta used a different strain of *B. stearothermophilus* and a different plating medium, which may have been responsible for the difference between his results and those of the other workers. Furthermore, in Busta's experiments the spores were severely heated in the presence of the inhibitor, whereas the other workers studied the growth of unheated spores: it is possible that the former procedure leads to an increased effect of the inhibitor.

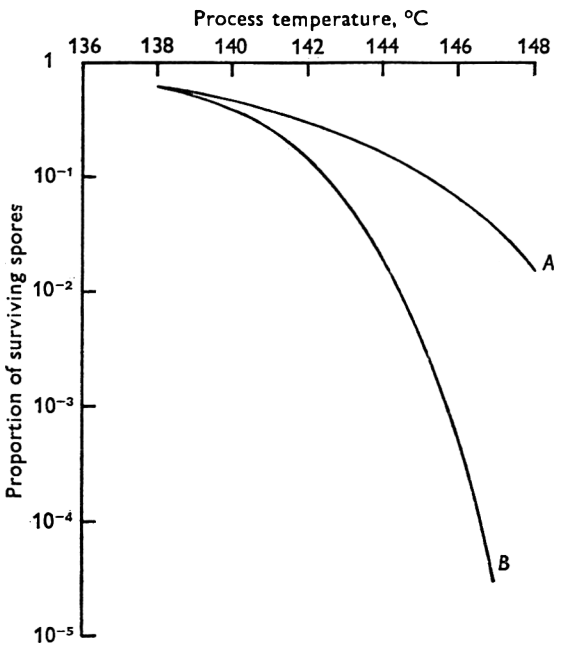

Fig. 1. Variation of sporicidal effect with process temperature. *A*, Derived curve $Q_{10} = 5{\cdot}5$; *B*, experimental curve $Q_{10} = 29$.

CONCLUSIONS

The apparent contradictions between the Q_{10} values for spore destruction which are shown by the study of the bacteriological results of Franklin *et al.* (1970) can be explained on the hypothesis that the colony counts for surviving *B. stearothermophilus* spores were influenced by the inhibitory factor in UHT-processed milk. It follows that even a colony count method does not give a reliable measure of the

numbers of *B. stearothermophilus* spores which survive a UHT-sterilization process. It will not be possible to distinguish between spores which have been destroyed by heat and those which have survived but are inhibited, until a method of counteracting the inhibitory factor has been found. Although this distinction may appear academic, it will become of practical importance if for any reason the effect of the inhibitor cannot be relied upon, or if true sporicidal effects of heat treatment processes are needed for comparisons.

This argument relates only to the determination of an absolute number of surviving spores. The comparisons of processing temperature given by Franklin *et al.* (1970) for the experimental UHT plant studied by them remain valid, because the inhibitory effect will be the same for the same proportion of survivors for both direct and indirect processing.

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The denaturation of α-lactalbumin and β-lactoglobulin in heated milk

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Summary. The rates of denaturation by heat of α-lactalbumin and β-lactoglobulin in skim-milk were measured by an immunodiffusion method over a wide range of temperatures. Both reactions showed an unusual temperature dependence.

The denaturation of α-lactalbumin is a first-order reaction; between 90 and 155 °C the kinetic constant k_1 in s^{-1} is given by the equation

$$\log k_1 = 7{\cdot}15 - 3{\cdot}60\ (10^3/T),$$

where T is the temperature in °K.

The denaturation of β-lactoglobulin in skim-milk is second order with respect to time, and the kinetic constant k_2 in $l\ g^{-1}\ s^{-1}$ is given by 2 equations, valid for different temperature ranges. Between 68 and 90 °C,

$$\log k_2 = 37{\cdot}95 - 14{\cdot}51\ (10^3/T).$$

Between 90 and 135 °C

$$\log k_2 = 5{\cdot}98 - 2{\cdot}86\ (10^3/T).$$

Results obtained by the immunodiffusion method agreed well with those found by salt fractionation of the milk proteins. The denaturation rates decreased when a specific reagent for sulphydryl groups was added, suggesting that such groups are involved in the denaturation of both proteins.

Knowledge of the effect of heat treatment on the whey proteins of milk is of importance in understanding the changes in the properties of milk that occur on such treatment. Of the 2 major whey proteins, β-lactoglobulin and α-lactalbumin, the former is of particular interest, since it is known that its −SH groups can, on heating, give rise to changes in flavour (Hutton & Patton, 1952) and cause alterations in the properties of κ-casein (Zittle, Thompson, Custer & Cerbulis, 1962). Larson & Rolleri (1955) determined the degree of denaturation of the individual whey proteins in milk samples heated for 15 min at a series of temperatures, but kinetic data on their rates of denaturation are lacking. Accordingly, we have measured their rates of denaturation over a wide range of temperatures, a task facilitated by use of the immunological methods of Larson & Twarog (1961) and Larson & Hageman (1963) which are particularly suitable for this type of work.

EXPERIMENTAL

Materials

β-Lactoglobulin and α-lactalbumin were prepared by the method of Aschaffenburg & Drewry (1957).

Anti-sera were obtained commercially from Antibodies Inc. (California) or prepared from rabbits given a course of injections of antigen similar to that recommended by Gell & Coombs (1963).

Skim-milk was prepared by our Experimental Dairy from bulk milk.

Heating

Samples of skim-milk were heated in sealed capillary tubes of length 10 cm, inside diam. 1·2 mm and outside diam. 1·7 mm. Each capillary, containing about 0·05 ml skim-milk, was heated by immersion in either a water-bath (68–100 °C) or an oil-bath (90–155 °C) maintained at the required temperature by a thermostat. When used at temperatures above 100 °C, capillaries were preheated for 1 min at 60 °C. At the end of the heating period the capillaries were cooled rapidly by immersion in iced water.

For the experiments in which proteins were separated by salting-out procedures, 250 ml of skim-milk were heated in a conical flask by immersion in a large pan of boiling water, swirling the contents rapidly until the required temperature was reached, when the flask was transferred to the constant temperature water-bath. After heating for the required time, the flask was cooled in an ice–water mixture.

Determination of residual native protein

α-Lactalbumin was determined by the immunodiffusion method of Larson & Hageman (1963), and β-lactoglobulin by the method of Larson & Twarog (1961) with some minor modifications. Application of these methods requires quantitative dilution of the heated milk samples. Accordingly, the length of the column of milk in each capillary was measured and the volume of milk calculated by multiplication with an appropriate factor obtained by determining the length filled by known weights of milk. The walls of the capillaries were scored with a diamond, and each capillary was placed in a tube with 2 ml of diluting medium; the capillary was crushed with a glass rod and the contents of the tube thoroughly mixed. For the determination of α-lactalbumin, the diluting medium was the buffered saline of Larson & Hageman (1963), but for β-lactoglobulin difficulties with the medium of Larson & Twarog (1961) led to the adoption of bovine plasma for dilution; this produced more consistent results. The diluted sample was then placed in the assay tubes over the column of agar containing antiserum, and each tube was sealed and incubated at 37 °C. The assay tubes for β-lactoglobulin were incubated in an inverted position. Without inversion, erratic results were obtained, probably caused by convective stirring of the antigen solution following density changes near the agar interface during incubation, as shown by Preer & Telfer (1957). After incubation for 16 h or more, the distance migrated by the precipitin front was measured with a low-power microscope, using a scale subdivided in 0·1 mm; the results, expressed as

distance divided by the square root of the incubation time, were used to determine the concentration of native protein by comparison with a calibration curve, as described by Larson & Twarog (1961).

Salt fractionation of the milk proteins was carried out as described by Aschaffenburg & Drewry (1959) except that, for some of the heated samples, double the normal volumes of filtrates were taken for determination of N by Kjeldahl analysis.

RESULTS

Accuracy of the immunodiffusion method

Larson & Twarog (1961) and Larson & Hageman (1963) showed that immunodiffusion methods could be used to measure the concentration of native β-lactoglobulin and α-lactalbumin in samples of heated milk, but the results obtained by Luz & Todd (1964), using several immunological methods, suggested that heat

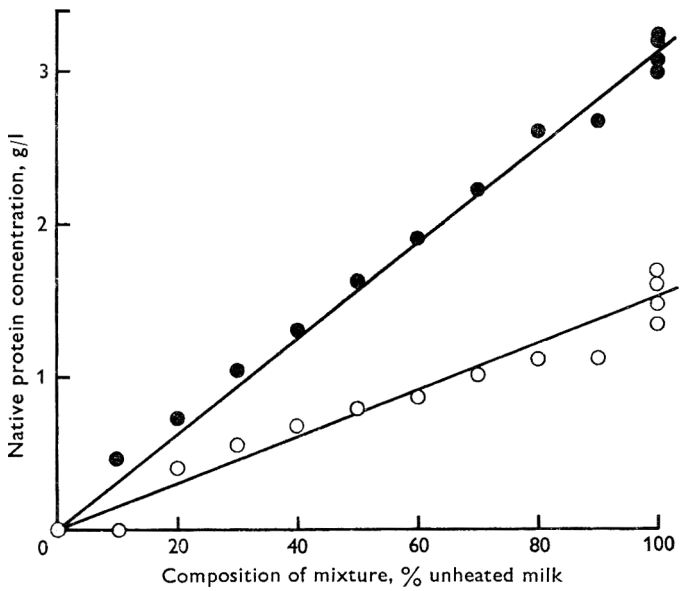

Fig. 1. Test of the method of measuring native protein concentration. The method was applied to mixtures of unheated milk and milk heated at 100 °C for 30 min. ○—○, α-Lactalbumin; ●—●, β-lactoglobulin.

denatured protein may still react with antiserum to the native protein. This would invalidate the immunodiffusion methods, whose use in these experiments depends on the ability of the antisera to discriminate between native and heat-denatured protein. The immunodiffusion method was therefore applied to mixtures of unheated milk and milk heated at 100 °C for 30 min; this heat-treatment should leave less than 1 % of the original whey protein undenatured. The results shown in Fig. 1 indicate that the antisera used were specific for native protein and were not affected by the presence of denatured protein, except possibly at low concentrations of unheated milk. Accordingly, the kinetic experiments to be described were restricted to the range where the results are most precise, namely 30–100 % native protein.

However, the absolute accuracy of the immunodiffusion method is uncertain since it consistently gave higher values than did the salt fractionation method when both methods were used on the same samples. For a series of 12 samples of unheated milk, it was found that β-lactoglobulin by immunodiffusion = $1{\cdot}09 \times \beta$-lactoglobulin by salt fractionation, with a standard error of $\pm 0{\cdot}03$. The kinetic constants for β-lactoglobulin given in this paper may be in error to the same extent, but the constants for α-lactalbumin would be unaffected even if there is a similar error in the immunodiffusion method for this protein.

Kinetics of denaturation

To determine the rate of denaturation of α-lactalbumin, 10–12 capillaries containing milk were heated at the required temperature for various periods and the residual native α-lactalbumin determined as described above. Fig. 2 shows the results

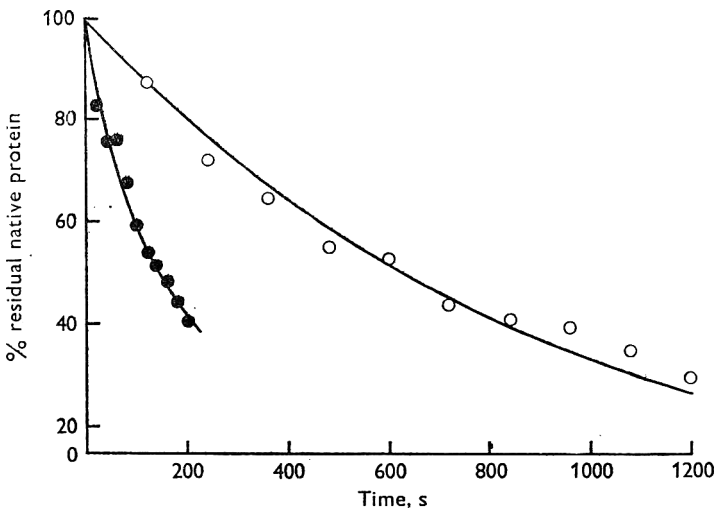

Fig. 2. Denaturation of α-lactalbumin and β-lactoglobulin in skim-milk at 85 °C. ○—○, α-lactalbumin; curve calculated from equation (2) in the text, with $k_1 = 1{\cdot}09 \times 10^{-3}\ s^{-1}$. ●—●, β-lactoglobulin; curve calculated from equation (4) in the text, with $k_2 = 2{\cdot}53 \times 10^{-3}\ l\ g^{-1}\ s^{-1}$, and $c_0 = 2{\cdot}72$ g/l.

obtained for one batch of skim-milk at 85 °C; at other temperatures, the results were similar except for changes in the time scale. The experimental points do not deviate significantly from a curve derived from the equation for first order kinetics,

$$-dc/dt = k_1 c, \tag{1}$$

where c is the concentration of native α-lactalbumin at time t and k_1 the reaction constant. The value of k_1 in s^{-1} was determined from the integrated form of this equation,

$$\log_{10}(c/c_0) = -k_1 t/2{\cdot}303, \tag{2}$$

where c_0 is the initial concentration, by plotting $\log c/c_0$ against time and measuring the slope of the straight line drawn through the experimental points; this is shown in Fig. 3 for the results given in Fig. 2.

Fig. 2 also shows a set of experimental points for the denaturation of β-lacto-

globulin at 85 °C. Curves derived from equations for first order kinetics are not capable of describing either these results or those at any other temperature. However, it was found that the experimental results at any temperature could be accurately described by the equation for a second-order reaction,

$$-dc/dt = k_2 c^2, \tag{3}$$

which becomes, on integration and rearrangement,

$$c_0/c = 1 + c_0 k_2 t. \tag{4}$$

The value of k_2 in $lg^{-1}\ s^{-1}$ was determined by plotting c_0/c against time, as in Fig. 4, and by measuring the slope of the straight line drawn through the experimental points. From equation (4) the curve for denaturation of β-lactoglobulin can be calculated; it can be seen in Fig. 2 that the experimental points do not deviate significantly from such a curve.

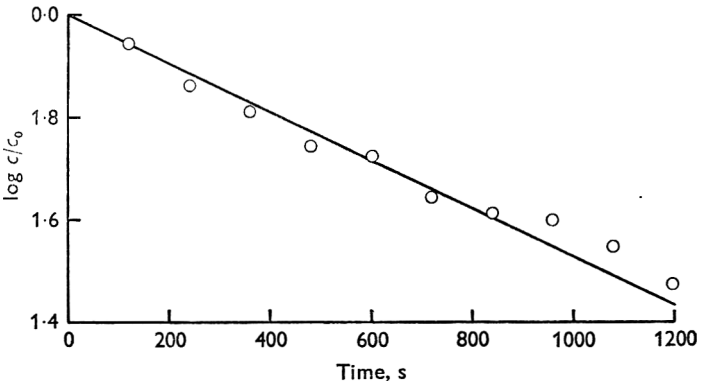

Fig. 3. First-order reaction plot for the denaturation of α-lactalbumin in skim-milk at 85 °C. The kinetic constant is found from the slope of the line.

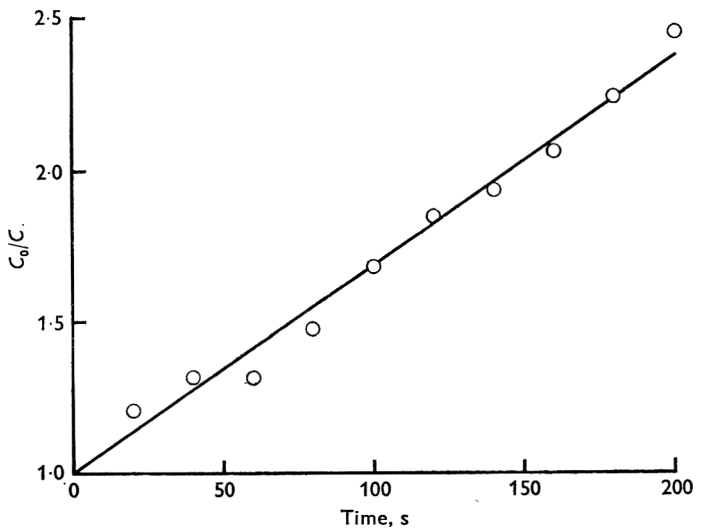

Fig. 4. Second-order reaction plot for the denaturation of β-lactoglobulin in skim-milk at 85 °C. The kinetic constant is found from the slope of the line.

Temperature dependence of the kinetic constants

Values of the kinetic constants for the denaturation of α-lactalbumin and β-lactoglobulin over a wide range of temperatures were found by the methods described above. Satisfactory results were obtained for reactions with half-times (i.e. the time for denaturation of half the original protein) ranging from 4 s to about 6 h. These results are shown in Fig. 5, where log k_1 for α-lactalbumin and log k_2 for β-lactoglobulin have been plotted against the reciprocal of the temperature in °K. Such Arrhenius plots for kinetic constants should give straight lines unless the reaction is complex. It is evident that for both these milk proteins a change in

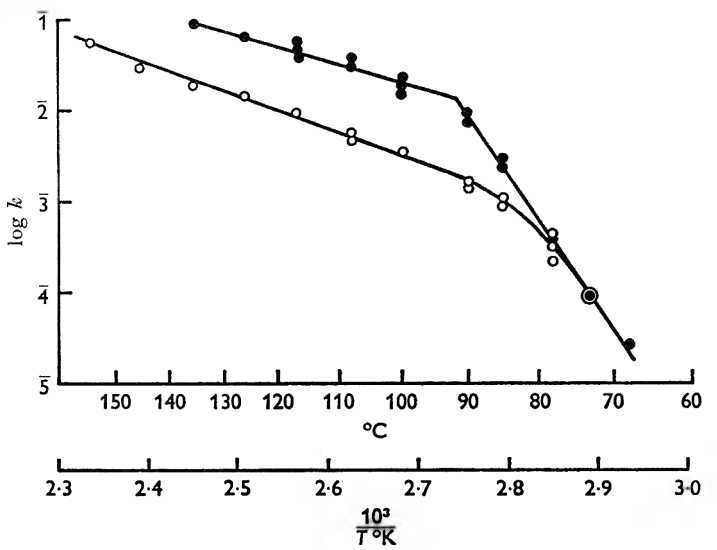

Fig. 5. Arrhenius plot of the kinetic constants. ○—○, α-Lactalbumin; ●—●, β-lactoglobulin. Straight lines calculated from equations (5), (6) and (7).

temperature dependence occurred at about 90 °C. Accordingly, linear equations valid for only limited temperature ranges have been fitted to the results by the usual least squares procedures, giving

$$\log k_2 = 37{\cdot}945 - 14{\cdot}514\ (10^3/T) \quad \pm 0{\cdot}063, \tag{5}$$

for β-lactoglobulin between 68 and 90 °C,

$$\log k_2 = 5{\cdot}980 - 2{\cdot}862\ (10^3/T) \quad \pm 0{\cdot}052, \tag{6}$$

for β-lactoglobulin between 90 and 135 °C, and

$$\log k_1 = 7{\cdot}150 - 3{\cdot}600\ (10^3/T) \quad \pm 0{\cdot}033, \tag{7}$$

for α-lactalbumin between 90 and 155 °C.

In these equations, T is in °K and the errors given are standard errors. The equations were used to calculate the straight lines drawn through the experimental points in Fig. 5; for α-lactalbumin at temperatures below 90 °C a curve has been drawn from which an estimate may be made of the appropriate value of log k.

Comparison with other methods

As a check on the equations and methods used, both the immunodiffusion method of analysis and the salt fractionation method of Aschaffenburg & Drewry (1959) were applied to samples of skim-milk heated for various periods at 78 °C. For this experiment the milk was not heated in capillaries but in 250-ml portions, as described earlier; the temperature of 78 °C was the highest possible that would give heating times substantially longer than the time taken to heat such a large volume of milk to the chosen temperature. The results are shown in Fig. 6 and in general there is good agreement between the rates of denaturation measured by the 2 analytical methods and those calculated from equations (4) and (5) for β-lactoglobulin and from

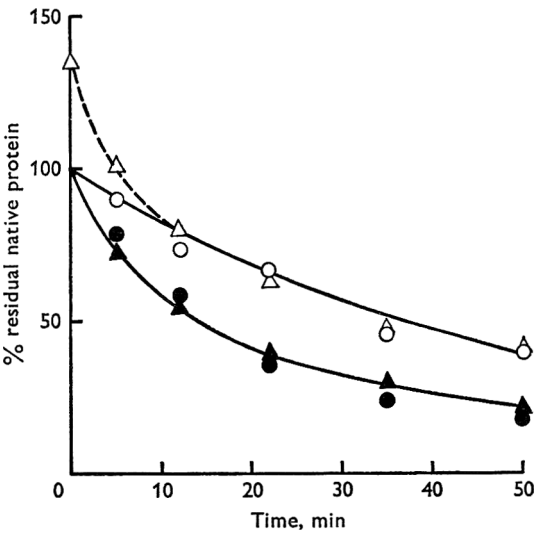

Fig. 6. Denaturation of β-lactoglobulin and α-lactalbumin in skim-milk at 78 °C: comparison of results for 2 analytical methods. ○—○, α-Lactalbumin by immunodiffusion: solid line calculated from equation (2) with $k_1 = 0{\cdot}311 \times 10^{-3}\ s^{-1}$; △—△, total albumin by salt fractionation calculated as % of α-lactalbumin in unheated milk; ●—●, β-lactoglobulin by immunodiffusion: curve calculated from equation (4) with $c_0k_2 = 1{\cdot}18 \times 10^{-3}\ s^{-1}$; ▲—▲, β-lactoglobulin by salt fractionation.

equation (2) for α-lactalbumin with a value for $k = 0{\cdot}310 \times 10^{-3}\ s^{-1}$ read from Fig. 5. One difficulty of the salt fractionation method is that it does not provide a specific measure of α-lactalbumin but only of residual albumin, i.e. total albumin less β-lactoglobulin, a value which includes serum albumin. Accordingly, the residual albumin has been calculated as a percentage of the α-lactalbumin found by immunodiffusion in the unheated milk. It is clear that the high values obtained by salt fractionation after short heating times reflect the presence of this serum albumin; for the longer heating times, agreement is better because of the absence of native serum albumin from these solutions. In one respect, however, agreement between the 2 methods was relatively poor: for the unheated milk, the immunodiffusion method gave 3·14 g/l β-lactoglobulin while the salt fractionation method gave 2·62 g/l. This difference also appeared, but to a lesser extent, in the values for the heated samples; good agreement was found only when, as in Fig. 6, the results from

the 2 methods were each expressed as a percentage of the appropriate value of the concentration of β-lactoglobulin in the unheated milk.

The validity of the equations derived from the immunodiffusion method can also be tested by applying them to the data of Larson & Rolleri (1955). These authors heated milk for 30 min at 8 different temperatures and used the electrophoretic diagrams of the whey proteins to estimate the concentration of each component after heating. Their results for α-lactalbumin and β-lactoglobulin, calculated as % native protein, are shown in Table 1, with the corresponding values calculated from the equations given above. In general, agreement is satisfactory.

Table 1. *Denaturation of β-lactoglobulin and α-lactalbumin in milk*

(Comparison of the residual native protein found in milk heated for 30 min at various temperatures by Larson & Rolleri (1955) with that calculated from equations in the text for the same heat treatment.)

	% Residual native protein			
	β-Lactoglobulin		α-Lactalbumin	
Temperature, °C	Found	Calculated	Found	Calculated
62	92	98	—	—
66	89	93	—	—
70	70	81	—	—
74	47	59	75	80
77	32	38	51	64
82	7	14	32	32
96	0	1	0	1

Factors affecting the rate of denaturation

Genetic variation of β-lactoglobulin

Gough & Jenness (1962) found that, of the 2 genetic variants described by Aschaffenburg & Drewry (1955), β-lactoglobulin B was denatured by heat more rapidly than β-lactoglobulin A, using samples of milk from cows homozygous for these variants. This difference in heat stability also appeared in experiments with the isolated proteins in buffer solutions, in which denaturation of both proteins followed first-order kinetics. Since mixed herd milk normally contains both variants, it appeared possible that 2 simultaneous first-order reactions might be a better description of β-lactoglobulin denaturation in milk than equation (4). To test this possibility, the rates of denaturation of α-lactalbumin and β-lactoglobulin at 100 °C were measured in skim-milk prepared from the milk of 3 individual cows producing β-lactoglobulin of the 3 common genetic types: A, B and AB. The results showed that the denaturation of all 3 genetic types followed second-order kinetics, with the constant k_2 for type AB having a value close to that found for mixed-herd milk; for types A and B, the constants were, respectively, about 50 % lower and higher.

However, in practice it seems likely that genetic variation may be safely ignored, since any bulk milk is likely to contain approximately equal amounts of the 2 genetic variants, as did the milk from the Institute herd used in the present work. It is known that the genes for both variants are almost equally common in Western breeds of cattle (Aschaffenburg, 1968).

Concentration of β-lactoglobulin

If the denaturation of β-lactoglobulin followed the kinetic equations for a normal second-order reaction, the time taken to reach 50 % denaturation should vary inversely with the initial concentration; for example, milk containing twice the normal concentration of β-lactoglobulin should reach 50 % denaturation in half the time taken by normal milk. To test this, crystalline β-lactoglobulin was dissolved in skim-milk and the rate of denaturation at 100 °C was compared with that of the β-lactoglobulin in the same milk without added protein. Addition of sufficient β-lactoglobulin A to double the concentration of β-lactoglobulin in the skim-milk left the half-time of the reaction unchanged; addition of a similar amount of β-lactoglobulin B to skim-milk reduced the half-time by less than 30 %. These results indicate that, with respect to concentration, the denaturation of β-lactoglobulin follows first-order kinetics, but that the reaction is second order with respect to time. Reactions of such hybrid character are unusual, but Haurowitz, DiMoia & Tekman (1952) found a similar effect in the heat denaturation of ovalbumin. Accordingly, equation (4) should not be expected to predict the results of experiments in which c_0 differs significantly from $3{\cdot}0 \pm 0{\cdot}5$ g/l.

The rate of denaturation of α-lactalbumin was found to be slightly faster than usual in skim-milk with added β-lactoglobulin, but the effect was not large. This is in agreement with some early results of Jenness (1958).

Addition of p-chloromercuribenzoate

When β-lactoglobulin is denatured by heat, its sulphydryl (−SH) groups become highly active and can be expected to react with disulphide bonds; in milk, such bonds are known to be present in α-lactalbumin and β-lactoglobulin, with smaller amounts in serum albumin, immunoglobulins and κ-casein. Preliminary reports (Jenness, 1958, and Chaudry & Humbert, 1968) indicated that this reaction was of importance in the denaturation of α-lactalbumin and β-lactoglobulin. To study this possibility, *p*-chloromercuribenzoate (PCMB), a specific reagent for −SH groups, was added to skim-milk before heating. The milk was made 0·28 mM in PCMB, giving a slight excess over the normal values for the total −SH content of skim-milk (Lyster, 1964*a*). The determination of native β-lactoglobulin by the immunodiffusion method is not affected by the reaction between PCMB and the −SH groups of β-lactoglobulin (Lyster, 1964*b*).

The addition of PCMB to skim-milk reduced the rate of denaturation of α-lactalbumin at all temperatures by factors ranging from about 25-fold at 85 °C to about 3-fold at 155 °C. The rate of denaturation of β-lactoglobulin was unaffected by PCMB at temperatures below 78 °C, but at higher temperatures it was reduced by amounts up to 100-fold. These large effects suggest strongly that, except for β-lactoglobulin at temperatures below 78 °C, reactions of the −SH group of β-lactoglobulin play an important role in the denaturation of both proteins. The products of the reactions are presumably aggregates of denatured α-lactalbumin and β-lactoglobulin formed by disulphide interchange; the aggregates may include other proteins containing disulphide bonds.

Whole milk

The rate of denaturation of β-lactoglobulin and α-lactalbumin in whole milk was measured at 2 temperatures, 78 and 117 °C, and compared with that of the corresponding skim-milk. No difference was observed with α-lactalbumin or with β-lactoglobulin heated at 117 °C. However, at 78 °C β-lactoglobulin was denatured slightly faster in whole milk than in skim-milk, k_2 being 18 % greater in whole milk. Clearly, the presence of fat has only a small effect which in magnitude is close to the limits of experimental error.

Changes in pH

To test the effect of pH changes, the pH of a sample of skim-milk was adjusted by adding small amounts of dilute NaOH or HCl before heating. For both proteins, the rate of denaturation at either 78 or 100 °C was independent of pH, within the range 6·2–6·9; outside this pH range the rates of denaturation increased.

Consecutive heat treatments

The results described above imply complicated mechanisms for the denaturation of both proteins, probably involving 2 or more successive steps. For such mechanisms, the effects of 2 consecutive heat treatments at different temperatures might not be simply additive. To test this possibility, samples of skim-milk were heated at one temperature long enough for about 60 % of the α-lactalbumin or β-lactoglobulin to be denatured, and then transferred to a bath at another temperature to determine the rate of denaturation at this second temperature. The 2 temperatures chosen were 78 and 117 °C, and it was found that preheating at either temperature had no significant effect on the subsequent rate of denaturation at the other temperature for either α-lactalbumin or β-lactoglobulin.

This result has important practical consequences, since it suggests that the equations given earlier will predict correctly the effect of heat treatments in which much of the denaturation occurs either in a preliminary holding tank or while the milk temperature is changing inside the plant; this happens, for example, in many UHT plants now in commercial use in practice.

DISCUSSION

Sensitivity and specificity are characteristic of immunochemical methods, and these properties were of particular value in determining the residual native protein in milk after heating. Because of the specificity of the method used, it was unnecessary to fractionate the samples; so that, although the method may be less accurate than the classical salt fractionation procedure, it is much more suitable for handling the rather large number of samples involved in kinetic measurements. Because of the sensitivity of the method, it was possible to heat very small volumes of milk; this allowed the experiments to cover a wide temperature range.

The results given above are generally compatible with the reaction mechanisms proposed by various workers for the heat denaturation of β-lactoglobulin on the basis of experiments with buffered solutions of the pure protein. In particular, the

effect of adding PCMB to the milk before heating is in agreement with the proposal of Pantaloni (1964) and Sawyer (1968) that there is an aggregation step in which –SH groups catalyse the formation of intermolecular disulphide bonds. However, milk is a complex medium, and at this stage a more detailed comparison would not appear to be justified.

The results presented above will, it is hoped, be of practical value in predicting the quantitative effect of heat treatment of milk on the 2 whey proteins from the time and temperature of heating; for example, the amount of denaturation to be expected on passing milk through a UHT plant of known time–temperature profile may be calculated. However, the form of the temperature-dependence of denaturation also suggests some conclusions of a more qualitative nature. Clearly, extrapolation to high temperatures from experimental results obtained at temperatures below 90 °C will lead to serious errors. In commercial heat treatments it is often important to know the effect of heat on the relative rates of 2 or more reactions; for example, it is desirable to find conditions for UHT treatment of milk in which bacteria and their spores are killed relatively quickly and flavour changes occur relatively slowly (Burton, 1969). Hutton & Patton (1952) suggested that protein denaturation preceded flavour changes in heated milk; if this is so, high temperatures will be advantageous provided the activation energy for the destruction of bacteria remains higher than that for protein denaturation.

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The interaction of bovine milk caseins with the detergent sodium dodecyl sulphate

I. The relationship between the composition and the size of the protein–detergent aggregate

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Summary. Study of the dissociation of high-molecular-weight aggregates of preparations of α_{s1}-, β-, κ-, and para-κ-casein by the detergent, sodium dodecyl sulphate (SDS), showed that there are differences in the aggregation properties of the individual caseins. Binding of detergent led first to the dissociation of casein aggregates and then to further interaction with the casein molecules. The amounts of detergent required to give the minimum sized protein–detergent aggregate when expressed as mg/mg casein were similar for κ-, para-κ- and α_{s1}-casein but much less for β-casein. However, expressed as mole/mole the requirement for κ- and α_{s1}-casein was similar but was twice that found for para-κ- and β-casein. The maximum amount of SDS bound was about twice that required for complete dissociation of the aggregates for κ-, para-κ- and α_{s1}-casein but was 13 times greater for β-casein.

Complete dissociation of κ-casein aggregates by SDS alone was not possible due to the presence of aggregates formed by disulphide linkages. These aggregates, which consisted of 3 ± 1 protein molecules, accounted for about one-third of the κ-casein in the preparations examined.

The colloidal properties of milk largely result from the presence of micelles which are formed from the interaction of aggregates of milk caseins with the ions and salts of calcium and phosphate. The chemistry of micelle formation is still largely unknown. Nevertheless, certain aspects, especially those involving casein characterization, have been studied in some detail. The property of a specific casein component, κ-casein, of conferring stabilizing properties on the casein complex was first reported by Waugh & von Hippel (1956). Since that time much work has been reported on the isolation and characterization of the casein proteins (see reviews by McKenzie, 1967; Hill & Wake, 1969). However, little is known about the mechanism of aggregation of the caseins. The work of Payens (1966) on the physico-chemical properties of the self-association systems of the caseins indicates that hydrophobic binding is probably important in aggregation and Ho & Chen (1967) have presented evidence for electrostatic binding between the α_{s1}-caseins. It is likely that several types of bonding are involved in casein aggregation and that the relative contribution depends upon the composition and configuration of the proteins.

Hill & Wake (1969) suggest that κ-casein has amphiphile properties and may act in a similar manner to a detergent. This idea is supported by the work of Noelken (1966), who showed that a detergent could confer stability on casein aggregates in a manner analogous to that of κ-casein.

The work reported in the present paper is an extension of the investigation on the behaviour of casein–detergent complexes previously reported (Cheeseman, 1968). Although little is known about the exact mode of action of detergents with a protein, they are thought to react with the hydrophobic regions of the protein (Reynolds, Herbert, Polet & Steinhardt, 1967) and a study of the reaction of a detergent with the caseins may give some indication of the hydrophobic properties of the individual caseins. The suggestion that κ-casein may behave in a manner analogous to a detergent means that such a study may also give some indication as to the mechanism of interaction of κ-casein with the other caseins.

MATERIALS AND METHODS

Preparation of κ-casein

κ-Casein was prepared for use in these experiments by the method of Zittle & Custer (1963) and lyophilized. Because para-κ-casein formed during the preparation and storage of κ-casein, the preparation was freshly purified before the start of each experiment. This was carried out by chromatography on a 2 × 20 cm column of DEAE cellulose (Whatman DE 52) equilibrated with 0·01 M-tris-HCl buffer of pH 7·0 containing 3·3 M-urea and 0·01 M-dithiothreitol (DTT). A solution of 0·5 g κ-casein in about 2 ml of buffer was loaded on to the top of the column; the buffer was passed through the column at a flow rate of 17 ml/h, and 10-ml fractions were collected. The para-κ-casein appeared in the column void volume and the κ-casein which was retained on the cellulose was eluted with buffer containing 0·3 M-NaCl. The contents of the tubes containing κ-casein were bulked, the solution dialysed to remove urea and κ-casein recovered by precipitation at pH 4·6 by the addition of dilute HCl.

A few preparations of κ-casein were purified by gel filtration in the presence of sodium dodecyl sulphate (SDS) by the method of Cheeseman (1968). Occasionally κ-casein prepared by this technique gave lower $s_{20,w}$ values than expected because of residual-bound SDS. To remove all traces of SDS the κ-casein–SDS complex which was soluble at pH 4·6 was precipitated from solution at this pH by addition of 10 vols acetone at 0 °C. The procedure of precipitation with acetone was repeated until the supernatant liquid was shown to be free of SDS using the rosaniline HCl reagent of Karush & Sonenberg (1950). Stock solutions of κ-casein were prepared by dissolving the wet precipitate in the minimum volume of 0·05 M-tris-HCl buffer of pH 7·6 containing 6 M-urea and dialysing the solution for 24 h at 4 °C against 0·05 M-tris-HCl buffer of pH 7·6 containing 0·1 M-NaCl and 1×10^{-4} M-EDTA. The concentration of κ-casein in the stock solutions was about 3 %, w/v, as determined from the absorbance at 280 nm ($E^{1\%}_{1\,cm}$ = 10·5; Garnier, Mocquot & Brignon, 1962). Sedimentation velocity analysis of 0·4 %, w/v, solutions of various preparations gave values for $s_{20,w}$ of 14–18S. These slight differences in aggregate size probably reflected minor variations in the method of preparation.

Preparation of para-κ-casein

A solution of κ-casein (1·4 %, w/v) at pH 6·0 was treated with rennin added at 0·004 %, w/v. The rennin solution was prepared from twice-crystallized rennin made in this laboratory. After 30-min reaction at 30 °C the precipitate of para-κ-casein which formed was centrifuged, the supernatant liquid removed and the sediment dispersed in 0·05 M-tris-HCl buffer of pH 9·0 for 20 min in order to destroy rennin activity. After the alkaline treatment the precipitate was recovered by centrifugation and washed in acetate buffer of pH 6·0 containing 0·1 M-NaCl and 1×10^{-4} M-EDTA, and finally dissolved in the same buffer containing the minimum quantity of SDS required to solubilize the precipitate. The solution, which contained about 0·8 %, w/v, para-κ-casein, was dialysed against the buffer at 4 °C until it became very slightly turbid; at this stage most of the aggregates had $s_{20,w}$ values of about 76S. Preliminary experiments in which κ-casein was treated with rennin in presence of SDS had shown that similar conditions were obtained at a protein:detergent ratio of 1:3·3. Addition of SDS to give a calculated ratio of 1:6·6 produced a clear solution containing aggregates with $s_{20,w}$ values of about 40S. This stock solution was used for subsequent experiments.

