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The Journal publishes not only papers read at meetings of the Society, but also papers on dairy technology by members and non-members

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Representative Research Papers with Shortened Titles:

CORRECTION

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A note on the serological typing of some strains of *Streptococcus cremoris*

B. Reiter, Constanza di Biase and F. H. S. Newbould

P. 128, footnote to table 4,

for 'phage lysis'

substitute 'phage lysin'

The effect of acidity, salt and copper and iron contamination on the keeping quality of butter

By A. K. R. McDOWELL
The Dairy Research Institute (N.Z.), Palmerston North, New Zealand

(*Received* 29 *November* 1963)

Summary. The keeping quality of salted butter made from acid cream was found to be satisfactory at pH values down to 6·0 provided that the butter was almost entirely free of 'contaminative' copper. Oily and fishy flavours developed during storage if the pH was lower than 6·0 or if the butter contained 0·02–0·03 ppm. of contaminative copper added as copper sulphate to the cream before churning.

The keeping quality of unsalted butter from acid cream was satisfactory at pH values down to 5·2 even when the butter contained 0·05 ppm. of added copper.

For good keeping quality in sweet cream salted butters it was found that the content of natural plus contaminative copper should not exceed 0·08 ppm. and in sweet cream unsalted butter 0·12 ppm. When the copper content was above these limiting values, oxidative defects occurred during storage.

When iron as ferrous sulphate was added to cream to give a total iron content above 0·50 ppm. in the butter, there was usually a metallic flavour in the fresh butter. In some butters this metallic flavour was still evident after storage, but in others it was either not present or it was masked by storage flavours. Iron in the proportions usually present in commercial butters was not found to have any pro-oxidative effect on the butterfat in the butter during storage.

When sweet or slightly acid creams containing copper or iron added as sulphate were churned, the distribution of the added copper or iron between the buttermilk and the butter indicated that the added metal ions were bound non-specifically to the serum and the fat-globule membrane proteins. When fully ripened cream containing added copper or iron was churned the proportion of the added copper or iron passing from the cream into the butter was much higher than for sweet or slightly acid cream because of the higher proportion of protein retained in the butter.

Butter made from good quality sweet cream is usually free of any major defect in flavour, even after a long period of cold storage. However, butter made from cream ripened with starter, is liable to develop oily and fishy flavours during storage. Thus the manufacture in New Zealand of butter for export has been confined to the sweet cream product.

Even in unsalted butter high acidity (low pH) in the serum can lower the keeping quality. Wiley (1939), for example found that unsalted butters of pH 5·7 and 5·0 were inferior in keeping quality to butter of pH 6·6.

In salted butter the effect of acidity in lowering the keeping quality is greatly increased (see, for example, Wiley, 1939; Huf, 1961), especially in the presence of trace amounts of copper. Recent investigations have shown that in acid butters copper in amounts very much smaller than these previously considered harmful can accelerate butterfat oxidation and deterioration in butter flavour (Kruisheer & Krol, 1955; Menger, 1961). In sweet cream butters a total content of 0·10 ppm., which was formerly accepted as a safe upper limit (McDowall, 1953; Pont, Rogers & Davies, 1961), is now considered to be too high, and it is stated that the maximum acceptable copper content should be 0·075 ppm. (Pont & Rogers, 1962). Kruisheer & Krol (1955) and Menger (1961) distinguish between content of 'natural' copper, which in butter made from milks of individual cows ranges from 0·02 to 0·10 ppm. and which is stated not to have any catalytic effect on fat oxidation, and content of 'contaminative' copper, which even when as low as 0·025 ppm. was stated to accelerate the oxidation of the fat and the development of undesirable flavours in fully ripened cream butters.

Iron also was thought to catalyse oxidation of butterfat and to hasten the deterioration in flavour of butter, especially of acid butter. It is now accepted, however, that the normal amounts of iron in commercial butter, 0·4–1·0 ppm., are too small to cause significant oxidation of the butterfat (Barnicoat, 1950; Menger, 1961), although iron in proportions greater than 0·5 ppm. may cause a metallic flavour in both fresh and stored butters (Mulder, Kruisher, den Herder & van Ginkel, 1949; Barnicoat, 1950; Menger, 1961). The metallic flavour is found only in the butter serum (Mulder *et al.* 1949) and is thought to be due to the intrinsic flavour of the dissolved iron (Menger, 1961).

In milk all copper or iron, whether natural or contaminative, is adsorbed by the proteins (King, Luick, Litman, Jennings & Dunkley, 1959; Menger, 1961). It may be assumed that copper or iron dissolved by cream from exposed metallic surfaces or added to cream, in experimental trials, in the form of metallic salts will also be adsorbed by the proteins. The distribution of contaminative copper and iron between the serum and the fat globule membrane proteins has been investigated in milk (King *et al.* 1959) but not in cream. In butter the content of each metal will include the proportion from the serum proteins in the retained buttermilk and that from the residual fat globule membrane proteins. Thus the distribution in cream is of considerable importance since the amounts retained in the butter will affect both its initial quality (iron) and its keeping quality (copper). There are, however, few published results for the copper or iron content of cream in relation to copper or iron content of the resulting buttermilk or butter.

Recent work has indicated that butter of very low copper content, irrespective of its pH, may be stored for long periods without serious deterioration in flavour (Kruisheer & Krol, 1955; Menger, 1961). The widespread replacement of tinned copper by stainless steel in the construction of butter factory equipment has led to a marked reduction in the copper content of New Zealand butter. It seemed possible, therefore, that for butter for long storage a lower limit of pH might be set than was formerly thought acceptable. The present paper describes an investigation of the relation of pH to the keeping quality of salted and unsalted butters almost free from copper and iron contamination, and of the effects of addition of small quantities of these metals as soluble salts on the keeping quality of acid and sweet cream butters.

EXPERIMENTAL

Butters were made from sweet cream (40 % fat) at the Institute experimental factory. The equipment used was almost wholly of stainless steel. In the manufacture of normal pH, sweet cream butter the acidity of the cream before Vacreator treatment was adjusted by adding sodium bicarbonate until the residual acidity was equivalent to 0·08 % lactic acid. Except in trial 4 (*b*), cream for the manufacture of acid butters was acidified by adding N lactic acid solution after Vacreator treatment and cooling. Direct acidification with lactic acid was used in preference to ripening with starter not only because the final acidity could then be controlled with greater accuracy but also in order to eliminate possible confusing effects of products of starter growth on the butter quality. Copper additions to the creams were in the form of 1 % solutions of copper sulphate and iron additions were as freshly prepared 1 % solutions of ferrous sulphate.

The creams, 350–400 lb/batch, were churned in a stainless steel, rollerless churn. Salt when required was added to the butter at the granule stage. Each trial consisted of 3 or 4 churnings from the same bulk cream. Two 56-lb boxes from each churning were placed in storage at 14 °F and were withdrawn, one after 4 months, the other after 8 months, for grading and analysis. The fresh and the stored butters were graded by a combined panel of Dairy Division official graders and Institute staff members.

Peroxide values were determined on butterfats from the fresh and stored butters by the method of Loftus Hills & Thiel (1946) and fat aldehyde values by the procedure described by McDowell (1955).

Copper and iron were estimated in creams, buttermilks and butters by methods based on acid extraction and filtration as described by McDowell (1947), except that for copper estimation the amount of butter was increased to 20 g and that the sodium diethyl-dithiocarbamate reagent was replaced by zinc dibenzyl-dithiocarbamate.

The investigation comprised the following trials:

(1) *Effect of acidity and of salt content*

Unsalted butters were prepared from creams varying in acidity from 0·15 to 0·30 % lactic acid in the same trial. Salted butters in which the salt content varied over the range 0–1·5 % were prepared from creams of 0·15 or 0·20 % acidity.

(2) *Effect of addition of copper*

(*a*) *Sweet cream butter.* Unsalted butters and salted butters containing 1·5 % of salt were prepared from sweet creams to which various quantities of copper as copper salt had been added.

(*b*) *Acid cream butter.* Unsalted and salted butters (1·5 %) were prepared from creams of varied acidity but containing the same proportion of copper salt, and from creams of the same acidity to which varied amounts of copper salt had been added.

(3) *Effect of addition of iron*

(*a*) *Sweet cream butter.* Unsalted and salted butters (1·5 %) were prepared from neutralized sweet creams containing 2·0 and 4·0 ppm. of added iron.

(*b*) *Acid cream butter.* In each trial 2 butters, one unsalted, and one salted (1·5 %), were made from cream of 0·15 or 0·20 % acidity, and 2 butters, one unsalted and one salted (1·5 %), from cream of the same acidity but containing added iron salt.

(4) *Partition of the copper and iron from the cream between the butter milk and the butter*

(*a*) *Sweet cream and acid treated cream butters.* All creams, buttermilks and butters were sampled and analysed for copper and iron, to permit calculation of the ratios for metal content of butter or of buttermilk to metal content of cream.

(*b*) *Ripened cream butters.* The effect of increasing acidity on the partition of the metals after churning was investigated in a trial in which high acid cream was produced by the method used in normal buttermaking practice, i.e. ripening with starter, in preference to addition of lactic acid. Copper (0·2 ppm.) and iron (2·0 ppm.) were added to each of 3 vats of pasteurized cream. Starter was added to 2 of the vats in sufficient quantity to cause lowering of the pH values of the creams to about 5·2 and 4·6, respectively, after holding overnight at 66 °F. The creams were churned and the butters salted and worked under normal conditions. The creams, buttermilks and butters were analysed for copper and iron contents and for protein contents. The curd contents of the butters were also estimated.

The butters were graded under the New Zealand Department of Agriculture grading system on the following basis—'premium' finest, 94 points and over; finest, 93 points and under 94; first grade, 90 points and under 93; second grade, 80 points and under 90; third grade, under 80 points.

RESULTS

The peroxide values of all the fresh butters were within the range 0·05–0·10 and are not given in the tables of results. Since the fat aldehyde values of the fresh and stored butters showed a very high correlation with the peroxide values they, also, are omitted from the tables.

The copper contents of most of the butters from cream containing no added copper salt were below 0·07 ppm., and the maximum was 0·08 ppm. The iron contents of all butters from creams containing no added iron salt were below 0·40 ppm. Since these results included the natural copper content of 0·02–0·05 ppm. and the natural iron content of 0·15–0·20 ppm. (Mulder *et al.* 1949; Menger, 1961) it was assumed that the contents of contaminative metals were very low.

(1) *Effect of acidity and of salt content with butters of low copper content*

In 3 trials the acidity of the cream was increased over the range 0·07–0·30 % lactic acid to give unsalted butters varying in pH from 7·07 to 5·24. There was no increase in fat oxidation and there were no oily or fishy flavours in any of the butters even after 8-months storage.

The effect of increasing quantities of salt on butters of low copper content from cream of 0·15 % acidity (butter pH 6·1–6·3) is shown as the average results for 6 trials in Table 1. Though there were slight increases in peroxide value with increasing salt content the loss in grade score for all butters after 8-months storage was only 0·4 points, with no suggestion of undesirable flavours. In 2 further trials in which the acidity of the cream was increased to 0·20 % (butter pH 5·6–5·8) the average results

(Table 1) indicate that there was little loss in grade score of any of the butters after 4-months storage, but that after 8 months, butters with 1·0 and 1·5% salt were lowered in grade because of the appearance of oily flavours. Even in these butters, however, the peroxide values were only slightly higher than at 4 months.

The results indicate that if the copper content is low unsalted butter may be made with pH as low as 5·2 and salted butter with pH as low as 6·0 without undergoing flavour deterioration during storage. Since in the presence of salt even 0·02 ppm. of added copper increased the rate of butterfat oxidation in low pH butters (see section 2 (*b*) below), and since there were probably traces of contaminative copper present in all the butters, it is possible that in the absence of any contaminative copper salted butter with pH below 6·0 would not develop oxidative defects during storage.

Table 1. *Effect of salt on the keeping quality of butter of low copper content from creams of* 0·15 *and* 0·20% *acidity*

Cream acidity, %		Time of storage, months	Salt contents of butters: Control (unsalted)	0·5%	1·0%	1·5%	S.E. of means
0·15	Cu content of butter, ppm.	0	0·05	0·05	0·06	0·05	—
	pH of butter	0	6·28	6·23	6·16	6·09	±0·016
	Average grade score of butter	0	93·8	93·7	93·7	93·6	±0·11
		4	93·5	93·5	93·5	93·4	
		8	93·4	93·3	93·3	93·2	
	Peroxide value of butterfat	4	0·09	0·09	0·10	0·11	
		8	0·11	0·14	0·15	0·18	
0·20	Cu content of butter, ppm.	0	0·06	0·07	0·07	0·07	
	pH of butter	0	5·78	5·71	5·63	5·60	±0·12
	Average grade score of butter and remarks on quality	0	93·8	93·8	93·8	93·8	±0·24
		4	93·3	93·3	93·3	93·3	
		8	93·3	93·3	92·0 (oily flavour)	91·8 (oily flavour)	
	Peroxide value of butterfat	4	0·06	0·06	0·07	0·07	
		8	0·16	0·17	0·22	0·22	

(2) *Effect of addition of copper*

(*a*) *Sweet cream butter.* Increase in the copper contents of *unsalted* butters from 0·03 ppm. (control) to 0·07 ppm. and to 0·11 ppm. by the addition of 0·2 and 0·4 ppm. of copper to the respective creams had no observable organoleptic effect on the keeping quality, though there were slight increases in peroxide value with increasing copper content.

The effect on butter of normal salt content (1·5%) of the addition of copper to the cream is shown in the results of a typical trial in Table 2. There were considerable increases in peroxide values of the stored butters with increasing copper content. An increase in copper content from 0·04 ppm. (control) to 0·08 ppm. had no observable organoleptic effect on the butter but further increases to 0·12 and 0·18 ppm. caused the appearance of oily and fishy flavours.

(*b*) *Acid cream butters.* The results of one trial indicated that the addition of 0·2 ppm. of copper to creams of 0·15, 0·20, 0·25 and 0·30 % acidity (butter pH 6·27, 6·01, 5·72 and 5·38) had no effect on the keeping quality of the *unsalted* butters (average copper content 0·09 ppm.) or on the fat oxidation values of the butterfats from them. In a further trial, however, the addition of 0·5 ppm. of copper, to cream of 0·20 % acidity caused the appearance of a fishy flavour in the *unsalted* butter (copper content 0·14 ppm.) and oxidation of the butterfat after 8-months storage.

Table 2. *Effect of addition of copper on the keeping quality of salted butter from neutralized sweet cream*

	Time of storage, months	Butter from cream containing added Cu, ppm. of cream			
		0 (control)	0·2	0·4	0·6
Cu content of butter, ppm.	0	0·04	0·08	0·12	0·18
pH of butter	0	6·76	6·77	6·77	6·76
Average grade score of butter and remarks on quality	0	94·0	94·0	94·0	94·0
	4	93·5	93·5	91·0, oily	89·0, fishy
	8	93·5	93·5	89·0, oily, inclined fishy	87·0, fishy
Peroxide value of butterfat	4	0·09	0·24	0·46	1·06
	8	0·10	0·28	0·72	1·80

The effect of addition of only 0·1 ppm. of copper to cream on *salted* butter from creams of either 0·15 or 0·20 % acidity (pH of butters 6·05 and 5·64, respectively), is shown in the results of one trial in Table 3. The addition of copper to increase the copper content of the butters by only 0·02–0·03 ppm. caused development of oily flavours during 4-months storage, and slight oxidation of the butterfat as indicated by peroxide value. The appearance of fishy flavours in the butter after 8-months storage indicated a further deterioration in quality.

The addition of 0·2 ppm. of copper to cream of 0·15, 0·20, 0·25 and 0·30 % acidity (butter pH 6·04–4·99) caused the development in the *salted* butters of oily or fishy flavours during 4-months storage and of fishy flavours during 8-months storage. The intensity of the flavour and the extent of oxidation of the butterfat increased with decreasing pH of the butter. Further increases in amount of added copper to cream of 0·15 or 0·20 % acidity to give *salted* butters (pH 6·0 and 5·8, respectively), with copper contents of 0·14 and 0·22 ppm. caused stronger oily or fishy flavours over 4 months and stronger fishy flavours over 8 months than in butter containing smaller quantities of added copper. These changes in flavour of the butter were accompanied by marked increases in peroxide value of the butterfat.

(3) *Effect of addition of iron*

(*a*) *Sweet cream butter.* The addition of 2·0 ppm. of iron as iron salt to cream of low copper content usually produced a metallic flavour in the salted or unsalted butter. The flavour was retained in some samples during storage but in others it was masked by storage flavours or it disappeared altogether. In a typical trial the grade score for 2 fresh butters with total iron contents of 0·69 and 1·07 ppm., respectively, was

reduced from 93·5 (control butter, iron content 0·31 ppm.) to 92·5, because of metallic flavours. After 8-months storage, however, all 3 butters scored 93 points and all were described as 'harsh'. There was no increase in peroxide values of the butters during storage.

Table 3. *Effect of addition of copper on the keeping quality of salted butters from creams of* 0·15 *and* 0·20 % *acidity*

		Cream acidity 0·15 %		Cream acidity 0·20 %	
	Time of storage, months	Control	0·1 ppm. of Cu added to cream	Control	0·1 ppm. of Cu added to cream
Cu content of butter, ppm.	0	0·04	0·06	0·04	0·07
pH of butter	0	6·07	6·05	5·68	5·64
Average grade score of butter and remarks on quality	0	94·0	94·0	94·0	93·5, harsh
	4	93·0	91·0, oily	93·5	93·0, suggestion of oily flavour
	8	93·0	90·0, oily, inclined fishy	93·0	88·0, fishy
Peroxide value of butterfat	4	0·10	0·17	0·15	0·31
	8	0·11	0·24	0·22	2·12

Table 4. *Effect of addition of iron on the keeping quality of unsalted and salted butters from cream of* 0·20 % *acidity*

		Unsalted butter		Salted butter	
	Time of storage, months	Control	2·0 ppm. Fe added to cream	Control	2·0 ppm. Fe added to cream
Fe content of butter ppm.	0	0·30	0·75	0·32	0·64
pH of butter	0	5·86	5·86	5·71	5·71
Average grade score of butter and remarks on quality	0	94·0	93·0, metallic	94·0	92·5, metallic
	4	93·5	93·0, metallic	93·5	92·5, flat and metallic
	8	93·5	93·0, not as bright as (1)	93·5	93·0, not as bright as (3)
Peroxide value of butterfat	4	0·06	0·06	0·07	0·08
	8	0·10	0·14	0·15	0·18

(*b*) *Acid cream butters.* The effect on the butters of iron added to cream acidified to either 0·15 or 0·20 % acidity was very similar to that on sweet cream butter. Nearly all the stored butters containing added iron were inferior in quality to the control butter either because of the metallic flavour itself or because of a lack of 'brightness'. The effect of added iron on butterfat oxidation was negligible. Results for a trial which was typical of those for added iron in acid cream butters are shown in Table 4. The results reported in this paper indicate that the total iron content of any type of creamery butter should not exceed 0·50 ppm. if the metallic flavour is to be avoided.

(4) *Partition of the copper and iron from the cream between the buttermilk and the butter*

(*a*) *Sweet cream and acid cream butters.* The average ratios of copper or iron contents of the buttermilk or butter from sweet cream to copper or iron content of the cream (approximately 40 % fat) are shown in Table 5. If all the copper and iron in cream were bound to the serum proteins and none to the fat globule membrane proteins the butter/cream ratio for a typical butter containing 10 % buttermilk after washing (McDowall & McDowell, 1949) would be 0·17. This is much lower than the ratio of 0·43 for copper and 0·55 for iron for butters from creams containing no added metal salt, but only a little lower than the ratio of 0·23 for added copper and 0·23 for added iron for butters from creams containing added metal salts (see Table 5). Thus it appears that in cream free from added metal salts a high proportion of the copper and iron is attached to the fat globule membrane proteins. Since this is the normal distribution for natural copper and iron in cream (King *et al.* 1959; Menger, 1961), and since the ratios for these metals are so much higher than those for added metals, it may be concluded that in these creams the proportions of natural copper and iron were high and the proportions of contaminative metals were low.

Similarly, it appears that in creams with added metal salts only a small proportion of the added metal was attached to the membrane proteins. Since the membrane proteins comprise a much larger proportion of the total protein in butter than in cream the ratio for protein content of butter to protein content of cream was compared with that for the added metals. The average protein ratio for 9 creams and butters was found to be 0·26 and thus was fairly close to the ratio of 0·23 for copper or iron. The conclusion may be drawn that copper and iron in metal salts added to cream were distributed fairly evenly amongst all the proteins in the cream.

The proportion of copper from sweet or slightly acid cream retained in the butter is, apparently, much lower than that reported by Allan (1950) for commercial samples or by Barnicoat (1950) for experimental samples. Allan himself suggested that in his investigation further contamination might have occurred after the buttermilk had been run off. It appears that copper contamination also may have occurred during churning in Barnicoat's experiments. Since Allan (1950) gives no results for copper content of the creams and Barnicoat (1950) no results for the buttermilks, neither suggestion can be confirmed. The proportion of iron retained in the butters in the present investigation was similar to that found by Barnicoat (1950), but much lower than that calculated from Allan's results (1950).

The proportions of the metals retained in the butters from creams of 0·15 and 0·20 % acidity were very similar to those for sweet cream. Results for copper contents of butters from creams of 0·30 % acidity, however, indicated that at higher acidities a higher proportion of the metal was retained.

(*b*) *Ripened cream butter.* The results (Table 5) indicated that higher proportions of copper, iron and protein were retained in the ripened cream butter, pH 5·27, than in the sweet cream butter, and that much higher proportions were retained in the fully ripened cream butter, pH 4·66. Ratios for copper, iron and protein contents of each ripened cream butter to copper, iron and protein contents of sweet cream butter were 1·38, 1·51 and 1·39, respectively, for the butter of pH 5·27 and 2·25, 2·25 and 2·26, respectively, for the butter of pH 4·66. Thus for the same butters the 3 ratios were similar.

ripened cream between the buttermilk and the butter

			Ratio of metal or protein content			
			Butter milk/cream		Butter/cream	
			Average*	Range of values	Average	Range of values
Sweet cream	Cu	No addition to cream	1·46 (6)	(1·44–1·50)	0·43	(0·33–0·56)
		Addition to cream (total Cu)	1·58 (12)	(1·47–1·63)	0·27	(0·23–0·36)
		Addition to cream (added Cu, estimated by difference)	1·54 (12)	(1·46–1·58)	0·23	(0·18–0·26)
	Fe	No addition to cream	1·36 (4)	(1·27–1·38)	0·55	(0·51–0·60)
		Addition to cream (total Fe)	1·57 (8)	(1·45–1·63)	0·33	(0·29–0·38)
		Addition to cream (added Fe, estimated by difference)	1·53 (8)	(1·42–1·58)	0·23	(0·19–0·29)
Sweet and ripened cream	Total Cu (addition of 0·2 ppm. to cream)	Sweet cream (butter, pH 6·56)	1·50	—	0·31	—
		Ripened cream (butter, pH 5·27)	1·30	—	0·48	—
		Fully ripened cream (butter, pH 4·66)	0·92	—	0·69	—
	Total Fe (addition of 2·0 ppm. to cream)	Sweet cream (butter, pH 6·56)	1·67	—	0·31	—
		Ripened cream (butter, pH 5·27)	1·41	—	0·47	—
		Fully ripened cream (butter, pH 4·66)	1·17	—	0·70	—
	Total protein	Sweet cream (butter, pH 6·6)	1·59	—	0·26	—
		Ripened cream (butter, pH 5·27)	1·49	—	0·37	—
		Fully ripened cream (butter, pH 4·66)	1·28	—	0·60	—

* Number of samples in parentheses.

The increasing quantities of copper and iron retained in the butter as the pH of the cream decreased from 5·3 to 4·6 were probably due to the retention of increasing quantities of precipitated casein; the amount of protein retained was far higher than could be accounted for by a greater retention of fat globule membrane protein alone, and the agreement between the ratios of metal content for acid cream butter and sweet cream butter and those for protein content was close.

The greater proportions of the metals retained in fully ripened cream butter in comparison with sweet or slightly acid cream butter is of considerable interest and does not appear to have been reported previously. It seems probable that the findings of earlier workers that the keeping quality of fully ripened cream butter compared very unfavourably with that of sweet cream butter made from the same bulk cream, which almost certainly contained adventitious copper, were due partly to the higher copper content of the acid cream butter.

DISCUSSION

Dairy products containing lipids are all liable to oxidation during storage. In milk and other fluid products the phospholipids are more prone to oxidation but in dried products the triglycerides are more readily attacked (Patton, 1962). In butter, which has been described as 'an aqueous concentration of phospholipids dispersed in fat' (Patton, 1962), both the fat and the phospholipids are susceptible to oxidation. There is considerable evidence to show that fishy flavour in butter from washed cream is due to phospholipid oxidation (Koops & Pette, 1956; Tarassuk, Koops & Pette, 1959) but there is no evidence that in butter from normal (unwashed) cream the butterfat itself is not also the source of the flavour (Forss, Dunstone & Stark, 1960).

It is apparent from the results of this investigation that provided the copper content is fairly low, little or no lipid oxidation occurs in unsalted butter. This might be explained by the inhibitory effect of protein on the metal (Lea, 1936). Further additions of copper probably overcome the initial inhibition (Lea, 1936) and the rate of lipid oxidation then increases with increasing copper content.

The higher rate of lipid oxidation and rate of development of fishy flavours in low pH butters as compared with the rate in sweet cream butters might be explained by the greater retention of copper as the pH decreases from 5·3 to 4·6. This does not explain, however, why butters with the same copper content deteriorate more rapidly as the pH is reduced progressively from 6·8 to 4·6. Koops, Tarassuk & Pette (1959) investigated this problem and found that in butter from washed cream containing added acid the development of fishy flavour was due to oxidation of the phospholipids in the fat globule membrane and that the reaction was catalysed by copper bound to the membrane proteins. The maximum consumption of oxygen and the maximum intensity of fishy flavour occurred at pH 3·8–3·9 and they suggested that since at this pH the electrostatic attraction between the positively charged membrane proteins and the negatively charged cephalin fraction of the membrane phospholipids is at its greatest (Payens, 1959) there will be an intimate contact between the two which will favour the phospholipid oxidation. With increasing pH, the protein also will become negatively charged and the association between protein and phospholipid will be weaker, thus explaining the gradual decrease in oxygen consumption and in intensity of fishy flavour as the pH of washed cream butter rises from 3·9 to 6·0. The theory may not be applicable to normal butter, however, since

the proportion of contaminative copper bound to the membrane protein may be too small to catalyse oxidation of the phospholipids.

The present study has shown that salt in increasing quantities up to 1·5 % promotes lipid oxidation in the presence of contaminative copper but not necessarily in its complete absence. The catalytic effect of salt in experiments with acid salt solutions dispersed in butterfat was explained by Loftus Hills & Conochie (1946) as due to chlorine which was formed by a reaction between fat hydroperoxides and hydrogen and chloride ions

$$R—O—O—H + 2Cl' + 2H^+ \rightarrow Cl_2 + H_2O + R—O—H,$$

and which induced further oxidation in the fat. These authors suggested that phospholipids in butter, especially those at the fat-water interface, would react similarly. The theory, which also accounts for the effect of increasing acidity in salted butter, offers a reasonable explanation of the peroxidizing effect of salt on the butter lipids.

In salted acid butter, protein apparently has no inhibitory effect on the activity of copper since the addition of only 0·02–0·03 ppm. of the metal to butter almost free from contaminative copper catalyses lipid oxidation. Evidently the combination of protein-bound copper, a high concentration of chloride ions and the presence of hydrogen ions creates particularly favourable conditions for lipid oxidation in stored butter. The exact nature of the combined catalytic effect has, however, not been elucidated.

Since there are considerable variations in the natural copper content of butter (Menger, 1961) it is not possible to set a definite limit to the total amount of copper permissible in butter intended for storage. The indications are, however, that the copper contents of salted acid butters and salted sweet cream butters should not exceed 0·06 and 0·08 ppm., respectively. Limits for unsalted acid and sweet cream butters would be approximately 0·10 and 0·12 ppm.

The results of the present study do not confirm those of an earlier investigation at this Institute on the addition of copper to acid and sweet cream butters (Barnicoat, 1950).

The development of a metallic flavour in fresh butter after the addition of an iron salt to the cream was thought by Barnicoat & Palmer (1939) and by Barnicoat (1950) to be due to phospholipid oxidation, since the flavour was very similar to that found in washed or synthetic creams. Recent work shows that the flavours (metallic, fishy, etc.) which develop in washed cream and in washed cream butter are undoubtedly due to phospholipid oxidation (Forss *et al.* 1960), but there is no evidence that a similar oxidation occurs on addition of iron salts to normal (unwashed) cream. The present author, using the thiobarbituric acid test to measure phospholipid oxidation (Dunkley & Jennings, 1951), found that there actually was an increase in intensity of colour in the test immediately after the addition of iron salts to milk, skim-milk, cream or butter (unpublished results). If the increase were due to phospholipid oxidation a further increase might be expected after holding the samples at 40 °F but the results were the same 4–5 days later. Thus it *appeared* that the initial increase in colour was due solely to the effect of iron on the reaction conditions for the test itself and not to a catalytic effect on phospholipid oxidation in the sample. Added iron therefore does not appear to have a catalytic effect either on phospholipid or on

butterfat oxidation, in keeping with the view (Menger, 1961) that the flavour defect in butter is due to the intrinsic flavour of the iron salt itself.

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With 1 plate

Histochemical study on the ripening of Parmesan cheese

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Summary. The results reported suggest that it is possible to follow the ripening of Parmesan cheese by means of histochemical reactions for proteins, glycoproteins, acid mucopolysaccharides and lipids containing choline.

The blue colour, formed when the proteins of the curd were stained with Bonhag's reagent, slowly turned to violet and then to red as ripening progressed.

Glycoproteins and acid mucopolysaccharides, although present in very small amounts, appeared to be a constant characteristic of the cheese.

Lipids containing choline, which were absent from the curd, were clearly visible in 3 to 7-month-old cheese. They then decreased and were absent in the ripened cheese.

In previous experiments on the histochemical analysis of various Italian cheeses (Bolcato, Accolti Gill & Bianco, 1961) some interesting results were obtained by staining sections with Bonhag's reagent for proteins. The sections not only acquired the characteristic blue colour that proteins give with this reagent, but also on the blue background red spots appeared which varied in number and size depending on the type of cheese. After further ripening it was observed that stained sections of Parmesan cheese had only a red colour. This suggested the possibility that Bonhag's reactions for proteins might give some useful information on the ripening of Parmesan cheese. While investigating this aspect we also studied the histochemical reactions of this type of cheese for glycoproteins, acid mucopolysaccharides, and lipids containing choline.

MATERIALS AND METHODS

Typical Parmesan cheeses (Parmigiano-reggiano, without formaldehyde) of different ages from the same dairy were used. The samples examined were curd, salted curd, and cheese aged 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 17 months. The experiment was repeated 3 times using cheese made at 3 different dairies.

Pieces of the samples not more than 4 mm thick were sectioned on the freezing microtome. The sections, 15–20 μm thick, were transferred to slides and left to air-dry to ensure adequate fixation before staining.

The proteins were stained by Bonhag's method (Pearse, 1960, p. 792) except that the sections were immersed in the reagent for 25 instead of 40 min.

Glycoproteins were recognized by the various shades of purplish red stain produced by the periodic acid Schiff technique (PAS) described by McManus (Pearse, 1960, p. 832). The reaction is based on the fact that aqueous periodic acid oxidizes 1,2-glycol groups in tissues to produce aldehydes that form colour with Schiff's reagent.

Acid mucopolysaccharides were made visible with Alcian blue. This dye stains acid mucopolysaccharides of epithelial and connective tissues a turquoise-blue but does not stain most neutral mucoproteins at low pH. Its selectivity depends on the use of short staining; with longer staining nearly all the tissue components become coloured. The specificity of the stain for strongly acidic groups may be increased by lowering the pH of the staining bath (Adams & Sloper, 1956). The sections were stained with Alcian Blue according to Mowry's procedure as described by McManus & Mowry (1960), the time of immersion in the dye solution (acetic acid concentration, 3%) being decreased from 30 to 10 min.

Clearer histochemical preparations for glycoproteins and acid mucopolysaccharides were obtained if sections were defatted for 8–10 h in xylene.

Lipids containing choline were stained by the phosphomolybdic acid method of Landing and others (Pearse, 1960, p. 851). By this reaction insoluble choline-phosphomolybdic complexes are formed which are subsequently rendered visible by conversion of the phosphomolybdenum moiety to molydenum blue. Sections were first defatted for 5–10 min in xylene in which these lipids are very slowly soluble (Pearse, 1960, p. 315).

RESULTS

Proteins. The sections of the curd treated with Bonhag's reagent had a blue background on which appeared some small red spots (Plate 1*a*). After salting the curd and during the following months, the red spots enlarged and sometimes increased in number while the background changed slowly from blue to violet (Plate 1*b*). In the sections of the 6- to 7-month-old cheese, the spots were so enlarged that the background appeared coloured red with some violet spots superimposed, and in the 9- and 17-month-old sections the whole field was completely red. Thus the stained sections changed from blue to red as ripening progressed. That completely red sections arise only from ripened cheese was confirmed by examination of a further 12 different samples of ripe Parmesan. It seems possible, therefore, that the approximate degree of ripening can be estimated from the number and size of the red spots in the first months, and afterwards from the intensity of the diffuse red colour. It is, however, preferable to consider the size of the spots, for their number may decrease during ripening as adjacent red areas enlarge and coalesce. This may occur especially in the sections of 4- and 5-month-old cheese, depending on the number and closeness of the spots (Table 1).

Two purchased samples of ripened Parmesan cheese, one of which was used in earlier work (Bolcato *et al.* 1961) did not agree with the above observations. These 2 samples of cheese were without a trade mark and probably, therefore, not genuine Parmesan. The histochemistry of cheese may therefore have some diagnostic value.

Glycoproteins. The purple spots on the pale pink background of the sections of curds stained for glycoproteins were very small and numbered 15–20 per section (Table 2). With the progress of ripening the fine spots gave way to spots of a larger size numbering 5–7 per section in 7-month-old cheese (Plate 1*c*). Subsequently, the spots decreased in number and were either absent or numbered only 1 per section in the ripened cheese (17-month-old). From observations made on numerous samples of ripened genuine Parmesan it is concluded that the presence of very small amounts of glycoproteins is a constant characteristic of the typical ripened product.

Acid mucopolysaccharides. The Alcian Blue dye formed tiny turquoise blue spots on the sections of the curd. Subsequently, as for glycoprotein, the tiny spots were replaced by spots of a larger size numbering 3–5 per section (Plate 1*d*), which decreased in the sections of 7-, 9- and 11-month-old cheese and were absent in the ripened cheese (Table 3). The lack of acid mucopolysaccharides appears to be another constant characteristic of a genuine ripe Parmesan cheese.

Table 1. *Changes in colour of sections of* 3 *Parmesan cheeses stained for proteins at various stages of ripening and number of coloured spots per microscopic field*

(Mean of 3 observations.)