Preparation of α_{s1}-casein

The α-complex was obtained from bulked herd raw milk by the urea fractionation method of Hipp, Groves, Custer & McMeekin (1952). An initial fractionation by ethanol precipitation (Zittle & Custer, 1963) was followed by fractionation on a 3 × 30 cm column of DEAE cellulose (Whatman DE 52) equilibrated with 0·01 M-tris-HCl buffer of pH 7·0 containing 3·3 M-urea. A solution of 0·5 g α-casein in about 2 ml buffer was loaded on to the top of the column; buffer was passed through the column at a flow rate of 25 ml/h and 10 ml fractions were collected. Increasing concentrations of NaCl (0·1, 0·175 and 0·225 M) were added stepwise to the eluting buffer to fractionate the α_{s1}-casein. Fractions containing predominantly α_{s1}-casein were bulked and the protein recovered. The process of fractionation was repeated and the final product was shown to be free of contaminants by starch gel electrophoresis (SGE) at pH 8·6 in tris-glycine-urea buffer and to be genotype B. The α_{s1}-casein solutions obtained from the column were dialysed against distilled water to remove urea, precipitated by the addition of N-HCl to pH 4·6, the precipitate redissolved in distilled water by addition of dilute alkali and the solution lyophilized. Stock solutions of α_{s1}-casein were prepared by dissolving the lyophilized material in 0·05 M-tris-HCl buffer of pH 7·5, containing 0·1 M-NaCl and 1×10^{-4} M-EDTA. Concentration was determined from the absorbance at 280 nm ($E^{1\%}_{1\,cm} = 10{\cdot}0$; Thompson & Kiddy, 1964). Sedimentation velocity analysis of a 0·4 %, w/v, solution of α_{s1}-casein gave a single peak with an $s_{20,w}$ of 6·2S.

Preparation of β-casein

β-Casein genotype A/B was prepared from raw skim-milk from an individual cow by the method of Aschaffenburg (1963) and was freed from contaminants by chromatography on DEAE cellulose in the same manner as the α_{s1}-casein except that 0·2 M-NaCl was used instead of 0·225 M-NaCl. Stock solutions of β-casein were prepared in the same manner as described for α_{s1}-casein. Sedimentation velocity analysis

of a 0·36 %, w/v, solution of β-casein gave 2 peaks with $s_{20,w}$ values of 3 and 18·2S. Concentration was measured from the absorbance at 280 nm using $E^{1\%}_{1\,cm} = 5{\cdot}39$ determined for this preparation of β-casein.

Ultracentrifugation

Sedimentation velocity analyses and protein–SDS binding studies were made using a Beckman Model E analytical ultracentrifuge equipped with a phase plate and RTIC unit. Schlieren optics were used and runs were carried out at 22·5 °C in the An–D rotor at 59780 rev/min with cells having 4° sector, 12 mm aluminium centrepieces and standard and wedge quartz windows. Sedimentation coefficients were determined from the slope of a plot of the natural logarithm of the maximum ordinate against time and corrected to $s_{20,w}$. No corrections were made for radial dilution in the ultracentrifuge cell or for the influence of detergent on solvent viscosity and density. It has been suggested by Edelhoch & Lippoldt (1960) that the effect of the detergent on density is not significant since most of the detergent is bound to the protein. The concentration of protein and protein–detergent complex was determined from the areas under the schlieren peaks and the initial concentration of solutions (c_0) was determined using a synthetic boundary cell.

Chemicals

Dithiothreitol (DTT), AR grade, was obtained from Calbiochem, Los Angeles, U.S.A. Sodium dodecyl sulphate (SDS), specially pure grade, rosaniline HCl and all other chemicals (AR grade) were obtained from BDH Ltd, Poole, England. Glass-distilled water was used in the preparation of all the solutions.

Preparation of protein–SDS complexes

Stock solutions of SDS were prepared containing 5 %, w/v, in 0·05 M-tris-HCl buffer of pH 7·6 and 0·1 M-NaCl. Portions, 5–160 μl, of these solutions were added to 1-ml portions of the appropriate casein solutions to give SDS concentrations of 0·25–8 mg/ml. The solutions were kept for 30 min at room temperature with occasional mixing. When DTT was used it was added after the initial period and the mixture allowed to stand for a further 30 min at room temperature before ultracentrifugation.

Binding of SDS to caseins

Solutions (1 ml) of casein containing 0·5–6 mg protein were dialysed for 48 h at room temperature against buffer containing concentrations of SDS as shown in Table 1. The amount of bound SDS was determined by measurement of the schlieren peak areas obtained from sedimentation velocity runs at 59780 rev/min. The zero time concentration was obtained by extrapolation of measured peak areas to $\frac{2}{3}$ speed. The area due to the protein as determined from a c_0 experiment was subtracted and the resultant area, attributed to bound SDS, was plotted against concentration of protein. A determination of the slope of this plot gave the amount of SDS bound/mg protein.

The effect of concentration on the sedimentation coefficient of the protein–SDS complex

The protein:SDS ratio examined (see Table 2) provided SDS in amount equal to or slightly greater than that required for maximum binding. For κ-casein a lower ratio was also examined, as was also the effect of adding DTT. Protein–SDS complexes were prepared as described above and progressively diluted with the appropriate buffer. The solution obtained at each dilution was examined by sedimentation velocity analysis at 59780 rev/min.

Table 1. *Conditions used for the study of the total binding of sodium dodecyl sulphate (SDS) to casein*

Casein	pH of buffer*	SDS concentration in buffer, mg/ml
κ	6·5, 7·6	2·0, 6·0
para-κ	6·0	2·0
α_{s1}	6·5, 7·6	2·0, 6·0
β	6·5, 7·6	2·0, 6·0

* Buffers (tris and acetate) were 0·05 M containing 0·1 M-NaCl, 1×10^{-4} M-EDTA and, in experiments with κ-casein, 1×10^{-3} M-DTT.

Table 2. *Conditions used for the study of the effect of concentration on the sedimentation coefficient*

Casein	Protein:sodium dodecyl sulphate ratio examined, w/w	Ratio required for maximum binding, w/w	pH of buffer*
κ	1:0·4	1:1·1	6·5, 7·6
	1:1·3		
α_{s1}	1:1·5	1:1·2	6·5
β	1:2·9	1:3·4	6·5

* Buffers (tris and acetate) were 0·05 M containing 0·1 M-NaCl, 1×10^{-4} M-EDTA.

RESULTS

The effect of SDS on the sedimentation rates of individual caseins

κ-Casein

The effect of detergent concentration on the sedimentation coefficient of κ-casein is shown in Fig. 1. Increase in concentration of SDS gave lower $s_{20,w}$ values. The faster moving component was designated 11S and the slower component 3S, these being the average uncorrected $s_{20,w}$ values obtained at the higher SDS concentrations. Calculations for the molecular composition of κ-casein–SDS aggregates were based on mol. wts of 19000 (Woychik, Kalan & Noelken, 1966) and 288. At a molar ratio of 1:40 a third component with a sedimentation coefficient of about 7S was obtained. The proportion of this component increased as the amount of SDS increased, and this increase was paralleled by a decrease in the amount of 11S component. The effect of SDS concentration upon the proportion of the slow component is shown

in Fig. 2. At low SDS concentrations the 11S component was the major constituent. Approximately equal amounts of the 3S and 11S components were obtained at a 1:20 molar ratio of κ-casein:SDS. At molar ratios greater than 1:30 a constant ratio of about 2:1 was obtained.

The effect of adding DTT to solutions containing SDS and κ-casein is shown in Figs 1 and 2. In the presence of DTT the faster moving component reached a minimum $s_{20,W}$ value of 8S compared with the mixture of 11S and 7S obtained in the absence of DTT. Similarly, in the presence of DTT almost complete dissociation into the 3S component was obtained at 1:40 molar ratio whereas at the same ratio and in the absence of DTT only about two-thirds of the aggregate was dissociated (Fig. 2).

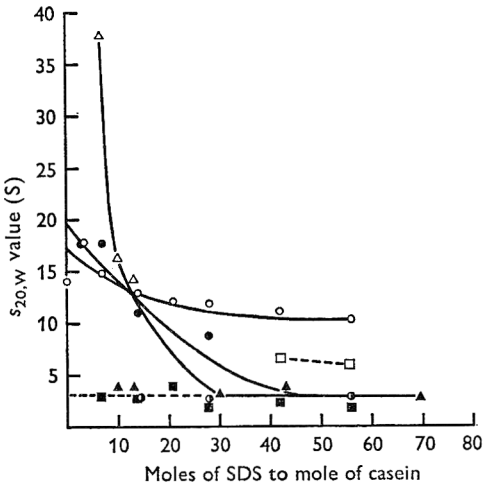

Fig. 1

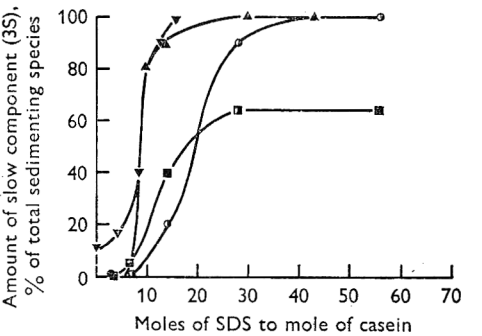

Fig. 2

Fig. 1. Relationship of $s_{20,W}$ value of κ- and para-κ-casein aggregates to concentration of sodium dodeeyl sulphate (SDS). In the absence of SDS κ-casein gave only one aggregate and para-κ-casein was not soluble. The addition of SDS brought about the formation of 2 or more aggregates. The size of the largest aggregate decreased with the addition of SDS and there was a concomitant increase in the proportion of the smallest aggregate (3S) (see Fig. 2). The fast sedimenting species in each mixture is shown as: κ-casein, ○; κ-casein + DTT, ●; para-κ-casein, △. The slow species (3S) is shown as: κ-casein, ■; κ-casein + DTT, ◑; para-κ-casein, ▲. Symbol, □, indicates a κ-casein aggregate of intermediate size (7S) which occurred at higher concentrations of SDS. Initial protein concentrations used in the experiments were: κ-casein, 0·4 %, w/v; para-κ-casein, 0·8 %, w/v.

Fig. 2. Relationship of the amount of the slow sedimenting species (3S), as a percent of the total sedimenting species, to concentration of sodium dodecyl sulphate. The solutions examined were: κ-casein, 0·4 %, w/v, ■; κ-casein, 0·4 %, w/v + DTT, ◑; para-κ-casein, 0·8 %, w/v, ▲; β-casein 0·36 %, w/v, ▼.

Para-κ-casein

The plot of sedimentation coefficient of para-κ-casein against increasing concentrations of detergent is shown in Fig. 1. Calculation of the ratio of para-κ-casein to SDS is based upon a mol. wt of 10700 for para-κ-casein. In the complete absence of SDS para-κ-casein was insoluble. At very low concentrations of SDS large aggregates were formed. At a molar ratio of 1:7 a single peak of about 40S was obtained. As the concentration of SDS was increased to between 10 and 15 molar excess, 2 schlieren

boundaries were obtained. At excess concentrations greater than 20 moles of SDS most of the complex formed a single peak with a sedimentation coefficient of 3S. The approximate percentage composition of the components with increasing SDS concentration is shown in Fig. 2. The addition of DTT to solutions of para-κ-casein and SDS had no effect upon the sedimentation coefficient of the complex or upon the relative proportions of the fast and slow components.

α_{s1}-Casein

The effect of detergent concentration on the sedimentation coefficient of α_{s1}-casein is shown in Fig. 3. Increase in concentration of SDS gave lower $s_{20,w}$ values. The initial value in the absence of detergent was 6·25S. With increasing concentrations of SDS this value fell to a minimum of 2·6S at a molar ratio of 1:50 and then remained constant. Calculations for the molecular composition of α_{s1}-casein–SDS aggregates were based on the mol. wt of 24600 for α_{s1}-casein (Noelken, 1966).

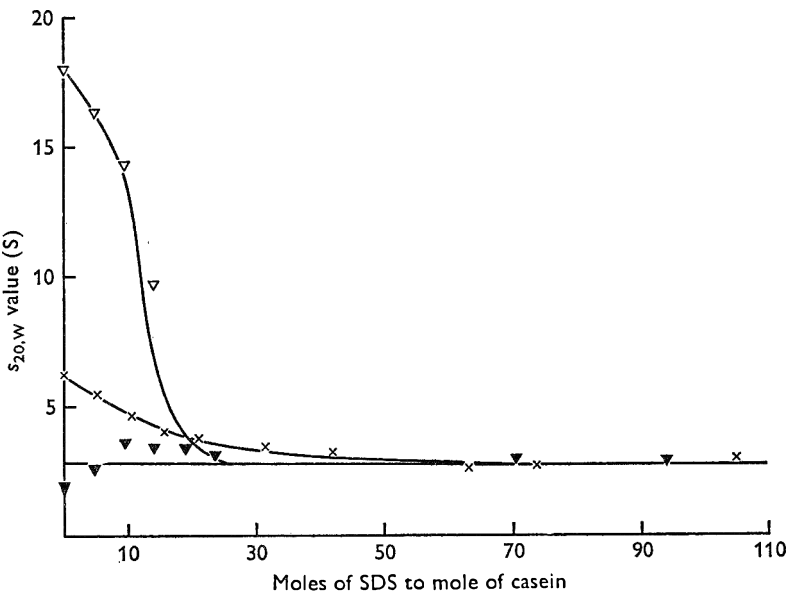

Fig. 3. Relationship of the $s_{20,w}$ values of α_{s1}- and β-casein aggregates to concentration of sodium dodecyl sulphate (SDS). α_{s1}-Casein forms only 1 aggregate in the presence and absence of SDS while β-casein forms 2 aggregates. Casein solutions examined were: α_{s1}-casein, 0·4 %, w/v, × ; β-casein, 0·36 %, w/v, fast sedimenting aggregate, ▽, slow sedimenting aggregate, ▼.

β-Casein

The effect of detergent concentration on the sedimentation coefficient of β-casein is shown in Fig. 3. With increase in concentration of SDS the $s_{20,w}$ values fell to reach a minimum of 3S at a molar ratio of 1:18. In the absence of SDS, 2 sedimenting boundaries were also formed. The relative proportions of these 2 boundaries depended upon the temperature at which the run was carried out. At 22·5 °C the faster component was predominant. The approximate composition of the components with increase in SDS concentration when examined at 22·5 °C is shown in Fig. 2.

Calculations for the molecular composition of β-casein–SDS aggregates were based on the mol. wt of 24100 for β-casein (Pion, Garnier, Ribadeau Dumas, de Koning & van Rooyen, 1965).

The effect of concentration on the sedimentation coefficient of the protein–SDS complexes

κ-Casein

Two molar ratios of κ-casein to SDS were examined, 1:28 (1:0·4, w/w), which gave 2 components of 11S and 3S, and 1:85 (1:1·3, w/w) which gave 11S, 7S and 3S components (cf. Fig. 1). The $s_{20,w}$ values obtained at various concentrations of κ-casein have been plotted in Figs 4 and 5. The faster-moving component, which comprised about 35% of the total area under the schlieren peaks, was found to be very

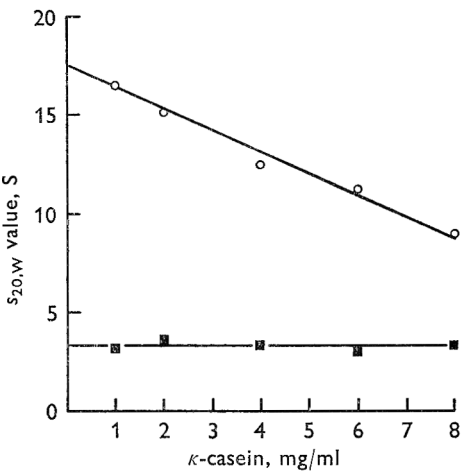

Fig. 4

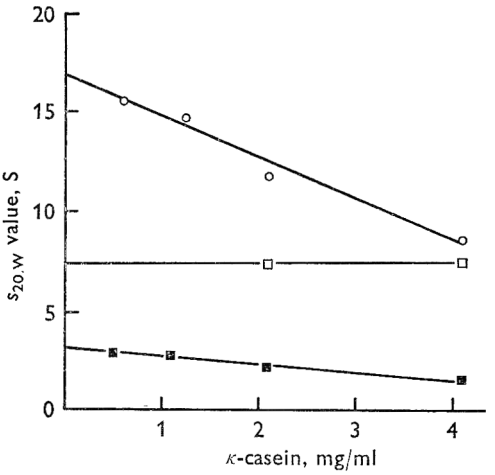

Fig. 5

Fig. 4. Effect of κ-casein concentration on the $s_{20,w}$ value of the aggregates using a molar ratio of κ-casein to sodium dodecyl sulphate of 1:28 (1:0·4, w/w). Two sedimenting species were obtained at each concentration examined: the fast sedimenting species is shown as ○; the slow sedimenting species, ■. Extrapolation to zero concentration gave $s^0_{20,w}$ values of 17·5S and 3S, respectively.

Fig. 5. Effect of κ-casein concentration on the $s_{20,w}$ value of the aggregates using the molar ratio of κ-casein to sodium dodecyl sulphate of 1:85 (1:1·3, w/w). At κ-casein concentrations of 2 mg/ml and higher, 3 sedimenting species were obtained. The fast species is shown as ○; the intermediate, □; and the slow, ■. Extrapolation to zero concentration gave $s^0_{20,w}$ values of 17·5S, 6S and 3S respectively.

dependent on concentration. The slower moving components were less so. The effect of concentration was greater with the higher SDS ratio. Extrapolation to zero concentration gave $s^0_{20,w}$ values of 17·5, 6·5 and 3S for the data obtained at the higher SDS concentration.

α_{s1}-Casein

A molar ratio of protein to SDS of 1:128 (1:1·5, w/w) was used. The $s_{20,w}$ values obtained at various α_{s1}-casein concentrations are plotted in Fig. 6. There was slight decrease in the apparent S value as the concentration increased. Extrapolation to zero concentration gave an $s^0_{20,w}$ value of 3S.

β-Casein

A molar ratio of β-casein to SDS of 1:245 (1:2·9, w/w) was examined over the range of β-casein concentrations from 0·4 to 2·6 mg/ml. The $s_{20,w}$ values obtained at the various concentrations of β-casein are plotted in Fig. 6. The slope of the plot shows slight concentration-dependence. Variation in the $s_{20,w}$ value calculated was greater than with other caseins but extrapolation to zero concentration gave an $s^0_{20,w}$ value of 2·85S.

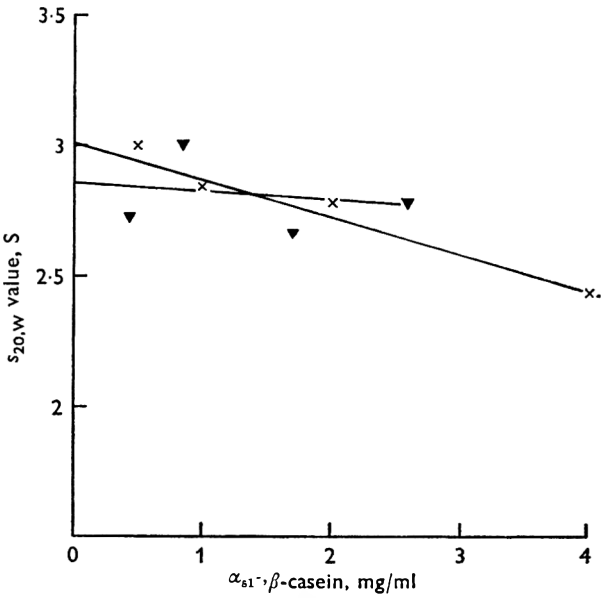

Fig. 6. Effect of casein concentration on the $s_{20,w}$ values of the aggregates using a molar ratio of α_{s1}-casein to sodium dodecyl sulphate (SDS) of 1:128 (1:1·5, w/w) and a molar ratio of β-casein to SDS of 1:245 (1:2·9, w/w). A single sedimenting species was obtained for both caseins with these amounts of detergents. α_{s1}-Casein, ×; β-casein, ▼.

Binding of SDS to caseins

κ-Casein

The maximum binding of SDS to κ-casein was determined at several concentrations of κ-casein over the range 0·5 to 4 mg/ml. The maximum binding obtained as determined from the plot shown in Fig. 7 was 73 moles of SDS to 1 mole of κ-casein (1·1:1, w/w). This was almost twice the amount required to obtain the minimum S value (Table 3).

Para-κ-casein

The maximum binding of SDS to para-κ-casein was determined at several concentrations of para-κ-casein over the range 0·5 to 4 mg/ml. The maximum binding obtained as determined from the plot shown in Fig. 7 was 32 moles of SDS to 1 mole of para-κ-casein (0·9:1, w/w). Again this was almost twice the amount required to obtain the minimum S value (Table 3).

α_{s1}-Casein

The maximum binding of SDS to α_{s1}-casein was determined at several concentrations of α_{s1}-casein over the range 0·5 to 4 mg/ml. The maximum binding as determined from the plot shown in Fig. 7 was 107 moles of SDS to 1 mole of α_{s1}-casein (1·2:1, w/w). As with the κ-casein, this was about twice the amount required to obtain the minimum S value (Table 3).

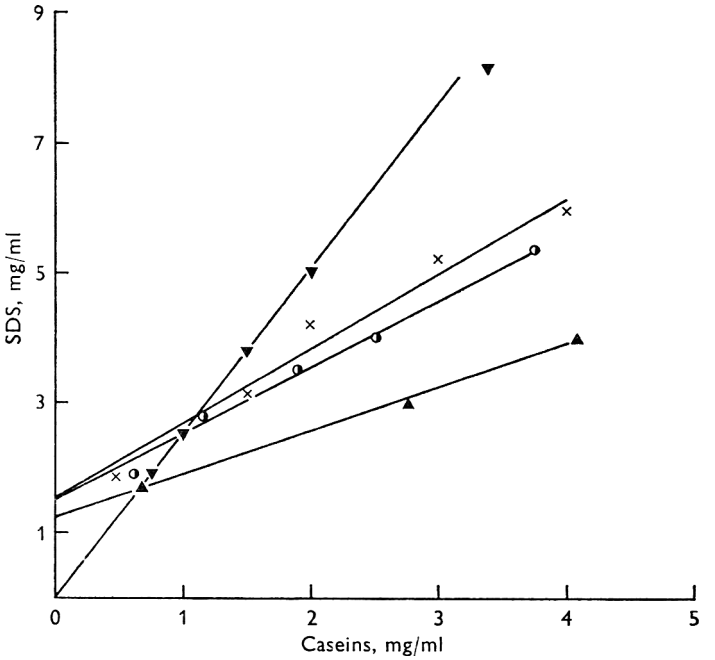

Fig. 7. Determination of total binding of sodium dodecyl sulphate (SDS) to the caseins. The amount of SDS bound was determined at several protein concentrations after dialysis against excess SDS and the total binding of SDS estimated from the slope of the plot of the concentration of SDS and casein. The amount of SDS bound in the aggregates was calculated from the area of the schlieren peak obtained during ultracentrifugation by subtraction of the area due to the protein alone. The plot for β-casein shows a higher slope than do those for the other caseins and thus reflects the greater binding capacity of this protein. α_{s1}-Casein, ×; β-casein, ▼; κ-casein + DTT, ◑; para-κ-casein, ▲.

Table 3. *The amounts of sodium dodecyl sulphate (SDS) required to give (a) minimum sedimentation coefficient and (b) maximum binding*

		Minimum S-value SDS concentration required			Total binding of SDS	
Casein	Initial S-value	Moles/mole casein	Mg/mg casein,	$s_{20,w}$	Moles/mole casein	Mg/mg casein
κ	18	—	—	3, 11, 7	—	—
κ (+DTT)	21	40	0·6	3	73	1·1
Para-κ	40	19	0·5	3	32	0·9
α_{s1}	6·2	50	0·6	3	107	1·2
β	18·2	18	0·2	3	244	3·4

β-Casein

Maximum binding of SDS to β-casein was determined at several concentrations of β-casein over the range 0·64–3·4 mg/ml. The maximum binding obtained as determined from the plot shown in Fig. 7 was 244 moles of SDS to 1 mole of β-casein (3·4:1, w/w). This was about 13 times the amount required to obtain the minimum S value (table 3).

DISCUSSION

The reaction of SDS with the casein aggregates appeared to proceed in 2 stages. The first stage was the production of protein–SDS complexes, the size of which decreased with the increase in proportion of the SDS until a complex with an $s_{20,w}$ value of about 3 was obtained. The second stage consisted of further binding of SDS to the protein until a maximum amount was bound. A possible explanation of the dissociation of the casein aggregates is that in binding to hydrophobic regions of the casein molecules the detergent competes with casein molecules for these sites and in so doing causes dissociation of the aggregate and also prevents its reformation. The aggregate undergoes reorganization to produce a more stable configuration containing a smaller number of protein molecules. The casein molecules which are displaced, because they are bound to the equivalent amount of detergent, associate with similar molecules to reform aggregates of a size commensurate with the protein and detergent composition. When complexes containing protein monomers are obtained further binding of detergent occurs until all available binding sites are occupied.

There was some variation in the behaviour of the individual caseins. κ-Casein formed 2 sizes of aggregates, about 35 % of 6 to 10S and the remainder of about 3S. The aggregates of 6 to 10S could be dissociated by SDS to the 3S complex only in the presence of a reducing agent (DTT), thus indicating that they had been formed by intermolecular disulphide bonding. It appears likely that there is a narrow size range of these aggregates and that only 2, 3 or 4 κ-casein molecules are involved. It is not possible to say whether these disulphide-linked κ-casein aggregates exist in the casein micelles in milk or whether they are artifacts brought about during the isolation of κ-casein.

The total binding of SDS to para-κ-casein was similar to but slightly lower than that to κ-casein. The lower value may indicate that the amount of SDS used to solubilize the para-κ-casein was underestimated by about 20 %. Dissociation of the para-κ-casein aggregates, however, required considerably more detergent for an equivalent complex size. A para-κ-casein–SDS ratio of 1:0·2, w/w gave aggregates of 30–40S whereas a κ-casein–SDS complex having the same weight composition had aggregates of 3S (20 %) and 13·4S (80 %). There would appear to be a considerable increase in hydrophobic properties of the para-κ-casein molecules to give rise to aggregates which are less readily dissociable by the detergent. It may be possible that structural re-arrangement of the molecule initiated by the enzymic removal of the predominantly hydrophilic portion produced this increase in hydrophobic property, or that the removal of the hydrophilic portion was in itself sufficient to enhance the hydrophobic nature of the para-κ-casein molecule. Although relatively more SDS was required to cause the initial dissociation of the para-κ-casein aggregates,

a rapid dissociation into protein monomer–SDS complex occurred at 10–20 molar excess of SDS. Alternatively, an alteration of charge distribution which is likely to occur when the macropeptide is removed from κ-casein by the action of rennin may affect formation of the complexes.

Molecules of α_{s1}-casein in the absence of detergent formed the smallest aggregates (6·25S), but they appeared to be strongly associated as complete dissociation to the 3S complex did not occur until about 50 molecules of SDS were bound per molecule of protein. These results, summarized in Table 3, show that this protein required more SDS than did β-casein (18 moles), κ-casein (40 moles) and para-κ-casein (19 moles), and it is suggested that the hydrophobic intermolecular binding is strongest in α_{s1}-casein.

Measurement of the minimum S observed was often difficult at the higher SDS concentrations due to unbound detergent forming micelles of about 1·8S. However, the results would suggest that 3S was probably the highest value obtained for the fully dissociated casein–SDS complex. This $s_{20,W}$ value suggests that the mol. wt of the complex would be in the region of 40 000–50 000, which means that a single molecule of casein would be involved. Molecular weights of this order are similar to those determined by Pitt-Rivers & Impiombato (1968) for micelle molecular weight of protein–SDS complexes which were found to be in the region of 34 000–41 000. However, as SDS forms about half of the complex, the hydrodynamic properties of the complex may well be quite different from those of the protein and the suggestion that the caseins occur as a monomer in the protein–detergent micelle cannot be substantiated on the ultracentrifugation evidence alone. Results from gel filtration experiments (Cheeseman, 1968) also suggested that the caseins occur as monomers in the protein–detergent complex.

The sizes of the aggregates are dependent upon protein concentration (Waugh & von Hippel, 1956; Payens, 1966), and when sufficient SDS was present to produce minimum aggregate size an increase in concentration of both protein and SDS gave an apparent reduction in S value. This effect is shown in Fig. 6 in particular with the α_{s1}-casein complexes and also in Fig. 5 for the κ-casein complexes having a protein: SDS ratio of 1:83. Non-reduced κ-casein complexes appeared to be very dependent on protein concentration but extrapolation to zero concentration for each of the protein:SDS ratios examined gave an $s_{20,W}$ value of about 17S (Figs 4 and 5).

The values shown in Table 3 for total binding of SDS to α_{s1}-casein and reduced κ-casein are similar to those obtained by Pitt-Rivers & Impiombato (1968) for a number of proteins. The value for total binding of SDS to para-κ-casein is somewhat lower but this could be due to an underestimation of the amount of SDS required initially to solubilize the para-κ-casein. The value obtained for β-casein appears to be considerably higher than any previously reported. This latter result was unexpected, especially as the 3S complex was obtained with about a third of the SDS required with the other caseins.

The work here reported shows that there are differences in the detergent-binding properties of the different caseins and it is suggested that these differences may reflect variations in hydrophobic properties due to differences in binding sites or configuration or both. The following paper reports on the concomitant spectral changes which occur during detergent binding.

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The interaction of bovine milk caseins with the detergent sodium dodecyl sulphate

II. The effect of detergent binding on spectral properties of caseins

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Summary. The dissociation of casein aggregates by the detergent sodium dodecyl sulphate (SDS) gave rise to difference spectra and these spectra were characteristic for each of the different types of casein. Increase in absorption by the chromophore groups, tyrosine and tryptophan, when α_{s1}- and β-casein aggregates were dissociated indicated binding of the detergent at regions of the molecule containing these residues. A decrease in absorption when κ-casein was dissociated indicated that the tyrosine and tryptophan residues were not in the region of the molecule to which the detergent was bound and that in the κ-casein aggregate these residues were in a more hydrophobic environment. Peaks on the difference spectra were obtained at 280 and 288 nm for α_{s1}-casein and 284 and 291 nm for β-casein and troughs at 278 and 286 nm for κ-casein. The difference spectrum reached a maximum value when the α_{s1}- and β-casein aggregates were dissociated and the further binding of SDS did not alter this value. The large negative change in the difference spectrum of κ-casein did not occur until after most of the aggregates were dissociated and did not reach a maximum until binding with SDS was complete. The value obtained for ΔOD was found to be temperature-dependent for β-casein–SDS interaction, but not for α_{s1}- and κ-casein. Changes in spectra were also observed when α_{s1}- and κ-casein interacted to form aggregates. The data obtained confirmed the importance of hydrophobic binding in casein aggregate formation and indicated the possible involvement of tyrosine and tryptophan residues in this binding.

In the previous paper, Cheeseman & Jeffcoat (1970) reported on the relationship between composition and size of the complexes of casein with sodium dodecyl sulphate (SDS) and showed that there were differences between the individual caseins in the contribution of hydrophobic binding in aggregate formation.

Binding of detergent can also be followed by difference spectrophotometry (Hayashi, Imoto, Funatsu & Funatsu, 1965) if the detergent affects the environment of the chromophore groups, tryptophan and tyrosine. The occurrence of specific difference spectra can indicate the nature of the group or groups involved, and if the molecular composition is known this will permit determination of the number of groups involved. The treatment of lysozyme with SDS results in changes in the optical rotatory dispersion (ORD) and circular dichroism, and Glazer & Simmons (1966)

concluded that these results indicated a change in the protein structure brought about by the binding of the detergent.

From a study of the ORD properties of the caseins, Herskovits (1966) suggested that α-helical organization does not occur in casein or casein aggregates in aqueous solution. It is, however, possible that the ordered aggregation of casein molecules, particularly if the aggregates are non-linear, could give rise to an environment within-the-aggregate similar to that found in the interior of globular proteins and dissociation may bring about changes in the local environment of those chromophore amino acids situated in the interior of the aggregate.

The present paper deals with the measurement and interpretation of the difference spectra which occur when casein aggregates interact with the detergent SDS. This information together with that obtained on the composition and size of the casein–SDS complexes may suggest possible mechanisms of casein interaction and aggregation.

MATERIALS AND METHODS

Model compounds

Acetyl tyrosine ethyl ester and acetyl tryptophan ethyl ester (Mann Research Laboratories, New York) were used as model compounds. Solutions of these esters were prepared in 0·02 M-tris-HCl buffer of pH 6·5 to give an optical density of 1·5–1·9 at 280 nm.

Preparation of caseins

The methods for the preparation of the caseins have been given in the previous paper (Cheeseman & Jeffcoat, 1970). The same preparations were used for the work reported here. Solutions of casein were prepared in 0·02 M-tris-HCl buffer of pH 6·5 to give an optical density of 1·5–1·9 at 280 nm. In some experiments dithiothreitol (10^{-3} M) was added to κ-casein solutions to maintain reducing conditions. Molar concentrations were determined as previously described (Cheeseman & Jeffcoat, 1970).

Measurement of spectral change

This was done by the method of Herskovits & Laskowski (1962) using a Unicam SP 700. Two pairs of stoppered 1-cm cells, made of 'Spectrosil', were used for the protein solutions and buffers. The arrangement of cells and solutions in the cell holder is shown in Table 1. Addition of concentrated solution of SDS to solutions of casein and model compounds was by means of a micropipette. Buffer was added to reference solutions to compensate for volume changes caused by addition of SDS solutions to the samples. Care was taken at all stages to avoid evaporation. The ΔOD values obtained were corrected for the change in protein concentration. For studies at temperatures other than ambient an electrically heated cell holder was used. Water at 0 °C was circulated through the cell holder for the experiments carried out at 3 °C.

Chemicals

Dithiothreitol (DTT), A grade, was obtained from Calbiochem, Los Angeles. SDS, specially pure grade, and all other chemicals (AR grade) were obtained from BDH Ltd, Poole, England. De-ionized water was used in the preparation of all the solutions.

Ultracentrifugation

Details of methods of ultracentrifuge analyses have been given in the previous paper (Cheeseman & Jeffcoat, 1970).

Table 1. *Arrangement of solutions in the spectrophotometer for measurement of the difference spectra*

Reference beam		Sample beam	
Cell 1	Casein solution*	Cell 3	Casein solution + SDS
Cell 2	Buffer + SDS	Cell 4	Buffer

* Concentration adjusted to compensate for volume changes due to addition of sodium dodecyl sulphate to Cell 3.

RESULTS

Model compounds

The spectral changes obtained with the model compounds and SDS are shown in Fig. 1. Acetyl tryptophan ethyl ester (Fig. 1*a*) gave peaks at 284 and 292 nm which reached a maximum at a ratio of 1 mole of tryptophan to 50 moles of SDS. Acetyl tyrosine ethyl ester (Fig. 1*b*) gave peaks at 278 and 284 nm and a trough at 265 nm, and these changes also reached a maximum at a ratio of 1 mole of tyrosine to 50 moles of SDS. The relationship of ΔOD with increasing concentration of SDS for these model compounds is shown in Fig. 1*c*, *d*.

Caseins

Figs 2*a*, *b* show the spectral changes obtained for α_{s1}- and β-casein with SDS. Peaks occurred at 280 and 288 nm for the α_{s1}- casein and at 284 and 291 nm for β-casein. The relationship of ΔOD with increasing concentration of SDS is shown in Fig. 3*a* for α_{s1}-casein and in Fig. 3*b* for β-casein. The maximum values were obtained with α_{s1}-casein at a protein:SDS molar ratio of 1:10 for the 288 nm peak and at a 1:50 ratio for the 280 nm peak. The β-casein peaks at 284 and 291 nm obtained maximum values at a 1:12 ratio. An example of the difference spectra when SDS binds to κ-casein is shown in Fig. 2*c*. Troughs were obtained at 278 and 286 nm and the change in the 278 nm absorption was about 60 % of that at 286 nm. The relationship of ΔOD with increasing concentration of SDS is shown in Fig. 3*c* for κ-casein and a maximum perturbation was obtained at a ratio of 1 mole of κ-casein to 120 moles of SDS. Addition of DTT (10^{-3} M) to κ-casein solutions did not affect the rate of spectral change during SDS binding nor the maximum ΔOD obtained.

The effect of temperature on the spectral change was examined also at 3 and 50 °C. Little change was found for the α_{s1}- and κ-casein–SDS difference spectra at these temperatures. However, significant differences occurred with β-casein and an example of the results obtained is shown in Fig. 3*d*.