Sample age, months	Parmesan Su	Parmesan Mn	Parmesan S.Ma
Curd	Pale blue background, 10 red spots of ~ 10 μm diam.	Pale blue background, 22 red spots, ~ 10–15 μm diam.	Pale blue background, 20 red spots ~ 10–15 μm diam.
2	Blue-violet background, 21 red spots, of ~ 30 μm diam.	Blue-violet background, 30 red spots 20–30 μm diam.	Blue-violet background, 22 red spots of ~ 30 μm diam.
3	Violet background, red spots irregular size 30–100 μm diam.	Violet background, 17 red spots of ~ 40 μm diam.	Violet background, ~ 20 red spots of 30–60 μm diam.
4	Some spots coalesced	Some spots coalesced	—
5	Background, red-violet. Spots coalesced to form red-coloured patches	Some large spots visible on the red-violet background	Red-violet background, spots 30–80 μm diam. Some spots coalesced
6	Red background with some large violet spots		Some large spots visible on the red background
7–17	The blood-red background became more intense as the violet tinge disappeared		

Table 2. *Number and size of the spots of stained glycoproteins in sections* (~ 30 mm²) *of* 3 *Parmesan cheeses during ripening*

Sample age, months	Parmesan Su			Parmesan Mn			Parmesan S.Mn		
	⩽ 15 μ	15–40 μ	⩾ 50 μ	⩽ 15 μ	15–40 μ	⩾ 50 μ	⩽ 15 μ	15–40 μ	⩾ 15 μ
Curd	15	0	0	12	3	0	20	4	0
1	2	2	0	—	—	—	—	—	—
3	0	6	0	3	2	0	0	5	0
5	0	4	2	0	6	0	0	6	0
7	0	4	3	2	0	3	0	4	2
9	0	1	0	0	1	0	0	1	0
11	0	1	0	2	0	0	0	0	0
17	0	1	0	1	0	0	0	0	0

Lipids-containing choline. A clear positive result was obtained on sections of 3- to 7-month-old cheese (Table 4) with the phosphomolybdic acid reagent. Plate 1*e* illustrates one of these sections where the numerous small blue spots indicate the lipid containing choline. These lipids were absent in the curd sections, increased to a maximum after about 5 months ripening and decreased to zero again in the ripened cheese. An intermediate stage, when the blue spots were on the decrease, is shown in Plate 1*f*.

Similar experiments on the ripening of Pecorino (Pugliesi) cheese gave quite different results (unpublished). In this cheese glycoproteins and acid mucopoly-

saccharides were always present in greater quantities and no lipids containing choline appeared during the ripening. Most probably these differences are related to the different quality of the milk or of the microbial flora involved in the ripening.

Table 3. *Number and size of the spots of acid mucopolysaccharides in sections* (~ 30 mm^2) *of 2 Parmesan cheeses during ripening*

Sample age, months	Parmesan Mn			Parmesan S.Ma		
	$\leqslant 15$ μm	15–40 μm	$\geqslant 50$ μm	$\leqslant 15$ μm	15–40 μm	$\geqslant 50$ μm
Curd	~ 100	0	0	~ 120	0	0
1	2	2	0	0	5	0
3	5	0	0	0	5	0
5	3	2	0	0	2	3
7	2	0	0	2	0	0
9	2	0	0	1	0	0
11	2	0	0	0	0	0
17	0	0	0	0	0	0

Table 4. *Appearance and disappearance of lipids containing choline during the ripening of 3 typical Parmesan cheeses*

(Number of spots (~ 20 μ diam.) per microscopical field.)

Cheese	Curd	Sample age, months						
		1–2	3	5–7	8	9	11	17
Parmesan Su	0	0	20	~ 250	—	70	0	0
Parmesan Mn	0	0	~ 233	~ 240	~ 175	80	12	0
Parmesan S.Ma	0	0	~ 220	~ 270	~ 190	95	5	0

DISCUSSION

It is difficult to explain the facts reported in the present paper because cheese is in continuous transformation owing to the microbial and enzymic changes that occur during ripening. It is supposed that the glycoproteins, acid mucopolysaccharides and the lipids containing choline, are formed by breakdown of the macromolecules of the curd and, by further biochemical reactions, are later decomposed into components which were unable to react with the histochemical reagents used. For instance, choline may have been oxidized to betaine by bacteria (Cromwell & Rennie, 1954; Bremer, Figard & Greenberg, 1960).

The application of histochemical techniques to the study of the composition and ripening of cheeses appears to be worth further investigation to extend and explain the results obtained. It would, for example, be interesting to discover why the blue colour that the proteins of the curd formed with Bonhag's reagent, slowly changed through violet into red as the ripening progressed. Experiments still in progress suggest that the products of casein degradation are involved.

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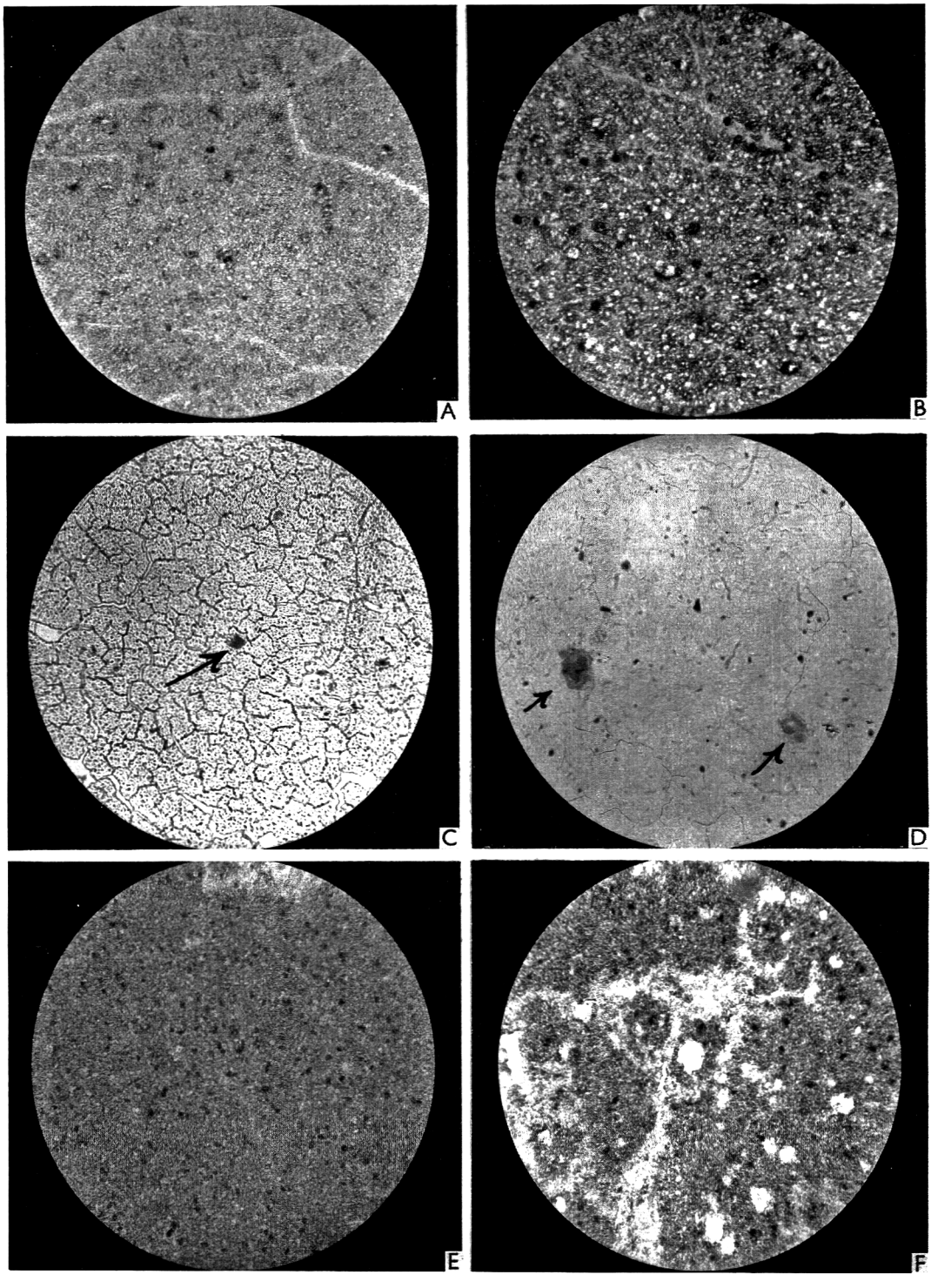
A
B
C
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E
F

EXPLANATION OF PLATE

PLATE 1

Sections of Parmesan curd and cheese at various stages of ripening treated with specific stains for protein, glycoprotein, mucopolysaccharide, or lipid containing choline (magnification, 50 ×).

(*a*) Protein of cheese curd, small red spots on blue background.

(*b*) Protein of 5-month-old cheese, red spots on violet background.

(*c*) Glycoprotein of 7-month-old cheese, purple spots on pink background.

(*d*) Acid mucopolysaccharide of 5-month-old cheese, turquoise-blue spots.

(*e*) Lipid containing choline in 5-month-old cheese, small blue spots.

(*f*) Lipid containing choline in 8-month-old cheese, blue spots.

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Attempts to relate the selenium content of heat-treated milks with their nutritive properties

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Summary. Fink (1954) and Fink & Schlie (1955*a*) fed rats on a diet intentionally marginal in vitamin E and the sulphur-amino acids, in which dried milk supplied the protein (10 %). They were able to relate the development of fatal liver necrosis in rats to heat damage of dried milks. Using their method, no liver necrosis developed in our rats with 2 spray-dried skim-milks, evaporated milk and milk subjected to ultra-high temperature treatment, with or without subsequent in-bottle sterilization. With a roller-dried skim-milk sent to us from Germany by Fink and found by him to be of 'middle degree necrogenity', no liver necrosis occurred in hooded or albino rats bred in our laboratory, but the lesion developed in albino rats from an outside source. It was found that this milk had a lower selenium content than 2 British spray-dried milks. With rats given a necrogenic diet, differences were observed between and within strains in their susceptibility to liver necrosis and in their ability to utilize dietary selenium.

It has been found at this Institute (Shillam, Dawson & Roy, 1960; Shillam, Roy & Ingram, 1962*a*) that spray-dried skim-milk preheated at 74 °F for 30 min (milk A) and milk subjected to the ultra-high temperature (UHT) process, i.e. milk heated for a few seconds at temperatures between 130 and 150 °C, were less satisfactory for the artificial rearing of calves than raw or pasteurized milk or spray-dried skim-milk preheated at 77 °C for 15 sec (milk B). These results were puzzling since it has been shown (cf. Kon, 1958) that the UHT process causes only small losses of nutrients no greater than those resulting from pasteurization. Since fatal liver necrosis has been observed in rats given low-protein (10 %) diets when certain dried skim-milks, but not when liquid skim-milk, supplied the protein (Fink, 1954; Fink & Schlie, 1955*a*), it seemed that tests of this type might help to throw light on the calf findings.

The diet used by Fink & Schlie (1955*b*) is intentionally marginal in vitamin E and the sulphur-amino acids, factors known to be protective against liver necrosis (cf. Naftalin, 1954). Subsequently work in Schwarz's laboratory (Schwarz, 1960) showed that the protective action of the sulphur-amino acids is due to contamination with traces of selenium whose activity varies with the form in which it is administered. The work in these two laboratories on the relationships between selenium, liver necrosis and heat treatment of milk has been well summarized by Shillam & Roy (1963).

Using Fink's technique we have tested a series of milks including milks A and B of the calf experiments and a sample of roller-dried skim-milk sent from Germany by

the late Prof. Fink and described by him as being of 'middle degree necrogenity'. Many of the dried milks tested by Fink (1957) had been subjected to more severe preheating than is normal in this country, and in the first consideration of the problem it was evident that losses of the sulphur-amino acids might account for his findings. To test this possibility the biological value of the proteins of the German sample was determined, with milk B included in the test for comparison.

Initially the tests were done with hooded Norwegian rats, but they showed no fatal liver necrosis with any of the milks. Fink (personal communication) recommended the use of albino rats since he had found that they were more susceptible to necrosis than hooded rats. Further tests were, therefore, done with albinos on milks A and B as well as on the German sample.

While these experiments were in progress Schwarz & Foltz (1957) reported their finding that selenium could prevent liver necrosis, and later Fink (1960) found that the necrogenic activity of a series of dried milks he tested was related to their selenium content. The preventive action of selenium has been confirmed and its concentration determined in 3 of the milks. Later Shillam, Roy & Ingram (1962*b*) found that the poor performance of calves given milk A or UHT milk was associated with denaturation of the whey proteins and that the addition of selenium to milk A was of no benefit to calves (Shillam & Roy, 1963). It thus became evident that the findings with calves at this Institute and with rats in Germany were connected with heat damage to different milk nutrients. Consequently, no correlation could be expected between the calf and rat experiments.

MATERIALS AND METHODS

Experiments on liver necrosis in rats

Rats. Male rats were used in litter-mate comparisons in all the experiments. Hooded Norwegian rats were from our colony (McKinlay, 1951). Albino rats were kindly supplied by Glaxo Research Ltd. for one experiment, but subsequently we used albinos of our own breeding from a stock obtained from the Ministry of Supply, Allington Farm, Porton. The hooded and albino rats bred here were weaned at 21 days and immediately given the experimental diets; the 'Glaxo' albinos were given our stock diet (McKinlay, 1951) for 4 days after arrival, and then the experimental diets. In 2 experiments albino dams were given a low-vitamin E diet during lactation to reduce the stores of this factor in their young.

Diets. Table 1 gives the composition of the control necrogenic diet (Naftalin, 1951), the basal diet (Fink & Schlie, 1955*b*) used for testing the milks and the low-vitamin E diet given to mother rats in some experiments to reduce the vitamin E stores of the young. For feeding, milks were given with the basal diet in the proportion 1 part milk solids and 3 parts basal diet. There were tested: spray-dried samples A and B, the German roller-dried sample, evaporated milk, milk sterilized by the UHT process with or without subsequent in-bottle sterilization and then condensed to about 20% total solids content, and liquid skim-milk. The last was offered separately from the basal diet in the calculated proportion and no water was given for drinking. The other milks were mixed with the basal diet, the dried milks weekly and the condensed samples daily. Fink (personal communication) found that a mixture of B vitamins

could satisfactorily replace the yeast used in his milk diets. Accordingly in expts. 6, 7 and 8 (Table 2) the yeast in the necrogenic diet and in the basal milk diet was replaced by an equivalent quantity of starch or sugar and a mixture of synthetic B vitamins. In expt. 8 the preventive effect, on the development of liver necrosis, of tocopherol and selenium supplements to the necrogenic diet was studied. Control groups of rats were given the necrogenic diet in all the experiments involving dried milks but not in the experiments with the evaporated and UHT milks.

Table 1. *Percentage composition of necrogenic diet, basal diet for milk tests and low-vitamin E diet*

Component	Necrogenic diet	Basal diet for milk tests	Low-vitamin E diet
Unextracted casein (Glaxo Research Ltd.)	8·0	—	25·0
Yeast, dried brewers'	3·0	2·0	—
Lard	7·0	—	7·0
Sugar	76·0	—	62·0
Dextrinized wheat starch	—	90·0	—
Salts (de Loureiro, 1931)	4·0	4·4	4·0
Cod-liver oil	2·0	3·6	—
Vitamin mixture	0·008*	—	0·018†
Vitamin A and D mixture‡	—	—	2·0

* Contained (parts): thiamine hydrochloride, 0·3; riboflavine, 0·3; calcium pantothenate, 2·0; pyridoxine hydrochloride, 0·3; inositol, 0·5; nicotinic acid, 4·0.

† Contained (parts): thiamine hydrochloride, 0·5; riboflavine, 1·1; calcium pantothenate, 3·5; pyridoxine hydrochloride, 0·5; inositol, 5·5; nicotinic acid, 6·0; folic acid, 0·06; biotin, 0·06.

‡ Consisted of 1000 i.u. vitamin A and 100 i.u. vitamin D_3/ml arachis oil.

Table 2. *Information about rats given a necrogenic diet; incidence of liver necrosis*

Expt. no.	Breed	Mother's diet during lactation	Source of B vitamins	Duration of expt., days	No. of rats	Rats that died: No.	Rats that died: Days of survival, mean and range
1	Hooded Norway	Stock	Yeast	96–97	12	7	60; 53–66
2	Hooded Norway	Stock	Yeast	300	12	2	103; 76, 130
3	Hooded Norway	Stock	Yeast	100	12	10	62; 50–81
4	Albino (Glaxo)	Stock (Glaxo)	Yeast	109	11	8	36; 26–61
5	Albino (Shinfield)	Stock	Yeast	144–148	10	6	92; 81–109
6	Albino (Shinfield)	Stock	Yeast	104	12	12	68; 42–104
6	Albino (Shinfield)	Stock	Vitamin mixture*	94	12	12	62; 42–94
7	Albino (Shinfield)	Low vitamin E	Vitamin mixture*	111–120	8	8	27; 16–43
8	Albino (Shinfield)	Low vitamin E	Vitamin mixture*	51–56	5	4	20; 16–26

* The mixture contained (parts): thiamine hydrochloride, 0·1; riboflavine, 0·4; pyridoxine hydrochloride, 0·1; calcium pantothenate, 0·75; inositol, 2·5; nicotinic acid, 1·0; folic acid, 0·03; biotin, 0·03. 0·01 parts of this mixture replaced the B vitamins of 3 parts of yeast.

Determination of the biological value and true digestibility of milk proteins

The biological value and true digestibility of the proteins of the German roller-dried milk and of the spray-dried sample B were determined at an 8% level of protein intake with 6 pairs of litter-mate weanling female rats by the balance sheet

method of Mitchell (Mitchell, 1923–24; Mitchell & Carman, 1926) as used in this laboratory (Henry, Kon & Watson, 1937; Henry, Kon, Lea & White, 1947–48; Henry & Kon, 1956).

Determination of selenium in milk diets

The selenium content of spray-dried milks A and B and of the roller-dried German milk was measured by digestion of the samples with a mixture of nitric and sulphuric acids in the presence of mercuric oxide followed by distillation of the selenium as bromide and its reduction to elementary selenium (Association of Official Agricultural Chemists, 1960). Since the selenium concentration in milk is low the subsequent steps of the method were modified as follows: the selenium precipitate was left to stand overnight and was then separated off on a no. 4 porosity sintered glass filter, rinsed with water and dissolved in 5 ml hydrobromic acid–bromine solution (5 ml HBr sp.gr. 1·46 and 10 ml saturated bromine water diluted to 100 ml with water). Selenium was determined colorimetrically by Cheng's (1956) method. Blank determinations were also made.

Even with this modification the method was found to be not sufficiently accurate for determining the low concentrations of selenium in milk. These milks were, therefore, reassayed by the United Kingdom Atomic Energy Authority Laboratories, Wantage, using an activation technique. At the same time the diet containing the German milk and the necrogenic diet were assayed.