The possibility that on mixing α_{s1}- and κ-caseins the aggregate formed might give rise to a spectrum differing from that obtained from the solutions of the individual caseins was examined as follows. Solutions of α_{s1}- and κ-casein were prepared to equimolar concentrations to give a combined absorption of 1·5 to 1·9 at 280 nm. Lower concentrations of κ-casein to give the proportionate composition existing in

milk were not investigated as it was thought desirable to follow aggregate size by analytical ultracentrifugation using schlieren optics, which therefore placed a lower limit on concentration. The sedimentation velocity analyses of the solutions are given in Table 2. A small difference spectrum showing positive ΔOD in the region 275–290 nm was obtained when the mixture was compared with the individual casein solutions, but it did not exhibit any characteristic peaks as had been found when SDS reacted with the caseins. In an attempt to form aggregates of α_{s1}- and κ-casein

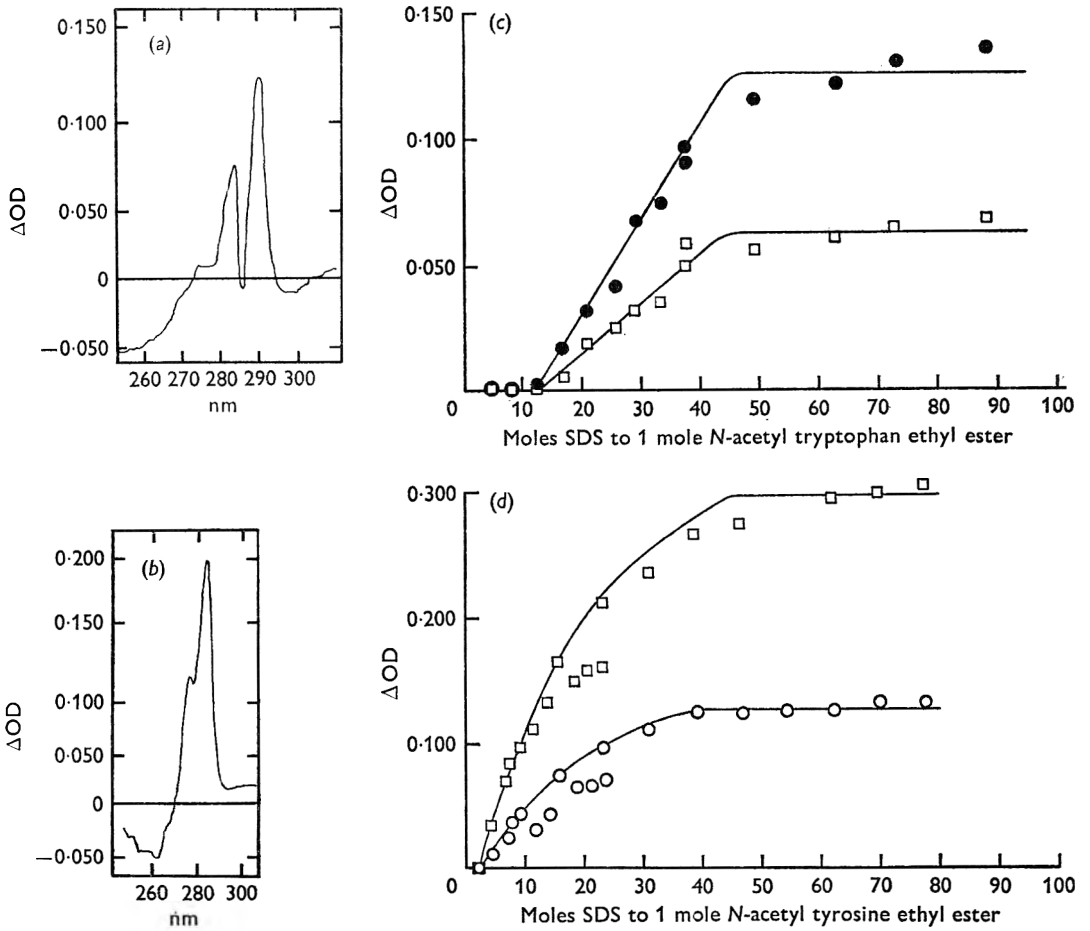

Fig. 1. Examples of the difference spectra obtained when the model compounds interact with sodium dodecyl sulphate (SDS) and in ΔOD as a function of increased concentration of SDS. (*a*) The difference spectrum obtained when SDS (12 mM) was added to a solution of *N*-acetyl tryptophan ethyl ester (0·24 mM). Peaks are at 284 and 292 nm. (*b*) The difference spectrum obtained when SDS (71 mM) was added to a solution of *N*-acetyl tyrosine ethyl ester (1·17 mM). Peaks are at 278 and 284 nm and a trough at 265 nm. (*c*) The relationship between ΔOD and concentration of SDS from the difference spectrum obtained with *N*-acetyl tryptophan ethyl ester (0·28 mM) and SDS. Plots for 284 (□) and 292 (●) nm are recorded. Data presented in terms of moles of SDS to one mole tryptophan. (*d*) The relationship between ΔOD and concentration of SDS from the difference spectrum obtained with *N*-acetyl tyrosine ethyl ester (1·48 mM) and SDS. Plots for 278 (○) and 284 (□) nm are recorded. Data presented in terms of moles of SDS to one mole tyrosine. The figure recorded after each reactant was the final concentration in the reaction mixture for (*a*) and (*b*), and the initial concentration for (*c*) and (*d*).

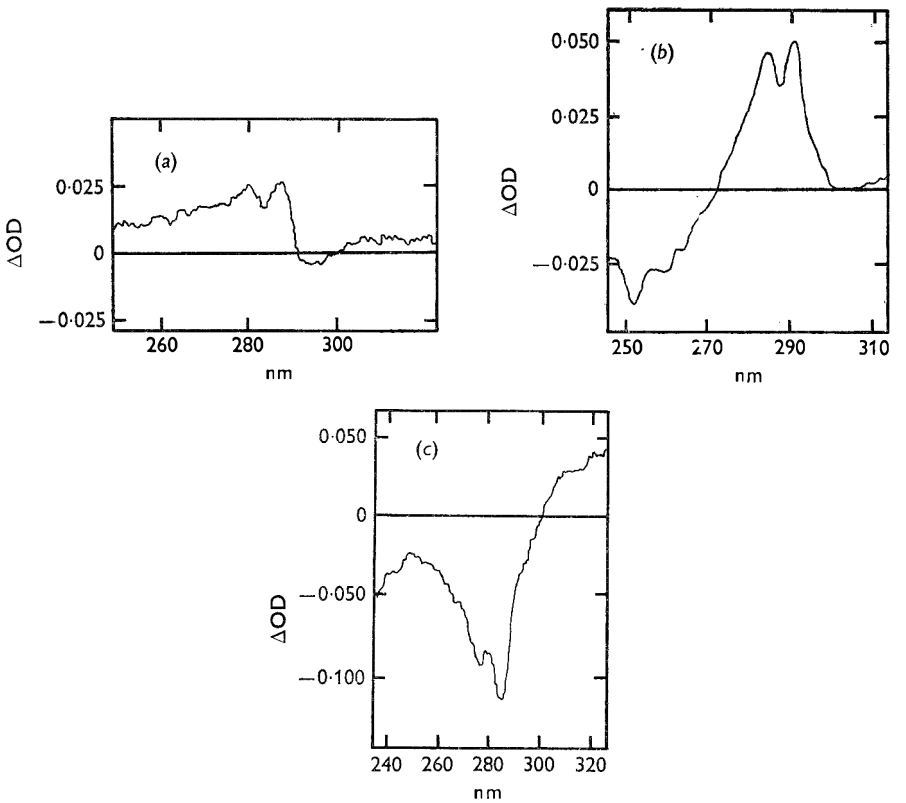

Fig. 2. Examples of the difference spectra obtained when caseins interact with sodium dodecyl sulphate (SDS). (*a*) α_{s1}-casein (0·07 mM) and SDS (2·63 mM). Peaks are at 280 and 288 nm. (*b*) β-casein (0·13 mM) and SDS (7·64 mM). Peaks are at 284 and 291 nm. (*c*) κ-casein (0·07 mM) and SDS (9·05 mM). Troughs are at 278 and 286 nm. The figure recorded after each reactant was the final concentration in the reaction mixture.

Table 2. *Sedimentation coefficients ($S_{20,w}$ values) obtained by sedimentation velocity analysis. α_{s1}- and κ-casein and equi-molar mixtures*

	α_{s1}	κ	$\alpha_{s1}+\kappa$ (0·5 h after mixing)	$\alpha_{s1}+\kappa$ (7 days after mixing)
Untreated	5·1	18·5 (1·5)*	(21·1)† 3·9	13·0 (1·5)*
Treated with sodium dodecyl sulphate (SDS) and then dialysed against buffer at 4 °C for 7 days	4·7	14·5 (1·5)*	(16·4)† 4·4‡	11·2 (1·5)*§

* Comprised about 10 % of sedimenting species.
† Comprised 10–20 % of sedimenting species.
‡ The α_{s1} and κ were mixed after dialysis of the SDS.
§ The α_{s1} and κ were mixed before dialysis of the SDS.
Casein concentration about 0·9 %, w/v, α_{s1}-casein and 0·7 %, w/v, κ-casein in 0·05 M-tris-HCl buffer at pH 6·5 containing 0·1 M-NaCl, 10^{-4} M-EDTA and 10^{-3} M-DTT. Conditions of ultracentrifugation as in Cheeseman & Jeffcoat (1970).

from the protein monomers the solutions were first treated with SDS to give the 3S complex, mixed in equal proportions and the mixture exhaustively dialysed at 4 °C against a buffer to remove the SDS. The reformation after removal of SDS of aggregates with the same S values as were obtained without SDS treatment was confirmed by sedimentation velocity analysis (see Table 2). The difference spectrum was not the same as that found without pre-treatment with SDS. The mixed aggregate exhibited somewhat less absorption in the 260–290 region than did the combined single solutions, with a definite trough at 278 nm.

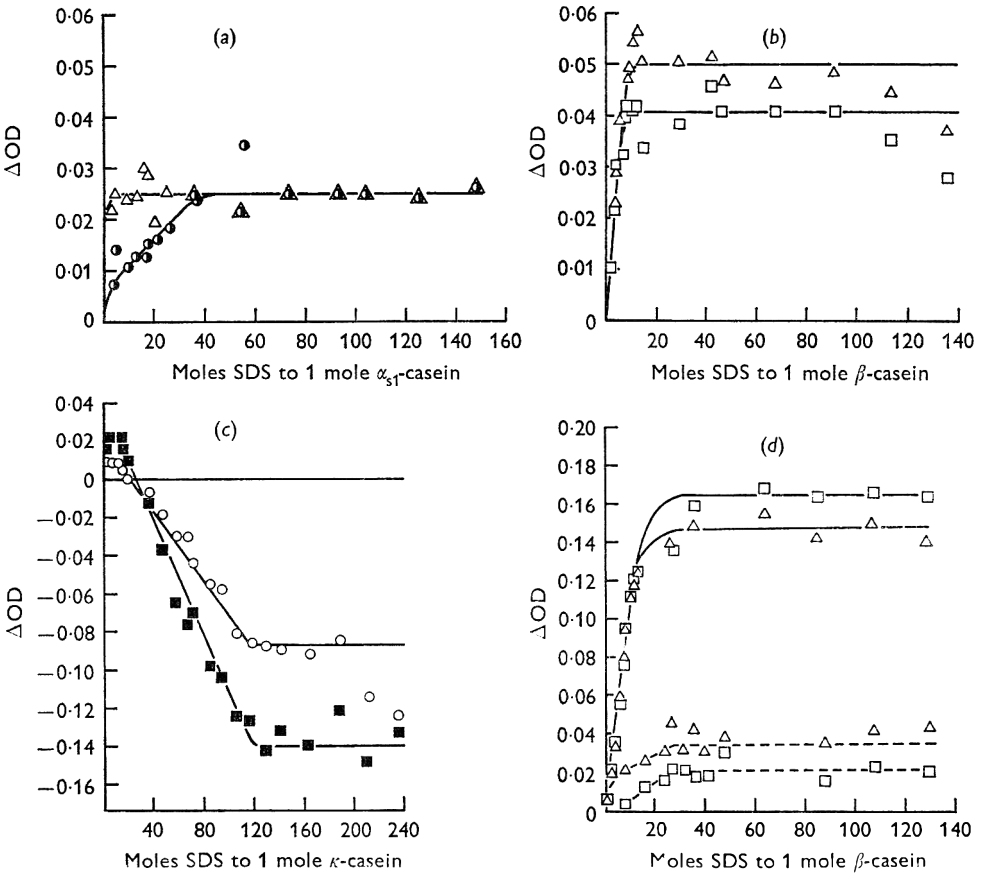

Fig. 3. The relationship between ΔOD and concentration of sodium dodecyl sulphate (mole/mole of casein). Data obtained at room temperature for (*a*), (*b*) and (*c*). (*a*) α_{s1}-Casein. Plots recorded for 280 (◑) and 288 (△) nm. (*b*) β-Casein. Plots recorded for 284 (□) and 291 (△) nm. (c) κ-Casein. Plots recorded for 278 (○) and 286 (■) nm. (*d*) β-Casein. Experiments carried out at 3 °C, ——, and 50 °C, – – – –. Plots recorded for 284 (□) and 291 (△) nm.

DISCUSSION

Comparison of the difference spectra obtained from the model compounds and SDS and from the caseins and SDS indicates that the interpretation of the difference spectra obtained from the protein–detergent interaction is difficult. In the present work, it is not possible unequivocally to identify the actual groups involved due to red or blue shifts in peaks of maximum absorbance and this therefore limits the

usefulness of the results. It is likely, however, that the absorbance peak (or trough) at higher wavelength in each difference spectrum is principally due to tryptophan, and the absorbance peak at the lower wavelength is due to tyrosine. In the interpretation of the results it must also be noted that the observed spectrum is the sum of the individual spectra of the chromophore groups and that individual contributions could be negative, positive or show no change.

The α_{s1}- and β-caseins showed a positive difference spectrum and this type of spectrum is considered to indicate a change in the environment of the chromophore groups to one that is more hydrophobic (see Yanari & Bovey, 1960). κ-Casein on the contrary exhibited a negative spectrum, suggesting that chromophore groups changed from a hydrophobic environment to one that is more hydrophilic. If these observations are considered in conjunction with the results obtained in the previous paper (Cheeseman & Jeffcoat, 1970), it is possible to relate the spectral change and the dissociation of the casein aggregates. At least 2 factors may be implicated; one is the possibility that the dissociation of the aggregate *per se* causes changes in the environment of the chromophore groups and the other is that the adsorption of the detergent on to the protein at or near the site of these groups may also cause change in their environment.

The recent work of Leslie, Irons & Chapman (1969) on high resolution nuclear magnetic resonance studies of α_{s1}-, β- and κ-caseins has shown that in the κ-casein aggregates the aromatic and most of the non-polar residues are restricted in motion. Disruption of the aggregates by urea freed the residues. Our results would be consistent with this finding.

The maximal spectral change appeared to occur when the aggregates of α_{s1}- and β-casein were dissociated. This was, however, by no means near the level of SDS at which the protein binding was fully saturated. This result suggested that the detergent reacted initially with the regions of the protein which contained most of the chromophore groups and it also follows that these regions of the α_{s1}- and β-casein molecules are those most likely to be involved in aggregation formation through hydrophobic bonding. There was some difference between the amount of SDS required to give maximal spectral change for the peaks at 288 and 280 nm for α_{s1}-casein and this may suggest that some of the groups contributing to the 280 nm peak did not fully interact with SDS until after dissociation of the aggregate (see Fig. 3*a*). If the 280 nm peak is due to absorbance by tyrosine, then this would imply that some tyrosine residues were not involved in the region of the α_{s1}-casein responsible for hydrophobic bonding.

Maximum spectral change did not occur with κ-casein until complete saturation with SDS had occurred. A slight positive spectrum was found during the dissociation of the aggregate. The extensive loss of absorption during binding of SDS to the κ-casein which occurred after 60 % or more of the κ-casein was in the monomeric form (see Fig. 2, Cheeseman & Jeffcoat, 1970) suggested that SDS was bound to the polypeptide chain in regions remote from the chromophore groups and that some alteration of structure occurred during the binding. This observation may indicate that the molecular structure of native monomeric κ-casein restricts the motion of many of the tyrosine and tryptophan residues in the molecule.

Examination of the dependence of spectral change on temperature showed that

with α_{s1}- and κ-casein there was little difference between results obtained at 3, 20 and 50 °C. With β-casein, however, there was a large dependence on temperature. More SDS was required to give a maximum ΔOD at 50 °C compared with the amount required at 20 °C and the maximum ΔOD obtained, particularly for the 284 nm peak, was lower than that at 20 °C. At 3 °C the ΔOD was about 3 times greater than at 20 °C and the 284 nm peak was greater than the 291 nm peak which was the opposite of the result at 20 and 50 °C. The aggregation of β-casein is temperature-dependent (Payens, 1966), but binding by SDS gave a minimum sized β-casein–SDS complex irrespective of the temperature. It would be expected that at 3 °C little or none of the β-casein was in the aggregated form and thus little SDS would be required to bring about complete dissociation. However, the results suggest that whereas a maximum ΔOD was obtained at 20 °C with β-casein:SDS molar ratio of 1:12 at 3 °C a maximum ΔOD was not obtained until the ratio of 1:24. A possible interpretation of this result is that temperature-induced changes in the native β-casein monomer give rise to a molecular structure that requires the binding of a greater number of molecules of SDS before the environment of the chromophore groups approaches that obtained at the higher temperatures.

A temperature-dependent conformational change in β-casein was demonstrated by Garnier (1966), who suggested from the results of difference spectra and of optical rotational measurements that at 5 °C the polypeptide chain was partially folded in a left-handed type poly-L-proline II helix and at 40 °C there was a decrease in this type of helix and an increase in the α-helical conformation. This intra-molecular configurational change could be the reason for the temperature-dependence of the β-casein–SDS spectrum reported in this work.

There were differences in the SDS binding properties of the individual caseins and while α_{s1}- and β-casein were similar but not identical in their reactions, κ-casein gave a very different response. An interpretation of this difference in behaviour could be that the tyrosine and tryptophan residues of κ-casein were not extensively involved in the binding with SDS. As SDS is considered to react primarily with hydrophobic regions of the molecule, this would suggest that most of these groups are in a region of the molecule which is not strongly hydrophobic and if the κ-casein association is principally hydrophobic then these residues are probably not involved in the self-association of κ-casein. The present knowledge of amino acid sequence of κ-casein is not yet sufficiently detailed for it to be possible to say where these aromatic residues occur in the polypeptide chain (see Jollès, Jollès & Alais, 1969), except that they occur in the para-κ-casein part of the molecule obtained after the action of rennin on κ-casein. Para-κ-casein strongly associates, and from the results reported in this paper this association is probably strongly hydrophobic. It may be suggested from the interpretation of our results that the hydrophobic sites of para-κ-casein are localized in a particular region or regions of the molecule and that hydrophobic binding is not a property of the entire molecule. Complete elucidation of the sequence of κ-casein and para-κ-casein will be required before this suggestion can be confirmed.

The occurrence of a difference spectrum when α_{s1}- and κ-caseins were mixed suggested that there was a change in the absorbance of the tryptophan and tyrosine residues when the new aggregate was formed. The 2 methods used for α_{s1}–κ-casein aggregate formation produced different spectra and may indicate that the sequence

of formation of the aggregate was important in the final arrangement of the individual casein molecules within the aggregate. These preliminary observations with the casein mixtures will require more extensive investigation to establish the validity of the apparent changes in spectra.

The effect of the negative charge of the dodecyl sulphate on the dissociation of casein aggregates has not been considered in this discussion. It is, however, possible that some of the differences observed in the spectrum could also be due to a charge effect on the molecular structure. Although this may affect the rate of dissociation of the casein aggregates it is unlikely to be of importance in the binding of the SDS to the hydrophobic region of the protein. In preliminary studies using a non-ionic detergent, Triton X-100, it was found that a greater amount of this detergent compared with SDS was required per mg casein to cause aggregate dissociation.

The importance of hydrophobic binding in aggregate formation and some involvement of tyrosine and tryptophan residues in the region of the molecule concerned with binding has been demonstrated by the interaction of SDS with individual caseins. It is, however, important to know the relative contributions of the tyrosine and tryptophan to these observed difference spectra, and work is continuing to obtain more information on this aspect.

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Structure of the casein micelle

The accessibility of the subunits to various reagents

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Summary. Carboxypeptidase A (CPA, mol. wt = 34600) and 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, mol. wt = 270) were shown to react with the 3 casein subunits, α_{s1}-, β- and κ-caseins, which constitute the micelle. Virtually all of the C-terminal amino acids were released by CPA from these subunits without disrupting the micellar structure.

It was verified that the rate of dissociation of the micelle into low molecular weight complexes is considerably slower than the rates at which these reagents act on micellar components. It is concluded that dansyl chloride, CPA, and probably also rennin, whose molecular weight is similar to that of CPA, are able to penetrate to the interior of the micelles. This was confirmed in a quite independent way with a non-reacting agent, myoglobin (mol. wt = 17000).

It is postulated that the casein micelle has a sponge-like structure in which the 3 different subunits are distributed fairly uniformly.

Micellar fractions obtained by differential centrifugation from skim-milk and colostrum were examined for their content of α_{s1}-, β-, κ- and para-κ-caseins by the use of CPA as described by Ribadeau Dumas (1968). The results confirm that there is a real difference in κ-casein content of the micelles, according to their rate of sedimentation.

Caseins in milk have the remarkable property of forming micelles, i.e. copolymers of very high molecular weight (3×10^6–3×10^9) in presence of calcium ions (Nitschmann, 1949). The casein micelles in cow's milk are essentially composed of 3 different subunits, α_{s1}-, β- and κ-caseins, having molecular weights of respectively, 23700, 24200 and 18700–23000.

It is now well established, from the work of Waugh and Von Hippel (1956), that one of the subunits, κ-casein, plays a key role in micelle formation since in its absence α_{s1}- and β-caseins form insoluble, unlimited aggregates with Ca^{2+} at room temperature and neutral pH.

Since the publication by Waugh & Noble (1965) of a tentative scheme for the micelle structure, other different micelle models have been proposed (see the review by

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Rose, 1969). As several authors have assumed a superficial location of the κ-casein, we decided to reinvestigate the micelle structure in search of direct experimental proofs of a non-uniform distribution of the 3 main casein subunits throughout the micelle. To this end, we examined the accessibility of the subunits to various reagents or compounds having different molecular weights: 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) (mol. wt = 270); myoglobin (mol. wt = 17000); carboxypeptidase A (CPA) (mol. wt = 34600). This latter reagent was particularly useful. Its action on each casein subunit can be easily identified and followed, as was shown by one of us (Ribadeau Dumas, 1968). Our experiments have led to the conclusion that the micelles are completely permeable to all these compounds and that the casein subunits are probably distributed uniformly throughout the body of the micelle.

A preliminary account of this work appeared recently (Ribadeau Dumas & Garnier, 1969).

MATERIALS AND METHODS

Skim-milk. Milk was obtained from individual French Friesian cows and kept at 37 °C, in presence of toluene. It was skimmed by centrifugation for 60 min at 1000 *g* and 35 °C, and utilized as soon as possible.

Whole caseins. Whole casein from individual skim-milk and colostrum was obtained by precipitating at pH 4·7 with N-HCl (the colostrum having been first diluted 3-fold). The precipitates were washed twice with distilled water and dissolved in water by adjusting the pH to 7·0 with N-NaOH. The procedure was repeated twice and the purified caseins, in solution at pH 7·0, were finally freeze-dried.

Micelle-free milk and 'soluble casein'. These were prepared by centrifuging skim-milk at 105000 *g* for 60 min. These conditions were chosen after it had been observed that the protein content of the supernatant (determined by absorbance measurement at 278 nm after addition of 10% (v/v) 1·0 M-K_3 citrate–0·1 M-imidazole-HCl buffer of pH 6·7) reaches an approximately constant level after 40-min centrifugation. The soluble casein was obtained from the supernatant (micelle-free milk) by precipitation at pH 4·7 as described above. Micellar fractions were also prepared under conditions of centrifugation indicated below.

Whole casein determination. Whole casein was usually determined by measuring the absorbance at 278 nm in presence of the citrate–imidazole buffer as described above. The average $E^{1\%}_{278\ nm}$ value for whole casein was 6·7. For determining the casein content of milk, the absorbance of the whey, obtained by precipitating the casein at pH 4·7, was subtracted from the value obtained for the milk. In some instances the casein content was determined by the method of Rowland (1938).

Action of dansyl chloride on casein and reconstituted micelles. Three ml of whole casein solution (2·5% in 0·1 M-NaCl) at pH 7·0 were placed in a water-bath at 37 °C under nitrogen. To this solution were added 20 μl of distilled water or 3 M-$CaCl_2$ (final concentration 0·02 M), at constant pH. After equilibrating for 30 min, 100 μl of dansyl chloride solution in acetone were added, containing 1·8, 3·8 or 4·7 mg of dansyl chloride. The pH was kept constant at 7·0 during the reaction by addition of 0·2 N-NaOH with a microsyringe. The consumption of NaOH was followed as a function of time. Each experiment was carried out in duplicate.

Starch gel electrophoresis of DNS-casein. This was carried out by the method of Wake & Baldwin (1961) as modified by Schmidt (1964). Dansylated-proteins were detected after electrophoresis by UV irradiation of the gels, and by subsequent staining with Amido black 10 B.

Myoglobin assay. Horse heart myoglobin was added at different concentrations to skim-milk and to skim-milk that had been concentrated 3-fold by batchwise treatment with Sephadex G 50 fine. The optical density at 408 nm was recorded for the citrate treated milks (0·2 M K_3-citrate, 0·02 M imidazole-HCl, pH 6·7, at final concentration), i.e. D_m, and for the citrate treated supernatants, i.e. D_s, after 60 min centrifugation at 105000 *g* and at 37 °C. The values of D_s were corrected from the myoglobin self sedimentation in the micelle-free milks, estimated from a separate control experiment.

Assay with CPA. A method for determining α_{s1}-, β- and κ-caseins in whole casein by using CPA was described in an earlier publication (Ribadeau Dumas, 1968). The following modifications were used when the determination of α_{s1}-, β- and κ-caseins in the micelles of native milk was necessary.

(*a*) The skim-milk (3 ml) was maintained in a water-bath at 37 °C in presence of toluene and 10 μl of Worthington DFP-treated CPA suspension (50 mg/ml) were added after 45-min equilibration. Portions (600 μl) of digest were pipetted after 2, 4 and 6 h. The reaction was stopped by addition of 300 μl 15 % trichloroacetic acid. After centrifugation, 600 μl of the supernatant were analysed for acidic and neutral amino acids.

(*b*) A 10-ml portion of the same milk was centrifuged for 60 min at 105000 *g* and 37 °C. The supernatant was treated with CPA (1 μl/ml) and analysed as in (*a*).

The difference of the figures obtained in (*a*) and (*b*) gives the amount of amino acid released by CPA from the micelles. An example of the calculation is given in Table 4. For further explanation see Ribadeau Dumas (1968).

A comparative study of the action of CPA on micelles and citrate-disrupted micelles was carried out as described above with the following modification. The 105000 *g* sediment obtained from 100 ml skim-milk was suspended in 100 ml of 0·01 M-$CaCl_2$ at constant pH 6·7 by stirring at 37 °C for 2 h. The suspension was again centrifuged and the sediment resuspended to give a suspension of washed micelles in 0·01 M-$CaCl_2$ at pH 6·7. CPA digestion was carried out as described in (*a*) above, both on the micellar suspension and on the same suspension after the addition of 20 μl 0·1 M potassium citrate/ml at constant pH. Samples were taken after 30 min, 1, 2 and 18 h. The amounts of α_{s1}-, β- and κ-caseins attacked by the enzyme as a function of time were calculated as above although this method of calculation can be applied only when the release of all the amino acids approaches 100 %.

Amino acid analyses were carried out with a Beckman Unichrom amino acid analyser, as described by Spackman, Stein & Moore (1958). This instrument was equipped for accelerated, sensitive analysis (Technical Bulletin, 1964; 1965), and with an automated integrator.

RESULTS

Water content of 105000 g sediment of micelles

The dry weights of sediments obtained by centrifuging 5 individual skim-milks for 60 min at 105000 *g* and 37 °C were determined after drying at 100 °C for 24 h under vacuum. The results are reported in the second column of Table 1.

Table 1. *'Soluble' casein and water content of micelles from individual milks*

Milk	Water content of the micelles, % (w/w)	Soluble casein,* % whole casein	Soluble casein after 30 min†, % whole casein
1	64·7	4·2	—
2	72·5	2·4	—
3	65·9	2·6	—
4	63·9	3·1	—
5	62·7	2·4	0·8
6	—	3·4	1·1
Average	66·0	3·0	—

* Casein which was not centrifuged at 105000 *g* for 60 min.

† The milks were centrifuged at 105000*g* for 60 min. The acid wheys remaining after precipitation of the soluble casein at pH 4·7 were readjusted to pH 6·7 (initial pH of milks). The sediments of micelles were then resuspended in their corresponding solutions, stirred for 30 min, and the soluble casein estimated again.

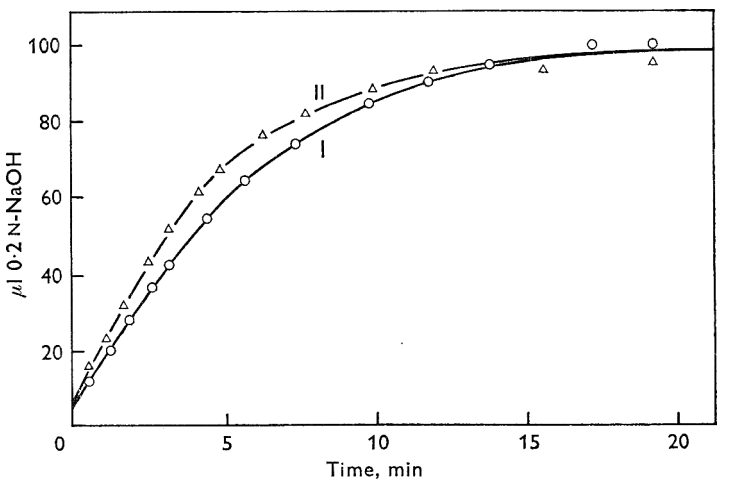

Fig. 1. Action of dansyl chloride on whole casein (I) and whole casein in the micellar state (II). Uptake of 0·2 N-NaOH expressed as a function of time. Concentration of casein solution: 2·5% in 0·1 M-NaCl or 0·02 M-$CaCl_2$ + 0·1 M-NaCl; pH 7·0. Amount of dansyl chloride/ml of casein solution: 4·68 μmoles.

Study of the equilibrium between micelles and soluble casein at 37 °C and at constant concentration of inorganic ions

Six different individual skim-milks were analysed for soluble casein at 37 °C, as described above. The average value was 3·0 g soluble casein/100 g whole casein (Table 1).

Micelle-free milks and sediments of micelles were prepared from 2 of the skim-

milks as described above. The casein contents of the micelle-free milks were estimated and the acid wheys, remaining after precipitation of the soluble casein at pH 4·7, were readjusted to pH 6·7 with N-NaOH. The sediments of micelles were then re-suspended in their corresponding solutions, stirred for 30 min, and the soluble casein estimated again (see last column of Table 1).

Penetration of dansyl chloride into the micelles

Fig. 1 shows the course of dansyl chloride action on whole casein and on the micelles which formed from the same casein in the presence of $CaCl_2$, determined from the proton release during the reaction.

The results of an experiment made on casein with and without $CaCl_2$, in which 3 different levels of dansyl chloride were used, are indicated in Table 2, V_0 being the initial velocity and H the height of the plateau correlated to the extent of dansylation.

Table 2. *Penetration of dansyl chloride into the micelle*

Amount of dansyl chloride (μmoles) added/ ml of casein solution (2·5 %)	Amount of $CaCl_2$ added, μmoles/ml	V_0, μmoles of NaOH per min per ml	H, μmoles of NaOH/ml
2·25	0	0·75	3·2
2·25	20	0·82	2·8
4·68	0	0·74	6·1
4·68	20	1·00	6·1
10·25	0	0·87	15·1
10·25	20	1·10	15·1

Table 3. *Penetration of myoglobin into the micelle*

		Ratios D_m/D_s o.d. at 408 nm, theoretical*			
	Observed	1	2	3	Myoglobin, mg/ml
1 Skim-milk	0·99	0·97	0·90	0·93	10·3
	0·96	0·97	0·90	0·93	5·1
	0·96	0·97	0·90	0·93	2·0
	0·99	0·97	0·90	0·93	1·1
2 Concentrated	0·90	0·89	0·70	0·77	2·0
skim-milk	0·89	0·89	0·70	0·77	2·0

* 1. Full penetration assuming 0·5 ml of bound water/ml protein and a total water content of 3·1 ml/ml protein (see Table 1).
2. No penetration assuming closely packed distorted spheres with no external water.
3. No penetration assuming closely packed rigid spheres with 0·25 ml of external water/ml protein. Volume of the precipitates: 0·096 ml/ml milk in 1 and 0·299 ml/ml milk in 2.

The initial velocity was always greater for the action of dansyl chloride on micelles. However there was no significant difference when the amount of dansyl chloride was increased. The height of the plateau, H, was the same in the presence and in the absence of calcium.

In every case the micelles no longer precipitated when rennin was added. This finding confirms the observation of Hill & Craker (1968) that the blocking of 2–3 lysyl

residues in κ-casein by dansyl chloride prevents clot formation after the release by rennin of the casein macropeptide.

Starch gel electrophoresis showed that all the caseins bound dansyl chloride even at low concentration of reagent. There was no preferential binding to any of the 3 main casein subunits.

Penetration of myoglobin into the micelle

The observed ratios D_m/D_s were not affected by the myoglobin concentration (see Table 3), indicating that adsorption, if any, was negligible. Although it is not possible to define how the micelles are packed at the bottom of the centrifuge tube, the observed ratios were significantly different (estimated error $\pm 2\%$) from the theoretical ratios calculated for 'no penetration' (Table 3, columns 2 and 3), especially with the concentrated skim-milk. They were in fact quite close to the values calculated for a full penetration (Table 3, column 1).

Penetration of carboxypeptidase A into the micelle

As was shown in an earlier publication (Ribadeau Dumas, 1968), CPA attacks the C-terminal ends of α_{s1}-, β- and κ-caseins almost as well with isolated fractions as with whole casein in solution without calcium. The same observation has been made now as far as native milk micelles are concerned.

Table 4. *Amino acid analyses of skim-milk and of the* 105000 *g supernatant after CPA action**

Amino acids	Skim-milk† a, μmoles/ml	105000 *g* supernatant b, μmoles/ml	Difference a–b, μmoles/ml
Thr	0·407	0·041	0·366
Ser	0·174	0·034	0·140
Gln + Asn	0·283	0·041	0·242
Ala	0·202	0·047	0·155
Val	0·846	0·051	0·795
Ile	0·859	0·193	0·666
Leu	0·615	0·100	0·515
Tyr	0·151	0·014	0·137
Phe	0·026	0·010	0·016

* Milk 1 of Table 5.

† Average values for 3 times of CPA action: 2, 4 and 6 h corresponding to a plateau value for the liberation of the amino acids.

Calculation of α_{s1}-, β-, κ- and para-κ-casein contents:

α_{s1}: (Leu-Tyr-Phe) × 23·7 = (0·515–0·137–0·016) 23·7	8·58	mg/ml of milk
β: (Val-Thr) × 24·2 = (0·795–0·366) × 24·2	10·38	
κ: Ala × 20·0 = 0·155 × 20·0	3·10	
para-κ: Phe × 13·5 = 0·016 × 13·5	0·21	
	Total 22·27	

We first determined the amounts of α_{s1}-, β-, κ- and para-κ-caseins in the micelles of milk which are accessible to CPA by treating with this enzyme 4 different individual skim-milks and the corresponding micelle-free milks obtained by centrifugation at 105000 *g* for 60 min, until a plateau was reached in the liberation of amino acids. An example of an experiment carried out on one milk is shown in Table 4 and the results of experiments on several milks are compared in Table 5.

In order to obtain more accurate kinetic information on the rate of release of the various amino acids, we carried out comparative experiments on washed micelles and citrate-disrupted micelles (see p. 271). The results of one such experiment are shown in Fig. 2.

Table 5. *Penetration of CPA into the micelle*

	Milk			
	1	2	3	4
(*a*) Total amount of casein ($\alpha_{s1}+\beta+\kappa+$ para-κ) estimated (mg/ml) by CPA analysis	22·2	22·6	27·7	26·6
(*b*) Total amount of casein estimated by Rowland's method (1938), mg/ml	25·5	25·0	29·1	26·8
(*c*) Amounts of casein components, % of b				
α_{s1}	33·2	37·6	34·7	41·3
β	40·4	36·9	48·1	45·4
κ	12·1	14·4	10·9	10·9
para-κ	0·6	0·3	0·4	0·6
Molar ratio $\alpha_{s1}/\beta/\kappa+$ para-κ	2·1/2·5/1	2·1/2·0/1	2·5/3·4/1	2·9/3·1/1

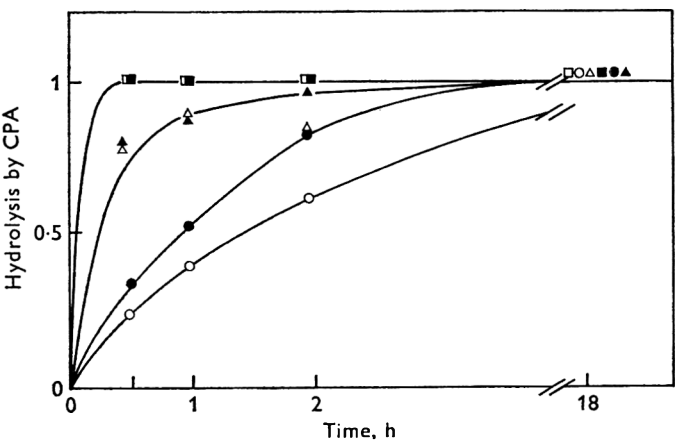

Fig. 2. Comparison of the action of CPA on α_{s1}-, β- and κ-caseins, in washed micelle suspension and in citrate solution. Ratios of the total molar amounts of α_{s1}-, β- and κ-caseins obtained after 18 h of CPA action. Black points correspond to experiments made on micelles and open points to experiments made after disruption of micelles by citrate. □, ■, β-casein; △, ▲, κ-casein; ○, ●, α_{s1}-casein. For conditions, see text.

Content of α_{s1}-, β- and κ-caseins in different micellar fractions

Micellar fractions, some already prepared and studied in an earlier publication (Ribadeau Dumas & Veaux, 1964) were tested for their contents of α_{s1}-, β-, κ- and para-κ-caseins. These fractions were obtained from normal skim-milk and skim colostrum by differential centrifugation followed by elimination of the inorganic ions and lyophilization of each fraction at neutral pH, as described earlier. Two fractions from normal milk were tested: soluble casein which was not sedimented during centrifugation at 30000*g* for 135 min (fraction S), and the micellar fraction which

sedimented in 30 min under the same g force (fraction M). In addition, 3 fractions from colostrum were studied: C_1 = fast sedimenting micelles (30 000 g, 135 min); C_3 = slow sedimenting micelles (30 000 g, 135 min and 105 000 g, 75 min) and C_4, the soluble casein (upper supernatant at 30 000 g, 135 min) (Ribadeau Dumas & Veaux, 1964). The results are presented in Table 6.

These results clearly establish that κ-casein content increases with the sedimentation coefficient of the micelles as was suggested before from the evidence of N-acetylneuraminic acid and stabilizing power estimations (Sullivan, Fitzpatrick & Stanton, 1959; Waugh & Noble, 1965). On the other hand the ratio α_{s1}/β increased enormously in colostrum from C_1 to C_4 but did not seem to vary in normal milk according to the sedimentation coefficient of the different micellar fractions. However, variations of this ratio are observed from one individual milk to another.

Table 6. *Content of α_{s1}-, β- and κ-caseins in different micellar fractions*

Casein	κ, %	N-acetyl-neuraminic acid content, %	para-κ, %	Molar ratio α_{s1}/β/ κ+para-κ	α_{s1}, %	β, %	Molar ratio α_{s1}/β
M	9·5	—	1·2	2·0/2·9/1	27·8	40·3	0·7
S	18·9	—	1·4	1·3/1·7/1	32·4	44·5	0·7
Whole colostrum casein	13·4	0·85*	4·7	1·2/0·9/1	30·0	21·6	1·5
C_1*	10·5	0·67*	3·5	1·8/1·7/1	33·1	32·0	1·0
C_3*	15·7	1·17*	3·9	1·2/0·7/1	31·5	19·3	1·7
C_4*	22·5	1·52*	6·7	1·0/0·3/1	40·7	14·0	2·9

M and S, fractions from one normal milk; M, casein obtained from the micellar fraction which sedimented during centrifugation at 30 000 g for 30 min; S, 'soluble' casein which was not sedimented in 135 min under the same g force; C_1 to C_4, fractions from one colostrum; C_1 and C_3, caseins obtained from micellar fractions of decreasing sedimentation coefficients; C_4, soluble casein.

* Values from Ribadeau Dumas and Veaux (1964).

DISCUSSION

Very low levels of rennin are able to coagulate milk. However, κ-casein, the enzyme's target, which is attacked during milk clotting, represents only about 10–15 % of whole casein and is located in micelles which are of large bulk as compared to the size of the enzyme molecule.

Three hypotheses are possible for explaining the high rate of rennin action on milk: 1, equilibrium is quickly established between micellar and soluble caseins in milk, and the rennin acts only on soluble casein in which κ-casein would be easily accessible; 2, κ-casein is on the surface of the micelles; 3, κ-casein is distributed within the micelles, but is freely accessible to the enzyme, due to a sponge-like structure of the particles.

It was shown (see Table 1) that after 30 min soluble casein-free micellar suspensions reform only one third of the original soluble casein, that is about 1 % of the total casein content. On the other hand, CPA is able to react with more than 70 % of each casein subunit within 5 min, without any apparent change in the micellar integrity of milk. This rules out the first hypothesis since the rate at which the equilibrium is established appears to be considerably slower than the rate of CPA or rennin action. A similar finding was recently reported by Rose (1968), from different experiments.

Steric hindrance does not prevent the penetration of rennin into the micelle, as is clearly indicated by the ease of penetration by dansyl chloride, myoglobin and CPA. CPA in particular is very similar to rennin in size and electric charge.

Dansyl chloride mainly reacts with the ϵ-NH_2 groups of the lysine residues. The number of lysyl residues is broadly similar for the 3 casein subunits (respectively 11·8, 9·1 and 9·6 for 20000 *g* of α_{s1}-, β- and κ-caseins). Although electrophoresis of dansyl chloride-treated milk gave only qualitative data, no preferential binding of the reagent to any of the casein subunits was observed. Furthermore, binding was independent of the degree of aggregation and indeed occurred faster with the micelles. The reaction was completed in 15 min, and hence was much more rapid than the dissociation of micelles to soluble casein.

Surprisingly the C-terminal residues of each casein subunit were accessible to CPA in the micellar state: if to the values shown on Table 4 or 5 we add the α_{s2}- and α_{s3}-caseins (approximately 10 % of whole casein), it is apparent that virtually all the casein molecules of milk are accessible to the enzyme.

These experiments suggest that the casein micelles in milk have a sponge-like or a lattice structure in which the channels or cavities are penetrable by large molecules such as that of CPA.

Kinetic studies of the action of CPA cannot provide any indication as to the location of κ-casein in the micelles, since free diffusion through a micelle of 100 mμ in diameter would take only 10^{-4}–10^{-5} s (an approximate calculation was made of the diffusion rate of CPA, whose molecular dimensions and partial specific volume are known, through a micelle of 100 mμ in diam., in the conditions of viscosity and temperature of the experiment shown in Fig. 2). This is a considerably shorter time than is needed for the enzymic reaction, which can be estimated from Fig. 2. The hypothesis of an outer location (see above) is at first sight plausible, since it would explain the increase in κ-casein content in slower sedimenting micelles. But it can no longer be retained with sponge-like micelles, since nothing would then prevent the complete aggregation of α_{s1}- and β-Ca-caseinates in the bulk of the micelle. This certainly does not occur since, as has been found by Waugh & Noble (1965), hydration of α_{s1}-Ca-caseinate is about half that of the micelles. On the other hand, the special structure of the micelles probably excludes any straightforward relationship between sedimentation coefficient and size since they would behave as porous bodies for which the usual assumptions made for sedimenting impermeable particles would no longer apply.

On the basis of these results we have recently proposed (Garnier & Ribadeau Dumas, 1969) a model in which the essential features of micelle behaviour can be readily explained by the location of κ-casein at the nodes of a network made by the casein polymer.

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Milk enzymes—their distribution and activity

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Summary. The distribution and activity of alkaline phosphatase (E.C. 3.1.3.1), acid phosphatase (E.C. 3.1.3.2), catalase (E.C. 1.11.1.6), xanthine oxidase (E.C. 1.2.3.2), aldolase (E.C. 4.1.2.7 and 4.1.2.13), ribonuclease (E.C. 2.7.7.16) and carbonic anhydrase (E.C. 4.2.1.1) were studied in the major components of bovine milk. Fractionation was accomplished by centrifugation of milk, skim-milk and buttermilk, and ammonium sulphate precipitation of skim-milk serum. The range of activities found for the enzymes studied are tabulated together with the activities of some of the enzymes in mastitic milk, and the significance of the results obtained is discussed. No carbonic anhydrase activity was detected in any of the samples tested. The other enzymes studied were found to have a greater proportion of their total activity located in the skim-milk fraction. However, all of these enzymes except ribonuclease had a higher specific activity in the fat fraction.

Milk produced from normal healthy cows contains a wide variety of enzymes. These enzymes are believed to be derived from the secretory epithelial cells of the mammary gland, and their presence may be regarded as due to a 'spilling over' from these cells and serum during the milk secretion process. Several reviews have been published which describe some of the known properties of milk enzymes and propose explanations of their significance (McKenzie, 1967; Shahani, 1966; Corbin & Whittier, 1965). One fact appears to stand out in these reviews, namely that for most of the enzymes the distribution between the major components of milk has not been fully investigated. Knowledge of this distribution in milk would be beneficial to research investigations in the following ways. Firstly, it would indicate the best starting point for further isolation and purification of selected enzymes. Secondly, the possible existence of isoenzymes could be better investigated. Thirdly, the significance of the enzymes in dairy technology could be studied in more detail. Finally, determination of the activity of an enzyme might serve as a possible method for detection of udder diseases and other disorders. For these reasons the distribution and activity of the following enzymes in milk have been studied: alkaline phosphatase (E.C. 3.1.3.1); acid phosphatase (E.C. 3.1.3.2); catalase (E.C. 1.11.1.6); xanthine oxidase (E.C. 1.2.3.2); aldolase (E.C. 4.1.2.7 and 4.1.2.13); ribonuclease (E.C. 2.7.7.16); carbonic anhydrase (E.C. 4.2.1.1).

MATERIALS AND METHODS

Fractionation procedure

Fresh bulk raw milk was obtained from a local dairy herd of 50 animals, mainly of Friesian breed. Milk was collected from the bulk vat at 35–37 °C and was transported to the laboratory in sealed stainless steel containers. The temperature on receipt was 33–35 °C. The milk was immediately fractionated as shown in Fig. 1. The whole

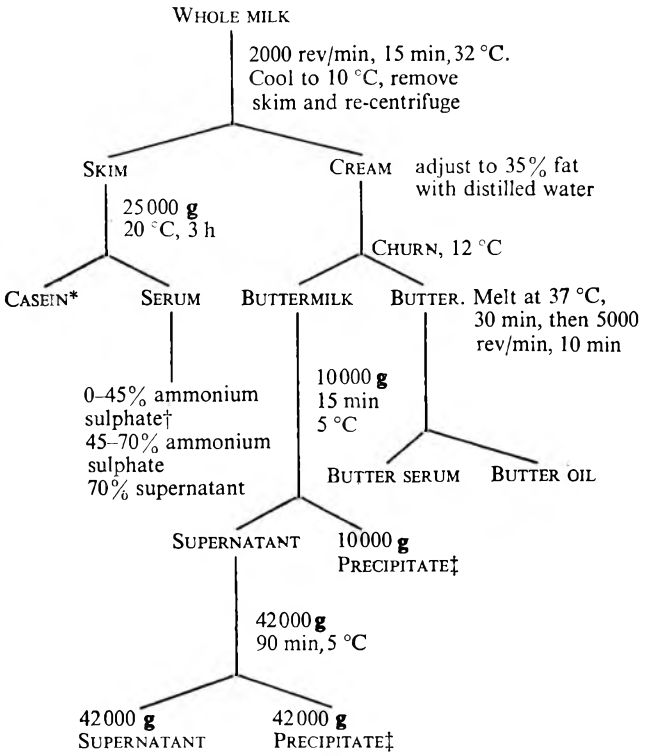

Fig. 1. Fractionation procedure. * Suspended in 0·1 M-NaCl. † All dialysed against 0·1 M-NaCl before assaying. ‡ Suspended in 0·05 M-NaCl.

milk was separated in an M.S.E. Mistral 6 L refrigerated centrifuge at 2000 rev/min for 15 min at 32 °C, using a No. 91301 swing-out head. The centrifuge bottles were then cooled to 10 °C in order to facilitate separation of the two layers, and the skim-milk was removed by means of a siphon and re-centrifuged at 10 °C to remove the last traces of fat. The skim-milk was then centrifuged for 3 h at 25000 *g* and at 15–20 °C, in a Sorvall model SS-4 centrifuge with an SS-34 angle head rotor. This treatment removed most of the micellar casein. Buttermilk was centrifuged at 42000 *g* for 90 min at 5 °C in an M.S.E. model superspeed 50 T.C. ultracentrifuge with a 10 × 100 ml angle head rotor. Other centrifugations were performed in an International model HR-1 refrigerated centrifuge. The cream was churned in a laboratory buttermaker (Dolby, 1954).

Chemicals

Hypoxanthine, *p*-nitrophenyl phosphate, yeast RNA, and fructose 1,6-diphosphate were obtained from the Sigma Chemical Company. Ammonium sulphate was of special enzyme grade (heavy metal free) from Mann Research Laboratories Inc., 136 Liberty St., New York. All other chemicals and reagents were of the highest purity available.

Assay procedures

Xanthine oxidase was assayed by monitoring the decrease in oxygen content of the test solution, using a Beckman Process Oxygen Analyser Model 778, connected to a Hitachi QPD54 strip chart recorder. Assays were carried out in the presence of 10^{-4} M hypoxanthine, 10^{-4} M-EDTA, and 0·1 M phosphate buffer of pH 7·8. The temperature of the reaction vessel was thermostatically controlled at $25 \pm 0{\cdot}5$ °C. One unit of activity was defined as a decrease in oxygen content of 1 μmole/min at 25 °C under the specified conditions.

Catalase activity was determined by monitoring the production of oxygen from 0·16 M hydrogen peroxide, in 0·10 M phosphate buffer of pH 7·0 at 25 °C. The instrument used was the same as that described for xanthine oxidase. One unit was defined as an increase of 1 μmole of oxygen/min under the specified conditions.

Acid phosphatase was assayed by a method adapted from that of Malveaux & San Clemente (1969). The test material was incubated for 1 h in presence of $3{\cdot}8 \times 10^{-3}$ M *p*-nitrophenyl phosphate in 0·4 M acetate buffer of pH 4·0. The unit of activity was defined as that amount of enzyme required to produce 1 μmole of *p*-nitrophenol/min at 37 °C.

Alkaline phosphatase activity was determined by the method of Garen & Levinthal (1960), modified by the use of an initial substrate concentration of $3{\cdot}16 \times 10^{-3}$ M *p*-nitrophenyl phosphate in 0·2 M carbonate buffer of pH 10·2. One unit of activity was the amount of enzyme required to produce 1 μmole of *p*-nitrophenol/min at 25 °C.

Ribonuclease was assayed by the method of Kalnitsky, Hummel & Dierks (1959). One unit of activity was equivalent to that amount of acid soluble oligonucleotides which caused an increase in absorbance of 1·00 at 260 mμ.

Aldolase was assayed by a slight modification of the method of Sibley & Lehninger (1949). This involved using 1·0 ml of enzyme solution, 0·6 ml of 0·5 M tris buffer of pH 8·6, 0·2 ml of 0·56 M hydrazine of pH 8·6, the reaction being started by the addition of 0·2 ml of 0·05 M fructose 1,6-diphosphate. One unit of activity was equivalent to 0·0445 μmoles of fructose 1,6-diphosphate split/h at 37 °C.

Carbonic anhydrase was assayed by the method of Wilbur & Anderson (1948). The fall in pH was monitored automatically by the connexion of a Hitachi QPD54 strip chart recorder to a Metrohm E350B pH meter.

Protein was determined by the method of Gornall, Bardawill & David (1949).

RESULTS

The fractionation procedure adopted is shown in Fig. 1. It resulted from many attempts to develop a quick and satisfactory method whereby all the fractions could be obtained within 48 h. The procedure made possible the complete assay of all enzymes within 5 days of the collection of bulk-milk samples. The reproducibility of this procedure was good as is shown in Table 1.

Table 1. *Protein distribution in different milk fractions*

(Protein was determined by the Biuret method. In samples with a high fat content, 2 ml of diethyl ether were added before reading the absorbance.)

Fraction	Protein recovery, %	
	Range	Average
Whole milk	100	100
Skim-milk	94·5 –96·5	95·6
Cream, 35 % fat	3·4 – 4·8	4·1
Buttermilk	2·5 – 3·5	3·1
Butter serum	0·9 – 1·1	1·0
10000*g* precipitate	0·08– 0·24	0·16
42000*g* precipitate	0·8 – 1·0	0·94
42000*g* supernatant	0·6 – 0·8	0·72
Serum	13·3 –19·0	13·8
Casein	62·0 –75·0	68·0
0–45 % Ammonium sulphate precipitate	5·0 – 6·5	5·5
45–70 % Ammonium sulphate precipitate	4·2 – 6·2	5·4
70 % Ammonium sulphate supernatant	1·4 – 2·8	2·1

Table 2. *Range of enzyme activities in normal and in mastitic whole milk*

Enzyme	Activity, units/ml*
Catalase (normal milk)	7·5–36·0
Catalase (mastitic milk)	175·0†
Xanthine oxidase (normal milk)	15·6–21·4
Xanthine oxidase (mastitic milk)	9·4†
Aldolase (normal milk)	5·04–8·7
Aldolase (mastitic milk)	56·5†
Ribonuclease	19·2–35·4
Acid phosphatase	0·0026–0·0037
Alkaline phosphatase	0·18–0·27

* Catalase, xanthine oxidase, acid phosphatase and alkaline phosphatase activities are expressed in μmoles product formed/min. Aldolase and ribonuclease activities are expressed in units as described in the text. The activities expressed for ribonuclease, acid phosphatase, and alkaline phosphatase are from normal milk.

† Bulked milk from infected animals.

The aim of the initial centrifugation of whole milk was to obtain a cream containing very little skim-milk protein. This would produce a clear separation between skim-milk enzymes and those associated with the fat globules. The cream obtained had a fat content of 65–70 %. Assay of cream and skim-milk layers for a number of enzymes after separation at 32 °C and at 10 °C showed no significant change in the partition of the enzymes between these 2 fractions.

The range of enzyme activities in the raw whole milks that were used is shown in Table 2. Milk from individual animals, shown by bacteriological analysis to have mastitis, was bulked and assayed for catalase, xanthine oxidase and aldolase, and the results are shown in Table 2.

Table 3 shows for each enzyme the percentage distribution and purification factor for the individual fractions, relative to whole milk.

For xanthine oxidase, 54 % of the whole milk activity was located in the skim-milk and 21 % in the cream. Of the cream xanthine oxidase 65 % was released on churning into the buttermilk, giving an overall purification factor of 5·7. Centrifugation of buttermilk resulted in a 40 % recovery of the activity in the 42000 *g* precipitate, but the purification factor of 1·9 was approximately one-third of that of the original buttermilk. In skim-milk, the enzyme was not associated with the casein, but precipitated on 45 % ammonium sulphate saturation of the serum.

In the case of catalase, skim-milk contained 73 % of the whole milk activity and cream contained 24 %. However, this enzyme, like xanthine oxidase, had a higher specific activity in cream than in skim-milk. Recoveries of greater than 100 % were obtained when the cream was churned and the buttermilk and butter serum recovered. The 42000 *g* precipitate gave the best recovery of buttermilk activity while the 10000 *g* precipitate had the highest specific activity. Of the whole milk units 59 % were present in the serum, while 8 % were found in the casein fraction. This enzyme, like xanthine oxidase, was precipitated in the 45 % ammonium sulphate fraction of serum, with 39 % recovery and a purification factor of 4·6.

Aldolase had 66 % of its whole milk activity associated with skim-milk and 19 % with cream. Buttermilk contained 10·6 % of the whole milk activity while the butter serum contained 1·5 % of the activity. The results show that the highest recovery obtained on centrifugation of buttermilk was in the 42000 *g* supernatant. In this fraction 63 % of the buttermilk activity or 6·7 % of the whole milk activity was recovered with a purification factor of 14. Serum contained 44 % of the whole milk activity, while the casein fraction contained 15·8 %. The precipitation of the enzyme with ammonium sulphate resulted in significant recoveries of activity in all fractions. The most unusual result was that 90 % of the original serum activity (44 % of the whole milk activity) was found in the 70 % ammonium sulphate supernatant.

Unlike the three preceding enzymes, ribonuclease had a lower specific activity in cream than in whole milk. Recoveries of greater than 100 % were found when whole milk was separated into skim-milk and cream. Very little activity was associated with the cream. Serum and casein contained 54 % and 25·5 % of the whole milk activity respectively. The 45–70 % ammonium sulphate precipitate of serum contained 12·4 % of the whole milk activity, while the 70 % supernatant contained 62 % of the activity and gave a 13-fold purification. Aldolase similarly showed high recovery and purification in the 70 % ammonium sulphate supernatant.

For acid phosphatase, 43 % of the whole milk activity was located in the skim-milk, but the specific activity of the enzyme was higher in cream. On churning the cream, the enzyme was released into the buttermilk with a recovery of 108 % and purification factor of 8·1. Centrifugation of the buttermilk reduced the purification factor and about 60 % of the activity was lost when the buttermilk was separated into the 3 fractions. Serum and casein contained respectively 16·4 % and 18 % of the

Table 3. *Distribution and purification of enzymes in milk. The % distribution values are relative to the values found for whole milk*

(Figures are averages of values obtained over several experiments.)

	Xanthine oxidase		Catalase		Aldolase		Ribonuclease		Alkaline phosphatase		Acid phosphatas	
Fraction	Distribution, %	Purification factor	Distribution, %	Purification factor	Distribution, %	Purification factor	Distribution, %	Purification factor	Distribution, %	Purification factor	Distribution, %	Purific tion fa
Whole milk	100	1	100	1	100	1	100	1	100	1	100	1
Skim-milk	54	0·6	73	0·7	66	0·6	106	1	47	0·5	43	0·4
Cream	21	4·7	24	11·9	19	4·6	3·3	0·7	31	6·3	23	5·4
Buttermilk	13·6	5·7	25	25·0	10·6	3·6	1·6	0·4	31	9·2	24·8	8·1
Butter serum	3·3	2·7	3·0	5·1	1·5	2·7	0·7	0·6	7·5	6·2	2·7	3·1
Buttermilk:												
10000 *g* precipitate	1·4	4·5	6·0	89·0	0·7	3·4	0·1	0·6	1·5	7·4	1·7	5·5
42000 *g* precipitate	5·6	1·9	10·6	32·0	3·8	4·7	0·32	0·5	9·4	8·7	3·0	1·4
42000 *g* supernatant	3·8	1·2	3·5	17·0	6·7	14·0	1·0	1·5	17	27·0	5·2	5·2
Casein	2·7	—	8·0	0·1	15·8	0·2	25·5	0·3	8·0	0·1	18	0·2
Skim-milk serum	26·5	1·9	59·0	3·1	49	2·3	54	3·2	30·5	2·3	16·4	1
Precipitate of serum at ammonium sulphate concentration: 0–45 %	13·8	2·0	39·0	4·6	13·2	2·5	4·3	0·7	23·5	4·5	8·7	1·2
45–70 %	1·6	—	5·3	0·3	20	1·9	12·4	1·8	5·1	2·2	4·9	0·7
70 % ammonium sulphate supernatant	—	—	—	—	44	8·7	62	13·0	—	—	1·6	3·3

activity of the whole milk. Ammonium sulphate precipitation of serum gave 8·7 % recovery of the whole milk activity in the 0–45 % fraction. A further 4·9 % of the activity was found in the 45–70 % precipitate. A 3·3-fold purification was obtained with the 70 % ammonium sulphate supernatant of skim-milk serum.

The distribution of alkaline phosphatase was similar to that of the acid phosphatase. Although most of the activity of the whole milk was found in the skim-milk fraction, the specific activity of the enzyme was higher in cream than in skim-milk. Churning the cream resulted in greater than 100 % recoveries of activity in the buttermilk. The enzyme was purified 9·2- and 6·2-fold in the buttermilk and butter serum respectively. On centrifugation of buttermilk, 54 % of the original buttermilk activity (or 17 % of the whole milk activity) was recovered in the 42000 *g* supernatant, with a 27-fold purification factor. Skim-milk serum contained 30 % of the whole milk activity and the casein fraction 8 %. Most of the activity in the serum was precipitated at 45 % ammonium sulphate saturation, with 23 % recovery of the whole milk activity and a 4·5-fold purification. Some activity was also detected in the 45–70 % ammonium sulphate precipitate.

No carbonic anhydrase activity could be detected in any of the milks tested.

DISCUSSION

The enzyme activities in the bulk milk used varied considerably for catalase, but much less for the other enzymes.

The high activity of catalase and aldolase in mastitic milk indicates that these enzymes could possibly serve as a diagnostic aid in the early detection of udder diseases. These results also suggest that catalase and aldolase may appear in milk as a result of direct 'spillage' from the blood serum. In contrast, xanthine oxidase activity did not increase in mastitic milk, and this finding is consistent with this enzyme being a 'true' milk enzyme, synthesized in the udder and fulfilling a particular function in the milk.

Bovine milk is rich in xanthine oxidase, and the enzyme has been implicated in the development of oxidized flavours in milk (Aurand, Chu, Singleton & Shen, 1967). For this reason an investigation into its distribution in various milk fractions is of special interest. The present results are in general agreement with those of previous workers who have studied the distribution of xanthine oxidase in skim-milk and cream (Ball 1939; Zittle, Dellamonica, Custer & Rudd, 1956; Pereira, Kristoffersen & Harper, 1962).

Zittle *et al.* (1956) found that after centrifuging buttermilk at 105000 *g* for 30 min approximately 70 % of the xanthine oxidase activity was concentrated in the microsomal pellet. The results presented here are similar, in that approximately 51 % of the buttermilk activity was associated with the microsomal component.

Hwang, Ramachandran & Whitney (1967) found that all the major milk proteins were activators and inhibitors of xanthine oxidase, depending upon their concentrations in the particular fraction being studied. The presence of a non-dialysable inhibitor in the protein-free milk fraction was also detected. These findings could account for the low recoveries found for casein, and for the ammonium sulphate fractions. Thus, the true distribution of activity of xanthine oxidase as it exists

naturally in milk may be different from that found from activities in the individual separated fractions.

The finding that 3·3 % of this enzyme's activity remained associated with the fat globules after churning indicates that it may be important in the production of certain oxidized flavours in butter.

Catalase has never been isolated and purified from milk and little is known about its heat stability, its distribution, or its structural properties. It is known that catalase oxidizes unsaturated fatty acids (George, 1952). Its action results from the non-enzymic activity of its haem group. Therefore, a knowledge of the distribution of this enzyme should assist in clarifying its importance in relation to oxidative defects in milk and other dairy products. This enzyme's distribution was found to be very similar to that of xanthine oxidase, both being associated in high concentrations with fat globules and the lipo-protein particles of buttermilk. Thus, catalase activity could also be significant in the degradation of unsaturated fatty acids in milk and related products. McMeekin & Polis (1949) stated that catalase was associated with casein because both were precipitated at 45 % ammonium sulphate saturation. The results presented here, however, indicate that very little catalase activity was associated with casein, and in fact by removal of the casein by centrifugation the enzyme can be purified. As catalase is removed from serum at 45 % ammonium sulphate saturation, this would account for the observation of McMeekin & Polis.

Aldolase has been reported only once previously in bovine milk, by Polis & Shmukler (1950), the activity being approximately at the same level as that of blood serum. They found that the enzyme was precipitated in the 50–60 % ammonium sulphate fraction. Herskovits, Masters, Wassarman & Kaplan (1967) recently showed that aldolase exists in multiple forms. This could explain the wide distribution found in the ammonium sulphate fractions of serum.

Morton (1956) has shown that the microsomes from bulk milk contain about 0·25 % nucleic acid, which apparently serves as binding material for the other constituents of the microsomes. Brunner (1965) has postulated that a breakdown of the nucleic acid by ribonuclease could affect the stability of the microsomes and consequently of the fat globules. Ribonuclease has been isolated and purified from milk by Bingham & Zittle (1964) and was found to be identical with pancreatic ribonuclease A. These workers found that this enzyme was present in the fat-free protein fraction of milk. In the present investigation also, the activity of the enzyme in the cream and buttermilk fractions was found to be extremely low. Therefore, it would seem that the significance of the action of this enzyme in microsomal particles should be investigated in more detail before any definite statement can be made about its role and significance in milk.

The absence of alkaline phosphatase activity in pasteurized milk is used as an indication of effective pasteurization, and this was one of the reasons why a study of the distribution and concentration of this enzyme in various milk constituents was undertaken. Results obtained in this investigation on whole milk agree with those obtained by Morton (1953*a*), but centrifugation of buttermilk showed a different pattern from that found by Morton (1953*b*), who found that 83 % of the alkaline phosphatase activity of buttermilk was associated with the microsomal precipitate, whereas the present findings showed that the bulk of the activity was associated with

the supernatant of centrifuged buttermilk. A similar discrepancy exists in the results from centrifuged skim-milk. However, Zittle *et al.* (1956) found similar results to those reported here for the activity distribution in centrifuged buttermilk. Copius Peereboom (1968) suggests that there are 2 types of alkaline phosphatase in cream. One type, α alkaline phosphatase, is associated with the outer lipo-protein layer of the fat globule membrane, while the other type, β alkaline phosphatase, remains more closely associated with the fat globule surface. On churning cream both these enzymes would be released into the buttermilk, and they could possibly be associated with different types of particles. This may explain the inconsistencies of distribution of the alkaline phosphatase reported in buttermilk fractions. Another factor affecting the distribution could be the severity of churning which could affect the physico-chemical structure of the fat globule membrane, causing the release of a different range of lipoprotein particles from one experiment to another.

Acid phosphatase is one of the most heat-stable of the enzymes found in milk and it has been reported that acid phosphatase possesses activity towards casein (Bingham & Zittle, 1963; Zittle, 1964). In this investigation it was found that the casein fraction contained high acid phosphatase activity, and this close association of enzyme and substrate could be of interest in the elucidation of this enzyme's significance in milk. The low recovery of activity in the 3 centrifuged buttermilk fractions may possibly indicate the presence of an activator in the buttermilk. The results are consistent with the activator being associated with a particular size of particle which could be separated from the enzyme by centrifugation. The wide range of activity found in the ammonium sulphate fractions of serum may indicate that there is more than one type of acid phosphatase in milk, but further investigations would be necessary to clarify this point.

Although carbonic anhydrase could not be detected in the bulk milk tested, this enzyme may well be present in milk containing large numbers of red blood cells, as happens in severe cases of mastitis.

In all, the present investigation has shown that only one of the enzymes studied, ribonuclease, is principally found in the non-fat fraction of whole milk. The other enzymes were distributed between the skim-milk and cream in varying degrees.

The fractionation of buttermilk by centrifugation showed that the lipoprotein particles exist in a wide range of sizes and that certain enzymes appeared to associate themselves with particular sized particles. For example, xanthine oxidase and catalase appeared to be attached to a particle that can be removed from buttermilk after centrifugation for 90 min at 42000 *g*. The remaining 4 enzymes were mainly associated with smaller particles which could not be removed by centrifugation at 42000 *g* for 90 min. Zittle *et al.* (1956) came to a similar conclusion in a study of xanthine oxidase and alkaline phosphatase distribution. Further work remains to be done to determine whether these particles in buttermilk exist in discrete sizes or in a complete range of sizes.

The investigation has indicated some possible procedures for the purification of the enzymes of milk. The results indicate that catalase may be purified 100-fold by applying appropriate centrifugation procedures to buttermilk. Similar conclusions apply to xanthine oxidase. Useful indications are also provided for the other enzymes studied.

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Apparatus and milking techniques used in lactation studies with sheep

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Summary. A description is given of a simple milking apparatus for collecting the yields of individual ewes, and of the milking procedure used in studies of lactation in ewes of 'mutton' breeds. The incidence of mastitis, level of milk yield, some of the non-nutritional factors affecting the milk yield and the coefficients of variation of milk yield and composition are discussed in relation to data from 6 experiments in which ewes of mutton breeds were machine-milked twice daily for 6–9 weeks of lactation.

Ewes are milked in large numbers in Europe and in the Mediterranean region, to produce milk which is largely used for manufacturing into cheese and yoghurt (Boyazoglu, 1963). Spedding (1962) has suggested machine milking as a means for measuring milk yield in lactating ewes and this procedure was used by Graham (1964). The results of a number of experiments at the Grassland Research Institute indicate that machine milking can be a useful experimental technique in studies of lactating ewes from 'mutton' breeds, particularly when accurate measurements both of total milk yield and milk composition are required. But it is important that the limitations of the technique should be clearly realized, and in particular the influence on the level and variability of milk yield.

Morag, Gibb & Fox (1967) described a complex milking parlour for ewes, based on the 'arrête de poisson' type of parlour used in the Roquefort region of France (Bosc, 1962). The first section of the present paper describes a simple milking machine unit that has been developed with the assistance of Alfa-Laval (U.K.) Ltd. This unit has been used for daily milkings in a 3-a-side chute parlour in which the ewes were milked from the side. It has also been used for the removal and collection of milk when estimating milk secretion rate by the technique described by McCance (1959). The procedures adopted for the daily milking of ewes of mutton breeds are also described.

Milking equipment

Fig. 1 shows the milking equipment and gives Alfa-Laval (U.K.) Ltd numbers for standard parts. The equipment consists of (i) 2 teatcups and claw; (ii) collecting jar and mounting; (iii) vacuum tap and magnetic pulsator. All of these are mounted as a unit on the 1-in. B.S.P. vacuum piping. Rubber tubing of $\frac{5}{16}$ in. int. diam. is used for the milk and pulse tubes except for a short piece of $\frac{3}{8}$ in. tubing from the milk

outlet of the claw-piece linked by a reducing coupling to the long milk tube. An air admission hole of 0·025 in. diam. is drilled in this coupling.

The collecting unit was designed to collect the milk from an individual ewe into a screw-top (Kilner) jar of appropriate size. The numbered and weighed jars are reweighed after milking, thus eliminating the need to measure, record and sample during milking. The collecting unit is mounted on a plate which is bolted into a pipe mounting on the vacuum pipe.

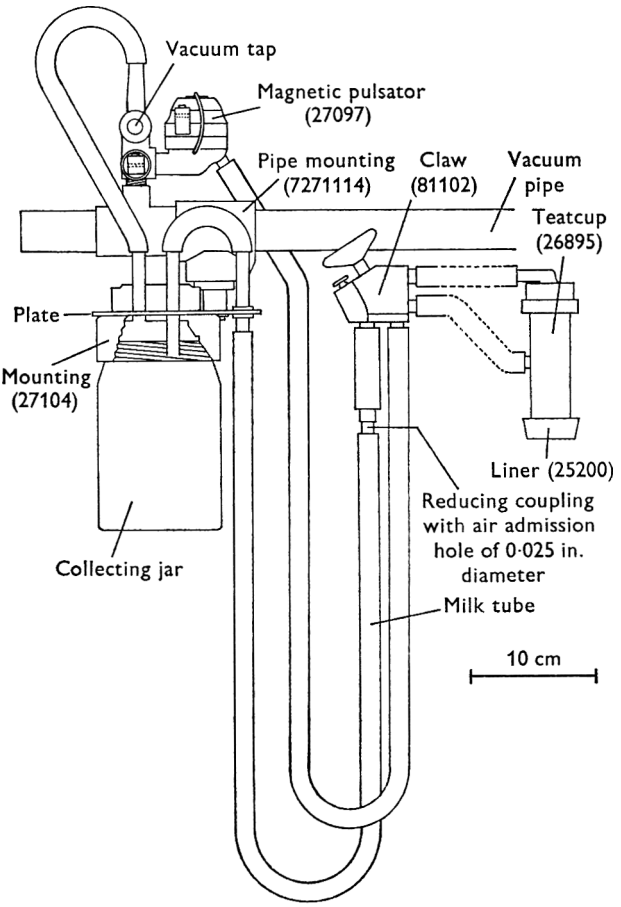

Fig. 1. Milking apparatus used in lactation studies in sheep.

Operating details of the milking machine

Vacuum level

A vacuum level of 13·5 in. (34·3 cm) of mercury was used.

Pulsation rate and pulsator ratio

A pulsation rate of 170 pulsations/min was used on the machines at this Institute. The pulsator ratio (as defined by Thiel, Claesson & Rabold, 1969) was 0·54. This resulted in the liner being open for approximately 40 % of the pulsation cycle.

Work in France in the 1930s indicated that the minimum milking time was

achieved with a pulsation rate of 175–180 pulsations/min (Galtier, cited by Bosc, 1966), although there was little difference in milking time with pulsation rates between 150 and 200 pulsations/min.

A pulsator ratio of 0·33 was used in the original milking machines developed in France (Bosc, 1966). The ratio was later changed to 0·5. The measurements on the Institute machine would suggest that a pulsator ratio of 0·5 results in the liner being open for about 40 % of the pulsation cycle. Therefore, the milking time would probably be decreased by increasing the pulsator ratio. Dimov, Goranov, Garanicheva & Dobrev (1965) found that the shortest milking time with the lowest stripping percentage resulted from using a milking machine with pulsation rate of 150 pulsations/min and a pulsator ratio of 0·66.

Milking procedure

The following procedures were adopted when milking ewes twice daily throughout lactation.

Training period

Because nutritional treatments were imposed during late pregnancy the ewes were always brought into individual pens close to the milking parlour approximately 6 weeks before lambing. When the ewes were taken out of their pens for weighing they were made to walk through the parlour. Approximately a month before lambing was due, the milking machine was run as the ewes passed through the parlour to accustom them to the noise. Then each day for about 2 weeks before lambing the ewes were held in the milking stalls and their udders massaged.

This procedure may not be necessary but it appears sensible to accustom the animals as much as possible to the milking parlour.

Suckling period

The lambs were left with the ewe for 12–16 h after parturition, in order to obtain colostrum. They were then removed and reared on milk substitute. The ewe was milked at the next milking. Although the ewes were very rarely disturbed or frightened, even at the first milking, only small quantities of milk were obtained at the first and second milkings.

Milking routine

The ewes were milked twice daily at 16- and 8-h intervals. Approximately 66 % of the total daily yield was obtained at the morning milking following the 16-h interval. The routine described here includes the measures taken to control the incidence of mastitis. The problem is discussed more fully below.

The ewe's udder was washed with an individual paper towel that had been dipped in an iodophor solution (Iosan CCT, CIBA Laboratories Ltd, Horsham, Sussex) diluted to contain 100 ppm available iodine, and then dried with a second paper towel. New towels were used for each ewe. Udder washing was carried out to stimulate milk ejection rather than for reasons of hygiene and may not be essential. In Europe, ewes are not normally washed before milking by machine. The teatcups

were then placed on the teats. As soon as the flow of milk ceased, machine stripping with udder massage was carried out. The ewes were not hand-stripped, although in Europe it is customary to hand-strip after machine milking. Morag *et al.* (1967) used a milking routine in which machine-stripping replaced hand-stripping and found it to be satisfactory. At the end of milking the teats were immediately dipped in an iodophore solution containing 5000 ppm available iodine.

Discussion of results obtained with the machine

Six experiments were carried out with the machine between 1963 and 1968. In each experiment the machine was used twice daily for 6–9 weeks of lactation, starting from 8–16 h post-partum when the lamb was removed from the ewe. Details of expts 1–4 have been given by Treacher (1967, 1970); the results of expts 5 and 6 have not been published hitherto.