RESULTS

Table 2 shows the incidence of fatal liver necrosis in rats given the necrogenic diets in the different experiments. Except in expt. 2, in which it was low, the incidence did not vary greatly between experiments, but the mean survival time was influenced by both the breed of rat and its vitamin E stores. The survival time of the Glaxo albinos (expt. 4) was less than that of either the hooded or the albino rats bred in this laboratory whose mothers ate our stock diet during lactation (expts. 1, 3, 5 and 6). When this stock diet was replaced by a low-vitamin E diet (expts. 7 and 8) the survival time was markedly reduced. In agreement with Fink's findings (personal communication), expt. 6 showed that under our conditions the yeast in the necrogenic diet could be satisfactorily replaced by a mixture of B vitamins. In expt. 8, 4 of the 5 rats given the necrogenic diet died of liver necrosis but there were no deaths in litter-mates given this diet for 51–56 days either with a weekly supplement (given in 2 equal doses) of 9·2 mg DL-α-tocopherol or with 0·1 or 0·2 ppm. selenium (as sodium selenite) added to the drinking water. The livers of the rats given the tocopherol supplement appeared normal apart from being rather pale. Rats given 0·1 ppm. selenium in their drinking water had yellow patches on the liver, with the exception of one which had a pale but otherwise normal liver. This rat may have been less susceptible to liver changes since a litter-mate that was the only survivor of the 5 rats given the necrogenic diet had yellow patches on its liver when killed. Liver abnormalities were much less marked with the higher selenium supplement.

Table 3 shows that no deaths from liver necrosis occurred when the diet contained any of the milks, except in expt. 4 with Glaxo albinos where of 11 rats given the diet containing the German roller-dried milk 7 died. Their livers had marked haemorrhagic

lesions. The German milk was tested on three other occasions (expts. 3, 5 and 7) but no deaths occurred.

Table 4 gives values for selenium in the spray-dried milks A and B, in the roller-dried German milk, in the diet in which the latter milk supplied 10% protein and in the necrogenic diet. It shows that the selenium content, determined by the activation process, of the German milk was considerably less than that of the other 2 milks and that the 2 diets contained essentially the same concentration of selenium. Although accurate determinations of the selenium content of the milks by the chemical method were not possible the results were of the same order as by the more accurate activation technique.

Table 3. *Tests for necrogenic properties of liquid skim-milk and of heated milks*

Expt. no.*	Milk tested	Result of test
1	Liquid	No necrosis within 97 days
	A	Histological examination of livers showed possible early signs of necrosis
2	Liquid, A	No necrosis in 6 rats within 180 days or in 6 rats within 300 days
3	Liquid, German	No necrosis within 100 days
4	Liquid	No necrosis within 109 days
	German	7 out of 11 rats died of necrosis; mean survival time 39 days (33–42 days)
5	B, German	No necrosis within 144–148 days
7	A, B	No necrosis within 111–120 days
9	Liquid, Evaporated, UHT, UHT with in-bottle sterilization	No necrosis within 1 year in groups of 12 hooded Norway rats with normal vitamin E reserves

* For experimental details in expts. 1–7 see Table 2.

Table 4. *Selenium content (ppm.) of milk powders and diets determined* (A) *by an activation process* (*see p.* 242) *and* (B) *by the method of the Association of Official Agricultural Chemists* (1960)

Sample	A*	B
Spray-dried skim-milk preheated at 74 °C for 30 min	0·27 0·24	0·19–0·28 (5)
Spray-dried skim-milk preheated at 77 °C for 15 sec	0·34 0·30	0·17–0·25 (5)
German roller-dried skim-milk	0·095 0·104	Too low to detect (6)
German milk diet (expt. 7)	0·030 0·021	—
Necrogenic diet (expt. 7)	0·044 0·025	—

Figures in parentheses are the numbers of determinations.

* Two portions of each sample and 2 selenium standards were irradiated. Each portion was compared with each of the 2 standards and the value quoted is the mean.

The biological value of the proteins of the German milk was 91·8 ± 1·06 compared with 90·8 ± 0·70 for milk B. This finding indicates no loss of the sulphur-amino acids in the German milk in spite of the probable drastic preheating treatment. The corresponding true digestibilities of the milk proteins were 93·9 ± 0·67 and 94·7 ± 1·02.

DISCUSSION

Naftalin (1954) has stressed the difficulties in producing liver necrosis in rats even when experimental conditions are constant and appear to be optimal for the appearance of the lesions. The different results with the necrogenic diet in expts. 1 and 2, in which experimental conditions were as similar as possible, support this view.

Schwarz (1960) has pointed out that determination of selenium is not necessarily a measure of its protective action against necrosis since some nutrients contain the element in a biologically inert form, and that animal assay is at present the only method for assessing this protective action. The necrogenic diet and the diet containing the German milk had essentially the same selenium content. Both diets were given in expts. 3, 4, 5 and 7. Although fatal liver necrosis invariably developed with the necrogenic diet, with the diet containing the German milk it occurred only with the Glaxo albinos (expt. 4) and not with albinos of our own breeding (expts. 5 and 7) or with our hooded Norwegian rats (expt. 3). Although the composition of the pre-weaning diet of the Glaxo rats is not known, this finding was surprising since the occurrence of fatal necrosis in this group when on the necrogenic diet was within the range observed for the hooded and albino rats used in expts. 3, 5 and 7. The results of these 3 experiments suggest strongly that selenium was more available from the milk diet than from the necrogenic diet, either because of the form in which it was present or because of the influence of other dietary factors. Moreover, expt. 8 showed that although, at the levels tested, selenium supplements prevented death, the livers were abnormal in appearance. In this connexion it should be noted that Bunyan, Green & Diplock (1963) have shown that although supplements of 0·05 and 0·50 ppm. of selenium to a necrogenic diet prevented liver necrosis, neither level reduced lipid peroxidation in the liver.

It is clear that the results of these experiments with rats have no bearing on the earlier calf findings at this Institute (Shillam *et al.* 1960, 1962*a*). In addition, the results provide further evidence that there are differences both between and within strains of rats in their susceptibility to liver necrosis and in their ability to utilize dietary selenium. The results of the selenium analyses of the milks support Fink's (1960) observation that the selenium content of heated milks is related to the severity of the heat treatment.

I am grateful to Dr Joyce Toothill for the chemical analysis of the milks for selenium and for help with some of the rat-feeding tests; to Mr H. Burton and Miss H. R. Chapman for the preparation of the UHT milks and to Mr A. Wagstaff for the determination of total solids in these milks; to the late Mr E. Capstick, United Dairies Ltd., for the gift of the evaporated milk; to Glaxo Research Ltd., for the albino rats used in expt. 4; to Miss M. V. Chapman for help with the care and feeding of the rats; to Drs G. K. Benson and C. A. E. Briggs for the histological examination of the livers and to Dr S. K. Kon for much helpful advice.

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Storage of chilled milk in relation to butter quality

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Summary. Butter made from cream separated from milk accumulated in a refrigerated tank from morning and evening milkings for 3 days was compared with butter from similar milk unchilled and separated and pasteurized for churning daily.

When the milk was of good quality there was no difference in the butters either when fresh or after storage for 4 and 8 months.

When the milk was of poor quality the butter from the chilled milk was superior in flavour, both when fresh and after storage, to the butter from the unchilled milk. The defects in the butter from the unchilled milk were probably due to bacterial growth in the milk during the short period of holding of the unchilled milk.

It is now generally accepted that milk produced under good sanitary conditions at the farm will show no deterioration in bacteriological quality if it is chilled soon after milking (Marth, Hunter & Frazier, 1954; Atherton & Bradfield, 1957; Marth & Frazier, 1957). Furthermore, the accumulation of milk in a refrigerated farm tank may be extended to 48 or even to 72 h with little increase in bacterial growth provided the bulk milk is recooled to 38–40 °F within 3–4 h after the addition of water-cooled milk from each milking (Marth *et al.* 1954; Atherton & Bradfield, 1957; Marth & Frazier, 1957; Howey, 1962; Klupsch, 1963).

Although the warming and re-cooling during accumulation of the supply over a 2- or 3-day period may have little effect on the bacteriological quality of the milk it could promote lipase activity and the production of unclean or rancid flavours in the chilled milk. Van Demark & March (1957) and Howey (1962), however, found that provided the blend temperature of the cooled and added milk was below 60 °F there was little evidence of lipase activity.

During prolonged storage of chilled pasteurized milk oxidation of the phospholipids and the butterfat can occur with development of an 'oxidized' flavour. There is, however, no evidence to suggest that with raw milk the defect will appear over a 2- or 3-day storage period (Storgårds, 1962).

Since there is little deterioration in the bacteriological and chemical quality of stored chilled milk it is not surprising that after separation at the dairy factory the skim-milk is suitable for the production of either skim-milk powder or casein (Brazendale, 1962). There appears, however, to be no information regarding the quality of the cream or the butter. In the present investigation butter made from cream

separated from milk accumulated over 3 days in a refrigerated tank was compared for quality and keeping quality with butter from cream from the same milk unchilled and separated daily.

EXPERIMENTAL

Milk water-cooled over a surface cooler was collected from 4 farms after each milking over a period extending from the evening milking on one day to the morning milking 3 days later. The milk was delivered to the Institute experimental factory and a portion, approximately one quarter, of the mixed milk was added to a refrigerated tank of 320 gal capacity. The tank was not insulated but was fitted with a refrigerated coil on its base (Scott, 1960). It was also fitted with a small slow-speed agitator. The temperature of the milk when added to the tank ranged from 65 to 80 °F. The first batch was cooled to 40 °F in approximately 4 h. The temperature after the addition of the second batch rose to 55–59 °F and the bulk milk was recooled to 40 °F in 4–8 h. With the addition of subsequent batches the temperature rose only to 44–49 °F but recooling was continued for 6–12 h. A short time after the addition of the final batch the bulk milk was heated to 85 °F and separated. The acidity of the cream was adjusted to 0·08% lactic acid, and the cream was pasteurized (tandem Vacreator using 3½–4 lb of steam/gal of cream with holding times of 1–2 sec at 205–207 °F and of 15 sec at 187 °F), cooled and held overnight for churning and manufacture into butter.

The evening milk remaining each day after transfer of the required amount to the refrigerated tank was held overnight at atmospheric temperature and then mixed with the morning milk remaining after transfer. The mixed milk was heated to 85 °F and separated. The acidity of the cream was adjusted and the cream pasteurized, cooled and held for churning the following day. In each trial, therefore, 3 'control' butters were made for the 1 chilled milk butter.

Free acidity was estimated in the fat from the fresh and stored milks. The milk sample (14 ml) was shaken with 14 ml of neutralized rectified spirit and then with 20 ml of a 2:3 ether–light petroleum mixture. After centrifuging, 15 ml of the extract were withdrawn, 4·5 ml of neutralized rectified spirit were added and the mixture was titrated to the phenolphthalein endpoint with 0·025 N-alcoholic potash. Results were recorded as ml of N-alkali/100 g of fat.

The creams were churned in a stainless steel rollerless churn. The butter was salted to give a normal salt content of 1·6%. Two 56-lb boxes of butter from each churning were stored at 14 °F and were withdrawn, one after 4 months and the other after 8 months, for grading and analysis. The fresh and the stored butters were examined by a panel of Department of Agriculture official butter graders and staff members.

Butterfats obtained from the stored butters by melting, decanting and filtering at 40 °F were examined by the peroxide test of Loftus Hills & Thiel (1946) and for free fat acidity.

Twelve trials were carried out during the 1960–61 and 1961–62 seasons. The first 6 trials, in 1960–61, were with good quality milk as normally received from the farms. The final 6 trials, in 1961–62, were with poor quality milk, obtained by restricting the washing of the milking machines at 2 of the farms to a rinse with cold water, for

2 days before the trial and during the 3 days of the trial. This milk was mixed at the factory with the normal milk from the other 2 farms.

Table 1. *Effect of accumulation and storage of good quality and poor quality chilled milk over 3 days on the quality of the fresh and the stored butters*

(Results for both good quality and poor quality milks are given as means of 6 trials in each case. Each trial involved 6 butters from chilled milk and 18 butters from unchilled milk.)

		Good quality milk		Poor quality milk		
	Butter storage time, months	Butter from unchilled milk (1)	Butter from stored chilled milk (2)	Butter from unchilled milk (3)	Butter from stored chilled milk (4)	Standard error of means
Copper content of butter, ppm.	0	0·08	0·08	0·05	0·05	—
Average grade score of butter	0	93·5	93·5	92·8*	93·4	±0·12
	4	93·4	93·4	92·8*	93·4	
	8	93·3	93·3	92·2†	93·1	
Free fat acidity of butterfat	0	0·48	0·75	0·70	0·76	—
Peroxide value of butterfat	0	0·10	0·10	0·07	0·06	—
	4	0·09	0·12	0·08	0·08	—
	8	0·13	0·21	0·09	0·11	—

* Unclean flavour; † unclean and musty flavour.

RESULTS

Trials with good quality milk. The bulk milk obtained by mixing the 4 farm milks was always of good quality, with methylene-blue reductase times usually of 7–9 h and rarely falling below 7 h. There was no evidence of an increase in free fat acidity of the chilled milk over the 3-day period of storage.

The raw creams from both the chilled and the unchilled milks were all low in acidity (0·08–0·10%), and usually were graded as 'finest'. Occasionally a slight staleness was observed in the chilled milk cream.

Results for the fresh and the stored butters are shown in columns (1) and (2) of Table 1, as the averages for 6 trials, i.e. for 6 butters from chilled and for 18 butters from unchilled milk. The copper contents of all the butters were reasonably low and were unlikely to affect the keeping quality (McDowell, 1964). The average peroxide value of the fresh butters from chilled milk was not significantly different from that of the fresh control butters. There was thus no evidence that butterfat oxidation occurred during storage of the chilled milk. The increases in peroxide values of the butters during storage were small and of the same order as found in good quality butters of low copper content. The free fat acidity of the chilled milk butter invariably was higher than that of the control butter. Since there was no apparent increase during storage of the milk the difference probably arose because of lipase activation during warming of the chilled milk before separation and during the interval between separation and pasteurization of the cream. The difference in free fat acidity had no apparent effect on the quality of the butter (see Table 1). The butters were all of excellent keeping quality, losing only 0·2 point in grade score after 8-months storage.

Trials with poor quality milk. The methylene-blue reductase times of the milks from the 2 farms with restricted milking machine washing varied from 5 h (in cool weather) to as low as 1 h or even less in hot summer weather. When mixed with the good quality farm milk (reductase test times of 6–9 h) the reductase test times again ranged from 5 to 1 h. The unchilled milk from each evening milking deteriorated overnight with considerable increases in acidity and decreases in reductase test times to as low as 15 min. The creams from the mixed evening and morning unchilled milk were thus of very poor quality and often were classed as 'second grade' because of presence of sour and 'unclean' flavours. The cream from the chilled milk was fairly low in acidity, about 0·10% compared with 0·15–0·25% for the unchilled milk cream, though usually it was classed as 'first grade' and not 'finest' because of unclean flavours. There was an increase in the acidity of the fat in the unchilled milk after standing overnight and of the fat in the chilled milk over the 3-day storage period. Since in the unchilled milk there was no temperature activation of the natural lipase it is probable that the increase was due to the action of fat-splitting bacteria. The increase in the chilled milk fat acidity also could have been due to these bacteria since there was no apparent increase during storage of good quality chilled milk.

Results for the butters are shown in columns (3) and (4) of Table 1. All these butters also were low in copper content. The acidities of the butterfats from the fresh butters were somewhat higher than the average acidity for butterfat from butter from unchilled milk of good quality (compare columns (3) and (4) with column (1)), but there was no further increase during storage of the butters. The low peroxide values of all the fresh butters and the very slight increases during storage indicate that deterioration in quality of the butter was not due to butterfat oxidation.

The chilled milk butter was of good quality when fresh and after 8-months storage (see Table 1). Some of the unchilled milk butters were of fair quality when fresh and also after 4-months storage but others were described as 'unclean' in flavour. Thus the average grade score, 92·8 fresh and at 4 months, was somewhat lower than that for the chilled milk butter, 93·4 fresh and at 4 months. After 8 months unclean flavours were still observed in some unchilled milk butters but the average grade score fell to 92·2 because of a predominant 'musty' flavour.

DISCUSSION

Under normal milking conditions at the farm the water-cooled milk is run directly into the refrigerated tank. In the present trials chilling did not commence until all the milks had been mixed at the factory and a portion had been transferred to the tank. Since the temperature of the milk as it left the farm would be maintained, or under hot summer conditions would be increased, during transport to the factory it is probable that the quality of the mixed milk in the final 6 trials was poorer than that of either of the 'poor quality' farm milks immediately after water cooling. In spite of this delay in chilling all the butters from chilled milk, were graded 'finest', i.e. a grade score of 93 or higher, both before and after storage. Thus it is reasonable to expect that butter from milk chilled at the farm also would be of similar quality.

Though milk produced under unsanitary conditions had little effect when chilled on the quality of the butter, the same milk when unchilled caused defects in butter flavour during storage. It is apparent, therefore, that any undue delay between collection of poor quality chilled milk, and pasteurization of the cream obtained from it at the factory might cause a deterioration in flavour of the butter. Since in commercial practice the cream is sometimes held in the neutralizing tanks for up to 6 h before pasteurization some deterioration in the quality of the butter could occur if the chilled milk is produced under poor sanitary conditions in the milking shed.

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A compound responsible for mushroom flavour in dairy products

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Summary. The 'mushroom compound' previously reported as a gas chromatographic fraction of oxidized dairy products has been identified as oct-1-en-3-ol. The alcohol was found to have a flavour threshold value of 1 part in 10^9 in water, 1 part in 10^8 in skim-milk, and 1 part in 10^7 in butterfat. A mechanism for the formation of oct-1-en-3-ol is discussed.

Mushroom flavours develop in dairy products under certain oxidizing conditions. Patton (1961) stated that butter can develop a remarkable range of oxidative flavour defects including 'mushroom-like' flavours. Mushroom flavour is frequently dominated by other associated flavour defects, such as oily, fishy, or metallic, which may mask the mushroom component. It can then only be clearly recognized after gas chromatographic separation.