Incidence of mastitis and methods of control

The incidence of mastitis infections in 6 experiments in which ewes were machine-milked daily through lactation is shown in Table 1. The pathogen was in all cases *Staphylococcus aureus*. Clinical infections were defined as those infections in which

Table 1. *Incidence of mastitis infections in machine-milked ewes*

Expt and year	Length of milking period, weeks	No. of ewes	Cases of infection		
			Clinical	Acute	Total
1 1963	9	24	0	6	6
2 1963	9	9	0	0	0
3 1964	6	31	2	6	8
4 1965	6	32	6	2	8
5 1966	6	20	1	0	1
6 1968	9	5	0	0	0
Total	—	121	9	14	23
% of total ewes infected			7·4	11·5	19·0

clots were found in the fore-milk but lactation continued, though at a lower level. In acute infections the ewe either died or the infected half of the udder became gangrenous within a few hours of infection. These cases usually showed no clinical symptoms at the milking before the infection was discovered. In 287 lactations of ewes milked in experiments by Plommet & Ricordeau (1960) the incidence of staphylococcal mastitis was 13 %.

After the outbreak of mastitis in the first experiment a routine for the control of mastitis was introduced similar to that used at the National Institute for Research in Dairying (1963). The milkers wore sterilized rubber gloves, each ewe's udder was washed with a fresh paper towel, the teats were dipped in disinfectant solution immediately after the milking was finished and finally the teatcups were disinfected with water at 95 °C for at least 5 s before being put on the next ewe. In subsequent experiments, the milkers did not wear rubber gloves and the teatcups were not sterilized between ewes unless there had been a case of mastitis, when the full routine was re-introduced.

No firm conclusion can be drawn on the efficacy of the control routine in reducing the incidence of mastitis in machine-milked ewes. There were no cases of mastitis in expt 2 and a reduced number of acute cases in expt 4, but no reduction occurred in expt 3. No measurements were made of subclinical infections. However, it seems probable that the high incidence of mastitis in expts 1, 3 and 4 was related to the housing conditions, as the incidence was greatly reduced in expts 5 and 6 which were carried out in a new animal house, in which the ewes were housed on perforated metal floors, instead of on sawdust as in the earlier experiments.

Level of milk production

The results of these experiments are set out in Table 2. In general, they support the findings of Ricordeau & Denamur (1962) that the milk yield of ewes under machine milking is 60–80 % of the yield of suckled ewes. Although no direct comparisons were made in these experiments, the milk yield approximated to or was a little lower than the level that would be expected in similar ewes suckling single lambs. For example, the yield of 1·73 kg/day obtained by Treacher (1970) at peak of lactation in machine-milked Scottish Half-bred ewes can be compared with the yield of 1·98 kg/day obtained by Wallace (1948) from ewes of the same breed but fed at a higher level both in pregnancy and lactation. There are no published figures for the milk yield of suckled Dorset Horn ewes.

This reduction in the milk yield of milked ewes, as compared with that of ewes suckling lambs, does not appear to be influenced by frequency of milking. Morag (1969) found no difference and Semjan (1961) a difference of 5 % between the yields of ewes milked twice or three times daily. However, Gaal (1957) reported a 23 % higher milk yield on milking 3 times daily.

The effect on milk yield of eliminating hand-stripping from the milking routine is difficult to assess. In European countries where machine-milked ewes are generally hand-stripped, approximately 10 % of the total yield is obtained in the hand-strippings (cf. Ricordeau, Martinet & Denamur, 1963). However, the reduction in milk yield resulting from omitting hand-stripping from the milking routine may be greater than the actual quantity of milk that would have been obtained by hand-stripping. Thus, Ricordeau *et al.* (1963) observed a reduction of 29 % with mature ewes, although Bosc, Flamant & Ricordeau (1967) found a reduction of only 7 % with ewe lambs. Dimov, Taner & Shalichev (1966) found no difference between the milk yield of low yielding ewes that were machine- or hand-stripped. These results suggest therefore that omitting hand-stripping from the routine of ewes that had not previously been milked is unlikely to reduce milk yield by more than 10 %.

Variation in milk yield and milk composition in machine-milked ewes

The data in Table 2 indicate that there were considerable differences between experiments in the size of the coefficient of variation of total milk yield. Age and weight variation in the groups and nutrition in pregnancy appear to have influenced the size of the coefficient of variation. In expts 1 and 5, with groups of ewes matched for age and previous treatment, the coefficients of variation were similar to those found for suckled ewes in the experiments of Barnicoat, Murray, Roberts & Wilson (1957) and Owen & Ingleton (1963). In expts 2 and 4 there was more variation within

Table 2. *Means and coefficients of variation (c.v.) for milk yield and composition*

Reference	Breed and no. of ewes	Length of lactation, weeks	Total milk yield		Fat		Solids-not-fat		Crude protein		Lactose	
			Mean, kg	c.v., %	Mean, %	c.v., %	Mean, %	c.v., %	Mean, %	c.v., %	Mean, %	c.v., %
			Machine-milked ewes									
eacher (1967)	Dorset Horn 23*	9	83·1	20·1	6·83	10·4	10·84	2·5	5·13	4·9	4·87	2·6
eacher (1967)	Dorset Horn 8	9	90·4	35·9	6·55	9·6	10·67	2·7		—		—
eacher (1970)	Scottish Half-bred 21	6	41·5	52·1	5·46	13·8	11·76	3·7	6·16	6·1	4·70	5·9
eacher (1967)	Dorset Horn 23	6	57·9	31·1	7·77	7·6	10·40	3·6	4·98	6·9	4·69	5·5
T. Treacher (unpublished)	Dorset Horn 18	6	51·0	25·4	7·75	9·8	10·56	3·5	4·97	7·5	4·76	3·4
T. Treacher (unpublished)	Dorset Horn 5	9	91·3	48·1		—		—		—		—
eordeau & Labussière (1968)	Lacaune 101	†	30·1	26·3		—		—		—		—
			Suckled ewes									
ven‡	Clun 14	10	130·0	20·8	5·3	16·1	11·4	3·3	5·2	5·5	5·0	3·8
rdner & Hogue (1964)	Rambouillet and Columbia 20	13	175·3	15·8	6·9	13·8		—	5·12	2·0		—
rnicoat *et al.* (1957)	Romney 33	12	73·1	27·0		—		—		—		—

* No. of ewes completing lactation.
† Between days 45 and 85 of lactation.
‡ Yield from Owen & Ingleton (1963) and composition from Ashton *et al.* (1964).

the groups of ewes in age, previous history and liveweight, and the coefficients were higher. They were still higher in expts 3 and 6 in which the potential yields of some ewes were reduced by severe under-nutrition in late pregnancy (Treacher, 1970).

The data from Table 2 suggest that the degree of variation in selected groups of machine-milked ewes of mutton breeds can be similar to that in dairy cows (Lucas, 1960) but it can also be higher, particularly in ewes that have been malnourished during pregnancy. Ricordeau & Labussière (1968) found a coefficient of variation for milk yield of 26·3% in an experiment with machine-milked ewes of the Lacaune breed.

The coefficients of variation for milk constituents were very much lower than those for milk yield. The coefficients for solids-not-fat, crude protein and lactose content were very similar to those reported by Ashton, Owen & Ingleton (1964) for milk from suckling ewes, but those for fat were lower. When compared with data for dairy heifers (Broster & Tuck, 1967; Broster, Broster & Smith, 1969) the coefficients of variation for ewes are similar for solids-not-fat percentage and lower for fat percentage.

I wish to acknowledge the assistance of Alfa-Laval, Ltd in developing this machine, and in particular to thank their former technical manager Mr Corderoy.

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The composition of ewe's milk fat during early and late lactation

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Summary. The composition of ewe's milk during the first 4 days of lactation and on the 100th day of lactation was investigated. The total fat content was highest (17·9 %) on the day of parturition but decreased rapidly to reach a level on the 2nd day after parturition that was similar to that observed on the 100th day of lactation (9·9 %).

The concentration of octadecenoic acid, which was the major fatty acid of ewe's milk, was very much higher in early lactation than in late lactation. As the concentration of octadecenoic acid decreased the concentration of the shorter chain fatty acids (6:0–14:0) increased. The major octadecenoic acid was the *cis*-9 isomer. However, the proportion of the *trans*-11 isomer increased from 5·5 % of the total octadecenoic acid concentration in early lactation to 11·9 % in late lactation. Although linoleic acid remained a minor component of the fatty acids of the milk during lactation, its concentration increased from less than 1 % during early lactation to 1·4 % by the 100th day of lactation.

Investigations have shown that there are large differences between the plasma lipid composition of the newborn lamb and that of the adult sheep (Leat, 1966). These large differences are not confined to the concentrations of the various lipid fractions in the plasma, but occur also in the fatty acid compositions of these lipid fractions. In the plasma of the adult sheep the C_{18} polyunsaturated fatty acids constitute up to 40 % of the total fatty acids in the cholesteryl ester and phospholipid fractions, whereas in the corresponding plasma lipid fractions of the newborn lamb the C_{18} polyunsaturated fatty acids are present to the extent of only about 1 % of the total fatty acids. However, during the first 3 days after birth there are increases in the concentration of total plasma lipids, changes in the relative proportions of the major lipid components (R. C. Noble, W. Steele & J. H. Moore, unpublished observations) and increases in the C_{18} polyunsaturated fatty acid contents of the plasma lipids (Leat, 1964). These increases in the concentrations of the C_{18} polyunsaturated fatty acids of the plasma lipids occurred in spite of the fact that the diet of the lamb during this period consisted entirely of ewe's milk. The milk fat of other ruminants is known to contain very small concentrations of C_{18} polyunsaturated fatty acids (Jensen, Quinn, Carpenter & Sampugna, 1967), and since our unpublished results were similar to those of Leat (1964) it was of interest to determine the fatty acid composition of the milk of the ewes during the first 4 days of lactation. For comparison, the fatty acid composition of the ewe's milk was also determined during late lactation.

EXPERIMENTAL

Animals and diets

Six adult ewes of the Cheviot breed were given a diet of hay and proprietary concentrates; water was available *ad lib.* Milk samples were taken on the day of parturition and on the 3 consecutive days after parturition. Samples of milk were also obtained during late lactation (i.e. 100 days *post partum*).

Methods of analysis

The fat was extracted from the milk with chloroform–methanol (2:1, v/v), and the fatty acid composition of the extracted fat determined by the methods previously reported (Noble, Steele & Moore, 1969). The solvent was removed from a portion of the lipid extract by means of a rotary film evaporator connected to a supply of nitrogen, and the total fat content of the milk was determined gravimetrically. The 18:1 fatty acids in the milk fat were separated into *cis-* and *trans*-isomers by thin-layer chromatography on silica gel impregnated with silver nitrate (Morris, 1966). The positional distribution of the double bond in each of these fractions was determined on a gas-liquid chromatograph fitted with a single flame ionization detector using a support-coated open capillary column (1525 cm × 0·05 cm) with a stationary phase of diethylene glycol succinate (Perkin-Elmer Ltd, Beaconsfield, England). The retention volume of each of these isomers was identified by the use of authentic acids obtained from the Hormel Institute (Austin, Minnesota, U.S.A.). The identification of each isomer was further checked by oxidation of the methyl ester fractions (Chang & Sweeley, 1962) and analysis of the resultant mono- and dicarboxylic acids by gas chromatography as described by Moore & Williams (1966).

RESULTS

Immediately *post partum* there was 17·9 % fat in the milk. On the first day *post partum* this value had declined to 14·3 %, on the second and third days it was 10·4 and 10·6 % respectively, and on the hundredth day it was 9·85 %.

The fatty acid composition of the milk of the ewes at the various stages of lactation is given in Table 1. The major fatty acid in all of the milk samples was 18:1. However,

Table 1. *Composition* (%) *of the major fatty acids present in the ewe's milk fat*

	Days *post partum*					
Fatty acid	0	1	2	3	100	S.E.
4:0	1·64	2·21	2·18	3·05	2·40	±0·804
6:0	0·26	0·33	0·73	0·63	1·34	±0·175
8:0	0·56	1·44	1·67	1·24	1·81	±0·788
10:0	1·65	1·02	1·91	1·00	4·55	±0·462
12:0	1·14	0·94	1·24	1·11	2·73	±0·286
14:0	6·82	5·77	6·09	6·14	10·60	±1·511
16:0	25·8	24·3	24·6	24·4	24·2	±2·32
16:1	1·59	1·60	1·66	1·69	0·85	±0·407
18:0	10·3	10·7	11·5	10·6	10·5	±2·13
18:1	45·3	46·2	43·9	45·1	33·4	±3·39
18:2	0·65	0·68	0·86	0·77	1·41	±0·199

the concentration of this acid decreased from about 45 % of the total fatty acids in early lactation to about 33 % in late lactation; there was a concomitant increase in the concentrations of the fatty acids from 6:0 to 14:0. There did not appear to be any marked changes in the concentrations of any of the other major fatty acids. A feature of the fatty acid composition of the ewe's milk fat was the relatively high concentration of 10:0, particularly in the samples obtained during late lactation. The concentration of 18:2 in the milk fat immediately after parturition was less than 1 % of the total fatty acids and in late lactation it was still only 1·4 %.

Table 2 shows the composition of the total 18:1 fatty acids of ewe's milk on the day of parturition and at 100 days *post partum*. At both stages of lactation the major fatty acid was the *cis*-9 isomer. The proportion of the *trans*-11 isomer increased from 5·5 % of the total 18:1 fatty acids at parturition to 11·9 % in late lactation.

Table 2. *Compositions of the octadecenoic acid fraction in the ewe's milk*

(% of total octadecenoic acid)

	Days *post partum*	
Fatty acid	0	100
18:1 *trans*-9	1·83	Trace
18:1 *trans*-11	5·46	11·9
18:1 *cis*-9	92·7	88·2

DISCUSSION

The percentage of fat in the milk of the ewe at parturition observed in the present work is in good agreement with the values reported by Barnicoat, Logan & Grant (1949) and Perrin (1958). The results in Table 1 show that within 24 h of parturition the fat content of the milk had decreased considerably. Barnicoat *et al.* (1949) and Perrin (1958) described a similar finding. The fat content of the ewe's milk at 100 days after parturition was about 10 %. This is higher than the content of fat in ewe's milk in mid-lactation, as given by Ling, Kon & Porter (1961), but it has been shown by Barnicoat *et al.* (1949) that the fat content of ewe's milk 90–100 days *post partum* (late lactation) is greater than that observed in mid-lactation (50 days *post partum*) but similar to that found during the first week of lactation.

Ruminant milk fatty acids are known to be derived from 3 sources: (1) *de novo* synthesis in the mammary gland from acetate and β-hydroxybutyrate resulting in the formation of the fatty acids from 4:0 to 16:0; (2) the diet, which normally contains C_{16} and C_{18} fatty acids, and (3) the fat depots, which contain mainly 16:0, 18:0 and 18:1.

It can be seen from the results in Table 1 that the milk obtained from the ewes immediately after parturition contained very high concentrations of 18:1. In late lactation, the concentration of 18:1 had decreased and there were increases in the concentrations of the shorter chain fatty acids (6:0 to 14:0). Immediately after parturition, the output of milk fat per unit of food consumed is higher than it is during late lactation. Thus, it seems reasonable to suggest that during this very early part of lactation the depot fat assumes a particularly important role in donating fatty acids to the mammary gland for milk fat synthesis. The work of Smith &

Dastur (1938) with cows showed that during inanition the concentration of 18:1 in the milk fat was markedly increased whereas the concentrations of the shorter chain fatty acids were decreased. The concentrations of 18:1 in the ewe's milk in late lactation are considerably lower than those in early lactation (Table 1). Stull, Brown, Valdez & Tucker (1966) showed that in the cow there was a slight decrease in the concentration of 18:1 between early and late lactation but the concentrations of 16:1 and 18:0 were markedly reduced.

The concentration of 10:0 found in the ewe's milk fat both in early and late lactation (Table 1) is greater than that observed in cow's milk (Jensen *et al.* 1967). However, our findings are in good agreement with those of Hilditch & Jasperson (1944), who also reported that the concentration of 10:0 in the milk fat of the ewe in mid-lactation was higher than the concentration of this fatty acid in the milk fat of the cow.

According to Holman (1960) the minimum requirement for essential fatty acid by the young rat and the human infant is met when 1 % of the total dietary calories is provided by linoleic acid. However, if the values given by Barnicoat *et al.* (1949) are used to calculate the energy contents of ewe's milk during the early stages of lactation, it is evident from the results given in Table 1 that linoleic acid provides only about 0·4 and 0·5 % of the total calories available to the lambs on the day of parturition and on the 3rd day after parturition respectively. Nevertheless, Leat (1964) has shown that during the first 3 days after birth there are large increases in the concentration of linoleic acid in the plasma lipids of the lamb. It seems possible, therefore, that the dietary requirement of the lamb for linoleic acid is considerably less than that of the young rat and the human infant.

The difference in the concentration of 18:2 in the milk fat of the ewe observed between early and late lactation (Table 1) is consistent with the work of Stull *et al.* (1966), who reported that the concentration of this fatty acid in cow's milk fat increased as lactation proceeded.

The presence of *trans*-octadec-11-enoic acid in ruminant milk fat is well established (Garton, 1963). *Trans* acids arise from the metabolism of dietary C_{18} unsaturated fatty acids by rumen micro-organisms (Shorland, Weenink, Johns & McDonald, 1957). Thus, the increase in the relative proportions of *trans*-octadec-11-enoic acid in the ewe's milk observed in mid-lactation (Table 3) is probably a reflection of the increased contribution of dietary fat to milk fat synthesis at this stage of lactation.

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Section E. Diseases of dairy cattle. Brucellosis

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CONTENTS

	PAGE
Brucella phage	303
Phage–cell interactions	303
Use of phage for typing	305
Typing and classification	305
Other tests	308
Incidence of biotypes	308
General Bacteriology	309
Resistance and antibiotic sensitivity	309
Morphological structure	310
Spheroplasts	311
Media	312
Antigenic structure	312
Fluorescent antibody	314
Interferon	315
Virulence	315
Pathology and Pathogenesis	318
Cattle	318
Sheep	319
B. ovis in sheep	320
Pigs	321
Horses and donkeys	321
Rabbits	321
Dogs	321
Brucellosis in other animals	321
Vaccination	322
General	322
Experimental vaccination of laboratory animals	323
Vaccination of cattle	326
Vaccination of sheep and goats	328
Vaccination of sheep against *B. ovis*	329
Vaccination of reindeer	329
Vaccination of pigs	329
Vaccination of man	330
Diagnosis	330
Immunoglobulins	330
Ig production in brucellosis	332
Diagnosis in cattle	333
Diagnosis in sheep and goats	338
Diagnosis of *B. ovis*	339
Diagnosis in pigs	339
Diagnosis in man	339
Diagnosis in other animals	339
Incidence	340
Incidence of brucellosis in man	342
Control and eradication	343
Conclusions	344
References	344

Brucella phage

Much of the recent work on brucella phage has been concerned with studies on host range of various phage preparations and some fundamental work on phage–cell interactions. Earlier work has been recently documented by Parnas (1967*a*).

Phage–cell interactions

Calderone & Pickett (1965) studied in detail 11 brucella phages of Russian or Polish origin (including the reference Tb phage). They reported that phage formed

plaques on *B. suis*, which could only be seen if a perfectly clear medium was used. These observations have not been confirmed by other workers, e.g. Jones, Merz & Wilson (1968*a*); Ostrovskaya & Tolmacheva (1968), although Parnas & Sarnecka (1964) claimed that phage lysed and multiplied on *B. suis* forming a turbid type of plaque (which they described as type 5). Gargani (1965) reported that phages P and Tb lysed some cultures of *B. suis*; there was little adsorption of phage P but the phage retarded *Brucella* multiplication.

Calderone & Pickett (1965) found that the guanine/cytosine composition of the DNA of 11 phages studied was very similar (45·3–46·7 %) and the average Tm value (°C) was 88·6–89·2; this very close similarity between the phages was also found by antigenic studies using heterologous and homologous antiphage sera.

A detailed study of phage–cell interactions was made by Jones *et al.* (1968*a*), involving rates of adsorption, one step growth curves and lysis from without. The highest rate of adsorption occurred with *B. abortus* (and *B. neotomae*—see later) and large, clear plaques were produced. The rate of adsorption on to *B. suis* cells was very low, replication did not occur and plaques were not formed. A high ratio of phage to cells led to a killing of *B. suis* (lysis from without) confirming observations made previously (Morgan, 1963; Münter, 1963). That lysis from without was caused by phage particles was confirmed by Jones, Merz & Wilson (1968*b*) by treatment of phage with trypsin and by density gradient centrifugation. Jones *et al.* (1968*a*) found that adsorption of phage took place neither with *B. melitensis* nor with rough strains of *B. abortus*, but they reported that occasional cultures of *B. melitensis* showed lysis with $10^4 \times$ RTD (routine test dilution) of phage and rightly cautioned that, for this reason, too much reliance should not be placed on the use of phage alone (at both RTD and $10^4 \times$ RTD); furthermore, rough cultures of *B. abortus* did not show lysis from without, and susceptible cultures of *B. abortus* of intermediate colonial morphology showed a pattern similar to that of *B. suis*.

Whilst *B. neotomae* was not lysed at RTD, it showed complete lysis at $10^4 \times$ RTD; Jones *et al.* (1968*a*) showed that *B. neotomae* adsorbed phage at the same rate as *B. abortus*, but about half the bacterial cells were killed and only some went through an infective cycle, releasing mature phage; the final phage count was always considerably less than the phage input.

Merz (1965), Merz & Wilson (1966) and Jones *et al.* (1968*a*) continued their studies on the effect of phage on a variant of *B. abortus* 544, viz. 544A. They showed that phage stock contained wild-type phage as well as clear (C) and late-clearing (LC) mutant phages. When phage was adsorbed on to 544A, the wild-type phage was not able to lyse the cells and release phage because of the lack of enzyme responsible for breaking up the cell wall; eventually, variant phages arose which produced the enzyme for breaking up the cell wall, leading to more efficient replication.

The phage–cell interactions have also been studied by Ostrovskaya & Tolmacheva (1968); they also showed that phage adsorbed and replicated on *B. abortus*, adsorbed but did not replicate on *B. suis*, but did not adsorb on *B. melitensis*.

The heat resistance of phages has been reported by Münter (1964) and Edlinger (1964), who reported that a fraction of one phage (212) showed different heat resistance.

Studies on the inactivation of phages by extracts of *Brucella* have been made by

Tuszkiewicz (1965), Parnas & Zalichta (1965) and Parnas & Sidor (1966); acetone-killed cells of *B. abortus*, *B. melitensis* and *B. suis* inactivated phage; rather surprisingly, inactivation was strongest with *B. suis* and weakest with *B. melitensis*.

Use of phage for typing

The Tbilisi (Tb) phage is now regularly used at RTD and $10^4 \times$ RTD for routine typing as the reference phage (Report, 1967*b*); reference has already been made to the limitations of the use of phage as the sole means of typing. Most of the brucella phages studied lyse, at RTD, *B. abortus* but not *B. melitensis* nor *B. suis* (Cerbu, 1966; Bhambani & Krishna Murty, 1964; El-Naasan, 1967; Fara & Gariboldi, 1963; Steidten, 1965; Parnas, 1967*b*).

New phages have been reported by Massidda (1965), El-Naasan (1965, 1967), Parnas, Zalichta & Sidor-Wójtowicz (1968), Lazuga (1968) and Moreira-Jacob (1968). The latter isolated phages from cultures of *B. abortus* (A422), *B. melitensis* (M85) and *B. suis* (S708); the *suis* phage was capable of replication and forming plaques on cultures of *B. suis*. In unpublished work at this laboratory it has been confirmed that phage S708 can replicate on *B. suis* strain 1330 and form well-defined plaques; at RTD this phage preparation lysed cultures of *B. abortus* and of *B. suis*. Such a phage could well play an important part in the classification of *Brucella* and in differentiating the 3 classical species.

On the basis of lysis by these 3 phages Moreira-Jacob (1968) divided *Brucella* cultures into 4 lytic groups:

Group I. *B. melitensis*—lysed mostly by *Melitensis* phage M85; Group II. *B. abortus* biotypes 2 and 6—lysed mostly by M85 and A422; Group III. *B. abortus* biotypes 4 and 1—lysed by all 3 phages; Group IV. *B. suis* biotypes 1, 3 and 4, *B. neotomae*—lysed mostly by S708 phage.

El-Naasan (1967) isolated phages from a number of sources including dung, stomach contents of foetuses, urine of infected cows and, with much more difficulty, from *Brucella* cultures. All 10 phages studied were similar to known phages in host range and neutralization with antiphage sera. None of the phages was capable of lysing *B. suis*. However, Fritzsch & Abadjieff (1967), studying the lytic effect of 4 phages (212, P, F1 and 371) on 74 cultures of *B. abortus*, reported that phage 371 lysed all the cultures, and phage 212 had the least activity. Morgan (1963) found that phages 212 and 371 had an identical host range. Gargani & Pin (1965) compared the Tb with other phages from Poland; cultures of *B. melitensis* were uniformly insensitive but *B. abortus* and *B. suis* showed varying behaviour towards the various phages. Ostrovskaya & Kaitmasova (1966) examined 680 cultures of *Brucella* (320 *B. abortus*, 298 *B. melitensis* and 62 *B. suis*) using the Tb phage at RTD and $10^4 \times$ RTD; 96·6 % of *B. abortus* cultures were lysed by both concentrations; most of the cultures of *B. melitensis* and *B. suis* were phage-resistant.

Typing and classification

The subcommittee on the taxonomy of *Brucella* in its report to the International Committee on the Nomenclature of *Brucella* (Report, 1967*b*) decided that *B. neotomae* (Stoenner & Lackman, 1957) should be accepted as a new species. On the basis of oxidative metabolism, phage and conventional typing methods (Meyer, 1964*a*,

1966*a*; Morgan, 1964) it was suggested that *B. rangiferi tarandi* (Davydov, 1961) should constitute a new biotype (biotype 4) of *B. suis* although Parnas (1966) and Davydov (1965) consider that it should be accepted as a separate species. In the U.S.S.R., reindeer brucellosis has a wide geographical distribution, independent of other *brucella* infections in animals (Vershilova, Ivanov & Orlov, 1966). The position of *B. ovis* (Buddle, 1956), even at the generic level, was unresolved (but see later). Tudoriu (1966) reported that whilst most cultures of *B. ovis* were stable, 2 cultures became transformed into smooth, *B. melitensis*-like strains.

Calderone & Pickett (1965) reported on the guanine and cytosine content (G+C, expressed as a percentage of the total base), of *B. abortus*, *B. melitensis* and *B. suis* by plotting the experimentally obtained average Tm values of their nucleic acids. The values obtained were 57·9 % (G+C) for *B. melitensis*, 58·5 % for *B. suis* and 57·9 % for *B. abortus*. Hoyer & McCullough (1968*a*, *b*) confirmed these observations and extended them to include *B. ovis*, *B. neotomae* and *B. canis*; the % (G+C) values for *Francisella tularensis* and *Escherichia coli* were 36 and 50.

Of great interest was the work on homologues of the DNAs from the various *Brucella* species (including *B. ovis*, *B. neotomae* and *B. canis*) reported by Hoyer & McCullough (1968*a*, *b*). By allowing radioisotope-labelled, single-stranded DNA fragments of each of the *Brucella* species to react with homologous DNA in the presence of systematically varied amounts of homologous or heterologous unlabelled DNA fragments (competitors), they showed that, within the limits of the sensitivity of the procedure, there were equal and reciprocal polynucleotide homologies between *B. abortus*, *B. melitensis*, *B. suis*, *B. neotomae* and *B. canis*. The DNA of *B. ovis* did not compete as effectively as the DNA from the other species in either the *B. melitensis* or *B. suis* systems; its degree of effectiveness was 93–94 % assuming homologous DNA normalized to 100 %. In further work on DNA homology with *B. ovis*, these authors concluded that the *B. ovis* DNA had undergone a deletion or a series of deletions so that a proportion of its DNA was now missing compared with DNA of the other species.

On the basis of these results, *B. neotomae*, *B. canis* and *B. ovis* can be accepted as members of the genus *Brucella*.

At this stage, some special mention should be made of a new species of *Brucella*—*B. canis*—described by Carmichael & Bruner (1968). A disease in dogs, associated with abortion and early embryonic death in females and epididymitis and testicular degeneration in males, has been reported throughout the U.S.A.; beagles are the breed mainly, but not exclusively, involved (Anon, 1966; Carmichael & Bruner, 1968; Carmichael & Kenney, 1968; Carmichael, 1968); a somewhat similar organism has been described by Moore & Bennett (1967).

The organism could be isolated in pure culture from blood or vaginal discharge of affected bitches, and from the foetus and placenta. Prolonged (up to a year) bacteraemia is frequently accompanied by lymphadenitis and splenitis and, on culture, abundant growth of the organism can be obtained; the rate of isolation from semen has been low.

The disease is easily transmitted experimentally by inoculation and by oral, conjunctival and contact exposure; the incubation period can vary from 4 days (after intravenous inoculation) to 3 weeks (oral exposure). Puppies born of infected

mothers can be bacteraemic with enlarged lymph nodes. The infection rate is highest when dogs are housed in groups and the incidence can be as high as 25–40 %. Diagnosis is based on the recovery of the organism and/or an agglutination test using heat-killed cells and incubation in a water-bath at 50–52 °C; temperatures above 45 °C are important for the agglutination test. Human infection has also been reported (Carmichael, Barol, Broad & Freitag, 1968).

The organism is aerobic, forming colonies which, on prolonged incubation (5–7 days), are mucoid, and 1–1·5 mm in size. Reference has already been made to the work of Hoyer & McCullough (1968*b*) on content of (G+C) and on DNA homology, by which criteria it is indistinguishable from other species of *Brucella*. The organism has an oxidative metabolic pattern similar to *B. suis* biotypes 3 and 4 (although it does not oxidize erythritol); it is not lysed by phage at either RTD or $10^4 \times$ RTD and its growth is inhibited by basic fuchsin but not by thionine. Even on primary isolation, the organism is in the non-smooth phase and it does not agglutinate in sera prepared from other (smooth) species of *Brucella*—neither does it agglutinate in mono-specific sera; for confirmation of identity by serological tests, therefore, unabsorbed serum against rough *Brucella* should be used (Jones, Zanardi, Leong & Wilson, 1968).

In a detailed study of the antigenic relationship of *B. canis* to other species of *Brucella*, Diaz, Jones & Wilson (1968) showed that *B. canis* lacked the lipopolysaccharide endotoxin associated with the agglutinogen of smooth types of *Brucella*. By using whole-cell suspensions in agglutination and agglutinin-absorption tests, *B. canis* was shown to be similar to rough *B. abortus*, *B. melitensis* and *B. ovis*, e.g. *B. ovis* antiserum agglutinated *B. ovis* and *B. canis* antigens but not (smooth) *B. melitensis* antigen. By using soluble antigens in immunoelectrophoretic and gel diffusion studies, extensive cross reactions within the genus *Brucella* were shown but not with other Gram-negative bacteria.

It is important to distinguish between this disease caused by *B. canis* and the occasional recovery of *B. abortus*, *B. melitensis* or *B. suis* from dogs (e.g. Clegg & Rorrison, 1968; Kimberling, Luchsinger & Anderson, 1966; Nicoletti, Quinn & Minor, 1967) where dog-to-dog transmission does not occur.

It is generally recommended that 12 substrates should be used when examining cultures by oxidative metabolism tests (Report, 1967*b*; Morgan & Gower 1966; Wündt, 1967; Philippon, 1968; Vershilova & Ostrovskaya, 1964) and that all available tests should be used to identify atypical *Brucella* strains. A simplified metabolic test was developed by Ruiz Castañeda (1968).

A new sero-biotype of *Brucella*, *B. murium*, was described by Korol & Parnas (1967)—see also Parnas, Korol, Zalichta & Sidor-Wójtowicz (1968); this was isolated mainly from *Mus musculus* in areas where brucellosis of domestic animals and man was not present. A few cultures examined in the author's laboratory had an oxidative metabolic pattern similar to *B. neotomae*. According to Parnas *et al.* (1968), cultures of *B. murium* did not grow on basic fuchsin, but grew on thionine; agglutination occurred in monospecific abortus serum, but lysis did not occur with phage at either RTD or $10^4 \times$ RTD.

On the basis of antibiotic sensitivity, Fritzsch & Abadjieff (1966) divided 68 cultures of *B. abortus* into 9 types: 54 belonged to one type; cultures of *B. melitensis*

and *B. suis* differed from the *B. abortus* cultures in antibiotic sensitivity. Farrell & Robertson (1967) reported that cultures of *B. abortus* biotype 2 were antibiotic-sensitive (to polymyxin B sulphate, amphotericin B and bacitracin) as well as dye-sensitive; these 2 properties were not genetically related. On the basis of antibiotic sensitivity, these authors suggest that the dye-sensitive strains of *B. abortus* type 4 should be designated as a separate biotype.

Keppie, Witt & Smith (1967) showed that the growth of Strain 19 was inhibited by concentrations of erythritol greater than 1 μM/ml; lower concentrations neither inhibited nor stimulated growth. The growth of other CO_2-independent attenuated strains was moderately stimulated by erythritol (2–10 μM/ml). Jones, Montgomery & Wilson (1965) recommended that CO_2-independent cultures should be examined for sensitivity to penicillin, erythritol, thionine blue and Safranin O in order to identify them as the vaccine Strain 19. They found that erythritol-stimulated mutants in the US Strain 19 seed occurred in 1 in 10^5 cells; a similar frequency of Safranin O-resistant mutants also occurred although these 2 mutations were independent.

Other tests. A number of papers have appeared on the use of Safranin O for differentiating species within the genus *Brucella* (Balandin, 1965; Cedro, Forti de Menaldo & Cisale, 1966; Jones, 1964; Jones, *et al.* 1965; Krčméry & Nižnánsky, 1966); the growth of *B. suis* is inhibited by Safranin O but Jones (1964) also reported that the growth of a few cultures of *B. melitensis* (1 of 7 cultures examined) and of some aerobic cultures of *B. abortus* was also inhibited by Safranin O; a high frequency of resistant mutants was observed even in the cultures of *B. melitensis* that did not grow on the dye.

Using antigens prepared by successive freezing and thawing of *Brucella* cultures for a complement-fixation test, Gargani & Casadei (1967) were able to distinguish between the biotype 1 of each species and, further, it was possible to differentiate *B. abortus* 1 from *B. suis* 1.

Van Drimmelen & Van Niekerk (1967) confirmed that *B. ovis* is oxidase-negative, thus differentiating it from *B. melitensis* (*B. abortus* and *B. suis* are also oxidase-positive). Van Drimmelen (1964) also reported that *Brucella* cultures isolated from ram semen had a low urease activity and a high pH threshold, whilst *B. suis* had high urease activity (and a low pH threshold activity); intermediate values were obtained with the other species of *Brucella*. Studies on the urease, transaminase and deaminase activities have been reported by Todorov & Karaivanov (1966), Todorov, Karaivanov, Valerianov, Mermerski & Nikolov (1965) and Todorov, Karaivanov & Koleva-Todorova (1966). *B. suis* differed from *B. abortus* and *B. melitensis* in that it possessed no phenylalanine deaminase activity. By using 30 % (instead of 3 %) hydrogen peroxide and a dense (3×10^9 ml) cell suspension, Malikova (1965) found that *B. suis* showed peroxidase activity by the pyrogallol method but *B. melitensis* and *B. abortus* showed no activity. A comparison of some enzymic activities of *Brucella* was also reported by Merinov (1967).

Datsevich (1964) reported that strains of *B. abortus* contain volutin granules but *B. suis* and *B. melitensis*, with rare exceptions, do not; this method was also used by Kolomakin, Barinova & Litvinova (1969).

Incidence of biotypes. A large number of reports have appeared on the incidence of species and biotypes of *Brucella*: in the Soviet Union by Balandin, Prostetova &

Moskalenko (1964); Khoch & Davydov (1968) (in reindeer); Minkov (1966) (in sheep); Pinigin, Kokourov, Petukhova & Merinov (1968) (in yaks). Kaitmazova & Ostrovskaya (1967) examined 5219 cultures isolated during a 4-year period: all strains isolated from cattle in the north-western part of the USSR were *B. abortus* (87 % being biotype 1 and 13 % biotype 6); isolates from sheep in Turkmen SSR were all *B. melitensis* (90 % biotype 1 and 10 % biotype 3). However, in a mixed animal production area, a third of the cattle isolates were *B. melitensis* biotypes 1, 2 and 3, and the remainder biotypes 1, 3, 4, 5, 6, 7 and 9 of *B. abortus*; only *B. melitensis* strains were isolated from sheep. This pattern of mixed species/biotypes was not seen in another area with a mixed animal population. The possible evolution of *Brucella* was discussed by Aslanyan (1967).

Aldrick (1968) reported on the incidence of species in Australia and Papua–New Guinea, and Cook, Campbell & Barrow (1966) isolated 7 strains of *B. suis* biotype 3 from rodents (*Melomys cervinipes*) in North Queensland.

Mathur (1964*a*) has made a detailed study of the species and biotypes isolated from cows, buffaloes, goats, sheep and man in Karnal. Biotypes 1 and 3 of *B. melitensis* were isolated from sheep and goats (as well as a few *B. abortus*); significantly, all the human isolates were also *B. melitensis*. Only biotypes 1 and 3 of *B. abortus* were isolated from cattle and buffaloes—an observation also made by Pat & Panigrahi (1966) in Orissa.