Pont (1951) reported that when skim-milk was exposed to sunlight the burnt protein odour was frequently accompanied by a mushroom odour. Forss, Dunstone & Stark have reported studies on fish-oil flavours in butterfat (1960*a*) and in washed cream (1960*b*). The compounds responsible for fish-oil flavour when separated by gas chromatography gave a fraction which had a mushroom odour. Samuelsson (1962), commenting on these results, pointed out that sunlight flavour is often described as resembling mushroom flavour.

Mushroom flavour has been reported in other lipid-containing food which has undergone oxidation. Recently, Hoffmann (1962) isolated oct-1-en-3-ol from gas chromatographic fractions which had a mushroom odour; the fractions were isolated from mildly oxidized linoleic acid ester, soyabean oil, reverted linseed or rapeseed oil, and oxidized palm oil. Hoffmann (1961) considered that further decomposition of oct-1-en-3-ol could form cyclic acetals (1,3-dioxalans) which could be responsible for the mushroom odour. Diemair & Schams (1962), working on the volatile compounds from stored fatty fish, reported the presence of a gas chromatographic fraction with a smell of mushrooms.

Oct-1-en-3-ol had been isolated from mushrooms by Murahashi (1938). Honkanen & Moisio (1963) have isolated oct-1-en-3-ol from the volatile substances of clover and clover flowers and observed that it had a strong mushroom odour. Stark & Forss (1962) suggested that this compound was also responsible for the mushroom flavour of oxidized dairy products. This paper presents chemical evidence confirming this hypothesis.

EXPERIMENTAL

Source of the 'mushroom compound'

The 'mushroom compound' was isolated from 12 kg lots of oxidized butter prepared from washed or non-washed cream. The cream was treated with 5 ppm. copper and 50 ppm. ascorbic acid (Forss *et al.* 1960*b*). The butter-oil and serum were separated and the volatile compounds were removed by steam distillation at 45 °C and 15 mmHg. The combined distillates were treated with a concentrated solution of 2,4-dinitrophenylhydrazine dissolved in 1:1 H_2SO_4 and water; the final concentration of 2,4-dinitrophenylhydrazine in the distillate was 250 mg/l. and the acid concentration 0·1 N. The distillate was allowed to react overnight at a temperature of 4 °C, and the unreacted volatile compounds containing the 'mushroom compound' were steam distilled at 35 °C and 15 mmHg. This distillate was extracted with light petroleum and the extract concentrated in a distillation apparatus fitted with a fractionating column. The final volume of the light petroleum concentrate was approximately 2 ml.

Isolation and identification of the 'mushroom compound'

The methods used for isolation and identification of the 'mushroom compound' were similar to those already described (Forss *et al.* 1960*a*). The 2 stationary phases used in gas chromatography were 'Apiezon M' and diethylene glycol succinate. The 2-ml sample of light petroleum concentrate of the distillate was distilled on to an 'Apiezon M' column held at a temperature of 50 °C. When all the light petroleum had passed through the column the gas flow was turned off, the temperature of the column was raised to a final temperature of 105 °C in 5 min and the gas flow was restarted. Samples were trapped and re-run on the same column or on the diethylene glycol succinate column.

The infra-red spectra were measured on a Beckman Model IR 7 spectrophotometer fitted with scale expansion using a Beckman ultramicro cell of thickness 0·2 mm, volume 5 μl, with carbon tetrachloride as the solvent. The ultra-violet spectra were measured on a Beckman Model DK 2 Extended-UV-Range spectrophotometer.

Mass spectra were measured on a 60° sector, 30 cm radius single focusing high resolution instrument constructed by the C.S.I.R.O. Division of Chemical Physics. The temperatures of the ion chamber and the inlet system were not controlled and measurements were made on small samples which nearly always contained traces of water, carbon dioxide and low molecular weight aldehydes which had escaped reaction with the 2,4-dinitrophenylhydrazine. Mass measurements were made by comparison with a calibrating substance selected to give peaks straddling as closely as possible the unknown fragment.

Oct-1-en-3-ol was prepared by the method of Crabalona (1944), using propenal and n-amyl magnesium bromide. A sample of the alcohol was also obtained from a commercial laboratory.

RESULTS

Characterization of the 'mushroom compound'

Synthetic oct-1-en-3-ol was found to have a mushroom odour similar to that of the gas chromatographic fraction from oxidized butter. The retention times on 2 gas chromatographic columns of the isolated 'mushroom compound' and synthetic oct-1-en-3-ol were identical and are shown together with the data for a number of n-alkanals and alkan-1-ol in Table 1. The data suggested that the 'mushroom compound' and synthetic alcohol were structurally very similar.

The infra-red absorption spectra of carbon tetrachloride solutions of the 'mushroom compound' and synthetic oct-1-en-3-ol are shown in Fig. 1. The concentration of isolated 'mushroom compound' was approximately 50 μg/5 μl of carbon tetra-

Table 1. *Gas chromatography of the 'mushroom compound' on 2 columns with Apiezon M, and diethylene glycol succinate as stationary phases, respectively*

	Relative retention times	
Compound	'Apiezon M', 105 °C	Diethylene glycol succinate, 83 °C
Hexan-1-ol	48	58
n-Heptanal	53	29
Heptan-1-ol	96	98
'Mushroom compound'	100	100
Oct-1-en-3-ol	100	100
n-Octanal	110	50
Octan-1-ol	188	167
n-Nonanal	215	82
n-Decanal	430	139

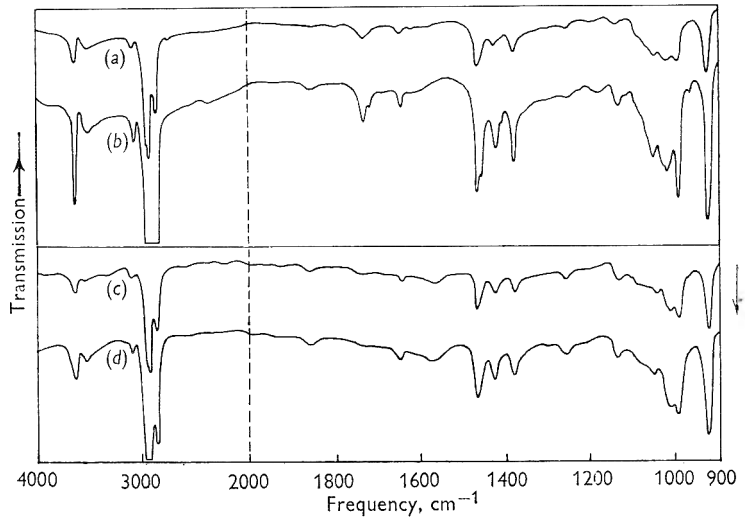


Fig. 1. Infra-red spectra of carbon tetrachloride solutions of: (*a*) isolated 'mushroom compound'; (*b*) scale of (*a*) expanded approximately 4 × ; (*c*) synthetic oct-1-en-3-ol; (*d*) scale of (*c*) expanded approximately 2 × .

chloride and scale expansion was used to increase band intensities. The more significant absorption frequencies occurred at 927, 990, 1380, 1425, 1470, 1645, 1855, 3090, 3630 cm^{-1}. The isolated 'mushroom compound' contained a trace amount of carbonyl material, resulting in weak absorption of the C=O band at 1733 cm^{-1}.

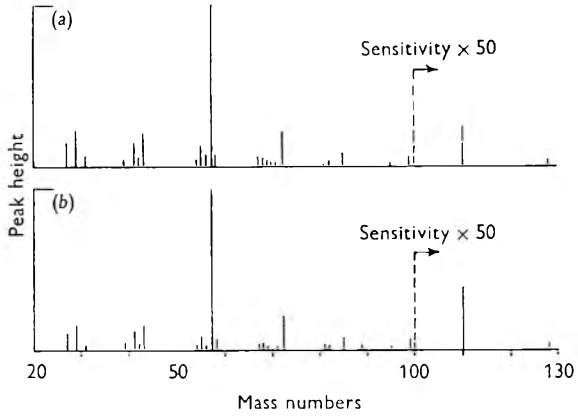


Fig. 2. Mass spectra of: (*a*) isolated 'mushroom compound'; (*b*) synthetic oct-1-en-3-ol.

Fig. 2 is a diagrammatic representation of the mass spectra of (*a*) 'mushroom compound', (*b*) synthetic oct-1-en-3-ol. The major peaks occurred at mass numbers of 99, 85, 83, 72, 57, 43, 41, 31, 29 and 27. Imperfections in the matching of the mass spectra of the 2 compounds appear to be due to the presence of a small amount of heptan-1-ol in the isolated mushroom fraction (the retention times of the 'mushroom compound' and heptan-1-ol are very similar on both gas chromatographic columns). The mass measurement of the parent peak gave a mass of 128·15, the mass for $C_8H_{16}O$ being 128·16. The height of the parent peak was approximately 0·1 % of the largest peak in the spectrum. Lowering the ionization voltage to 13 eV increased the relative height of the parent peak to approximately 1 % of the largest peak, while the overall spectrum became much weaker. At this ionizing voltage the re-arrangement ion at mass 72 became the largest peak in the spectrum.

Under extremely dry operating conditions using a gas chromatographic column with silicone oil as the stationary phase, the mushroom fraction was partially split into 2 additional fractions in approximately equal amounts. The ultra-violet spectra of these 2 fractions showed $\lambda_{max.}$ at 228 and 226 mμ, and mass spectrometric measurements gave a parent peak at mass 110 for both fractions. Similar results were obtained when oct-1-en-3-ol was treated with concentrated sulphuric acid. This suggested that the fractions were probably isomeric forms of octa-1,3-diene produced by dehydration of the alcohol. Dijkstra (1957) reported $\lambda_{max.}$ of 226 and 222 mμ for the *cis*- and *trans*-forms, respectively, of penta-1,3-diene.

Flavour evaluations

For flavour evaluation by a panel of experienced graders, solutions of isolated and synthetic oct-1-en-3-ol were prepared in distilled water, skim-milk, and deodorized butterfat. To determine the threshold concentration a series of increasing concentrations was submitted to the graders. They were asked to select the one in which the

flavour was just perceptible, and to comment on the odour and flavour. Oct-1-en-3-ol from either source had a flavour threshold value of one part in 10^9 in water, one part in 10^8 in skim-milk, and one part in 10^7 in butterfat. The term 'mushroom' was used by graders extensively throughout the trials to describe both odour and flavour. There was a tendency to describe the samples of butterfat as having a more typical mushroom flavour than either the water or the skim-milk samples. Samples of the alcohol in water and skim-milk at 100 times threshold concentration were submitted without comment to 18 people outside the laboratory. Of these, 16 described the flavour and odour as 'mushroom' while the other 2 likened it to mouldy bread.

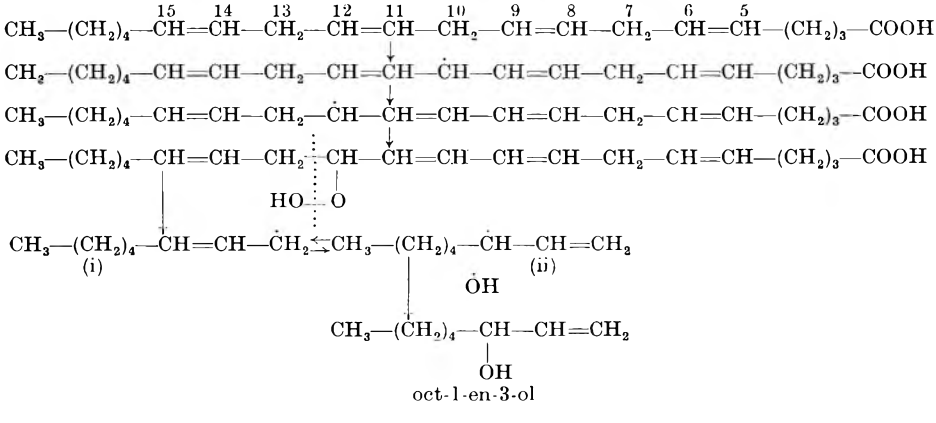
DISCUSSION

An earlier conclusion that 'mushroom compound' was carbonyl in nature (Forss *et al.* 1960*a*) is to be attributed to resemblance of the odour of very dilute solutions of oct-1-en-3-one to that of oct-1-en-3-ol, and to an observed tendency for the alcohol to be oxidized on treatment with 2,4-dinitrophenylhydrazine and sulphuric acid. Diemair & Schams (1962) concluded that a mushroom fraction from fatty fish was carbonyl in nature but their infra-red data suggest that they may also have been dealing with a vinyl carbinol.

Honkanen & Moisio (1963) suggested that oct-1-en-3-ol in dairy products could originate from clover eaten by the cows. Their experiments showed that oct-1-en-3-ol (1 g) fed to cows produced a level in the milk of 2 parts in 10^8 after 2–4 h. Flavour trials in this laboratory showed that this level is below threshold for butterfat and just above threshold for skim-milk. The 'mushroom compound' has not in our investigations been found in dairy products prior to oxidation.

The mechanism for the autoxidation of methyl linoleate based on the theory of Farmer, Koch & Sutton (1943) involves abstraction of hydrogen from the pentadiene system to form a resonance hybrid radical which reacts with oxygen mainly at the end positions. This theory is supported by the predominance among the carbonyl compounds isolated from autoxidized linoleic acid ester of compounds readily derived from attack at this point.

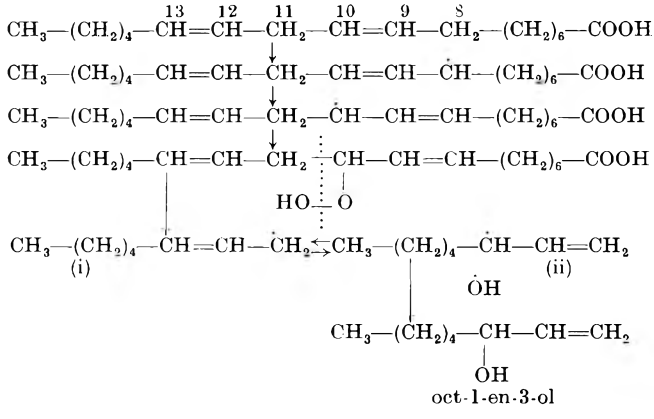
By analogy, the formation of oct-1-en-3-ol may be produced from arachidonic acid according to the following scheme:



Hydrogen abstraction at the methylene group on carbon atom 10 followed by allylic rearrangement would lead to the formation of a hydroperoxide on carbon atom 12. The decomposition of the hydroperoxide and chain scission between carbon atoms 12 and 13 would produce the alkyl radical (i). Allylic rearrangement to alkyl radical (ii) and reaction with an hydroxyl radical may produce oct-1-en-3-ol.

Hoffmann (1962) isolated oct-1-en-3-ol from linoleic acid ester and postulated the formation of a hemi-acetal from the C_{11} free radical 'via an unstable intermediate'. He suggested that the hemiacetal would undergo 'an intramolecular cyclic rearrangement followed by cleavage to the secondary unsaturated alcohol (by allylic rearrangement) oct-1-en-3-ol'.

However, the formation of oct-1-en-3-ol from autoxidized linoleic acid may be more readily explained by a mechanism similar to that proposed by Schepartz & Daubert (1950), and later by Hoffmann (1962), for the formation of hept-2-enal from the carbon atom 14 hydroperoxide. This involves hydrogen abstraction at a methylene group in an α-position to the pentadiene system:



We suggest that abstraction of hydrogen at the C_8-methylene group in linoleate followed by allylic rearrangement would lead to formation of a hydroperoxide on carbon atom 10 which, on decomposition of the hydroperoxide and chain scission between carbon atoms 10 and 11 would produce the alkyl radical (i). Allylic rearrangement to alkyl radical (ii) and reaction with an hydroxyl radical would produce oct-1-en-3-ol.

Other mechanisms, such as reduction of an octene hydroperoxide, could also give rise to octenol. We have evidence, however, that other alcohols are formed under the same conditions and their formation is most readily explained by the above mechanism.

The flavour threshold values of oct-1-en-3-ol and oct-1-en-3-one are comparable in water and skim-milk, but in butterfat the threshold for the alcohol is higher. The alcohol does not have the same objectionable flavour and odour characteristics as the ketone. It is possible that the alcohol plays an important part in the natural flavour of some other foods, including cooked meats, the fat of which contains much linoleic acid. Cooked pork, in particular, appears to have a mushroom component in its aroma. (Hornstein & Crowe (1960) concluded that the flavour difference between pork and beef may have its origin in the fat portion of these meats, and pork fat

contains 10 times as much linoleic acid as beef fat.) We have also observed a gas chromatographic fraction with a mushroom flavour from Cheddar cheese.

Demonstration of the significant contribution of an alcohol to oxidized flavour defects in dairy products directs attention to the role of non-carbonyl compounds in such flavours.

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Factors affecting the multiplication and survival of coagulase positive staphylococci in Cheddar cheese

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SUMMARY. The presence of a thermolabile inhibitor of coagulase-positive staphylococci in milk was confirmed. Starter streptococci significantly suppressed the multiplication of staphylococci in milk not only by their acid production but also by some other competitive effect.

Cheesemaking trials showed that staphylococci multiplied considerably more rapidly in 'slow' or 'sweet' cheese, where the starter was inhibited by phage, than in normal cheese. Little decrease in numbers occurred in 'sweet' cheese even after 18 months, in contrast to the rapid decline in the normal cheese.

Staphylococci subjected in the laboratory to sublethal heat treatments had a prolonged lag phase on all media and their % recovery on selective media was significantly lower than on optimal non-selective media. It is suggested that the low survival rate of the staphylococci in cheese made from milks heated at sublethal temperatures is due to the lag in recovery of heat-shocked cells and their inability to multiply in the unfavourable cheese curd.

INTRODUCTION

Cases of enterotoxin food poisoning caused by the consumption of cheese heavily infected with staphylococci have been reported previously (Report, 1959; Hendricks, Belknap & Hausler, 1959; Munch-Petersen, 1960). It is important, therefore, to establish the frequency and the extent of staphylococcal contamination of milks and cheese, the condition under which those organisms are most likely to proliferate and survive and the minimum heat treatment which would eliminate them from the cheesemilk.

Thatcher, Comtois, Ross & Erdman (1959) demonstrated the presence of enterotoxin in some Canadian cheeses by animal tests. However, until the immunodiffusion tests on the enterotoxins are sufficiently developed to be used as routine tests for milk and cheese (Read, Pritchard & Donnelly, 1963; Read, Pritchard & Black, 1964) it is considered by the Public Health Authorities that a food containing about 500000/g coagulase-positive staphylococci should be regarded as unfit for human consumption (Allison, 1949), whether or not it may contain enterotoxin.

Experimental work on the multiplication of staphylococci during cheesemaking (Mattick, Neave & Chapman 1959; Takahashi & Johns, 1959; Thatcher & Ross, 1960; Jezeski, Morris, Zottola, George & Busta, 1961; Roughley & McLeod, 1961)

has been previously described. Cheese made from 'low grade' milk, with a high total bacterial count, permitted less growth of the staphylococci than did high grade milk, probably because of the action of inhibitory organisms (Takahashi & Johns, 1959). Staphylococci died out rapidly at about pH 5·0 in cheese (Mattick *et al.* 1959) and in starter (Reiter, Vazquez & Newland, 1961). Mattick *et al.* (1959) also showed that a difference of 0·2 pH units in the range of pH 5·1–5·3 significantly affected the rate of reduction in numbers, the least rapid decrease being in the cheese of higher pH. At much higher pH's, e.g. pH 6·6 staphylococci multiplied and survived in much larger numbers and for longer periods, little decrease in numbers of viable organisms being found after 6 months (Sharpe, Neave & Reiter, 1962). The failure of staphylococci to die out in cheese of an unusually high pH (5·5–5·8) was also reported by McLeod, Roughley & Richards (1962). Although the pH of the cheese is therefore of great importance for the survival of the staphylococci, the enterotoxins if produced are not inactivated at low pH and appear to survive for many months (Miljkovic, 1960).

No doubt adequate heat treatment such as 155 °F for 15 sec (Jensen & Pedersen, 1960) of all cheese milk would eliminate the staphylococci from the cheese milk and cheese, but not all cheesemakers would accept such severe heating because of its effect on the flavour, body and texture of the cheese.

A survey of the incidence of staphylococci in commercial milk and cheese undertaken in conjunction with the National Agricultural Advisory Service (unpublished) showed that a high proportion of cheese made from raw milk contained, as expected, large numbers (more than 10^5/g) of coagulase-positive staphylococci. It was surprising, however, that all but the most perfunctory heat treatment (as low as 145 °F 'flash' to 15 sec holding time) of the cheesemilk resulted in cheese free or practically free from staphylococci when sampled as early as 1–2 weeks after making. As the heat tolerance of staphylococci is reported to be much higher (Jensen & Pedersen, 1960), it was decided to re-investigate the heat sensitivity of some strains of coagulase-positive staphylococci in the laboratory and to confirm the results with cheesemaking experiments.

The work reported here describes the effect of starter streptococci on the multiplication of staphylococci both in laboratory experiments and in cheese made on a 40 gal scale. In some cheesemaking experiments the starter was suppressed by its homologous phage during the cheesemaking, so that the multiplication of the staphylococci could be compared in cheeses of very different pH. The heat sensitivity of the staphylococci and the behaviour of heat-shocked cells both in laboratory tests and in cheesemaking trials were also investigated.

MATERIALS AND METHODS

Staphylococci

Staph. aureus 30 (NCDO 1501), phage type 42D, isolated from mastitis; *Staph. aureus* N1, isolated from a Cheddar cheese responsible for food poisoning.

Starter streptococci. *Str. lactis* ML_3 (NCDO 763) and *Str. cremoris* C_3 (NCDO 506).

Phages. Specific for streptococci ML_3 and C_3.

Selective media. The egg yolk glycine pyruvate tellurite medium (BP) of Baird-Parker (1962), which had previously been found to be selective and diagnostic for

Staph. aureus (Sharpe *et al.* 1962), was used in preference to the phenolphthalein phosphate medium (BK) of Barber & Kuper (1951), as many phosphatase positive strains detected on the latter medium were coagulase negative, confirming the observations of Thatcher & Simon (1956) and Sharpe *et al.* (1962). On the BP medium coagulase positive staphylococci could be recognized with some experience, but representative colonies from both milk and cheese samples were always confirmed by the coagulase test (method 2 of Williams & Harper, 1946). The BP medium showed the highest correlation (85·2 %) between colonies diagnosed by appearance and those which were confirmed to be coagulase positive and also gave the highest viable counts. Enumeration of staphylococci for experiments with pure cultures, when diagnostic media were not required, was made on a modified Chapman's medium (1946) in which the salt content was reduced to 6·5 % to avoid undue inhibition of the staphylococci.

Non-selective medium. Nutrient agar (NA) and nutrient agar containing 5 % horse blood (BA).

Cheese

Experimental cheeses were made at the Institute's dairy and at a small creamery, using cheesemilk heated at different temperatures in an APV pasteurizer with holding tube (17 sec).

Naturally infected milks

Samples of milk for laboratory experiments on heat tolerance were drawn from 4 cows known to be infected with different strains of *Staph. aureus*. Preliminary work showed that in each case only low numbers of staphylococci were present in the milks; samples were therefore used both directly after milking and also after 18 h incubation at 30 °C. For cheesemaking trials milk from a single herd was used.

Heat-resistance tests

The heat resistance of strains of staphylococci in milk using either inoculated laboratory strains or naturally infected milks were determined by the method of Stern & Procter (1954) as modified by Franklin, Williams & Clegg (1958), the numbers of survivors being estimated by colony counts using the Miles & Misra (1938) method. The media used to recover the survivors were blood agar, the selective BP medium, and salt nutrient agar. When only small numbers of staphylococci were present a dilution count of the number of survivors was made.

RESULTS

Multiplication of staphylococci in milk in the presence or absence of starter

Effect of heat treatment of milk on the subsequent multiplication of staphylococci

As staphylococci are known to be at least partially inhibited in raw milk (Smith, 1957), preliminary experiments to investigate their growth were made in raw milk, in milk heated to 161 °F for 17 sec and in steamed milks (30 min at 212 °F on 3 consecutive days), in the presence and absence of starter. Staphylococcus strain 30

was inoculated into the various milks either alone or with 1 % starter strain ML_3 and the staphylococci were enumerated immediately after inoculation and after 5 h incubation at 30 °C.

Table 1. *Growth of* Staph. aureus (30) *in raw and heat-treated milks in the presence or absence of* Str. lactis (ML_3)

	Numbers of staphylococci × 10^{-3}/ml milk					
Time of incubation,	Raw milk		Milk heat-treated at 161 °F/17 sec		Steamed milk	
h	0	+	0	+	0	+
0	12	18	19	14	20	21
5	24	15	30	18	1400	74

0 = Absence of starter. + = Presence of starter.

Table 1 shows the result of one such experiment, where strain 30 increased 70 times in the steamed milk but only twice or less in raw or pasteurized milk. This confirms the presence of a heat labile inhibitor which was not affected by the pasteurization temperature. In the presence of starter, however, the staphylococci were inhibited even in steamed milk, increasing only about $3\frac{1}{2}$ times. It was possible that the inhibition of growth in steamed milk in the presence of starter was due solely to the acid formed. The multiplication of the staphylococci was therefore observed with and without neutralization of the lactic acid formed.

Effect of pH

Four flasks (A–D) containing 250 ml of steamed milk were inoculated with strain 30 and two of the flasks (A and D) were also inoculated with the starter strain ML_3. The pH of the milk in flask B (staphylococci alone) was adjusted to that in flask A by the addition of 10 % lactic acid. The pH in flask D was adjusted to that of the milk in flask C (staphylococci alone) with 2N-NaOH. The volumes of milk were large enough to permit withdrawals to check the pH without changing appreciably the level of milk in the flasks. pH readings were taken every 30 min between 1 and 10 h, and again at 24 h.

Table 2. *Growth of* Staph. aureus *in steamed milk at* 30 °C *in the presence or absence of streptococci, and with and without the pH adjusted*

	A		B		C		D	
Time of enumeration,	Staph. + Str. pH unadjusted		Staph. alone, pH adjusted to that of A		Staph. alone, pH unadjusted		Staph. + Str. pH adjusted to that of C	
h	Nos.*	pH	Nos.*	pH	Nos.*	pH	Nos.*	pH
0	33	6·4	45	6·5	35	6·5	25	6·5
6	370	4·7	1200	4·7	3700	6·4	390	6·4
$7\frac{1}{2}$	170	4·5	4400	4·5	6700	6·3	400	6·4
10	140	4·5	1660	4·5	5600	6·3	390	6·4
24†	2·5	4·3	1400	4·1	23500	6·1	17·0	4·9

* Nos. = no. of viable staphylococci × 10^{-3}/ml milk.
† No further adjustment of pH made after 10 h.

It can be seen from Table 2 that the lactic acid produced by the streptococci did not affect the multiplication of the staphylococci (flasks A and D) up to 6 h. After 6 h the staphylococci began to die out as the pH decreased (flask A), but the numbers remained stationary as long as the pH was kept at 6·4 (flask D, 10 h). The staphylococci growing alone (flasks B and C) showed a 10 times higher rate of multiplication than in the mixed culture when the pH was maintained at 6·4 (flask D), and about 3 times higher without the adjustment of the pH. The staphylococci alone grew almost as well down to pH 4·5 at $7\frac{1}{2}$ h as at the higher pH (flasks B and C) but thereafter began to die out whereas at the higher pH growth continued. Similar observations were made in a second experiment. It is evident therefore that the streptococci compete with and retard the multiplication of the staphylococci (Jones, King, Fennell & Stone, 1957) and that this cannot be due entirely to the lactic acid formed (flasks B and C). However, attempts to demonstrate inhibitory substances produced by the starter failed both in liquid media and on agar.

Experimental cheeses

Paired cheeses of significantly different pH

Cheeses were made in pairs, each pair consisting of one in which the starter was working normally and another in which it was considerably suppressed by the addition of its homologous phage. Three pairs of cheeses were made, each pair from the same heat-treated milk using either starter streptococcus ML_3 and staphylococcus strain 30 (2 experiments) or starter streptococcus C_3 and staphylococcus strain N_1 (1 experiment).

Starter streptococci. The increase in the streptococcal count which also includes mechanical concentration of organisms in the curd from initially inoculated milk to milling was 100–1500-fold. In 2 of the 3 cheeses to which phage was added to inhibit the starter, the count either remained static, the original number in the milk being about 10^6 cells/ml, or dropped below the original level; the count in the phage-treated curd at milling was 10^3–10^4 times lower than in the control cheeses. In the third cheese to which phage was added the cheese was delayed for 4 h; in this time some acid was produced, which was shown to be due to phage-resistant mutants of the starter streptococcus. In this experiment there was only 100-fold difference at milling between the streptococcus count in the control and the phaged cheese. At 2 weeks the numbers present in all the phage-treated cheeses were only 10–20 times lower than in the controls, due to the continued growth of the phage-resistant mutants, which appreciably lowered the pH although not to that in the control cheeses. After 4 weeks ripening the numbers in all cheeses had dropped to below 10^6 cells/g.

Staphylococci. The multiplication and survival of staphylococci in all 3 pairs of cheeses followed very similar patterns, a typical result being shown in Fig. 1. In this pair of cheeses, inoculated with *Staph. aureus* N 1 and *Str. cremoris* C_3, the addition of the homologous phage and the consequent suppression of streptococcal growth resulted at milling in a much higher pH than in the control cheese (pH 6·6 and 4·95, respectively). Although this high pH gradually decreased during maturation it remained considerably higher than in the control cheese. The numbers of staphylococci increased rapidly in the phage-treated cheese during making, increased slightly still further during the first 2-weeks maturation, and then declined very slowly,

10^7/g viable organisms still being present after 72 weeks. In the control cheese the staphylococci multiplied at approximately $\frac{1}{5}$th of the rate in the phage-treated cheese and immediately began to decrease rapidly in numbers so that even after only 24 weeks less than $1{\cdot}5 \times 10^3$/g were present.

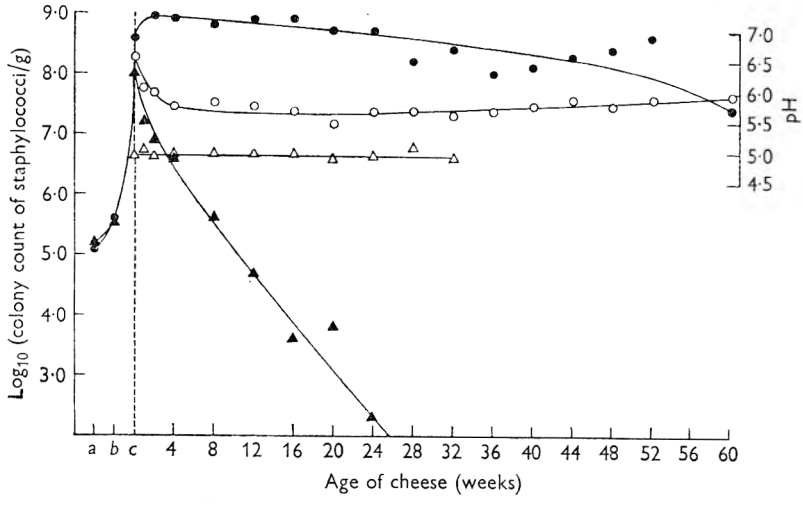

Fig. 1. The effect of starter failure due to bacteriophage on the multiplication of staphylococci during the making and maturation of Cheddar cheese. ●, Counts in slow cheese; ▲, counts in control cheese; ○, pH of slow cheese; △, pH of control cheese; *a*, inoculated milk; *b*, curd at cutting; *c*, curd at milling.

Very similar results were obtained with the other two pairs of cheeses, except that in the third pair, where milling was delayed until some acidity had developed in the phage-treated cheese, the staphylococci decreased more rapidly than in the other phage-treated cheeses, so that at 24 weeks only 1×10^4/g staphylococci were still present, and the control contained 1×10^2/g.

Cheese made from naturally infected raw milk

In order to relate the rate of multiplication during cheesemaking of staphylococci naturally occurring in milk to that of artificially introduced staphylococci 2 cheeses (SR 8 and SR 9) were made by the normal process from raw milk naturally infected with staphylococci (Fig. 2).

In both these cheeses the rate of multiplication of staphylococci during cheesemaking was similar to that in heat-treated milk inoculated with laboratory strains. However, the numbers of staphylococci declined much more slowly during maturation of the raw milk cheese than in cheese made from inoculated heat-treated milk. The viable numbers of organisms remained for a considerable period (2–12 weeks) almost stationary before beginning to decline more rapidly. This was observed in 2 experiments.

As controls for SR 8 and SR 9, cheeses were made from the same milk which had been heated to 155 °F for 17 sec; in these the numbers of staphylococci were reduced to less than 10/ml in the milk and the curd at milling contained less than 150/g.

Another experiment with raw-milk cheese using milk from another herd gave a 1×10^4 increase in numbers of staphylococci from the number/ml in the vat milk to the number/g curd at milling (pH 5·1). This was more than 10 times higher than the increase in other cheeses of normal pH, whether inoculated with a laboratory culture or naturally infected and even greater than in the phaged cheese. A large proportion of this milk was obtained from cows suffering or recently recovered from clinical mastitis. Jones *et al.* (1957) suggested that milk from a diseased udder may have a decreased natural inhibitory effect and Walker, Harmon & Stine (1961) found a greater increase of staphylococci during cheesemaking with mastitic milk than with normal milk. Our results here indicate that the natural inhibitory substances in normal milk may be important in suppressing the growth of staphylococci in raw milk used for cheesemaking; they were not investigated further.

Fig. 2. The multiplication of staphylococci during the making and maturation of Cheddar cheese, using naturally infected raw milk. ▲, Counts in cheese SR 9; △, counts in cheese SR 8; ●, pH in cheese SR 9; ○, pH in cheese SR 8; *a*, inoculated milk; *b*, curd at cutting; *c*, curd at milling.

Heat resistance of staphylococci in laboratory experiments and in cheesemaking trials

Laboratory experiments

Steamed milk was seeded with a suspension of *Staph. aureus* 30, heat treated at 3 different temperatures and plated on blood agar (BA) and on BP agar. As seen from Table 3 heat treatment for 17 sec at 155 °F resulted in no survivors, and killed about 99, 80 and 50 % at 150, 145 and 140 °F, respectively. It was further observed that visible colonies appeared on both media after a delay of 24 h, compared with unheated controls. This indicated a delayed multiplication of the heat-shocked cells. The different number of organisms recovered on the 2 media at 145 and 150 °F may be explained by the presence of inhibitory substances in the BP agar which suppress a higher number of heat-shocked cells than the non-inhibitory optimal BA medium; the presence of salt in the recovery medium caused even more marked inhibition of the sensitized cells. This is shown in Table 4 where blood agar is compared with

nutrient agar and with nutrient agar plus 6·5 % salt. The 3 strains of staphylococci used here differed somewhat in their heat sensitivity. Strain 8319, a human strain, was the most heat-sensitive but 3 other human strains were found to be as heat-resistant as the bovine strains.

Table 3. *Numbers of* Staph. aureus *surviving different heat treatments in milk, as determined on non-selective blood agar and selective Baird-Parker media*

	Numbers of staphylococci × 10^{-5}/ml milk				
		Milk treated for 17 sec at			
Medium	Control raw milk	140 °F (60 °C)	145 °F (62·7 °C)	150 °F (65·5 °C)	155 °F (68·5 °C)
BA	8500	4900	1800	33	< 0·0001
BP	7000	3000	850	0·25	< 0·0001
BP	0·024*	0·013*	< 0·0002*	< 0·0002*	< 0·0002*

BA = blood agar medium; BP = Baird-Parker medium.
* Numbers determined by dilution counts.