In Italy, Mazzetti (1967) observed that brucellosis in man was closely associated with the disease in sheep and to some extent in goats; *B. intermedia* (*B. melitensis* biotype 2) was the commonest strain; cattle strains were *B. abortus*. Similar observations were made by Mardones Sevilla (1968/9) in Spain. A close connexion between *Brucella* species and animal host was observed by Meyer (1964*b*, 1967*b*) in an examination of 550 strains of *Brucella* from various parts of the world. Dijkstra (1964) found that 79·5 % of cultures from aborted foetuses were *B. abortus* biotype 3. In an extensive examination of brucella strains isolated from man in Mexico, Ruiz Castañeda (1968) found 11 strains of *B. abortus* and 2 *B. suis*, whilst the remainder (2636) were *B. melitensis*. In South Africa, Van Drimmelen (1966) found that the commonest isolate from sheep was *B. ovis*; swine brucellosis had not been reported there, but Chambron (1965*a*) found *B. melitensis* in Senegal. *B. suis* biotype 2 was isolated from hares in Poland (Iwanow & Tropilo, 1966). All isolates from cattle in Britain have been biotypes of *B. abortus*, the majority being of biotype 1 (Morgan, 1968, 1969; Brodie, 1968*b*). *B. abortus* biotypes 1 and 2 were isolated by Nelson, Anderson, Kimberling & Pietz (1966) from problem herds and there was no correlation between biotype isolated and problem herd classification.

Detailed descriptions of techniques for the classification of *Brucella* have been published (Morgan & Gower, 1966; Alton & Jones, 1967).

General bacteriology

Resistance and antibiotic sensitivity

The minimal bactericidal dose of gamma rays for *B. suis* was 600000 γ; smaller bacteriostatic doses resulted in dissociation and appearance of L-forms (Genov, Dimitrov, Antonov & Fjodorov, 1967). Fumigation with a 20 % solution of

formaldehyde (15–22 ml/m^3) for 3–4 h was found to be effective for disinfecting buildings (Zharov, 1968) whilst Ivanova (1966) found that ethylene oxide (2 kg/m^3 for 2–3 h) was capable of disinfecting experimentally contaminated bales of wool. Tarakanov (1968) reported that *B. melitensis* survived for 121 days and *B. abortus* and *B. suis* for 110 days in experimentally infected wool, and Daminova (1967) found that the survival of *Brucella* in soil was influenced by the humidity and recommended that the period of resting *Brucella*-contaminated pastures should be extended to 3 months. The survival of *Brucella* mixed with faeces on external and internal walls of buildings was 1 day in summer and 53 in winter; survival in pond water could be as long as 3 months. Rankin & Taylor (1969) studied the survival of various bacteria in slurry: *B. abortus* could survive for up to 11 weeks but not beyond that period when the slurry was stored out of doors in winter. These observations are of practical significance in the control of brucellosis particularly where eradication campaigns are under way. (See also Döpel, 1968.)

Morphological changes caused by contact with heat, chemical and chemotherapeutic agents were studied by Bonaduce & Compagnucci (1963); significantly, agglutinating serum, even after 48 h, had no effect on morphology or on oxygen consumption.

Lysov & Trutnev (1967) reported that saliva from normal sheep inhibited the growth of *B. abortus* and was bactericidal after 24 h of exposure. Filtrate of abomasal contents killed *Brucella* in 10–30 min; this action was due to hydrochloric acid and not to pepsin.

The action of various antibiotics on *Brucella* has been reported by Richardson & Holt (1964*a*), Nagy & Zalay (1968), Murty & Varshney (1964), Uraleva (1965), Farrell & Robertson (1967), Ryan (1967) and Roux, Ramuz & Serre (1969). Richardson & Holt (1964*a*) found that 1·5 units of Synnematin B (penicillin N) inhibited the growth of *B. abortus* in vitro, but had no effect on intracellular *Brucella*; however, a combination of 4–10 units Synnematin B plus 1 μg/ml tetracycline was much more effective than either antibiotic alone so that such a combination might be effective for clinical treatment. Roux *et al.* (1969) found that when chlortetracycline was added to cultures of chick-embryo fibroblasts the intracellular concentration of the antibiotic reached a peak 40 times the extracellular concentration after 1–5 h and then dropped to 20 times. However, *B. melitensis*, sensitive to 1·5 μg of the antibiotic, multiplied in fibroblast cultures in the presence of 10 μg/ml extracellular antibiotic, and these authors suggest that intracellular *Brucella* cells were protected from the antibiotic by a surrounding membrane (see also Zanardi, 1968; Karlsbad, Kessel, de Petris & Monaco, 1964). Goldenbaum, Kessel & Fukui (1966) also reported a synergistic effect of chlorpromazine (5 μg/ml) and streptomycin on intracellular *B. abortus*. Nalidixic acid at 7·5–10·0 μg/ml was inhibitory to *Brucella* (Deitz, Bailey & Froelich, 1964); it was found to be effective in treating human brucellosis by Sharma (1965).

Morphological structure

The ultrastructure of *Brucella* has been studied by Vysotskii, Kurdina & Ostrovskaya (1967), Vysotskii & Dranovskaya (1967), Vysotskii (1968), de Petris, Karlsbad & Kessel (1964), Grund (1964), Zanardi (1968) and Karlsbad *et al.* (1964). Vysotskii

et al. (1967) found that R strains of *Brucella* differed from S variants in lacking a capsule-like substance; there was no species difference in the R cells, which were polymorphic. However, de Petris *et al.* (1964) found no difference between S and R variants of *B. abortus* grown on a lifeless medium and found no evidence of a capsule. The cell wall consisted of a triple-layered membrane 65–80 Å thick with an inner homogeneous layer of varying thickness. The triple-layered cytoplasmic membrane was 70 Å thick and a number of membranous structures 300–800 Å thick were associated with it. Karlsbad *et al.* (1964) found no differences in the structure of S variants of *Brucella* grown in monocytes or in lifeless medium; this was confirmed by Zanardi (1968). Grund (1964) found that very small, coccoid forms, 0·2 μm diam., were found as cultures grew older, and this was said to be the result of a reduction in metabolic activity.

Spheroplasts

Studies on the characterization and structure of spheroplasts and L-forms have been reported by Hines, Freeman & Pearson (1964*a*, *b*), Baughn & Freeman (1966), and Hatten & Sulkin (1965, 1966*a*, *b*). Hines *et al.* (1964*a*, *b*) found that *B. suis* showed the best conversion (99 %) and *B. melitensis* the greatest resistance (10 %) to the formation of spheroplasts on exposure to 2 % glycine for 48 h; there was no difference in the morphology of spheroplasts produced by penicillin or glycine; the use of both penicillin and glycine had a synergistic effect. Spheroplasts induced by glycine and glycine–penicillin were osmotically sensitive, would not reproduce, and only a small percentage reverted to bacillary form, whereas penicillin-induced spheroplasts did not reproduce but were not osmotically sensitive. The spheroplasts of *B. abortus* adsorbed brucella phage at a reduced rate but spheroplasts produced by a combination of glycine and penicillin failed to adsorb phage. Whilst spheroplasts lacked the cell wall endotoxin they reacted with antiserum produced against cell walls and whole cells, thus confirming that true protoplasts were not produced. In a subsequent paper, Hines *et al.* (1964*b*) produced some excellent electron photographs of *B. suis* and spheroplasts derived from *B. suis*. The fine structure of the cell wall was much clearer in spheroplasts than in normal cells and consisted of 2 electron-dense layers and a lighter middle layer; a similar multi-layered construction was found in the cytoplasmic membrane. Baughn & Freeman (1966), in an analysis of the antigenic structure of *B. suis* spheroplasts, found that those induced by glycine were the least complex with only 6 antigens detected by immunoelectrophoresis; penicillin-induced spheroplasts were the most complex with 8 antigens; however, no completely new antigens were detected in spheroplasts.

Hatten & Sulkin (1966*a*) isolated L-forms of *Brucella* (i.e. produced typical 'fried egg' type of colonies) from tissue cultures inoculated with *B. abortus*; T (tiny) colonies were also produced. The L-forms would grow only in the presence of 5–10 % added CO_2. The L-forms fluoresced with anti-*Brucella* conjugated serum. An interesting observation was that L-forms isolated soon after intracellular infection produced typical L colonies which, on subculture, gave rise to a bacterial colony typical of the parent strain. After longer periods of intracellular infection, T-forms mostly were isolated; these only occasionally reverted to the bacterial form, and when they did the colonies were atypical. This suggested that prolonged intracellular survival led to

stabilization of the L-forms. L-forms of *Brucella* were reported to be more regularly formed by *B. suis* (Khristoforov & Peshkov, 1969).

Media

The growth characteristics of *B. abortus* and *B. neotomae* on solid medium were studied by Gibby & Gibby (1964). At the exponential growth phase, generation times of 100 min were reported for each of the species tested; significantly, the logarithmic phase did not extend much beyond 18 h. There was a marked loss of viability with *B. neotomae* associated with progressive acidity, whereas with *B. abortus* there was a marked alkalinity as the culture aged. These authors found that erythritol had no effect on growth rate, but Keppie, Witt & Smith (1966) found that incorporation of erythritol at 1 μm/ml in Morris medium caused the earlier appearance of colonies of virulent strains of *B. abortus* and *B. melitensis*, whereas on Albimi agar only the growth of *B. melitensis* was stimulated. Erythritol had no effect in either medium on *B. suis*. It would be interesting to determine whether the strain of *B. abortus* (LiCP-11) used by Gibby & Gibby was a virulent one or not. Smith, Anderson, Keppie, Kent & Timmis (1965) extended the work reported by Keppie *et al.* (1966) and showed that analogues of erythritol inhibited the growth of *B. abortus*, *B. melitensis* and *B. suis* on solid media; growth was also inhibited in a tissue culture system and in infected guinea-pigs.

Huddleson (1964*a*), commenting on the failure of certain aerobic strains to grow on liver agar, found that growth did occur in 10 % CO_2 or after the addition of serum, crystalline bovine albumin or Tween 40.

A selective medium for the isolation of *Brucella* from milk was described by Ryan (1967); this consisted of a blood agar base containing penicillin (or bacitracin), polymixin B sulphate, Vancomycin, nalidixic acid, cetrimide, Actidione (cycloheximide) and Mycostatin (nystatin). Such a medium was claimed to be superior to Mair's medium.

Cabelli & Levin (1964) described a medium for the quantitative recovery of *Pasteurella* and *Brucella*, consisting of a modified peptic digest medium but with the concentration of cysteine decreased to 0·01 g/100 ml and substitution of brilliant green for penicillin. Painter, Deyoe & Lambert (1966), in a comparison of several media for the isolation of *Brucella*, recommended that the enrichment of media with serum was necessary for the maximum isolation of all strains. The addition of antiphage serum to nutrient agar was found to be of limited value by Keyhani (1968). Differences in growth potential of varieties of potatoes in Turkey were reported by Ünel & Sarisayin (1967). The construction of an installation suitable for large-scale production of *Brucella* cells in liquid medium was described by Scheibner (1966).

Antigenic structure

Of considerable interest has been the work of Ahvonen and his colleagues on the sharing of a surface antigen between *B. abortus* and *Yersinia enterolitica* (Ahvonen & Jansson, 1968; Ahvonen & Sievers, 1969; Ahvonen, Jansson & Aho, 1969). Although bovine brucellosis has been eradicated in Finland, a series of human patients was found with brucella antibodies in the range 100–1600 i.u. These sera were also found to agglutinate to a similar or higher titre *Y. enterolitica* type 9 but

were not at all active against other types of *Y. enterolitica* or *Y. pseudotuberculosis* types I–V. In a series of cross-absorption experiments, *Yersinia* antigen always removed both homologous and heterologous agglutinins but absorption with *Brucella* antigen, whilst reducing the titre, in many cases left antibodies detected by *Yersinia* antigen. The International Standard anti-*B. abortus* serum gave a titre of 1/1280 with both *Brucella* and *Yersinia* antigens. Ahvonen & Sievers (1969) reported that the clinical features of the disease in humans consisted of fever, diarrhoea and abdominal pain—clinically similar to infections with *Y. pseudotuberculosis* (Syn. *Pasteurella pseudotuberculosis*) and with *B. abortus*; human infections with *Y. enterolitica* are not rare, especially in some countries, and these authors were eventually able to isolate the organism from the stools of human patients; an association between *Yersinia* infection and acute and subacute arthritis has also been noted. The origin of these infections was not known; in animals, *Y. enterolitica* has been isolated especially from chinchillas. We have confirmed that a strain of *Y. enterolitica* type 9 received from Dr Ahvonen was agglutinated to titre by *B. abortus* antisera.

An important contribution to the study of the antigenic structure of members of the genus *Brucella* has been made by Diaz and his colleagues in a series of papers.

Diaz, Jones, Leong & Wilson (1968) prepared ether–water extracts of smooth *B. melitensis* and *B. abortus*. Extracts of *B. melitensis* contained a lipopolysaccharide protein component which was specific for the surface of smooth types of *Brucella* and correlated with the M agglutinogen of Wilson & Miles (1932), a polysaccharide protein component (component 1), and several protein components which were associated with internal antigens of both smooth and rough types of *Brucella*. Extracts of *B. abortus* revealed only the lipopolysaccharide protein component which was correlated with the A agglutinogen and component 1 (polysaccharide protein component); in gel diffusion tests, component 1 of both species showed identity whereas the M and A components showed only partial identity with unabsorbed sera and no cross reactions using monospecific antisera.

Reference has already been made to the work of Diaz, Jones & Wilson (1968) on the antigenic relationship between *B. canis* and other species of *Brucella*. *B. canis* lacked the lipopolysaccharide enterotoxin of smooth species of *Brucella* but showed extensive cross reactions with other rough and smooth *Brucella* on the basis of immuno-gel diffusion studies with water-soluble antigens obtained by ultrasonic treatment. Similarly, these authors (Diaz, Jones & Wilson, 1967) found extensive cross reactions between water-soluble antigens of *B. ovis* and smooth and rough *B. melitensis* as well as between *B. ovis* and rough *B. melitensis* using whole cells for agglutination and agglutinin-absorption tests. In a further extension of this work, Diaz, Jones, Leong & Wilson (1967) reported that the water-soluble antigen present in both smooth and rough types of *Brucella* sensitized tanned erythrocytes whereas only the agglutinogen found on the surface of smooth *Brucella* cells can attach to normal erythrocytes. A technique of producing antibodies to specific antigens for the isolation of pure antigens was described by Wolf & Live (1964).

Hindsill & Berman (1967) found that sonic extracts of *B. abortus* Strain 2308 formed 9 precipitin lines on immunoelectrophoresis, while, when using absorbed serum, one of these lines (Component IX) was missing, suggesting that this line represented a 'surface' antigen. Baughn & Freeman (1966) reported that ultrasonic

extracts of *B. suis* revealed 4 major and 9 minor precipitin lines on immunoelectrophoresis; 4 of the minor lines did not develop after absorption of the serum nor were they detected using spheroplast antigen preparations of *B. suis*.

Nagy (1967, 1968) studied the antigens of biotypes 1, 2, 4 and 5 of *B. abortus* using acetone-killed suspensions and antisera prepared in cattle and rabbits. He noted that, although there was considerable sharing of antigens, Strain 19 was void of a component present in all the virulent biotypes examined. A water-soluble, diffusible fraction was reported by Pathak & Singh (1967) which gave 1–3 precipitin lines with sera from infected animals. Parnas, Cegielka & Burdzy (1964) reported a difference in the number of precipitin lines in a streptomycin-independent and in streptomycin-resistant strains of *B. abortus*, a difference which was supported by spectroscopy. Three thermostable and 4 thermolabile precipitinogens were reported by Gajos (1967*a*) from *B. melitensis* Strain 16M.

Gargani & Casadei (1967), using extracts of *B. melitensis*, *B. abortus* and *B. suis* obtained by freezing and thawing, reported that it was possible to distinguish between the species by a complement-fixation test and suggested this as a method for species identification. Gajos (1967*b*) reported that ultrasonic extracts of *B. melitensis*, *B. abortus* and *B. suis* were specific when tested against homologous sera after absorption with heterologous antigens, thus making it possible to differentiate *B. abortus* from *B. suis*.

Misra & Panda (1964) found some cross reaction between *B. abortus* and *Vibrio cholera* but no reaction between *B. abortus* and *Salmonella pullorum* or *Proteus* OX19. Downey & Morter (1964) found no evidence of a serological relationship between *Leptospira pomona* and *B. abortus*, and repeated vaccination with *L. pomona* vaccines did not produce a rise in the brucella titre of calves previously vaccinated with Strain 19. Gangulee & Sen (1966) and Nicoletti & Holmes (1968) also found that the administration of haemorrhagic septicaemia vaccines had no effect on the brucella titres (Lacave & Le Garrec, 1964). Wober, Thiele & Urbaschek (1964) studied the free lipids of *B. abortus* and found that saturated acids, especially palmitic acid, predominated among the component fatty acids of the 7 phosphatides isolated by preparative thin layer chromatography.

A phenotypic colony variation was observed by Philippon & Renoux (1969) when colonies of *B. abortus* were isolated from infected lymph nodes of cattle or rabbit spleens. The colonies were heptagonal or irregular in shape, but on first subculture circular colonies were produced.

Fluorescent antibody

Meyer (1966*a*) concluded that immunofluorescent staining was a rapid and dependable technique for identifying species of *Brucella* although the organisms were not always detected by impression smears from known infected animals. Three strains of *B. suis* were not stained by any of the conjugated sera studied; neither were strains of *B. ovis*. Chernysheva *et al.* (1965) and Tchernycheva (1965) were able to detect 5000 *Brucella*/ml contaminated with 100×10^6/ml cells of *P. tularensis*, *P. pestis*, *E. coli* and *Bacillus anthracis* in smears stained with conjugated *Brucella* sera; tests were also positive for pond water containing 2000 *Brucella* cells/ml. Karasek (1965*a*, *b*), using fluorescent antibody staining, found that whilst streptomycin in

high concentrations prevented growth of the cells on culture it did not interfere with specific fluorescence; penicillin, tetracycline and chloramphenicol had no effect on either culture or staining.

Le Garrec, Gajos & Pilet (1967) found it impossible to evaluate the immunofluorescence technique for examining spleen and lymph nodes from infected mice because of fluorescence in control samples. Karasek & Jentzsch (1966), however, found the method satisfactory although they found that paraffin fixation of tissues was unsuitable and recommended the use of frozen sections.

An indirect fluorescent antibody procedure using soluble antigen fixed on to an artificial matrix and a mechanical means for reading the tests was devised by Toussaint & Anderson (1965). Two cellulose acetate paper disks were used for each test—one strip containing soluble antigen in 1 % bovine serum albumin and the other (control) disks containing bovine serum albumin only. The results of the tests were read on a fluorometer fitted with a chromatogram door. These authors used soluble antigen from *Schistosoma mansoni* and found that the method gave excellent results.

Interferon

Younger & Stinebring (1964) reported that the intravenous injection of large numbers of *Brucella* cells in chickens resulted in the appearance of a viral inhibitor indistinguishable from interferon; it was detected as early as 3 h after inoculation and reached a peak between 6 and 12 h. Keyhani (1969) also reported that the administration of brucella phage weekly, for 12 weeks, to chickens produced a significant degree of protection against challenge with Newcastle disease and it was suggested that this was due to the production of interferon by the phage.

Virulence

Whilst BCG-immune histiocytes, as well as some of their subcellular components such as ribosomes and ribosomal RNA, were effective in inducing cellular resistance in normal animals against both *Brucella* and *Mycobacterium*, the *Brucella*-immune histiocytes were found by Fong, Chin & Elberg (1964) to be incapable of transferring resistance to the cells of normal animals, neither could they achieve attenuation of virulent tubercle bacilli.

Factors concerned in host resistance to experimental brucellosis were discussed in an excellent article by Keppie (1969)—see also Keppie (1964). Whilst polymorphonuclear and macrophage phagocytes of normal cattle and guinea-pigs were able to destroy attenuated strains of *B. abortus*, they could only restrain the intracellular growth of virulent strains for a short (12–18 h) period; even the presence of immune serum had no effect on their survival (Smith & FitzGeorge, 1964*a*, *b*; FitzGeorge, Solotorovsky & Smith, 1967). It was also shown that organisms of a virulent strain grown in vivo were more resistant than organisms of the same strain grown in vitro. FitzGeorge & Smith (1966) further reported that growing cells in vitro in the presence of 20 % bovine allantoic fluid also produced organisms with an increased capacity for intracellular survival compared with the same strain from an unsupplemented medium. Allantoic fluid could not be replaced by erythritol. The extracellular bactericidal action of bovine white blood cells on *Brucella* could be inhibited by walls

of avirulent and virulent *Brucella* cells grown in vitro or in vivo, and it was postulated that differing amounts of this protective cell wall material could be one factor in determining virulence; cell wall preparations, however, had no effect on the intracellular survival of *Brucella*.

Ralston & Elberg (1968*a*, *b*; 1969) showed that immune serum conferred on macrophages from both immune and non-immune rabbits an enhanced capacity to attach, ingest, and restrict the multiplication of *Brucella*; in the absence of immune serum, immune macrophages killed significant numbers of *Brucella* in the first hour after exposure and only temporarily restricted further multiplication. These workers also noted a marked heterogeneity in the ability of macrophages to ingest and control the multiplication of *Brucella* cells; the proportion of macrophages involved and the extent of the restriction on bacterial growth were greatly affected by the extent of cellular immunity and the presence of immune serum (see also Dima, Vasilesco & Pop, 1965). Braude (1964) also reported that humoral factors played an important role in the first stage of phagocytosis but not in the second (intracellular digestion) stage.

Zanardi (1968) reported that *Brucella* cells were usually seen in vacuoles of guinea-pig phagocytes and were associated with an electron-dense material which appeared within 4 h of infection. Infection with cells of low virulence was followed by an initial drop in numbers of *Brucella* at 24 h with a subsequent rise in numbers. A vacuole was also seen surrounding smooth *Brucella* cells in guinea-pig monocytes (Karlsbad *et al.* 1964).

A growth-enhancing substance for *B. abortus* in bovine white blood cells (Williams, Keppie & Smith, 1964) was found by Burrin, Keppie & Smith (1966) to be associated with the lymphocytes and did not specifically stimulate virulent strains. Rezai (1968), however, isolated a factor from extracts of peritoneal macrophages from brucella-vaccinated guinea-pigs which inhibited the growth of *B. abortus*; such a factor could not be isolated from macrophages of non-immune guinea-pigs or from serum.

There was no difference in the intracellular survival of *B. abortus* in polymorphonuclear cells of normal or immune animals (Macrae & Smith, 1964) and immune serum had no effect (Smith & FitzGeorge, 1964*b*). *B. abortus* multiplied more slowly in macrophages of immune calves than in those of non-immune animals, and again, immune serum had no effect on the intracellular survival (FitzGeorge *et al.* 1967; Joubert & Valette, 1967), suggesting that immunity to brucellosis in calves depended more on cellular than on humoral mechanisms. However, Vershilova & Chernysheva (1967), working with *B. melitensis*-infected guinea-pigs, demonstrated that humoral factors played a significant role as indices of immunity. MacKaness & Blanden (1967), studying the build-up of infection in mice, found that maximum infection as well as delayed hypersensitivity occurred by the fourth day whereas macrophages did not show hyperactivity until 8 days when the number of organisms fell.

The bactericidal effect of fresh rabbit blood against *B. abortus* was studied by Joos & Hall (1968). Blood from control (non-immunized) rabbits had moderate bactericidal activity after a lag of about 2 min whilst the blood of some immunized rabbits had an immediate and strong bactericidal effect; in other rabbits, especially when superimmunized, the bactericidal activity was inhibited.

Richardson & Holt (1964*b*) found that *B. abortus* had a generation time of 4 h when cultured in uterine mucosal and foetal skin cells whereas only limited multiplication occurred in cultured calf spleen cells, with a generation time of 8 h. In all cases, there was a marked drop in the number of *Brucella* cells in tissue cells in the first 3 to 6 h; this may have been due to the addition of streptomycin at zero time.

Freeman, Pearson & Hines (1964) have extended their studies on the survival of smooth and rough types of *Brucella* in guinea-pig mononuclear phagocytes. They showed that rough, avirulent *Brucella* cells grew intracellularly but had a marked destructive effect on the monocytes, releasing the bacteria which were then killed by the antibiotics present in the tissue culture system; smooth, virulent cells had a much less destructive effect on guinea-pig monocytes. In a further paper (Freeman & Rumack, 1964), it was shown that rough *B. suis* were ingested at a greater rate than smooth cells. Spheroplasts from smooth cells treated with glycine (see Hines *et al.* 1964*a*, *b*) were also phagocytosed at a greater rate than smooth cells. Spheroplasts from both smooth and rough *B. suis* had a marked destructive effect on the monocytes; this effect was lost by treatment of the spheroplasts with formalin and it was postulated that the loss of surface components of the bacterial cells (as in S-R variation) resulted in cells of greater cytopathogenicity (Freeman & Rumack, 1964).

Jeunet, Cain & Good (1968) found that the isolated rat liver rapidly took up *B. melitensis* and *S. typhosa*, but not *B. abortus* cells; the addition of specific antibody led to uptake of *B. abortus*. In vivo, *B. abortus* cells are engulfed by the rat spleen.

Hindsill & Berman (1968) studied the effects of an extract of *B. abortus*, obtained by sonic disintegration, both by intradermal injections in vaccinated and non-vaccinated guinea-pigs and by the effect on macrophages from immune and non-immune guinea-pigs. Their fraction A had a marked cytotoxic effect on the macrophages and elicited a marked dermal response in immune guinea-pigs. A fraction B which was the acid-insoluble portion of fraction A was more toxic for normal than immune guinea-pigs and also produced a greater dermal reaction in normal than in immune guinea-pigs.

Baker (1965) and Baker & Wilson (1965*a*, *b*) used the ability of endotoxin to induce hypoferraemia in mice in order to measure the specific activity of endotoxin preparations of *B. abortus* and to determine the endotoxin content of several strains of *B. abortus* of varying virulence for guinea-pigs; there was no correlation between endotoxin content and virulence nor were there any differences in the chemical composition.

In an extension of this work, Leong, Diaz & Wilson (1968), using evidence from immuno-gel diffusion and other studies on an ether–water brucella endotoxin preparation, showed that the biologically active endotoxin migrated in the slow-diffusing component; destruction of this component by acid hydrolysis led to loss of toxicity.

Veda, Veda & Imaizumi (1966), in a study of the hyper-reactivity of mice to endotoxins after intravenous injection of *B. abortus*, reported that hyper-reactivity was moderate with endotoxin of virulent *B. abortus* and absent with endotoxin of Strain 19. Indeed, they found that in mice infected with different doses of 3 strains of *B. abortus* of varying virulence there was a close correlation between hyper-reactivity to endotoxin and the severity of infection and it varied with the virulence and dose

of *B. abortus* administered. Urbaschek (1964) reported that endotoxin from *B. abortus* increased the motility of the isolated rat uterus; the effect was greatly enhanced if the uteri were from rats treated with oestradiol.

Gibby & Gibby (1965) found that *B. neotomae* infection in the white mouse did not cause visible disease and the bacteria were rapidly eliminated. Injection of *B. neotomae* with 5% mucin produced a lethal disease and a different colonial form was obtained on isolation, but subcultures, even in low doses, caused a rapidly lethal disease, whether mucin was present or not. Stoenner (1965), however, reported that the ID_{50} of 6 strains of *B. neotomae* for mice was 8 organisms although they were less virulent for guinea-pigs (ID_{50} of 38500). In both species, the greatest concentration of organisms was in the spleen.

Grekova & Gubina (1965) studied the virulence for guinea-pigs of 3 strains of *Brucella* isolated from reindeer; one strain was of less virulence than the remaining 2 whose virulence was comparable to that produced by a fully virulent *B. melitensis*. (See also Meyer, 1966*b*.) A marked reduction in virulence by passage of *B. melitensis* in media containing neomycin and oxytetracycline (3 units each/ml) was reported by Ivanov & Kirillov (1964), and Klochkov (1964) found a stable, avirulent and non-agglutinogenic variant by exposure to UV light.

Sacco (1966, 1968), using various routes of challenge of *B. abortus* in guinea-pigs, found that the intramuscular was the most sensitive, whilst oral and urethral routes were the least sensitive. In a study of the susceptibility of selected wild, laboratory and domestic animals to various species of *Brucella* (Thorpe, Sidwell & Lundgren, 1967), mice were found to be relatively susceptible but rats, lagomorphs and squirrels were more resistant. All the *Brucella* species studied were capable of causing chronic infection in the susceptible species but there was no evidence of excretion of the organisms. Both mule deer and sheep were found to be resistant to *B. neotomae*. Although dogs and foxes could be infected with *B. suis* (but not cattle, sheep, cats and fowls), they were not considered likely to act as carriers (Milanov, Chilev, Krustev & Mateev, 1966). Pigs were also reported to be much less sensitive to infection with *B. abortus* and *B. melitensis* than to *B. suis* infection (Orlov, 1965*a*).

Mateev (1968) found that 9 of 35 strains of *B. suis* were very virulent for guinea-pigs, 2 were avirulent and the remainder were of moderate virulence.

FitzGeorge, Keppie & Smith (1965) found that there was a correlation between virulence of strains of *B. abortus* for guinea-pigs and their sensitivity to hydrogen peroxide but this was not found in strains of *B. melitensis* and *B. suis*. Dranovskaya (1966) found that the Michaelis constant of dehydrogenases of virulent *B. melitensis* was 10–100 times greater than in less virulent strains, whilst Vershilova, Dranovskaya & Larionova (1967) reported that the incorporation of 0·09 M lactate in the medium inhibited the growth of weakly virulent strains compared with virulent ones.

Pathology and pathogenesis

Cattle

The preferential growth of *B. abortus* in certain foetal tissues has been ascribed to the presence of a growth stimulant, erythritol, in these tissues (Smith *et al.* 1962; Keppie, Williams, Witt & Smith, 1965). It was further reported (Anderson & Smith, 1965; Williams *et al.* 1964; Keppie *et al.* 1967) that concentrations of erythritol

up to 1 μM/ml stimulated the growth of virulent strains of *B. abortus* much more than that of attenuated or avirulent strains; it was found that the growth of Strain 19 was inhibited by 2 μM/ml or more of erythritol, thus confirming the work of Jones *et al.* (1965). Crouch & Elberg (1967) also reported that the growth of the *B. melitensis* Rev. 1 vaccine strain was stimulated by erythritol. Meyer (1966*c*, 1967*a*), however, reported that utilization of erythritol was a characteristic of the genus and was unrelated to strain virulence.

Hignett & Nagy (1964) and Nagy & Hignett (1967) found that heavy exposure of very young calves to virulent *Brucella* by mouth for the first 15 days of life led to diminished serological responsiveness when challenged at 6 months of age. They further reported that neonatal infection led to a degree of immunity against subsequent exposure, and made the important point that calves infected with *B. abortus* at 7 months of age may become infected permanently. Morgan, MacKinnon, Lawson & Cullen (1969) found that some young calves born of infected dams reacted to the Rose Bengal plate (RBP) agglutination test and to the complement fixation (CF) test; the serum agglutination test (SAT) was sometimes negative and in any case became negative sooner than the CF and RBP tests which remained positive for up to 6 months. Hignett, Nagy & Ironside (1966) and Baker & Faull (1967) found no difference in the conception rates to first service in an infected herd compared with a non-infected herd. In a further paper (Hignett, Nagy & Ironside, 1967*a*), they reported on the cultural examination of milk, vaginal mucus, placental cotyledons, foetal fluids and lymph nodes from cattle of varying status of vaccination and infection; little success was obtained in isolating the organism from lymph nodes of known infected cattle and it was suggested that the Ringer's solution used may have had a deleterious effect on the organism. Nelson *et al.* (1966) again confirmed that *B. abortus* was recovered much more frequently from the supramammary lymph node than from other nodes and that recovery was also much more frequent from the right hind quarter.

Lambert, Deyoe & Painter (1964) studied the persistence of Strain 19 vaccine in 2 young bulls; one animal vaccinated at 6 months of age developed orchitis within 10 days and Strain 19 was isolated from the testes 2 months later; in the other animal, Strain 19 was isolated from the semen 12 months after vaccination. Rankin (1965), in a study of 12 naturally infected bulls, concluded that bulls could be infected in calfhood and retain infection into adult life, that they were rarely responsible for the spread of the disease to cows by natural service, and that brucellosis was not an important cause of infertility in bulls.

The reactive tissue changes in brucellosis of man and animals were discussed by Jacob (1964) and 2 staining techniques for demonstrating *Brucella* in tissue sections were described (Jacob, 1968). Bierer & Powell (1967) found a sharp fall in the white blood cell count immediately after calving or abortion but no changes which could be specifically attributed to infection with *Brucella*.

Sheep

An account of *B. abortus* infection in sheep was given by Luchsinger & Anderson (1967) in the U.S.A. and by Allsup (1969) in Britain. Luchsinger & Anderson (1967) reported that ewes aborted or gave birth to weak lambs during 2 consecutive lambing

seasons; the spread of infection was attributed to animal husbandry practices and ecological conditions; infected cattle were present on the farm. Allsup (1969) isolated *B. abortus* biotype 1 on 4 occasions from unconnected outbreaks of ovine abortion; there was little lateral spread within flocks and again, evidence of cattle infection was established on 2 of the farms.

In a continuation of their studies on the pathology of brucellosis in sheep, Collier & Molello (1964) inoculated sheep in advanced pregnancy with *B. abortus*, *B. melitensis* or *B. ovis*. *B. abortus* and *B. melitensis* induced comparable effects: a short period between inoculation and impending abortion, intense colonization of organisms in the placentomes, invasion of foetal tissues and many foetal deaths. Infection with *B. ovis* was much less virulent: foetal death did not occur until at least 45 days after inoculation, the organisms colonized the interplacentomal areas, and there was little evidence of foetal invasion.

Brucellosis in sheep in South Africa can be caused by *B. ovis*, *B. abortus*, *B. melitensis* and even *B. suis* (Van Drimmelen, 1966); the isolation of *B. suis* from a goat was also reported by Manley (1968).

Naimanov (1966) studied the sera of young lambs born of ewes infected with *B. melitensis*; brucella antibodies, detected by the CF test, could be present from 5 days of age onwards. Anreani (1967) concluded that *B. melitensis* persisted for a short time in the rams he studied.

B. ovis *in sheep*. A number of papers have appeared on the pathology of *B. ovis* infection in sheep and in laboratory animals (Epstein, Ciprián, Andreatta & Bacigalupo, 1964; Ciprián, Andreatta & Epstein, 1967; Biberstein, McGowan, Olander & Kennedy, 1964; Dimitrov, 1966; Shott, 1967). Primary lesions were found in the tail of the epididymes and interstitial tissue of the seminal vesicles and ampullae and, after a bacteraemic phase, the organisms were localized in these organs, together with the liver, spleen and kidney (Biberstein *et al.* 1964). No evidence of an autoimmune reaction was found by Shott (1967).

Studies on the pathological and immunological responses of foetal lambs to *B. ovis* have been made (Osburn, 1965, 1968; Osburn & Kennedy, 1966; Osburn & Hoskins, 1969; Biberstein, Kennedy, Robinson & McGowan, 1966). Although bacteraemia may occur at any time during gestation, localization of the organisms in the placenta did not occur before the 75th day; even after intrauterine inoculation of ewes at various stages of pregnancy, *B. ovis* required 3–7 weeks of activity in the pregnant uterus before abortions occurred. It was concluded that the foetal lamb became immunologically competent to *B. ovis* early in gestation and thereby became capable of surviving for long periods in utero. The variations in the inflammatory response in foetal lambs when sheep in varying stages of pregnancy were exposed to infection was thought to be due to the immaturity of the foetal immunological system.

Biberstein *et al.* (1966) demonstrated that foetuses of ewes infected with *B. ovis* produced antibodies to the organism and these were unrelated to the presence or absence of antibody in the dam. This was confirmed by Osburn & Kennedy (1966) and Osburn & Hoskins (1969) who showed that foetal lambs produced both IgM and IgG; CF antibody activity was associated with both immunoglobulins.

Pigs

Accounts of the lesions found in porcine brucellosis have been given by Zettl (1964), Fiedler (1965), Pulst (1964) and Englert, Weis & Osolina (1964). The importance of the boar in spreading the disease was stressed by Florent & Docquier (1965) and Vandeplassche *et al.* (1967). In an account of a swine brucellosis epizootic by Luchsinger, Anderson & Werring (1965) it was believed that the disease was introduced into 3 herds by the purchase of pregnant females; they also stressed the importance of mechanical spread in herds. Orlov (1965*a*) reported that pigs became susceptible at 4–5 months of age but were resistant to infection with *B. melitensis* and *B. abortus*. Meyer (1967*b*) in a study of the host–parasite relationships in brucellosis found that only 1 of 168 isolates from swine was *B. abortus*; the remainder were all *B. suis*, 146 being *B. suis* biotype 1. Hares were believed to have introduced infection into a herd by Kötsche & Stahl (1967). In a study cf the pathogenesis of brucellosis in swine, using *B. suis* biotypes 1 and 3, Deyoe (1967, 1968) found no evidence that the biotype used had any effect on the severity of the infection.

Horses and donkeys

Abortions due to *B. abortus* biotype 1 were described by McCaughey & Kerr (1967*a*) and Shortridge (1967) in mares and by Crossman & Bonson (1968) in a donkey.

Rabbits

Kniazeff, Elberg & Traum (1964) reported that rabbits in states of pregnancy, pseudopregnancy or under progesterone treatment in oestrus were more susceptible to generalized *Brucella* infection; even so, the organism could not be isolated from the uterus except in the latter half of pregnancy.