Table 4. *Recovery on different media of* Staph. aureus *before and after heat treatment for* 17 *sec at* 150 °*F*

	Numbers of staphylococci × 10^{-5}/ml milk					
	Controls, raw milk			Heated milk		
Strain of staphylococcus	BA	NA	SA	BA	NA	SA
30	3500	350	2500	1500	67	0·017
N 1	6700	5000	800	1700	5·9	5·9
8319	2700	2400	850	0·05	0·05	< 0·01

BA = blood agar medium. NA = nutrient agar medium. SA = salt agar medium.

The heat sensitivities of laboratory strains were compared with those of naturally occurring strains by heating milk samples withdrawn aseptically from mastitic udders. Milks from 2 cows infected with a different strain of staphylococcus were heated immediately and also after enrichment for 18 h at 30 °C (Table 5). The recovery on the BA and BP agar were again markedly different, but as before, heat treatment at temperatures within the range of 145 and 150 °F reduced the numbers of staphylococci appreciably. Similar results were obtained with 2 other milks.

Cheese trials

To investigate whether a lower heat treatment resulted in lower numbers of staphylococci occurring in cheese than was expected from heat tolerance tests in milk, raw milk known to be naturally infected with staphylococci, and portions of the same milk held at different temperatures were made into cheese.

Table 6 shows that in this instance even the lowest temperature used (145 °F for 17 sec) was quite adequate in reducing the numbers of staphylococci to a level below accurate determination, whereas in laboratory experiments (Tables 3 and 5) a temperature of 155 °F for 17 sec was necessary for this. However, in the above experiment it was necessary to enumerate the staphylococci on a selective medium to

Table 5. *Numbers of* Staph. aureus *naturally occurring in raw milks surviving different heat treatments as determined on selective and non-selective media*

Cow no.	Medium	Numbers of staphylococci × 10^{-5}/ml milk: Control, raw milk	Milk heat-treated for 17 sec at 140 °F	145 °F	150 °F	155 °F
763	BP	0·17*	0·00003	< 0·0002	< 0·0002	< 0·0002
	BA	1800†	120	1·2	0·01	0·01
	BP	1300	55	< 0·1	< 0·1	< 0·1
543	BP	—	—	—	—	—
	BA	1000†	200	1·4	0·035	< 0·1
	BP	450	8·5	0·015	< 0·1	< 0·1

BP = Baird-Parker medium. BA = blood agar medium.
* Milk sample heat-treated immediately.
† Milk sample heat-treated after 18 h incubation at 30 °C.

Table 6. *Proliferation of naturally occurring staphylococci in cheeses made from raw and heat-treated milks using Baird-Parker agar for enumeration*

Samples examined	Numbers of staphylococci × 10^{-3}/ml milk or /g cheese: Control, raw milk	Milk heat-treated for 17 sec at 155 °F	150 °F	145 °F
Milk in vat	93	< 0·1	< 0·1	< 0·1
Curd at milling	320 (5·25)	< 0·1 (5·2)	< 0·1 (5·2)	< 0·1 (5·25)
Cheese at:				
1 week	110 (4·9)	< 0·1 (4·85)	< 0·1 (4·85)	< 0·1 (4·9)
4 weeks	81 (5·0)	< 0·1 (4·95)	< 0·1 (4·95)	< 0·1 (5·0)

pH of curd or cheese in parentheses.

Table 7. *Proliferation of* Staph. aureus 30 *in pasteurized milk, (with and without reheating to* 145 °*F*/17 *sec) estimated by counts on selective and non-selective media*

Samples examined	Numbers of staphylococci × 10^{-3}/ml milk or /g cheese: Milk reheated 145 °F/17 sec BP	BA	No further heat treatment BP	BA
Milk in vat	< 0·1	< 0·1	90	150
Curd at milling	1·3	9	28000	35000
Cheese at				
1 week	0·5 (4·9)	1·5	10000 (5·0)	12000
4 weeks	0·95 (5·0)	0·95	10000 (5·0)	10000

BP = Baird-Parker medium; BA = blood agar medium; pH of cheese in parentheses.

avoid overgrowth with the rest of the bacterial flora which occurred on blood agar; this may therefore have prevented some of the heat-shocked cells from multiplying. To avoid this, single-herd milk of good quality was first heated to 161 °F for 17 sec to render it virtually sterile and then inoculated with strain 30. Half of it was made into cheese and the other half was reheated and held at 145 °F for 17 sec before making into cheese. While the recovery of survivors on the BP medium was somewhat lower (Table 7) than on the blood agar, the results were substantially the same,

heat treatment at 145 °F being sufficient to reduce the number of staphylococci from about 10^7 to 10^3/g when the cheeses were sampled at 1 week.

Cross-contamination in a highly infected small creamery

Cheese trials were undertaken to observe to what extent cross-contamination might influence the number of staphylococci present in cheese.

A creamery was selected which had a consistent record of very high staphylococcal counts in milk and cheese, and poor hygiene both in the dairy and in the surrounding environment. Single-herd milk was transported from the creamery premises to the Institute and portions of it were heat-treated at 3 different temperatures. Cheeses were made from raw and heat-treated milk both at the Institute and also at the creamery. For this latter purpose heat-treated milk was returned to the creamery and cheese made in a small 100 gal tank alongside a 750 gal raw milk cheese vat without precautions against cross-contamination. All practical precautions against cross-infection were taken at the experimental dairy of the Institute, each cheese being made by one person using separate sterile equipment.

Table 8. *Comparison of cheeses made at the N.I.R.D. and at a commercial creamery, from naturally infected milks with identical heat treatment*

	Numbers of staphylococci × 10^{-3}/ml milk or /g cheese*		
Heat treatment of milk (17 sec)	Expt. 1 155 °F	Expt. 2 150 °F	Expt. 3 145 °F
Samples examined			
Cheesemaking at N.I.R.D.			
Raw single-herd milk (A)	18	7·9	3·2
Curd at milling	64000 (5·05)	15000 (5·05)	22000 (5·1)
Cheese, 1 month	15000 (5·3)	11000 (5·15)	8700 (5·15)
Milk (A) heat-treated	< 0·01	< 0·01	< 0·01
Curd at milling	0·15 (5·05)	0·15 (5·05)	< 0·15 (5·05)
Cheese, 1 month	0·15 (4·95)	0·35 (5·1)	< 0·15 (4·95)
Cheesemaking at creamery			
Curd at milling from raw bulk milk	4400 (5·3)	15000 (5·3)	13000 (5·3)
Cheese, 1 month	2300 (5·4)	850 (5·15)	4200 (5·3)
Milk (A) heat-treated	< 0·01	< 0·01	< 0·01
Curd at milling	7·9 (5·1)	20 (5·1)	15 (5·15)
Cheese, 1 month	3·2 (4·95)	15 (5·1)	16 (4·9)

* Counts made on Baird-Parker medium.
Figures in parentheses refer to pH of curd or cheese.

The cheese made at the Institute from milk heat-treated at the lowest temperature (145 °F) contained less than 150 staph./g curd at milling while the same milk at the creamery resulted in cheese containing 16×10^3 staph./g the difference representing the measure of cross-contamination from the raw milk vat and any contamination derived from the equipment, personnel and air (Table 8). Although it was necessary to make the raw milk cheese on the farm from a different batch of milk, the results have been included as a comparison with the cheese made at the same time with the heat-treated milk.

The low heat treatment virtually eliminated the staphylococci from the cheese curd and thus confirmed the previous results.

DISCUSSION

From the work presented it appears that under normal cheesemaking conditions the staphylococci occurring in milk contend with the natural inhibitory substances found in raw and pasteurized milk, and with the acid production and other competitive effects of the large and rapidly multiplying starter population. In spite of these adverse circumstances and allowing for an approximately tenfold mechanical concentration of organisms in the curd the staphylococci multiplied about 20–50 times during the cheesemaking process. Similar increases in numbers were also found by Mattick *et al.* (1959), Takahashi & Johns (1959), McLeod *et al.* (1962) using milk inoculated with pure strains of staphylococci. Unlike Takahashi & Johns (1959), however, we detected no difference between the rate of multiplication of strains inoculated into milk and those naturally occurring in milk; only in one instance when a cheese was made from heavily infected mastitic milk did a more rapid multiplication occur of the naturally occurring organisms.

Preliminary findings (Sharpe *et al.* 1962) that inhibition of the starter by addition of homologous phage resulted in about fivefold increase in multiplication of staphylococci during cheesemaking were confirmed. The further multiplication that occurred in this 'sweet' cheese during the first few weeks of maturation, followed by a stationary phase and very slow decline in numbers over a considerable period, was in sharp contrast to the situation in cheese of normal acidity, where the number of these organisms declined rapidly during maturation and did not survive for more than a few months. Sweet cheese may therefore not only contain dangerous levels of enterotoxin but could also provide a considerable reservoir of contamination in the home. It is not known whether the high numbers of staphylococci in such cheeses even after 18 months are due to survival of the original organisms or to a stationary phase in which growing organisms might produce more enterotoxin. Clearly all sweet cheeses must be regarded as suspect unless proven to be free of staphylococci.

A preliminary survey on the contamination of single herd (35 samples) and market milk (15 samples) with coagulase positive staphylococci over a period of 12 months has shown that these milks contained regularly between 10^3 and 10^4 organisms/ml. These findings have since been confirmed by a wider survey of the N.A.A.S. (unpublished). If such milks had been made into cheese without any heat treatment, the cheeses would have contained between 10^5 and 10^6 staphylococci/g at the beginning of maturation if the multiplication rate had been similar to that observed in our experiments. In the case of a starter failure, due to bacteriophage, antibiotics or any other cause, five times these numbers would have been expected.

It was found, furthermore, that cheeses made from milks heat-treated far below the time and temperature combinations required in laboratory tests, were free from staphylococci when tested between 1 and 2 weeks after making. These observations confirm the findings of Takahashi & Johns (1959) who had reported that the use of cheesemilk 'flash' heated at 150 °F (65·5 °C) resulted in cheese free from staphylococci.

The findings that staphylococci exposed to sublethal temperatures exhibit a prolonged lag phase on all the agar media used and that the recovery of survivors is lower on selective media has also been observed by other workers in different contexts. Jackson & Woodbine (1963) observed a prolonged lag phase of heat shocked staphylo-

cocci in liquid media and several workers observed an inhibition of heat-treated organisms on salt-containing media (Kaufmann, Harmon, Pailthorp & Pflug, 1959; Busta & Jezeski, 1961, 1963; Stiles & Witter, 1963).

These factors suggest an explanation of the unexpectedly low numbers of staphylococci surviving in cheese curd after a heat treatment which laboratory experiments had shown to be quite ineffective. Before the heat-shocked cells recover from the lag period induced by heat treatment of the milk the cheesemaking process is completed, and the starter streptococci have lowered the pH sufficiently to suppress the growth of the heat-shocked organisms. In addition, the inhibitory effect of salt in the cheese curd on the heat-sensitized cells will also suppress growth. This combination of inhibitory effects is sufficient to explain the low survival rate of staphylococci in cheese curd, and allows the practical cheesemaker to employ lower heat treatment of the cheesemilk if he thinks that higher temperatures affect the quality of his cheese. Such findings may also apply to other organisms than staphylococci present in heat-treated milks.

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Analysis of milk by infra-red absorption

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Summary. The infra-red absorption of milk and its main constituents has been recorded and it has been found possible to determine quantitatively the fat, protein and lactose contents from measurements of the intensities of the respective absorption peaks at 5·73, 6·46 and 9·6 μm. Solids-not-fat (SNF) content can be obtained from a single intensity measurement at 7·9 μm where the fat shows no attenuation and where the protein and lactose extinction coefficients are approximately equal. Photometric errors due to natural variations in fat globule size distribution can be eliminated by prior homogenization. Based on these observations, an automatic infra-red milk analyser (IRMA) has been constructed, which can determine the fat, protein, lactose and SNF in a pre-warmed milk sample in under 1 min. Preliminary tests carried out on 50 milk samples from individual cows, representing 3 different breeds, gave standard deviations from the chemical analyses for the percentages of fat, protein, lactose and SNF of 0·10, 0·07, 0·07 and 0·19, respectively. The standard deviations for the percentages of fat and SNF on 60 farm bulk milk samples were found to be 0·08 and 0·12.

This paper presents the basic principles and preliminary results of a rapid method for the quantitative analysis of milk by means of infra-red absorption. The method has already been reported briefly (Goulden, 1961*a*) and is covered by a patent vested in the National Research Development Corporation (Goulden, 1961*b*). A commercial instrument employing this principle is now being developed and its constructional features have been described by Goulden, Shields & Haswell (1964).

THEORETICAL

Infra-red absorption spectroscopy is commonly used for the quantitative analysis of multicomponent systems, but the high water content and radiation scattering properties of milk make it a particularly difficult system for analysis by this method. As can be seen from the spectra shown in Fig. 1, even thin films of water absorb strongly throughout the 2–10 μm wavelength region. The water absorption bands are so intense that it is almost impossible to discern bands due to other milk constituents. When a double-beam spectrometer is used to cancel out automatically the water absorption, the characteristic bands of the other components become clearly visible as in the spectra shown in Fig. 2. These bands arise from particular components of milk and when recorded as optical densities (absorbancies), the band intensities are proportional to the concentration of each of the major constituents.

In addition to attenuation by absorption, a beam of radiation passing through milk is attenuated by scattering from both the fat globules and the protein micelles. The scattering contribution decreases both as the particle size/wavelength ratio decreases, and as the refractive index of the suspended particles approaches that of the suspending medium. In the visible region of the spectrum where the wavelengths are less than the diameters of most of the fat globules, scattering is intense and is responsible for the opacity of milk. Except in the near infra-red region, the wavelengths are larger than the diameters of the fat globules; so that in regions of no absorption, milk appears almost transparent. The reduction in fat globule diameters obtained by homogenization renders milk even more transparent, as can be seen from the spectra shown in Fig. 2. Increased scattering in the unhomogenized milk spectrum (Fig. 2*a*) is responsible for the pronounced rise in base-line at shorter wavelengths, where the fat globule sizes are of the same order as the wavelengths.

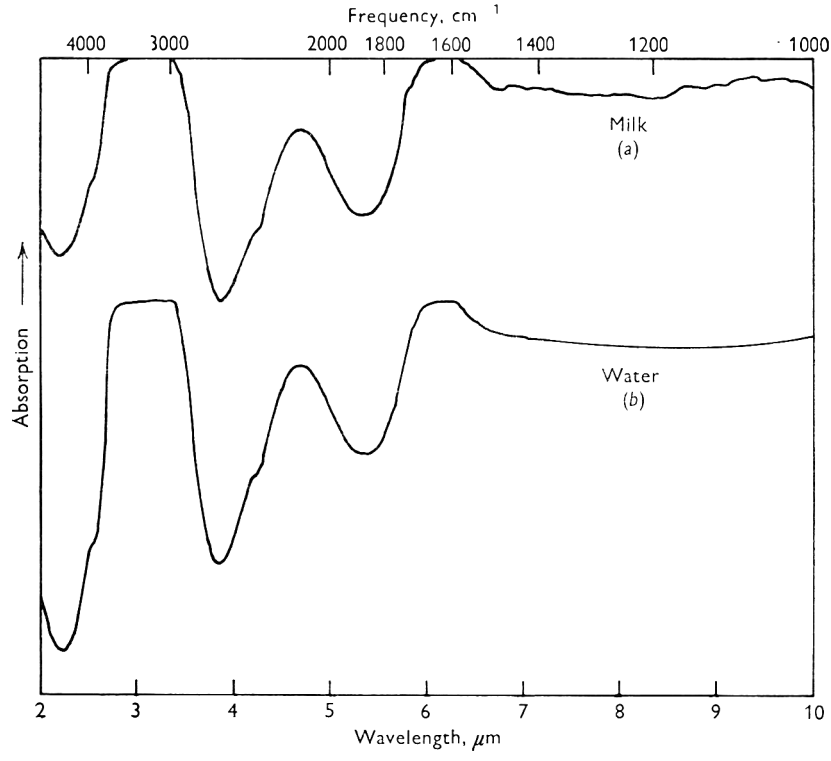

Fig. 1. Absorption spectra of (*a*) milk, and (*b*) water versus air, path length ~ 50 μm.

The determination of fat, protein and lactose contents will be considered first. This is a three-component system in which optical densities must in the general case be measured at 3 different wavelengths. For a fixed optical path length, the optical densities D_1, D_2, D_3 measured at the 3 wavelengths λ_1, λ_2, and λ_3 are given by:

$$\left.\begin{aligned} D_1 &= a_1x + b_1y + c_1z + d_1, \\ D_2 &= a_2x + b_2y + c_2z + d_2, \\ D_3 &= a_3x + b_3y + c_3z + d_3, \end{aligned}\right\} \qquad (1)$$

where a, b and c are the appropriate extinction coefficients of the 3 components present at concentrations x, y and z. For intensely absorbing solvents such as water, the a, b and c coefficients frequently have negative values due to displacement of solvent by the more weakly absorbing solute. In the special case of milk, d terms must be added to each equation to allow for the effects of polarization and displacement of water molecules by the mineral salts, even though these salts show no appreciable absorption themselves. Solution of these simultaneous equations leads to a four-term expression for the concentration of each component in the form

$$x = \alpha D_1 + \beta D_2 + \gamma D_3 + \delta. \tag{2}$$

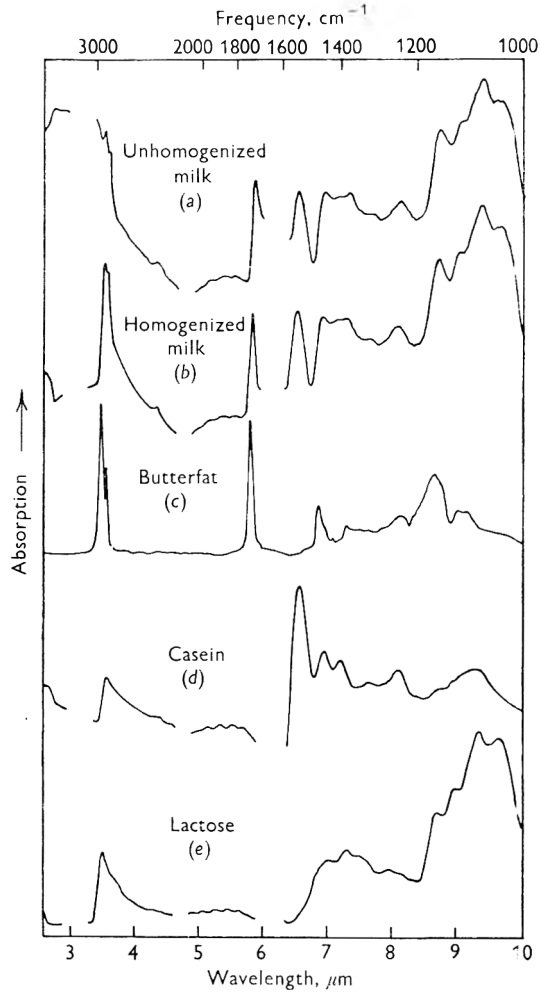

Fig. 2. Absorption spectra of milk and its components. (*a*) Unhomogenized milk; (*b*) homogenized milk; (*c*) butterfat; (*d*) sodium caseinate suspension; (*e*) lactose solution. (*a*), (*b*), (*d*) and (*e*) versus water, path length ~ 50 μm; (*c*) versus air, thin film.