Dogs

Reference has already been made to the disease in dogs caused by *B. canis* (Carmichael & Bruner, 1968). Infection of dogs with *B. abortus* has been reported by Clegg & Rorrison (1968) in this country, Kimberling *et al.* (1966) in the U.S.A., and McErlean (1966) in Ireland; in the latter case, *Brucella* was not isolated but the animal had a high titre to *Brucella* and *B. abortus* infection was known to occur on the farm. Nicoletti *et al.* (1967) reported the isolation of *B. suis* from a bitch which had aborted 5 months previously; pigs on a neighbouring farm were infected with *B. suis* and the dog's owner also developed a febrile illness with a high *Brucella* titre. The pathological changes occurring after experimental inoculation of dogs with *B. melitensis* were described by Kolesnik, Pinigin & Petukhov (1966); it was concluded that dogs were resistant to brucellosis.

Brucellosis in other animals

There is a growing literature on the incidence of brucella reactors and of the isolation of species of *Brucella* from wild animals. Meyer (1967*b*) reported on the species of *Brucella* typed from various animals.

Reports have appeared from the Soviet Union of the isolation of atypical species of *Brucella* from murine rodents (Dushina, Mitrofanova, Chudentsova & Savchenko,

1964; Korol, 1964, 1969; Korol & Parnas, 1967). Korol reported that, of 6 foci of murine brucellosis, only 2 coincided with infection in farm animals. Ivanov, Nikiforov & Kolesnikova (1968) reported that rats were more susceptible to *B. abortus* than to *B. melitensis* or *B. suis* infection; however, *B. abortus* could not be recovered beyond 40 days. Renoux (1968) reported that mice infected with *B. melitensis* Strain H38 developed a chronic disease although the antibody response was poor.

The importance of brucellosis in hares is the risk of spread to domestic animals, especially pigs (e.g. Kötsche & Stahl, 1967). The histopathology of the disease in hares has been described by Becker (1964) and Tropilo (1967). Olszewski & Wilczyński (1968) failed to infect hares orally with *B. abortus*.

Roth (1967) surveyed various game animals in Rhodesia for brucellosis; no evidence for endemic brucellosis was found. McCaughey & Fairley (1969) and McCaughey (1969) found that 4 of 32 fox sera reacted to the CF test (but were culturally negative). Szyfres & Tomé (1966) also reported evidence of infection with *B. abortus* in foxes in Argentina. In an extensive study of brucellosis in wild life in Utah, Thorpe, Sidwell, Bushman, Smart & Moyes (1965) isolated *B. neotomae* from the desert wood rat (15 isolations), from fleas on the desert wood rat (one isolation), *B. suis* from a black-tailed jack rabbit (one) and *B. melitensis* from the same species. Sixty-eight sera from 13 animal species contained antibodies for *Brucella*. *B. abortus* was isolated from one of 70 white-tailed deer and 3 of 170 mice by Corey, Paulissen & Swartz (1964) in Arkansas. Huntley and his colleagues (Neiland, King, Huntley & Skoog, 1968; Brody, Huntley, Overfield & Maynard, 1966) have continued their studies on the incidence, pathology and symptomatology of brucellosis in caribou in Alaska; the position in the USSR has been reviewed by Davydov (1965), Vershilova *et al.* (1966), and Khoch & Davydov (1968).

Vaccination

General

Methods for the large scale production of Strain 19 cells by continuous culture have been described by Collins, Boyce & Edgar (1966); Boyce & Edgar (1966); Boyce, Edgar & Maley (1967); Semcheva, Vinogradova & Larionova (1964); Szakmáry & Hoffmann (1964); Scheibner (1966) and Alton & Jones (1967). The inclusion of buffer salts and an antifoam agent in the culture medium were found to be important. The viability of cells surviving after freeze-drying was 10–15 % less in those grown by continuous culture than in those grown on solid medium (Boyce & Edgar, 1966). Carboxymethylcellulose is widely used to sediment the bacterial cells and was reported by Horwell (1967) to prolong the viability of *Brucella* cells. Leeman, de Weck & Schneider (1969) reported that hypersensitivity to carboxymethylcellulose in cattle caused anaphylactic reactions to certain drugs, e.g. certain commercial penicillin preparations which are said to contain carboxymethylcellulose to stabilize them or to facilitate solution. Orr (1964) reported anaphylactic shock in 1 of 4 calves following vaccination with Strain 19. Love, Pietz & Ranger (1966) studied the effect of storage at 4, 25 and 37·5 °C on Strain 19 vaccines from 3 commercial sources (designated A, B, and C). At 25 °C viable counts fell to an unsatisfactory level within 12 weeks, and within 3 days when stored at 37 °C. Even at 4 °C, only one preparation maintained its viable count throughout its dating period.

The 2,3,5-triphenyltetrazolium reduction test was reported to be satisfactory as an

index of viability of Strain 19 vaccine (Sprovieri & Freitas, 1967). Teklinski (1966) found that freeze-drying had no effect on the antigenicity of Strain 19 vaccine, compared with liquid vaccine. Keppie *et al.* (1969) found that the immunogenicity of *B. abortus* for mice was increased by adding 20 % allantoic fluid to the medium used for growing the cells. The growth of *B. melitensis* Rev. 1 vaccine by continuous culture was reported briefly by Morgan, Boyce & Casey (1970); they also reported that the viability of this vaccine was at least 50 % greater by preservation in liquid nitrogen than by freeze-drying, confirming the observations of Cedro, Quenedo, Forti de Menaldo & Cisale (1969).

Beck, Ellis, Fichtner, Laiho & Whitehead (1964) stressed that vaccination equipment, especially syringes, if employed without proper care in their cleansing and sterilization, could lead to the carry-over of Strain 19 vaccine from one animal to another. These authors filled a syringe with reconstituted Strain 19 vaccine, which was then expelled, and the syringe filled with sterile saline and used for both viable counting and inoculation into a cow (first dilution). This procedure was repeated up to a ninth dilution. Although Strain 19 could be recovered (321/ml) up to the eighth dilution, a direct effect on the antibody levels could be found only up to and including the fourth dilution which contained $4{\cdot}21 \times 10^3$ viable cells per ml. Irrespective of the number of Strain 19 cells which were administered, maximal antibody levels were reached 8 days later but their level and persistence did depend on the number of cells injected.

Pilet (1965) confirmed these findings using guinea-pigs; even after washing out the S19 contaminated syringe with 90 % alcohol, positive agglutination titres occurred when the syringe was used to inject sterile saline into guinea-pigs. Work at this laboratory (G. A. Cullen & M. J. Corbel, to be published) has also shown a similar pattern of results in cattle, even when the vaccine was heat-killed before the serial dilutions in the syringes were made. These results would indicate that only disposable syringes should be used for vaccinating cattle, especially in areas where a test and slaughter policy for brucellosis is under way.

Experimental vaccination of laboratory animals

Khaev (1966) found that the intradermal injection into guinea-pigs of small (1×10^5) doses of Strain 19 gave better antibody production than did the same dose given by subcutaneous injection, whilst Lu Shih-liang, Liu Te-cheng & Ch'en Cheng-jen (1965) found percutaneous vaccination (by skin scratches) was less effective than subcutaneous inoculation. Oral administration of vaccine was found to be ineffective by Krylova, Garanina, Davletshina, Salmakov & Sagitova (1967), whilst repeated vaccination (5 times over a 6–7 month period) led to a reduction in protection (Grekova, 1968). Rather surprisingly, Levaditi, Guillon & Marin-Aponte (1968) found that the immunogenicity of Strain 19 in guinea-pigs was impaired when it was incorporated in Freund's incomplete adjuvant.

Morgan (1968) reported that Strain 19 vaccine in guinea-pigs gave good protection (100-fold increase or more in the ID_{50}) against most of the strains of *B. abortus* biotype 1 examined; the protection against *B. abortus* biotype 2 was considerably lower. The vaccine gave adequate protection against some but not all of the other biotypes (4, 5 and 9) examined.

Gerzymisch, Hock & Strey (1967) found that antibody production to Strain 19 in rats depended on the quality of the dietary protein.

A number of reports have appeared, especially from the Soviet Union, of trials with various vaccine strains, e.g. Abidjanov (1965), Zhovanik (1966), Orlov & Klochkov (1967), Matvienko, Baiturina & Myakushina (1967), Klochkov (1965, 1967), Ostrovskaya (1965), Kharisov & Sakharova (1966), Kharisov, Sakharova & Krylov (1966), Trilenko (1965), Kirillov (1964, 1968), Ivanova & Tuzova (1969), Salmakov (1964), Ostrovskaya & Vershilova (1967), Taran, Nelyapin & Polyakova (1964), Taran, Nelyapin, Polyakova & Lunina (1964), Taran, Zamakhaeva, Abakin, Polyakova & Nelyapin (1965). Taran *et al.* (1965) worked with the well-known 104M vaccine strain which was shown to be more immunogenic than Strain 19. León (1966) used a strain obtained by passage in ox bile tryptose agar, whilst Quatrefages, Carrère and Petitdidier (1965) found that the efficiency of a killed vaccine was enhanced by the incorporation of a saponin.

Nishihara, Ahn, Taplan & Carpenter (1964) found that a vaccine prepared from *B. melitensis* irradiated with cobalt-60 produced a higher degree of protection in mice than vaccines prepared from heat-killed or chemically killed cells, but Babini & Luboz (1967) found that vaccines inactivated by X-rays were no better than formalin-killed cells.

The use of Rev. 1 vaccine in experimental animals has been reported by Alton (1969*a*, *b*), Ulasevich (1965), Elberg & Faunce (1964), Hadley (1967), Orlov, Ulasevich & Borisovich (1965) and Vershilova, Ostrovskaya & Grekova (1966). A vaccine dose as low as 260 cells of Rev. 1 was found by Elberg & Faunce (1964) to produce a high degree of immunity in cynomolgus monkeys. Alton also found that 100 cells of Rev. 1 produced nearly as good immunity in guinea-pigs as a dose of 10^8 cells. Vershilova, Ostrovskaya & Grekova (1966) found that populations of initial cultures of Rev. 1 and 104M vaccine were not homogeneous, but contained a proportion of dissociated cells; only 0·5 % of dissociated cells was found in the 19-BA vaccine.

The efficiency of combined vaccines has been reported by Yusupov (1965), Pilipenko (1966), Miller, Blackmon, Pate, Martin & Foster (1968), Gubina & Chernysheva (1964), Knyazeva (1965) and Boyakhchyan & Kalashyan (1967). Some workers, e.g. Gubina & Chernysheva (1964), report that anthrax and brucella combined vaccines produced a lower level of protection against *Brucella* but there may be species differences as well.

Studies on the immunogenicity of cell wall and other fractions of *Brucella* have been reported by Rasooly, Olitzki & Sulitzeanu (1965, 1966, 1967), Sulitzeanu (1965), Macrae & Smith (1964), Ellwood, Keppie & Smith (1967), Lembke & Milczewski (1966), Bobo & Foster (1964), Foster & Badakhsh (1965), Badakhsh & Foster (1966), Gargani & Pin (1964), Gargani & Guerra (1967), Sen, Ghosh & Singh (1968), and Roux, Asselineau, Serre & Lacave (1967). Work by Rasooly *et al.*, Macrae & Smith (1964) and Ellwood *et al.* (1967) has shown that the protective antigen and the agglutinogen were identical. Immunogenic material obtained from culture filtrates of *B. abortus* was found by Ellwood *et al.* (1967) to be identical with cell wall material, and these authors drew attention to the high phospholipid content of the immunogen; heptose was absent, and lysine was the predominant amino acid of the 8 which were detected. Rasooly *et al.* (1967) drew attention to the large variability in response of

guinea-pigs to challenge with *Brucella* and the consequent inability to obtain reasonable dose–response curves.

Bobo & Foster (1964) and Foster & Badakhsh (1965) studied the immunogenicity and toxicity of ether extracts of *B. abortus*—the so-called endotoxin-containing precipitate (ECP)—which were rendered non-toxic by treatment with lysozyme; this treatment had no adverse effect on the immunogenicity of the extract when tested in mice, and their work also indicated that the ECP with or without treatment with lysozyme can immunize swine. The work reported so far, using cell wall material or extracts, indicates the possibility of vaccinating both man and animals with purified extracts and not live cells.

Halliday (1968) studied the effect of passively acquired antibody on the agglutinin response of rats to *B. abortus*: agglutinin production was significantly inhibited by low concentrations of maternal antibody in young rats born of immune mothers; inhibition was also produced when rats from non-immune mothers were transferred to immune mothers. It was also found that, after feeding with brucella-immune serum, absorption of 7S immunoglobulins (Igs) occurred in preference to 19S and the degree of inhibition of agglutinin production was proportional to the amount of antibody absorbed; antiserum containing 19S Igs had little effect on agglutinin production.

Pilet, Mallick & Le Garrec (1967), on the other hand, found that the immunity of white rats born of vaccinated mothers was comparable to that produced in rats born of non-immune mothers although their previous work had suggested that the antibody response of young born of vaccinated mothers was less than that of control animals. Morris (1965, 1967) also reported that, in rats, only gamma 2 globulins were transmitted across the gut of the rat but showed incomplete antibody activity in the serum after absorption; this was attributed to a configurational change and not to selective transfer. In a previous paper, Halliday (1965) reported that newborn lambs did not preferentially absorb different immunoglobulins.

The use of brucella vaccines consisting of killed cells incorporated in an adjuvant is increasing both in cattle and in sheep and goats (see later). In the Report (Report 1964*b*) of a group convened by WHO, the following advantages were claimed for the use of adjuvant vaccines: 1, use of smaller amount of antigen in single and combined vaccinations; 2, attainment of a better immune response especially where high antibody levels are required, e.g. influenza; 3, fewer systemic reactions because of the reduced amount of antigen used or its slower release; 4, inclusion of many antigens in a single, combined vaccine; 5, reduction in the number of injections required to obtain effective immunity; 6, reduction in the total number of injections needed for the many immunizing agents now available.

A new metabolizable adjuvant for human use was described by Woodhour, Metzgar, Stim, Tytell & Hilleman (1964) and Hilleman (1964), who also discussed the possible modes of action of adjuvants. Talal, Herman, de Vaux St Cyr & Grabar (1964) studied the effects in mice of injecting Bayol F (a common ingredient of adjuvants) on its own or together with ovalbumin, or ovalbumin alone (as controls). Marked gamma globulin changes occurred in the 2 groups injected with Bayol F and later peritoneal plasmacytomas and myeloma globulins occurred after the ninth month.

The use of mass grinding of tissues prior to cultural examination has been advocated by Renoux (1964) and Pilet & Le Garrec (1967*a*, *b*, 1969), and Pilet & Le Garrec (1966) recommended the use of mice for evaluating brucella vaccines (post-mortem examination and spleen counts made 6–12 days after challenge; see also Sulitzeanu, 1965).

Vaccination of cattle

Strain 19 vaccine is still widely used for vaccinating cattle. Redman, Deyoe & King (1967) found that vaccination of calves at 2 or 3 months of age conferred as good protection as when vaccinations are made at 4–8 months of age and post-vaccinal antibodies receded to a negative status sooner in cattle vaccinated as calves at 2–3 months of age compared with those vaccinated at 4–8 months of age. Powell, Hendricks & Roebuck (1967) found that vaccination at 3 months of age also conferred good immunity to subsequent challenge in the first pregnancy. In Britain, only female calves between 91 and 180 days of age can be vaccinated with Strain 19 (Anon, 1967). The importance, especially during eradication campaigns which are accompanied by the use of S19 vaccination, of over-age vaccinations has been stressed by a number of people, especially in the U.S.A. (e.g. Schilf, 1966; Manthei, 1964; Wixom & Vanderwagen, 1966; and Fichandler, 1967). Reference has already been made to the potential dangers of using syringes contaminated with Strain 19. Murchison, Wood & Smith (1966) reported a greater use of Strain 19 vaccine in Britain in 1965 compared with 1960–1. Ivanov & Chulkov (1969) recommended the use of S19 vaccination together with other measures only in recently infected herds with a morbidity rate not exceeding 47 %.

The isolation of Strain 19 from the milk of Strain 19 vaccinated cattle has been reported by Jones *et al.* (1965) and Nicoletti & Fincher (1966) and from bulls by Lambert *et al.* (1964); the importance of checking the possible identity of CO_2-independent cultures with Strain 19 has been stressed. Nicoletti & Fincher (1966) also found that the use of various chemotherapeutic agents failed to eliminate the excretion of Strain 19 in the milk and the blood serological picture was unaffected; this latter finding was also confirmed by Schipper & Myers (1964).

Miller *et al.* (1968) reported that there was a marked depression of serum alkaline phosphatase activity for 2–11 days after vaccination with Strain 19; injection of the diluent only had no effect.

The effect of the route of inoculation of Strain 19 vaccine has been studied by Camy (1967), who reported a delay in the appearance of serum antibodies following intra-uterine inoculation, but sterility followed the injection of the full dose of vaccine. Kharissov, Sakharova & Abouzarov (1966) found that calves could be effectively immunized by inhalation of bacterial cells; $32–35 \times 10^9$ bacterial cells were inhaled during exposure for 45 min.

The simultaneous inoculation of Strain 19 with different vaccines had no adverse effect on the antibody response (Moya, Blood & Alvarez, 1964; Smith, Fastier & Hansen, 1965) although Gubina & Chernysheva (1966) reported that anthrax vaccine interfered with the response to Strain 19. Lokteva (1967) and Ruffo & Casillo (1965) confirmed that agglutinins appeared 1–2 weeks after adult vaccination with Strain 19 and disappeared $1\frac{1}{2}$–5 months later. Stepin (1966) found that the antibody levels in

inguinal and pelvic lymph nodes were higher in infected compared with Strain 19 vaccinated animals.

Lambert, Deyoe & Painter (1966) reported that post-vaccinal agglutinins persisted longer in bulls than in heifers vaccinated at similar ages; at post-mortem examination at 18 months of age, no gross or microscopic lesions were found nor was Strain 19 isolated, although in a previous paper (Lambert *et al.* 1964) Strain 19 was isolated from 2 vaccinated bulls.

Adjuvant vaccines containing either *B. abortus* Strain 45/20 or *B. melitensis* Strain H38 have been developed for use in cattle. The development of the 45/20 adjuvant vaccine was reviewed by Morgan & McDiarmid (1968) with special reference to the local reactions at the point of inoculation, the antibody response to vaccination and the resultant immunity, and the important part which the nature of the adjuvant used, plays on all 3 factors. With the adjuvants now available and in use, they concluded that the strategic use of 45/20 adjuvant vaccine, together with other measures of control mentioned in the article, could play a useful role in the control of bovine brucellosis. The circumstances under which the use of 45/20 adjuvant vaccine might be beneficial were specified in a joint statement by the Agriculture Departments of Great Britain (Anon, 1967). The reader is also referred to important papers by McDiarmid (1968), Roerink (1966, 1967*a*, *b*, 1969), Florent & de Keyser (1965), de Keyser & Florent (1967), Cunningham (1966, 1968); Cunningham & O'Reilly (1968), MacIntyre & Sizer (1969), and Powell *et al.* (1967); the latter noted that 2 injections of the vaccine at 3 and 6 months of age were less effective than 2 injections at 6 and 9 months of age. Although differences have been reported (e.g. de Keyser & Florent, 1967; Renoux, Nicolas, Imbert & Quechon, 1964*a*) especially on the degree of immunity following 45/20 vaccination, these may, in part, be due to the nature of the adjuvant used. Results of published work can be summarized as follows: with the adjuvants now used, the vaccine causes a not unacceptable local reaction; the antibody response depends on the previous brucella status of the animals and reactions to the CF test persist longer than reactions to the SAT; the immunity following 2 injections of the vaccine compares favourably with that produced by Strain 19 and the use of 45/20 adjuvant vaccine has no effect on the milk ring test (MRT).

The results of experimental and field use of another adjuvant vaccine containing killed virulent *B. melitensis* Strain H38 cells have been reported by Renoux and his colleagues (Renoux *et al.* 1964*a*, *b*, *c*; Renoux & Valette, 1967*a*, *b*; Valette & Renoux, 1967*a*, *b*; Renoux, Philippon & Plommet, 1969). In a comparative trial with 45/20 adjuvant, Strain 19 and Rev. 1 it was reported that the best protection was afforded by a single injection of the H38 adjuvant vaccine, followed by Rev. 1, Strain 19 and 45/20 adjuvant, in that order. It was also claimed that the antibodies following the use of the H38 adjuvant vaccine receded to negative levels more rapidly than after Strain 19 vaccination and the immunity lasted for at least 2 years.

It has also been reported (Renoux *et al.* 1964*c*; Valette & Renoux, 1967*b*) that the simultaneous administration of H38 adjuvant vaccine and Foot-and-Mouth disease vaccine had no adverse effect on the resultant immunity to brucella infection 223 days after vaccination. Combined use of the Foot-and-Mouth vaccine and 45/20 adjuvant vaccine resulted in a lesser degree of protection against *Brucella*.

The use of Rev. 1 vaccine in cattle against *B. abortus* challenge has been reported by Renoux *et al.* (1964*a*, *b*, referred to above) and Van Drimmelen & Horwell (1964); the latter reported that the vaccine had no adverse effect on calves or mature cows and was not excreted either in the milk or placenta after calving; similarly, its use was considered safe in both young and mature bulls.

Abdullin & Salmakov (1966) reported that the Strain 82 (isolated by Salmakov, 1964) protected 3 of 6 heifers against experimental challenge with *B. abortus* compared with 4 of 6 protected with Strain 19; serological tests were negative within 30 days after the use of the 82 strain. Jovanik (1965) reported on the use of the VIEV brucella vaccine in infected herds, alongside other control measures.

Vaccination of sheep and goats

Strain 19 vaccine is used for sheep, especially in the USSR (Bronnikov, 1965; Golotov, 1969; Kotchourine & Kossilov, 1965; Lokteva & Belyaeva, 1965; Orlov, 1965*b*; Orlov, Ulasevich & Ivanov, 1965; Cheremisin, Ivanova & Rastorgueva, 1969; Barcaccia & Tornimbeni, 1966; Lucchetti, 1966). Best results are achieved by revaccination; vaccination of adult sheep is effective in large farms at risk of infection. Bronnikov (1965) reported that, during the second half of pregnancy, 26 of 50 Strain 19 vaccinated ewes developed serological or allergic reactions although they were negative prior to insemination (12 months after vaccination); a somewhat similar observation was made by Le Pennec (1967). Immunization of sheep with Strain 19 by means of aerosol was found by Selivanov (1964, 1965) to be as effective as by subcutaneous inoculation. Yin, Shieh & Shi (1963) found that Strain 19 in goats gave poor protection against *B. melitensis* challenge but the immunity was enhanced by mixing the vaccine with aluminium hydroxide gel. Strain 19 was found to give no protection in goats against experimental challenge with *B. melitensis* Strain H38 by Morgan, Littlejohn, MacKinnon & Lawson (1966).

The use of Rev. 1 vaccine in both sheep and goats was reviewed by Alton & Elberg (1967); papers on the use of this vaccine have been published by Alton (1966, 1967, 1968), Alton, Elberg & Crouch (1967), Ceccarelli (1969), Entessar, Ardalan, Ebadi & Jones (1967), Ghosh, Sen & Singh (1968), Jones, Entessar & Ardalan (1964), Morgan *et al.* (1966), Ne'eman (1968*a*, *b*), Orlov, Ulasevich & Borisovich (1965); Orlov *et al.* (1965), Paltrinieri, Farina & Andreani (1968), and Ünel, Erdem, Williams & Stableforth (1969). It was concluded that Rev. 1 was stable, did not revert on continued passage, was safe for use in non-pregnant animals, and that one injection produced an immunity that lasted for up to $4\frac{1}{2}$ years. Within a year of vaccination, the majority (70 %) of animals are negative to the SAT and most, if not all, are negative to the CFT.

The H38 adjuvant vaccine was found by Ivanov, Kirillov & Popova (1965) to give good protection (12 of 16 animals immune) in sheep against experimental challenge although the CF titres were found to persist for much longer than with Strain 19 vaccine.

Studies on other vaccines have been reported by Trilenko, Tivelev, Subbotin & Fomenko (1965) and Trilenko (1967), who reported that the Strain K gave better protection in sheep than Strain 19 as well as not causing the appearance of serum agglutinins, whilst Ivanov & Kirillov (1968) also found their Strain 89/23, obtained

by passage in chick embryo, to be an effective vaccine for sheep. León & Guerrero (1968) found that the strain of *B. melitensis* obtained by passage in ox bile (see León, 1966) protected goats against experimental infection.

Lafenêtre, Carrère, Petitdidier, Vollhardt & Quatrefages (1964) and Lafenêtre, Carrère, Cortez, Vollhardt & Quatrefages (1964) reported that their live Strain B112 vaccine and 'NeoBrucel' (45/20 adjuvant) vaccine protected sheep. In unpublished work from this laboratory it was also found that 45/20 adjuvant vaccine gave good protection in goats against experimental *B. melitensis* challenge; there was no difference in the immunity following 1 or 2 doses of vaccine. Experiments are in progress to determine the duration of immunity produced by this vaccine.

Paltrinieri, Farina & Andreani (1967) and Gargani & Guerra (1967) studied the protection and the serological response of sheep inoculated with a frozen and thawed suspension of *B. melitensis* Strain H38; they drew attention to the early bacteriological recovery in natural infection. Using an extract as antigen, they found that the CF titre persisted much longer than the agglutination titre. A non-specific immunity to *Brucella* following BCG vaccination of sheep was reported by Cheng Hou-ching, Ts'ao Shu-tse & Lo Chung-yü (1965) but the number of animals used was very small. Cedro, Cisale, de Benedetti & Maubecin (1963), using simultaneous vaccination with an attenuated strain of *B. abortus* and heat-killed *B. melitensis* on 2 occasions in goats, reported that 1 of 12 vaccinated goats aborted but only 6 of the 11 controls.

León & Guerrero (1969) reported that innate immunity in goats was governed by a simple autosome recessive factor.

Vaccination of sheep against B. ovis. The Rev. 1 vaccine has been reported to give good protection lasting at least 4 years (Van Heerden, 1964). The other vaccine is killed *B. ovis*, either as 2 injections or one injection with Strain 19. Swift & Maki (1968) found that there was a difference in immunity depending on the strain used for preparing the vaccine. Ris (1967) found that rams, vaccinated 3 years previously with the combined *B. ovis* and Strain 19 vaccines, were all negative to the CF test for *B. ovis*.

Vaccination of reindeer

The vaccination of reindeer with Strain 19 has been reported by Golosov, Klimontov & Zabrodin (1964), Davydov & Lysko (1967) and Vashkevich (1964, 1966). Vashkevich found that reindeer reacted severely to Strain 19 at the full cattle dose; vaccination with a half or a tenth the cattle dose at 2, 6 or 14 months of age gave the best protection against challenge with a fully virulent reindeer strain. Golosov *et al.* (1964) reported that immunity was maximal 8 months after vaccination although agglutination and CF titres lasted for 2 years.

Vaccination of pigs

Edens & Foster (1966) continued their work with endotoxin-containing preparations (ECP) of *B. suis* to study its immunogenicity for pigs. Definite resistance was conferred and treatment of ECP with lysozyme had no deleterious effect on the degree of protection. Genev *et al.* (1966) reported that a strain, Dyulevo, which they had isolated was avirulent for pigs but the immunity produced, if any, was not tested.

Vaccination of man

The only vaccine in use against human brucellosis is the BA variant of Strain 19 (Vershilova, 1961) and its use was summarized by Vershilova (1965), who stated that vaccination in people at risk has brought about a 3–7-fold decrease in incidence. Pappagianis, Elberg & Crouch (1966) studied the effect of graded doses of Rev. 1 in human volunteers. Two of 3 volunteers given 10000 organisms developed fever and mild symptoms whilst the third gave a positive blood culture and mild symptoms. Doses of 1000 organisms induced no fever or symptoms and this low dose was accompanied by a delayed (9 months) agglutinin response; the CF antibody response appeared later than the agglutinin response. It was felt not possible to recommend the use of this vaccine for man.

Diagnosis

Immunoglobulins

Since the last review, considerable advances have been made on the immunoglobulins (Igs)—their nature, structure, formation, and factors affecting their production and persistence. The development of new techniques in electrophoresis, immunoelectrophoresis, density gradient centrifugation and column chromatography also led to considerable confusion in nomenclature and a WHO meeting on the Nomenclature of Human Immunoglobulins (Report, 1964*c*, 1966) recommended an agreed method of definition and nomenclature (see Cohen, 1965; Cohen, 1968; Fahey, Franklin, Kunkel, Osserman & Terry, 1967; Haber, 1968; Martin, 1969; the Report, 1968, of a WHO Scientific Group on the Genetics of the immune response). The reader is also referred to numerous papers on 'Antibodies' in the *Cold Spring Harbor Symposia on Quantitative Biology*, **32**, 1968.

Since the work of Porter (1962), Cohen & Porter (1964), and Edelman & Galley (1964), the structure of Igs was shown to consist of 2 heavy (H) and 2 light (L) chains, covalently linked by disulphide bonds.

There are 2 types of light chain in all the immunoglobulins, κ and λ, but they are never found together on the same molecule; in man, 70 % of immunoglobulins carry the κ chain and 30 % the λ chain: the heavy chains (γ for IgG, α for IgA, μ for IgM, δ for IgD and ϵ for IgE) of each Ig class differ in antigenic behaviour and carbohydrate content. The structure of the Igs has been studied in greatest detail in man, mice and some other laboratory animals, but it is believed that the structure is essentially similar in most animals. The essential basic structure of all Igs is $(H_2L_2)_n$, but although the L chains are common to all, the H chains are specific to each Ig class and n may also vary. Both IgG and IgA are monomers and each molecule can be referred to as $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$ for IgG or $\alpha_2\kappa_2$ or $\alpha_2\lambda_2$ for IgA (depending on whether the molecule contains κ or λ light chains). IgM is a pentamer and can be written $(\mu_2\kappa_2)_5$ or $(\mu_2\lambda_2)_5$. The carbohydrate content of IgA is 4 times that of IgG and that of IgM 5 times that of IgG.

IgA is of special biological interest because it is the principal protein of human colostrum and is also excreted in saliva, lachrymal glands and mucus glands of the intestine; in its exocrine form, IgA is attached to a secretory or transport piece. The results of immunofluorescent studies using specific anti α chain sera (Crabbé & Heremans, 1966) indicate that IgA is concentrated in plasma cells of the lamina

propria of the stomach, duodenum, jejunum, colon, appendix and rectum, whilst in the normal adult human 50 % of free IgG and IgA is concentrated in the vascular space. The loss of IgA from the vascular space is about 3 times greater than that of IgG and, when attached to the secretory piece, IgA is excreted against the concentration gradient into saliva, bronchial secretions and intestine. IgM is located almost entirely in the vascular space and is a particularly effective agglutinator.

In both cattle and sheep, fast IgG 1 seems to be the principal Ig in colostrum (Milstein & Feinstein, 1968; Sullivan, Prendergast, Antunes, Silverstein & Tomasi, 1969). The secretory fast IgG 1 was found by the latter authors to be identical with serum IgG_1 and no evidence of a secretory piece was found. However, Mach, Pahud & Isliker (1969) isolated from bovine colostrum an Ig which was defined as IgA on the basis of sedimentation constant (11S–), motility (β_2–γ_1), and hexose content (5–6 %). It was serologically distinct from IgG and IgM. These authors further reported the isolation of the secretory piece from normal milk. The IgA could not be detected in serum but was abundant in the saliva of adult cattle, but not of newborn calves. The presence of IgA was reported in swine colostrum by Porter (1969). Further considerable advances have been made on the structure of the H and L chains (Putnam, Titani, Wikler & Shinoda, 1968; Askonas & Williamson, 1968; Hood, Gray, Sanders & Dreyer, 1968). All light chains so far studied (in man) have a total length of 213–218 amino acid residues divided into variable (amino) and constant (carboxyl) halves. A similar pattern of variable and constant regions is believed to be present in the H chains.

The macrophage is also thought to play an important part in antibody production by engulfing and processing the antigen; this immunogenic material is then transferred through cytoplasmic bridges to lymph node cells where antibody is produced (Fishman & Adler 1968; Papermaster, 1968; Metcalf, 1968; Feldman & Gallily, 1968; Moore & Schoenberg, 1968).

The IgM (pentamer) molecule is sensitive to reduction with 2-mercaptoethanol (ME) and cysteine, is more heat and acid labile than IgG, has differences in molecular weight (as revealed e.g. by sucrose density centrifugation), electrophoretic mobility, ability to cross the placenta (in man) and in the pattern of appearance in the young; IgM production starts earlier than IgG, which reaches its maturity peak by 3 years of age (9 months for IgM); IgA production is much slower, reaching its maturity peak at adolescence.

In most animals, the antibody response to an antigenic stimulus consists of 2 phases —an initial IgM followed by IgG. The mechanism of the conversion of IgM to IgG is not known but it can be blocked, e.g. by 6-mercaptopurine (Sahiar & Schwartz, 1965).

There is also evidence that IgG inhibits 19S synthesis by neutralizing the antigen, thus leading to an IgG steady state (Möller & Wigzell, 1965; Jerne, 1967; Fink & LoSpalluto, 1967; Halliday, 1968; Uhr & Möller, 1968).

Studies on the in vitro production of antibodies (e.g. Stecher & Thorbecke, 1967; Richardson, 1969) also confirmed the same sequence of IgM followed by IgG synthesis, and found that the spleen was more important for IgM synthesis than lymph nodes. Stecher & Thorbecke (1967) also reported that the time of onset of IgM production in the spleen was correlated with the degree of maturity at birth.

Characteristics of the antibody response in sheep and goats have been reported by Aalund, Osebold & Murphy (1965), Freeman, Moore & Stavitsky (1966), and Jonas (1969); in cattle by Aalund (1968), Murphy, Aalund & Osebold (1964); in pigs by Tormo, Chordi, Rodriguez-Burgos & Diaz (1967), Kim, Bradley & Watson (1966); and in frogs by Alcock (1965).

Ig production in brucellosis

The pattern of Ig production in brucella vaccinated or infected cattle has been reported by Anderson, Jenness, Brumfield & Gough (1964), Rose & Amerault (1964), Rose & Roepke (1964), Rose, Lambert & Roepke (1964), Rose, Roepke & Briggs (1964), Huddleson (1964*b*), Jenness, Anderson & Gough (1965), Schimmel & Erler (1967), Erler & Schimmel (1967), Cunningham, O'Meara & Boggust (1968), Rice, Tailyour & Cochrane (1966), Rice, Cochrane & Tailyour (1966) and Rice, Alexander & Barrett (1967), in Strain 19-vaccinated sheep by Merriman & Rice (1969), in Strain 19-vaccinated pigs by Rice, L'Ecuyer & Merriman (1968), and in man by Heremans, Vaerman & Vaerman (1963), Reddin, Anderson, Jenness & Spink (1965), Zinneman, Seal & Hall (1965), Wilkinson (1966), Coghlan & Weir (1967) and Kerr, Payne, Robertson & Coombs (1967).

After Strain 19 vaccination, IgM antibody appears at about 5 days whilst IgG appears simultaneously or a few days later; they reach their peak at 28–42 days after vaccination compared with 13 days for IgM. Rice, Tailyour & Cochrane (1966) and Rice *et al.* (1967) also reported that both IgM and IgG from cattle were able to fix complement, but in pigs injected with either live or killed Strain 19 the IgM did not fix complement; these workers also reported that IgG values declined more slowly than reported by Rose and his colleagues and their results also differ from those of Anderson *et al.* (1964), who reported that IgM (on the basis of ME sensitivity) did not fix complement. The ability of both IgM and IgG from Strain 19-vaccinated cattle to fix complement has been confirmed at this laboratory (G. A. Cullen and M. J. Corbel, to be published). However, differences in the relative complement-fixing properties of IgG and IgM have been noted by a number of workers, e.g. Borsos & Rapp (1965). Cunniff & Stollar (1968) reported that 19S antibodies fix complement as effectively at 37 as at 4 °C, if not more so, but 7S antibodies fix complement better at 4 than at 37 °C; they also stressed that temperature dependence was a function of the antigen structure as well as antibody class. A similar pattern of Ig production based on ME sensitivity and density gradient centrifugation was reported by Reddin *et al.* (1965) in man after vaccination with Rev. 1 vaccine.

Following infection in both animals and man both IgM and IgG appear but, in contrast to the position following vaccination, the IgM values but not the IgG decline and later, and especially in chronic stages, the predominant and often the only Ig present is IgG. It is in this context, therefore, that the so-called supplementary tests are particularly useful, e.g. ME, CF, acid plate agglutination, heat inactivation.

In some animals, particularly man, sheep, and occasionally other species, the antibody, especially in the chronic stage, is non-agglutinating or 'incomplete' and can be detected using the antiglobulin (Coombs) test. Kerr *et al.* (1967) and Wilkinson (1966) found that the non-agglutinating brucella antibodies in chronic human

brucellosis were IgG and IgA. Zinneman *et al.* (1965) also isolated the blocking antibody from a chronic case in man and found it to be IgA.

Schubert & Colvin (1964) suggested that the blocking of agglutination observed in cases of human brucellosis was not caused by an incomplete antibody and blocking could be eliminated by increasing the density of the antigen non-specifically, e.g. by addition of *E. coli* or kaolin.

Cunningham *et al.* (1968) reported that an incomplete antibody persisted longer than the agglutinating and CF antibody after vaccination with Strain 19, 45/20, and after infection.

The serological tests available for the diagnosis of brucellosis were recently reviewed by Morgan (1967). However, it is stressed that no single serological test is exclusively superior to any of the others; they should rather be used in conjunction with each other—a point also stressed by Manthei (1968). Further, there are occasions when the serological tests, of whatever sophistication, are negative but the individual is culturally positive. Therefore, whenever possible both cultural and serological tests should be used especially in so-called problem herds during eradication programmes and in herds with acute infection (see also Gaumont, 1965; Hill, 1964; Nicoletti & Muraschi, 1966; Renoux, Philippon & Plommet, 1968; Camy, 1968; Nicolas, Pestre-Alexandre & Morange, 1968; Cedro *et al.* 1968; Goyon, 1965, 1969).

Descriptions of techniques used for diagnosis are available (Renoux & Gaumont, 1966; Alton & Jones, 1967; Report, 1964*a*; Kerr *et al.* 1968). Some of the current problems in diagnosis were also discussed by Manthei (1964, 1968), Morgan (1969) and Morgan *et al.* (1969).

Diagnosis in cattle

The Second International Standard anti *B. abortus* serum has been established to replace the First Standard (Davidson, Hebert & Morgan, 1969) and the International Unit defined as the activity contained in 0·09552 mg. Trials are in progess on the feasibility of using the second International Standard serum for standardizing the CF test.

Körmendy, Nagy & Stirling-Mócsy (1967) used as antigen the Strain 99 grown under different conditions (solid medium, shake culture and fermentor culture) and found no difference in sensitivity. Kötsche (1967) recommended the use of brucella antigen at pH 5·0 for the agglutination test in order to reduce non-specific reactions. In the Soviet Union, the SAT is used after incubation for 6 h at 37 °C followed by another 12 h at room temperature (Ivanov & Tavamaishvili, 1968) and this method was found to result in more doubtful reactions than incubation for 16 h followed by 1 h at room temperature. A technique allowing for the testing of haemolysed sera was described by Brochard (1967). Feeding or injecting carbolic acid solutions to cattle had no effect on the resultant titres (Ellis, Beck & Ruhland, 1968). Both Gangulee & Sen (1966) and Nicoletti & Holmes (1968) failed to produce cross reactions in cattle treated with haemorrhagic septicaemia vaccines. Hignett, Nagy & Ironside (1967*b*), however, reported that 8 of 9 cows with fluctuating SAT titres and injected with an unrelated organism (*Bacillus cereus*) showed a temporary increase to *Brucella* SAT after the second injection.

The large scale annual testing of sera has led to developments in automation both

for agglutination and CF testing (Joubert, Roumiantzeff & Valette, 1967). Gaillon, Ripault, Studievic & Dausset (1967) and Wagstaff, Firth, Booth & Bowley (1969) have also used the Auto Analyser apparatus for CF testing of sera for syphilis, with successful results.

Nicoletti & Muraschi (1966) reported that the SA test failed to identify 39 % of culturally-positive cattle and made the important point that the SA test alone cannot be relied upon for diagnosis, especially towards the latter part of eradication programmes; furthermore, they reported that 82 % of isolates in New York State in the period 1957–1962 were from vaccinated cattle.

Cedro, Cisale, Forti de Menaldo & Alvarez (1967) reported that 6 bulls from which *Brucella* species were isolated were negative to the agglutination test; the remaining 18 infected bulls reacted at 1/12·5 (4 cases), 1/25 (10 cases), 1/50 (3 cases) and 1/400 (1 case).

Le Pennec (1966) and Kretzschmar (1967) advocated the use of 5 % saline as diluent to reduce prozone phenomenon and to increase the sensitivity. Simintzis (1965*b*) claimed that agglutination tests on globulin, obtained by precipitation with ammonium sulphate, gave better results than tests on whole serum.

In a comparison of the rapid plate and tube agglutination test, Nicoletti & Muraschi (1966) reported that they correctly identified 66 and 61 % respectively.

Pat & Panda (1968) found that a plate test using tetrazolium-stained antigen gave comparable results with the tube test. A rapid whole blood test has been described by Krishna Murty & Kaushik (1964) and Hatfield (1967), whilst Fessel (1967) and El-Mossalami, Moursy & El-Naasan (1968) described a rapid slide test on muscle juice.

Of considerable significance in the diagnosis of brucellosis has been the development of a card test using an antigen stained with Rose Bengal dye (developed by Dr Pietz at Ames, Iowa). Details of the kit available are described by Nicoletti (1967), who also gave details of its use; the test was positive in all of 184 culturally positive cattle, whereas the tube test was positive in 48 %. In herds infected with field strains of *Brucella*, 46 % of 354 card test positive animals were culturally positive at a single attempt, whilst in herds from which field strains could not be isolated, 35 % of cattle with suspect tube titres were negative to the card test.

Morgan (1969) and Morgan *et al.* (1969), using the same antigen as used for the card test (but using sera and not plasma), compared the results of the Rose Bengal plate test (RBP) with those of the serum agglutination (SA) and CF tests on 6424 unselected cattle sera; since the vaccination status of the animals was often not stated, all sera with CF titres of 2/4 (numerator = degree of fixation; denominator = dilution) or more were considered as positive. The vast majority of sera with 'positive' CF titres were also positive to the RBP test, often irrespective of the SA titres, i.e. sera with SA titres of 1/40 could be negative to the RBP test and to the CF test but others, also with SA titres of 1/40, could be positive to the RBP and to the CF tests. All sera with antibody levels of 200 i.u. or over to the SA test were positive to the RBP test. Using the combined SA and CF tests, interpreted as laid down for the Brucellosis (Accredited Herds) Scheme in Britain, there was agreement between them and the results of the RBP test (both tests negative or both positive) in 90·8 % of the sera. Of the total sera examined, 31 (0·48 %) were positive on the combined SAT/CF tests but negative on the RBP test; nearly half of these had antibody levels

of between 100 and 200 i.u. on the SA test but negative on the CF test. In a further extension of this work (unpublished), on approximately 22 000 sera, agreement (i.e both combined SA/CF tests and the RBP tests negative or both positive) was found in 97 % of sera; 0·1 % of the sera were positive on the combined SA/CF tests but negative on the RBP tests. These authors concluded that the RBP was a most useful test, provided the positive sera were further subjected to a CF test.

In a report to the U.S. Livestock Sanitary Association, Dr W. C. Ray (personal communication) utilized the card test, alongside other tests, in experimentally infected cattle. It was again confirmed that the test was positive in all culturally positive animals and he also made the important point that the card test was positive sooner than the other tests in infected animals; this could be of considerable value in eradication programmes.

The complement-fixation (CF) test is now firmly established as an accurate diagnostic test; it has played and continues to play an important part in eradication campaigns (Brown, 1969; Granieri, 1966; Van Waveren, 1965; Nicoletti & Muraschi, 1966; Pilet, Toma & Bonneau, 1967; Goyon, 1966, 1967; Hill, 1964; Wiśniowski, 1964, 1967; Wiśniowski & Królak, 1965; Quesada & Traballesi, 1966; Gargani, 1964; Aleandri & Gargani, 1963; Preuss, 1965; Redaelli, 1965*a*, *b*; Kiss, 1966; Le Pennec & Goyon, 1965; Casillo & Bertoldini, 1964; Anderson *et al.* 1964; Kovačić, 1968; De Simone, 1969; Staak & Groocock, 1968; Christie, Kerr & McCaughey, 1968; Renoux *et al.* 1968). The test is particularly useful in sera with suspect titres to the SA test and for animals even with negative SA titres in infected herds, e.g. Nicoletti & Muraschi (1966) found the CF test better than the SA and other tests in 135 brucella-infected cattle and similar observations were made by Renoux *et al.* (1968). Pilet, Toma & Bonneau (1967) found that fixation at 4 °C with an acellular extract as antigen gave the highest titres. A method of eliminating anticomplementary activity in animal sera by adding porcine serum was described by Bucca & Alder (1969), and Valette & Joubert (1967) using the Technicon Autoanalyser reported that the results were reproducible and accurate. Sacco (1965) using Holth's CF test on placental extracts reported that the results were specific and useful. The conglutinating complement adsorption test was reported to be more accurate than the rapid plate or tube agglutination tests in cattle by Gulrajani, Misra & Verma (1968).

The heat-inactivation test has been reported by Bakshi & Narain (1964), Klein & Langkamp (1964), Beinhauer (1965), Schimmel, Erler & Erler (1965) and Pathak (1967) as a useful supplementary test.

Reference has also been made to the sensitivity of IgM to cleavage by sulphydryl reducing agents such as 2-mercaptoethanol (ME) into subunits which lack ability to agglutinate and to fix complement; sensitivity to ME is widely used as a presumptive test for IgM and has been used as a supplementary test for diagnosis of brucellosis (Anderson *et al.* 1964; Pathak, 1967; Rossi & Cantini, 1968; Nagy & Sorheim, 1969; Boycott, 1969). The test is useful especially in chronic stages of the disease where even low titres, consisting of ME resistant antibodies (by inference, IgG), are significant.

Rivanol (2-ethoxy-6, 9-diaminoacridine lactate) precipitates all serum proteins except gamma globulins (Saifer & Lepkin, 1959; Frommhagen & Martins, 1963; Huddleson, 1965; Patras & Stone, 1961*a*, *b*) and has been reported on for brucellosis diagnosis by Badnjević, Marić & Foršek (1964), Badnjević (1965) and Nicoletti &

Muraschi (1966). For the test, 3 vol. of 0·4 % (w/v) solution of Rivanol are added to 1 vol. serum and the precipitate removed by centrifugation. In the author's experience, it has been found easier to read the tests by treating the supernatant with activated charcoal before making serial serum dilutions and adding antigen. In recently vaccinated animals there is a drastic reduction in titre but not with infected animals.

The Coombs test has been described by Hajdu (1963, 1964*a*, 1965), Wiśniowski, Romaniukowa & Drozdzyńska (1964), Wiśniowski, Drozdzyńska & Królak (1968), Wiśniowski, Grajewski & Drozdzyńska (1969), Kuhlmann (1965), Le Pennec (1966), Le Pennec & Goyon (1965), and Cunningham (1967). Reference has already been made to the production and significance of incomplete antibodies detected by the antiglobulin test following vaccination with 45/20 adjuvant vaccine (Cunningham, 1967; Morgan & McDiarmid, 1968).

In cases where the SAT is negative or inconclusive, reactions to the antiglobulin test are considered significant.

The immunofluorescence test is being used for diagnosis and is claimed to be more sensitive than the agglutination test (Simintzis & Thivolet, 1965; Gentile, 1965; Hajdu, 1965, 1966; Mignani & Mammini, 1964; Jentzsch, 1964*a*, *b*, 1966; Karasek & Jentzsch, 1966). Simintzis & Thivolet (1965) found 4 sera that were positive to the agglutination test but negative to the fluorescent (FA) test, but prozone phenomenon did not occur with the FA test. Hajdu considers that the test is superior in sensitivity to the agglutination and CF tests. A fluorescein-stained complement technique, described by Jentzsch (1964*a*, *b*), was found to have a diagnostic accuracy of 96·4 % (Jentzsch, 1966).

The surface fixation test of Castaneda appears to be somewhat less sensitive than the tube agglutination test (Thomas & Willis, 1967; Entessar, 1965; Künter, 1965; Mathew, Bastain & Manjrekar, 1966; Morhain, 1966; Kretzschmar, 1967); the last author commented that the test required about a third as much labour as the tube agglutination test and that its sensitivity was not significantly less.

The milk ring test (MRT) is still widely used for screening milk from herds. The increasing use of bulk tanks (instead of churns) has raised some problems on the use and interpretation of the test. Hill (1966) and Worthington (1969) concluded that the test was not suitable for bulk tank samples but Sjollema (1967) stated that the use of MRT on bulk tank samples was nearly as accurate as on churn samples. A modification of the MRT on bulk tank samples used in the U.S.A. (E. A. Schilf, personal communication), and experimentally in the author's laboratory, is to examine 2 or 3 ml of bulk tank milk keeping the volume of antigen (0·03 ml) constant, thereby increasing the sensitivity of the test. The test is widely used in brucellosis eradication in Northern Ireland (Christie *et al.* 1968) and in the Brucellosis (Accredited Herds) Scheme in Britain (Simpson, 1968), and its limitations were discussed in detail by Christie *et al.* (1968). In the Netherlands, where nearly all herds are now *Brucella*-free (Jaartsveld & Jilesen, 1967), false positive MRT reactions were relatively more important; under these circumstances, the use of MRT dilution test, CF test, repeat test, and California mastitis test is advocated (see also Berthelon & Royal, 1966; Roushdy & Moursy, 1964; Nicoletti & Muraschi, 1966). Lauwers (1966) reported that where 3 successive herd MRTs were positive, 86·8 % contained serological reactions, whereas 97 % were free from reactors when 3 successive MRTs were negative.

Holmes (1967) described a modification of the MRT; one drop of the test milk was placed in a tube with 1 ml of known MRT-negative milk and one drop of antigen; the test was read after mixing and standing for 30 min at 37 °C; good results were claimed even with old milk samples.

The mechanism of the MRT and factors affecting it were studied by Hajdu (1964*b*), Rosaschino & Ruggeri (1966), Kenyon, Jenness & Anderson (1966), Scheibner (1967), and Anczykowski (1967, 1968*a*, *b*). Testing of freshly collected samples and vigorous shaking should be avoided.

Pietz, Anderson, Werring, Kenyon & Jenness (1967) reported that the regular MRT of fresh cream was slightly less sensitive than the test on milk and its sensitivity was further reduced as the pH dropped during storage. These authors described a modified MR test on cream which was not affected by the fall in pH. In a comparison of the 2 tests, the regular test was positive in 1·6 % of 1000 cream samples and the modified test in 3·3 %.

The value of the CF test on whey has been confirmed by Jaartsveld & Jilesen (1967), Robertson & Farrell (1968), and Farrell & Robertson (1968) and was more accurate in detecting excreting animals. Gromyko (1966) used the CF test on defatted whole milk, obtaining positive results in 109 of 199 blood-test-positive cows. However, as with all other tests, exceptions do occur and none of the milk serological tests, on its own, is guaranteed to identify every excreting animal. The use of the whey agglutination test has been reported by Lokteva (1965), Nicoletti & Muraschi (1966), Nagy, Hignett & Ironside (1967), Farrell & Robertson (1968) and Rickard (1965*a*); the latter authors found the test less sensitive than the whey CF test.

Cells grown in a fermentor were found to be satisfactory by Nagy & Körmendy (1968), and by Zinenko (1968), who used Congo red for staining the cells. Sethi, Garg & Arora (1968) reported favourably on the surface fixation test for milk and Zák (1966) found that the whey of brucella-infected cows had higher titres of incomplete than of agglutinating antibodies although the levels of incomplete antibodies in whey were always lower than the blood levels.

The isolation of *Brucella* from milk has been reported by Farrell & Robertson (1967, 1968), Barrow & Peel (1967), Barrow, Miller, Johnson & Hingston (1968) and Brodie (1968*a*).

The use of the MRT on buffalo milk has been reported by Mohanlingam, Vedanayakam & Varma (1965), Hamada *et al.* (1964), Roushdy & Moursy (1964) and Pat & Panigrahi (1965). The reaction seems to be slower in buffalo milk, probably because of its slow creaming ability.

The indirect haemagglutination test has been used by Khristoforov (1964*a*, *b*, 1965, 1966), Pathak (1967), Becht (1966) and Polyakov, Rassudov, Shoshiev, Lozovoi & Sagatovskii (1969) whilst Abdullin & Sakharova (1966) used carbon particles coated with brucella antibodies for detecting antigen in bovine serum and stomach contents of aborted foetuses.

The vaginal mucus agglutination test has been studied by Anczykowski, Murat & Walkowski (1966*a*, *b*, *c*), Simintzis (1965 *a*), Nagy *et al.* (1967) and Christie *et al* (1968). Nagy *et al.* (1967) found that mucus collected by the tampon method gave many false positive results but this was not found if a pipette was used.

A comparison of the results of cultural examination and of stained smears of foetal

materials and their relationship with the blood serological tests was made by Goyon (1969), who also reported that the serological tests could be negative although *Brucella* had been isolated. The need for a wide spectrum of tests in cases of bovine abortions is stressed.

The use of the immunofluorescence staining technique has been suggested by Karasek (1965*a*, *b*), Schweizer (1964) and Becht (1965) for the differential diagnosis between brucellosis and Q fever; the need so to differentiate was also stressed by Lapraik, MacKinnon & Slavin (1967).

V. L. Ivanov (1965) found that the injection of an attenuated, non-agglutinogenic strain (No. 21) of *B. abortus* intravenously into infected cattle caused a rise in temperature within 10–12 h, and 12–15 days later a rise in both SA and CF titres. The agar gel diffusion test, using a phenol extract of *B. melitensis*, was used to differentiate antibodies resulting from infection and from vaccination by Lo Chung-yu & Kan Mang-Lou (1965).

Diagnosis in sheep and goats

The known limitations of the SAT in the diagnosis of brucellosis in sheep and goats are well exemplified by Ünel (1968) and Ünel, Williams & Stableforth (1969); many infected sheep gave negative reactions to the SAT, so that it had not proved possible to eradicate the disease in state flocks by using the SAT alone and removing reactors. The CF, rapid plate, Rivanol and antiglobulin tests and the intradermal tests have been evaluated by a number of workers, e.g. Gaumont (1963), Doğuer (1964), Erdem & Ünel (1968), Ünel, Williams & Stableforth (1969), Panda & Pat (1967), Gelev (1968), Gargani & Guerra (1967), Le Pennec (1966, 1967) and Mathur (1966*a*, *b*, 1967*a*, *b*). It is recommended that a combination of tests should be used. Diagnostic criteria for the SAT, CFT and antiglobulin tests were suggested by Ünel, Williams & Stableforth (1969) who reported that the antiglobulin test was the most sensitive but even so did not identify all the infected animals. The pattern of antibody production after vaccination with Rev. 1 vaccine was reported by Erdem & Ünel (1968), after Strain 19 by Gannushkin & Bronnikov (1965) and after vaccination with a frozen and thawed suspension by Gargani & Guerra (1967). The use of 5% saline reduces the incidence of prozoning and its use is generally recommended.

In a small-scale trial with goats and sheep experimentally infected with *B. melitensis* Strain H38, Morgan *et al.* (1969) suggested that the Rose Bengal plate test might be of value. A whole blood agglutination test was evaluated by Dan'Shev & Koryukin (1965) and Makarov (1969) with results comparable to the tube and CF tests. Variations in titre during pregnancy were reported by Gannushkin & Bronnikov (1965).

The value of milk tests (MRT, whey agglutination and cultural tests) has been reported by Mathur (1967*a*, *b*) and Shivdekar & Pathak (1969); the agglutination test on whey was less sensitive than the MRT.

The use of the allergic test has been reported by Orlov & Kas'yanov (1968) and Nashkov & Khristoforov (1969); reading the tests at 72 h was recommended for increased sensitivity. The different preparations were non-agglutinogenic and non-allergenic; a new allergen was described by D'Ascani, Micozzi & Conti (1966).

Diagnosis of B. ovis

In addition to clinical examination, cultural isolation and the CF test, an indirect haemagglutination test was described by Ris (1964), who reviewed the value of other tests; the examination of stained smears detected most well-established cases but failed to detect early cases. Hughes & Claxton (1968) also found that clinical examination missed many infected rams; the CF test was found to be the most accurate of the tests they used. A gel precipitin test was described by Mathews & Trueblood (1967) whilst an intradermal test was advocated by Cedro, Cisale & de Benedetti (1964), Cedro, Cisale, de Benedetti & Forti de Menaldo (1965) and Cedro, Cisale, de Benedetti, Forti de Menaldo, Rovere & Rúiz y Ormaechea (1965).

Diagnosis in pigs

The complement fixation test, using serum inactivated at 62 °C, has been shown to be more sensitive than most other serological tests (Badnjević, 1964, 1965; Florent and Docquier, 1965; Zubov, 1963; Orlov, 1965*a*, *b*, *c*; Kötsche & Stahl, 1967; Glamba, 1967). Badnjević (1965) reported that the Rivanol test was as sensitive as the CF test although it is recommended by most people that a combination of tests should be used. Mateev & Krŭstev (1963) advocated the use of 5 % saline as diluent. The card test is under trial in the U.S.A. (Ladwig 1968). The allergic test (Zubov, 1963; Dannenberg & Henschel, 1964; Both & Kohl, 1967; Györffy, 1967) is recommended as a rapid herd screening test, followed by other serological tests on individual animals.

Diagnosis in man

Besides the SA test, the CF test, antiglobulin, mercaptoethanol, surface fixation and indirect immunofluorescence tests are recommended for human diagnosis (Potužnik, Svejnoch & Duniewicz, 1964; Chernysheva, Aslanyan, Scherbak & Mnatsakanyan, 1968; Brodie, 1968*b*; Kerr *et al.* 1968; Cox, 1968). Details of some of these tests were published by Kerr *et al.* (1968).

The isolation of *B. abortus* biotype 5 was reported by Robertson & Farrell (1967) and Barrow & Peel (1965), that of *B. canis* by Carmichael *et al.* (1968) and *B. melitensis* by Galbraith, Ross, de Mowbray & Payne (1969), who reported infection contracted from imported cheese in a family in Britain.

Diagnosis in other animals

B. canis infection in dogs (Carmichael & Bruner, 1968) can be diagnosed by cultural isolation of the organism from aborted foetuses, placenta, vaginal discharge and by blood culture. The agglutination test should be incubated at 50–52 °C before being read.

The antiglobulin and CF tests were recommended for use in horses by Rivera Garcia & Torres Barreto (1963), Rivera Garcia (1963) and McCaughey & Kerr (1967*b*). The CF test, preferably using overnight fixation at 4 °C, was found to be more sensitive than the agglutination test for buffaloes (Sadykhov, 1968*a*; Tevosov, 1967). The allergic test detected 5 of 13 *Brucella*-positive camels (Boháč, Polom, Jeřábek & Ládr (1968).

Incidence

In Britain, it was estimated that about 25% of dairy herds contained brucella-infected animals, although 62% of these contained only 1 or 2 infected animals/herd, (Leech *et al.* 1964). In a further survey of serological reactors (quoted by Simpson, 1968) it was estimated that 14% of cattle (20 months of age and over) would not pass the serological tests used. Local surveys on the incidence of brucellosis and of *B. abortus* in milk and cream have been reported by Ogden, Sellers & Crabb (1964), Baker & Faull (1967), Barrow *et al.* (1968), Tadayon (1968), Farrell & Robertson (1967, 1968) and Brodie (1968*b*). Allsup (1969) reported abortion in 4 flocks of sheep caused by *B. abortus*, but the incidence within flocks was low and sporadic with little lateral spread within flocks. Abortion in a donkey caused by *B. abortus* was reported by Crossman & Bonson (1968).

Cattle brucellosis has been eradicated from Czechoslovakia (Kouba, 1964, 1968, 1969) and Hungary (Tadar & Ösz, 1967).

Some references on the incidence of brucellosis in animals in various countries, are as follows:

Northern Ireland. Cattle: Christie *et al.* (1968).

Republic of Ireland. Cattle: Noonan (1967), O'Farrell & O'Reilly (1969).

Italy. Various species: Ademollo, Paltrinieri, Pellegrini & Farina (1965). Hares: Carlotto (1964).

Turkey. Cattle: Errol (1967), Doğuer & Berber (1965). Dogs: Anon (1965*a*).

USSR. Cattle: Anishchenko (1964), Mukhamedov (1965), M. M. Ivanov (1965), Grigorenko (1966), Kas'yanenko (1968). Sheep: Strikhanova (1964), Kadyrov (1965). *B. ovis*: Trilenko, Ginzburg & Ogorodnikova (1968). Yaks: Taranov (1968), Pinigin *et al.* (1968). Buffaloes: Sadykhov (1968*b*). Reindeer: Davydov (1965), Zabrodin (1968). Mink: Rementsova & Postricheva (1965). Steppe rodents: Korol (1964).

France. Cattle: Gaumont (1964), Goret (1964), Cortez (1964), Florio (1965). Goats: Gasse, Cortez & Lafenêtre (1963). Pigs: Duée & Moine (1966).

Netherlands. Cattle: van den Burg (1964). Pigs: Robijns (1965), Terpstra (1965), van den Born (1966).

Germany. Pigs: Englert *et al.* (1964), Pulst (1968), Fritzsche (1966).

Poland. Dogs: Bochdalek (1969). Hares: Tropilo & Mól (1965), Hay & Tropilo (1968). Foxes: Górski & Wawrzkiewicz (1964).

Greece. Various species: Dragonas, Stoforos & Efstathiou (1967).

Hungary. B. ovis: Szabó & Nyeredy (1967).

Bulgaria. B. ovis: Bakŭrdzhiev, Pandŭrov, Dimitrov & Zhekov (1964, 1965).

Czechoslovakia. Hares: Volejníček & Prášek (1964), Klimša (1965).

Yugoslavia. Various species: Talic (1967).

Roumania. Various species and man: Spînu, Vasilesco, Pop & Dobresco (1966). Man: Cerbu (1967).

Belgium. Pigs: Florent & Docquier (1965).

Spain. Various species: Vallejo Vicente (1967).

Portugal. Goats: Teixeira (1963).

Italy. Cattle: Mazzetti (1967), Maida (1968).

Egypt. Cattle and buffaloes: El-Ahwal, El-Magnid & Sokohi (1968).

Ghana. Cattle: Oppong (1966).

Morocco. Various species: Joubert & Fassi-Fehri (1966), Nguyen-Tharh-Cac & Fassi-Fehri (1968).

Nigeria. Various species: Adams & McKay (1966).

Rhodesia. Game animals: Roth (1965).

Senegal. Cattle: Chambron (1965*a*, *b*). Various species: Richard (1966).

Sudan. Various species: Abdulla (1966).

Tanzania. Various species: Mahlau (1967), Staak, Groocock & Sachs (1967). Buffalo: Staak, Sachs & Groocock (1968). Game animals: Sachs, Staak & Groocock (1968).

Uganda. Various species: Cox (1966).

West Africa. Various species: Beaupère (1966).

Republic of South Africa. Sheep: Van Drimmelen (1965).

Tunisia. Cattle and man: Chadli & Ben Osman (1968).

India. Cattle: Mathur (1966*a*; 1968), Kataria & Verma (1969), Basu, De & Bhattacharya (1966), Pande (1969). Sheep & goats: Mathur (1966*b*; 1967*a*, *b*; 1968), Arora (1969), Pande (1969). Various species: Prakash, Varmani & Ghose (1967).

Syria. Various species: Hirchert, Lange & Leonhardt (1966).

Iraq. Various species: El Shawi (1964).

Korea. Cattle and goats: Kim, Chung & Mun (1968).

USA. Cattle and pigs: Schilf (1968), Omohundro (1967), Editorial (1969). Pigs: Hendricks & Hausler (1967), Luchsinger *et al.* (1965). Dogs: Carmichael (1968), Carmichael & Bruner (1968). Wildlife: Thorpe *et al.* (1965). Caribou: Neiland *et al.* (1968).

Canada. Cattle: Rice (1969). Pigs: Malkin, Tailyour, Bhatia, Archibald & Dorward (1968).

Argentina. Cattle: Szyfres & Durán (1966), Porter (1964). Various species: de Benedetti, Gonzáles & Donadio (1967), Gimino & de Benedetti (1967). Foxes: Szyfres & Tomé (1966, 1967). Hares: Szyfres, Tomé & Mendieta (1968).

Brazil. B. ovis: Ramos *et al.* (1967). Cattle: Porter (1964).

Guatemala. Pigs: Gálvez (1969).

Peru. Various species: Escalante (1969), Porter (1964), Fernández (1966).

Uruguay. Pigs: Caffarena y Bachers, Agorio & Barriola (1967). *B. ovis*: Casas Olascoaga, Durán del Campo & Artigas Rivas (1968).

Haiti. Cattle: Laroche, Lacombe & Santiago Reyes (1966).

Malaysia. Pigs: Retnasabapathy & Chong Sue Kheng (1967).

Australia. Cattle: Turner (1966, 1969), Rammell (1967), Hughes, Young & Moyle (1968). Horses: Hutchins & Lepherd (1968).

New Zealand. Cattle: Rickard (1965*b*). *B. ovis*: Buddle (1965). Deer: Daniel (1966).

Incidence of brucellosis in man

The importance of human brucellosis is reflected in the numerous papers on the incidence of the disease, especially in those sections of the population most at risk (veterinary surgeons, farmers and farm workers, slaughterhouse men, etc.) and of the marked decrease or elimination of the disease following the eradication of the disease in animals. The reader is referred to excellent papers by Spink (1964) on various clinical aspects of the disease in man, including treatment, and McCullough (1964). Kerr, Coghlan, Payne & Robertson (1966) reported that 63 % of 309 veterinary surgeons had serological evidence of brucellosis and 27 % had clinical signs which taken together with the serological reactions were suggestive of chronic brucellosis. An increase in the percentage of serological reactors in veterinary students in Bristol as they approached their final 2 years was reported by Cayton, Osborne & Sylvester (1967), an observation also made on Toulouse veterinary students by Géral, Lasserre, Saurat, Lautié & Chantal (1968); 1·6 % of staff and students at Ghent were positive to SAT and CFT (Devos, Vuylsteek & Eylenbosch, 1969), 18 % of veterinary surgeons ond students at Illinois were SAT positive (Schnurrenberger, Martin, Sherrick & Yoder, 1967), 64 % of veterinary surgeons in Northern Ireland were positive to the SAT (McDevitt & McCaughey, 1969) and 74 of 80 in County Cork (Foley & Corridan, 1968) (see also Meenan, 1967). The incidence in veterinary surgeons in Belgium was reported by Lafontaine, Andre, van Oye, Thomas & de Berdt (1963). The occupational nature of the disease was reported by Henderson (1964, 1967), Boycott (1964) and Brodie (1968*a*). The incidence of human brucellosis in Malta has fallen from 2410 in 1946 to 56 in 1964. The decrease was most marked in the town areas (Agius, 1965). Regional differences in the incidence in humans in Italy were reported by Majori, Granzotto & Gargani (1966). In France, Pilet & Bourdon (1965) reported that the incidence had fallen from 3·3/100000 of the population in 1947 to 1·51/100000 in 1964. With the progress made in eradicating cattle brucellosis in the U.S.A., swine are becoming an increasing source of human infection (McCullough, 1964; Hendricks & Borts, 1964; Anon, 1965*b*; Spink, 1969). The importance of caribou as a source of human infection in Alaska has been shown by Brody *et al.* (1966). In New Zealand, Glass (1964) reported that 60 % of human cases were traceable to ingestion of cow's milk or milk products, the remainder to direct contact with infected cattle. In parts of the Soviet Union, Anishchenko (1964) and Simonyan (1969) reported that 67 % of human infections resulted from contact, largely from sheep and goats (see also Lamdik, 1966). The importance of the goat as source of human infection in India was discussed by Mathur (1964*a*, *b*), and of pigs in Brazil by de Almeida & Caldas (1963) and in Roumania by Spînu *et al.* (1966). The importance of spinal involvement in human brucellosis has been indicated by Araiz, Martinez-

Penuela & Gastearena (1965), Bullock & Arnesen (1964), Debono (1964), Farid & Omar (1965), Kerr *et al.* (1966), Mergol'd (1964) and Spink (1964).

Although combined antibiotic therapy (usually streptomycin and tetracycline) is recommended as the preferred method of treatment, Sharma (1965) suggested that nalidixic acid ('Negram', one g 4 times daily) could be effective and ampicillin was evaluated by Farid, Bassily & Omar (1964).

Control and eradication

In planning campaigns against disease, the Joint FAO/WHO Expert Committee on Zoonoses (Report, 1967*a*) stressed the importance of prevention, control and eradication. It defined prevention as measures aimed to protect man or animals and could frequently be independent of measures designed to bring the disease under control; control consists of measures of reducing the incidence of disease or infection and eradication as the total elimination of the aetiological agent; occasionally, the term eradication is used to denote elimination of the disease and *not* of the infective agent. The need for surveys and surveillance reports, and close co-operation between medical and veterinary services and the farming community, was stressed.

In Britain, where brucellosis is confined almost entirely to cattle, the control by vaccination with Strain 19 was extended, in May 1967, by the introduction of the Brucellosis (Accredited Herds) Scheme aimed primarily at providing a source of known, *Brucella*-free stock for the later stage of eradication. At the same time, vaccination with Strain 19 vaccine was confined to heifer calves between 91 and 180 days of age. The outline of the Scheme was presented by Simpson (1968) and progress was summarized by Brown (1969). Dairy herds are required to pass 3-monthly MRTs before becoming supervised; this is followed by an official blood test of all eligible animals. Compensation up to a maximum of £160/animal is paid for reactors after the herd becomes supervised. Once an application is accepted to join the Scheme, restrictions on the movement of animals are introduced. For beef herds, a blood test of the whole herd, which may be conducted in 2 parts, is made before the herd becomes supervised and, if clear, the official test is made not less than 6 months later. Various proposals for eradication have also been made, e.g. British Veterinary Association (1965) and eradication on a herd basis by Baker & Faull (1967), Walker (1968) and Craig & Wright (1967).

Good progress has been made in the eradication scheme in Northern Ireland (Christie *et al.* 1968; Christie, 1969); 96·5% of the 43 000 herds are on the register as brucellosis-free; the remainder consists of herds awaiting final test prior to registration, infected herds which have not yet reached certified status, and herds previously registered but which have become re-infected. In the Republic of Ireland (Hynes, 1967), control is based on calfhood vaccination in areas of high incidence, Certified Herds Scheme and area eradication. The progress in eradication in the U.S.A. in cattle and swine has been reported by Mingle (1964) and Schilf (1966, 1967, 1968); the goal is to attain Certified Brucellosis Freedom by 1975. The possibility of reducing the extent of the use of Strain 19 vaccine is being discussed with the possibility of the continuation of vaccination at owner's expense in those areas where it is not considered a wise use of public funds.

The use of Strain 19 to control the disease in herds and the potential dangers of

common pasturing, was stressed by Bürki & Schmid (1964), van den Burg (1964), Mészáros (1967), Hsieh, Hso, Yin, Kuo, Hseng & Yao (1964) whilst the procedures used in the Ukrainian and Latvian SSRs were described by Didovets, Zhovanik, Teitel & Bozhko (1967) and Iaunsleinis (1965).

In an outbreak of brucellosis in swine in Germany, slaughter of whole herds had occasionally to be resorted to (Fritzsche, 1966).

The position in France has been described in papers by Joubert, Bertrand & Ferney (1964), Berthelon (1965) and Lafenêtre (1965). Calfhood vaccination, hygienic measures and the gradual elimination of reactors were used by de la Fuente (1968) to control the disease in dairy herds.

Success in eradicating *B. ovis* infection in sheep in Tasmania by the use of the CF test and slaughter of reactors was described by Ryan (1964) and an eradication scheme for cattle brucellosis was discussed by Hopkirk (1966). Success in the eradication of brucellosis in a colony of 265 beagle dogs was described by Moore, Gupta & Conner (1968) by the use of blood culture and agglutination tests and the removal of infected dogs.

Since the last review, reports of the eradication of bovine brucellosis have come from Finland (Huhtala, 1963); Switzerland (Fritschi, 1964); Yugoslavia (Böhm, 1969); Czechoslovakia (Kouba 1965*a*, *b*).

Conclusions

In the period under review, considerable advances have been made in the study of immunoglobulins and these have been of value in the understanding of the use and interpretations of an increasing array of serological diagnostic tests.

Although many countries are continuing their efforts to eradicate bovine brucellosis, reports indicate the incidence of the disease in other animals, including disease in dogs caused by a new species of *Brucella—B. canis*, an organism also capable of infecting man. *B. ovis* infection in sheep has been successfully controlled and eradicated by the use of vaccination or test and slaughter policy. Brucellosis in sheep and goats continues to be a serious problem in many areas. Brucellosis in animals is reflected in the occurrence of the disease in man, especially in those sections of the population most at risk.

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Journal of Dairy Research
Volume 37, Number 2, June 1970

CONTENTS

ORIGINAL ARTICLES

A rapid bioassay for penicillin residues in milk
T. M. COGAN and P. F. FOX *page* 165

Influence of aggregation on the susceptibility of casein to proteolysis
P. F. FOX 173

The effect of the periodic feeding of linseed oil on the production and iodine value of milk fat
J. C. WADSWORTH 181

The effect of the modification of arginine side chains in casein on the coagulation of rennin-altered casein
R. D. HILL 187

The effects of intravenous infusions of insulin and of sodium succinate on milk secretion in the goat
J. A. F. ROOK and J. B. HOPWOOD 193

Extraction of the 260 nm-absorbing material from group N streptococci as a method for estimating cell growth
D. McC. HOGG and G. R. JAGO 199

Measurement of the quantity of lactose passing into mammary venous plasma and lymph in goats and in a cow
N. J. KUHN and J. L. LINZELL 203

Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization. I. An experimental ultra-high-temperature sterilizer and its characteristics
H. BURTON and A. G. PERKIN 209

Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization. II. The sporicidal efficiency of an experimental plant for direct and indirect processing
J. G. FRANKLIN, H. M. UNDERWOOD, A. G. PERKIN and H. BURTON 219

Comparison of milks processed by the direct and indirect methods of ultra-high-temperature sterilization. III. A note on the results for spore destruction obtained with an experimental ultra-high-temperature milk sterilizer
H. BURTON 227

The denaturation of α-lactalbumin and β-lactoglobulin in heated milk
R. L. J. LYSTER 233

The interaction of bovine milk caseins with the detergent sodium dodecyl sulphate. I. The relationship between the composition and the size of the protein–detergent aggregate
G. C. CHEESEMAN and JOAN JEFFCOAT 245

The interaction of bovine milk caseins with the detergent sodium dodecyl sulphate. II. The effect of detergent binding on spectral properties of caseins
G. C. CHEESEMAN and DOROTHY J. KNIGHT 259

Structure of the casein micelle. The accessibility of the subunits to various reagents
B. RIBADEAU DUMAS and J. GARNIER 269

Milk enzymes—their distribution and activity
B. J. KITCHEN, G. C. TAYLOR and I. C. WHITE 279

Apparatus and milking techniques used in lactation studies with sheep
T. T. TREACHER 289

The composition of ewe's milk fat during early and late lactation
R. C. NOBLE, W. STEELE and J. H. MOORE 297

Reviews of the progress of Dairy Science. Section E. Diseases of dairy cattle. Brucellosis.
W. J. BRINLEY MORGAN 303

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