If wavelengths can be selected at which β and γ are both small enough to be neglected and δ can be taken as constant, x will be a linear function of D_1. Even when either β or γ are of appreciable magnitude, in systems where the normal

concentrations of y and z do not vary over a large range, D_2 and D_3 can to a first approximation be regarded as constant and included in the intercept term δ. As shown later, such conditions apply at the wavelengths selected for analysis of the fat and lactose.

At the wavelength selected for protein determination (λ_2), where the fat makes an appreciable contribution to the total absorption, it can be deduced from equations (1) and (2) that

$$y = D_2/b_2 - (a_2/b_2)\,x + \text{a constant}. \tag{3}$$

Substitution of the appropriate coefficients from Table 2 shows that $a_2/b_2 = -\frac{9}{40} = -0{\cdot}225$. In practice, the magnitude of the protein correction term can easily be calculated from this ratio and the fat content (x) which will already have been determined.

In place of the separate protein and lactose contents, the SNF is frequently required. With sufficient accuracy, this can be taken as the sum of the protein and lactose contents, together with a small additional term to account for the mineral matter. Variations in the latter are small enough to allow this term to be taken as constant. To determine SNF from a single optical density measurement, a wavelength should ideally be selected at which the fat contribution is very small, or constant with change in fat content, and where the lactose and protein extinction coefficients are equal. The latter requirement ensures that the measured optical density is independent of variations in the protein to lactose ratio.

Although it is unusual to find a wavelength where the optical density does not increase with higher solute concentrations, such a condition can be realized in a system containing a highly absorbing solvent. It represents the condition where the increase in total attenuation due to absorption and scattering is exactly counterbalanced by the increase in transmission due to displacement of absorbing solvent. For D_1 in equation (1), such conditions are satisfied when $a_1 = 0$ and $b_1 = c_1$, so that D_1 is proportional to $(y+z)$.

APPARATUS AND METHODS

Several different spectrometers were used to obtain the data presented here. Preliminary quantitative studies and the spectra shown in Figs. 1 and 2 were recorded with a Grubb Parsons S3A double-beam infra-red spectrometer. The data reported in Tables 1 and 2 and Figs. 3 and 4 were obtained with a grating-prism-filter spectrometer specially designed for aqueous solution studies in the 5–10 μm region. This instrument was a prototype of the Grubb Parsons–N.I.R.D. Infra-Red Milk Analyser (IRMA) and was fitted with both a normal wavelength scanning mechanism and a cam mechanism for the selection of a number of pre-set wavelengths. In this instrument, the stray radiation at 6·46 μm was found to be less than 0·03 % of the total signal without water in the beam.

Fixed path length cells with calcium fluoride windows were used for most of this investigation, since the transmission of this material exceeded 95 % at wavelengths less than 8 μm. Although the transmission fell to 40 % at 9·6 μm adequate resolution was obtained with monochromator slits wide enough to provide sufficient energy for operation of the servo system.

Milk samples were homogenized at 40–50 °C using either a Rannie 22 gal/h homogenizer or an Ormerod power operated laboratory homogenizer specially modified for use in IRMA (Goulden, 1962). Where necessary, mean fat globule diameters of homogenized milks were determined by a spectroturbidimetric method (Goulden & Phipps, 1960). Samples were analysed for fat content by the Gerber method (British Standards Institution, 1955), for total solids by the gravimetric method (British Standards Institution, 1951), SNF being obtained by difference. Protein was determined by the Kjeldahl method (total N × 6·38) and lactose by a modification of the method of Hinton & Macara (1927).

A number of test samples was made up to check the linearity of instrument response and to determine interaction coefficients. Milks with differing fat contents were prepared by the addition of cream or separated milk to a bulk milk sample. Protein suspensions were made in water by redispersing freeze-dried sodium caseinate prepared by the method of Scott Blair & Oosthuizen (1962). Aqueous lactose solutions were prepared from 'Analar' grade lactose monohydrate.

The ratio (R) of the protein to lactose extinction coefficient recorded as a function of wavelength in Fig. 3*c* was obtained by the use of a double-beam technique in which the lactose bands were automatically subtracted from those of separated milk to leave only the protein bands. This difference spectrum was obtained after filling the reference cell with a lactose solution of exactly the same concentration as that present in the separated milk contained in the sample cell.

RESULTS AND DISCUSSION

Choice of analytical wavelengths

Fig. 2 illustrates the spectra of milk and its 3 major constituents. For the determination of fat content, the best wavelength can be seen to be that of the triglyceride carbonyl band at 5·73 μm (1745 cm^{-1}), since the protein and lactose show no absorption at this wavelength. The intensity of the amide II band at 6·46 μm (1548 cm^{-1}) can be used as a measure of the total protein content. For lactose, 9·6 μm (1042 cm^{-1}) is a convenient wavelength where lactose has a much stronger absorption than protein.

As already indicated, the optimum conditions for SNF determination occur at wavelengths where the fat attenuation is zero and where the protein and lactose extinction coefficients are equal. The attenuation of fat in the emulsified state is not quite the same as the absorption of fat in the liquid form, but includes an additional negative contribution due to the water displacement effect. The total fat attenuation spectrum can be calculated from the slopes of the optical density versus fat concentration lines for a series of milk samples made up with differing fat contents. Fig. 3*b* illustrates part of the resulting spectrum calculated for the 6·75–8·5 μm region and can be compared with the absorption of liquid fat shown in Fig. 3*a*. Comparison of these 2 spectra shows that except near intense fat absorption bands or at longer wavelengths where the water absorption is lower, the total fat attenuation is negative. As frequently observed in systems where scattering is present, the fat absorption bands are displaced to longer wavelengths.

From Fig. 3*b*, it can be seen that the condition of zero fat attenuation occurs near 6·9 and 7·95 μm, whilst that for equal protein and lactose extinction occurs at 7·32

and 7·65 μm. Thus the 2 optimum theoretical conditions for SNF determination cannot be realized at any one wavelength. From considerations of fat attenuation and R values alone, it appears that the best wavelength for SNF determination lies within the 7·65–7·9 μm region. As can be seen from the milk spectra in Fig. 2, the absorption of milk decreases within this region and to reduce instrumental difficulties in measuring accurately such weak optical densities, the SNF was determined from the optical density at 7·9 μm. At this wavelength, the fat attenuation is almost zero and although R has a value of 1·4, satisfactory methods for the determination of SNF in milk are already available based upon measurements of density or refractive index, where the ratios of the corresponding coefficients can be calculated to be 0·67 and 1·43, respectively (Goulden, unpublished).

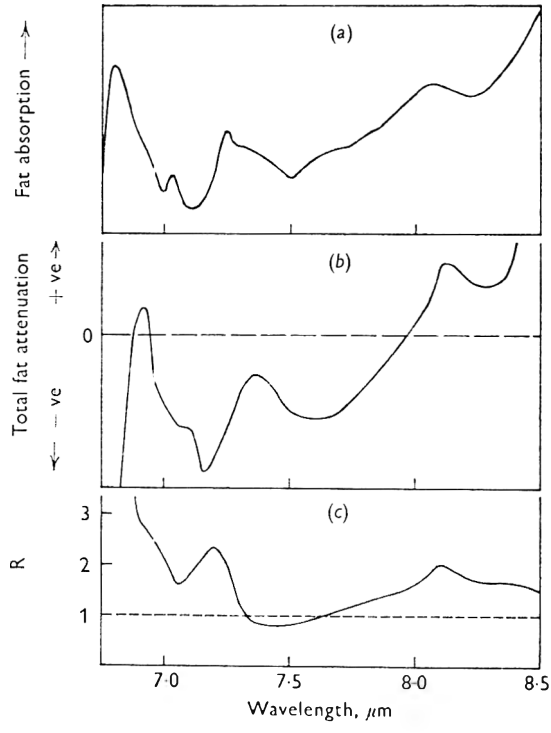

Fig. 3. (*a*) Fat absorption spectrum; (*b*) calculated total fat attenuation; (*c*) ratio (R) of milk protein to lactose extinction. Liquid butterfat was used for spectrum (*a*) and butterfat in emulsion form for spectrum (*b*).

Determination of optimum path length

The choice of optimum path length for infra-red spectroscopy of aqueous solutions is governed by 2 major opposing factors: increased path length ensures higher optical densities but reduces the energy passing through the cell with a consequent decrease in instrument response. In any particular instrument, the resolution and aperture of the monochromator, as well as the desired speed of response of the null-balance servo system, all affect the choice of cell path length. For the instruments used here, an optimum path length of about 40 μm was found and Table 1 records the transmission of water measured at the 4 analytical wavelengths.

Table 1. *Transmission of water at analytical wavelengths*

Milk component to be determined	Wavelength, μm	Transmission of water, % (path length 37·5 μm)
Fat	5·73	15
Protein	6·46	8
Lactose	9·60	11
SNF	7·90	13

Determination of interaction coefficients

Linearity of instrument response and the applicability of the Beer–Lambert Law are confirmed by the linear relationships shown in Fig. 4. The series of samples with varying fat content differs from the other two in that both major components of separated milk are present at virtually constant concentrations. Negative slopes of the fat lines in Figs. 4*b* and *c* are due to the displacement of water by the fat globules, whilst the positive slope of the fat line in Fig. 4*a* represents the sum of both absorption and scattering effects. The negative intercept of the fat line in Fig. 4*a* arises also from the water displacement effect, since at zero fat concentration, protein and lactose are still present in the separated milk. Such displacement effects are absent in the lactose and sodium caseinate solutions, the latter providing a good approximation to the absorption of either the casein complex or the soluble proteins. The micelles of the casein complex in milk have diameters of the order of 0·1 μm and therefore cause negligible scattering at infra-red wavelengths.

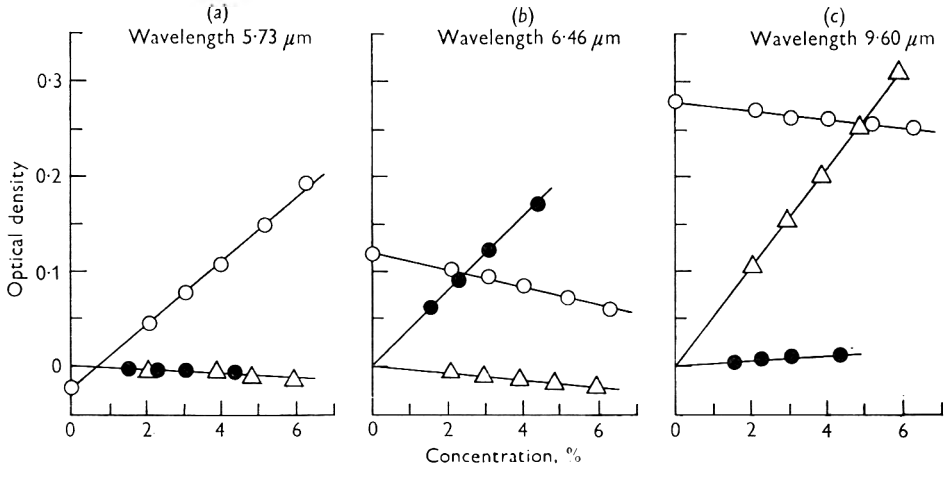

Fig. 4. Relationships at analytical wavelengths between optical densities and concentration of fat (○), protein (●) and lactose (△).

Table 2 records the slopes of all the lines in Fig. 4; these slopes being the relative values of the *a*, *b* and *c* coefficients in equation (1). Table 3 lists the calculated possible errors expected in the determination of each component due to neglect of interaction terms. The ranges of variation of each component about the mean value were

deduced from a study of the chemically determined composition of milk samples from individual cows used for testing the accuracy of IRMA.

From the information assembled in the last column of Table 3, it can be seen that apart from the effect of fat on the protein absorption, all other interaction coefficients lead to calculated error ranges of 5 % or less of the actual component value. Thus to a good approximation, both fat and lactose contents can be determined from single optical density measurements made at the appropriate wavelengths 5·73 and 9·6 μm. In the case of protein, the lactose interference effect can be neglected, leaving a two-component system for analysis at the wavelengths 5·73 and 6·46 μm. Since at 6·46 μm, the protein extinction coefficient is several times larger than that of the fat, a convenient analytical procedure is to correct each protein reading with the aid of the fat content already obtained, using the experimentally determined correlation between fat content and deviation of uncorrected protein readings from their chemically determined values. The use of this method will be illustrated later.

Table 2. *Relative extinction coefficients of fat, protein and lactose*

(Calculated from Fig. 4.)

	Wavelength, μm		
Component	5·73	6·46	9·60
Fat	+34	−9	−5
Protein	−1·6	+40	+2·6
Lactose	−2·3	−3·8	+52

Table 3. *Calculated possible IRMA errors (%) in measured fat, protein and lactose due to neglect of interaction effects*

Main component	Approximate range of variation about mean, %	Interfering component	Ratio of extinction coefficient of main component to that of interfering component	Calculated % error due to variation of each interfering component
Fat	±50	Protein	−21	±1·5
		Lactose	−15	±0·7
Protein	±30	Fat	−4·4	±12
		Lactose	−10	± 1
Lactose	±10	Fat	−10	± 5
		Protein	+20	±1·5

Effects due to changes of homogenization pressure and of fat globule size variation

When absorbing materials are present in the form of particles or globules rather than in true solution, their absorption maxima are known to be displaced to longer wavelengths. Although an increase in base-line absorption due to scattering may cause the peak optical densities of a suspension to appear greater than those of the corresponding solution, the optical density measured relative to the base-line is always less in the presence of scattering. In general, both effects decrease with decreasing particle size; so that for well-homogenized milk with a small mean fat globule diameter (sample 7, Table 4), the wavelength of the fat absorption peak at 5·725 μm was found to be exactly the same as that measured for butterfat in the liquid state.

The data of Table 4 illustrate the effect of increasing homogenization pressure and consequent reduction in mean fat globule diameter on the optical density of the fat band in milk, as measured at a wavelength of 5·732 μm and expressed as mV instrument output. Since with IRMA, optical densities are measured at fixed wavelengths, shifts in the wavelength of maximum absorption could give rise to changes in the fat signal, apart from effects due to changes in peak intensity. Thus it can be seen that although the intensity of the fat band measured at a fixed wavelength is unaffected by small changes in fat globule size of the homogenized milk, it is important to select the appropriate wavelength for the fat globule size range of interest.

Table 4. *Effects of changes of homogenization pressure on the intensity of the fat absorption band*

Sample no.	1	2	3	4	5	6	7
Homogenizer	*	A	B	B	C	A	A
Mean fat globule diam., μm	—	1·25	1·15	1·00	0·75	0·65	0·50
Wavelength of fat peak, μm	5·74	5·73	5·73	5·73	5·73	5·725	5·725
Output at 5·732 μm, mV.	26·9	19·2	19·2	18·9	18·3	17·7	17·2

* Sample 1, milk before homogenization.

Since IRMA incorporates a low-pressure homogenizer producing mean fat globule diameters in the 1·0–1·3 μm range, effects due to small changes in homogenization efficiency can be minimized by selection of a wavelength close to 5·73 μm. Samples 6 and 7 (Table 4) correspond respectively to homogenization pressures of 2000 and 3000 lb/in^2, so that for the analysis of commercially produced homogenized milk, a slightly shorter wavelength is required. Confirmation of these effects was provided by the results of a further experiment carried out at a wavelength of 5·718 μm. At this wavelength, samples 6 and 7 gave identical readings, although with samples 1–5 greater differences in intensity were observed than those reported for a wavelength of 5·73 μm. The use of a high-pressure homogenizer in IRMA, together with a wavelength of 5·725 μm, would enable both raw and commercially homogenized milks to be analysed using the same calibration relationship. Although not recorded in Table 4, no changes in the intensities measured at the protein, SNF and lactose analytical wavelengths were observed for samples 2–7.

In an earlier communication (Goulden, 1961*a*), it was reported that changes in the mean diameter of the fat globules from 1·0 to 0·5 μm lead to no detectable changes in the intensity of the fat band. This observation was based upon peak intensity measurements made with a conventional wavelength-scanning infra-red spectrometer; a measurement procedure which is less sensitive to effects due to variations in fat globule size.

The distribution of the fat globule sizes in milk is known to vary according to the breed of cow, period of lactation and other factors. To assess the magnitude of errors due to natural variation in fat globule size distributions, the optical density of the fat band was measured at a fixed wavelength for 24 milk samples from individual cows. The coefficient of variation of the error in fat content as determined by IRMA was found to be 2·7 % when the samples were homogenized and rose to 3·3 % when the unhomogenized milks were used. These samples were examined within a few hours of

collection and it is expected that after storage for several days, clumping of the fat globules in the unhomogenized milks would lead to even greater errors in the fat content as determined by IRMA.

Effects due to variation in sample temperature

Since the absorption of water is known to be temperature-dependent, the absorption of aqueous solutions will vary with temperature, particularly at wavelengths close to the 6·2 μm water absorption peak. The magnitude of errors due to sample temperature variation was calculated from changes in IRMA readings following repeated filling of the sample cell with homogenized milk at a temperature differing from the equilibrium temperature of the water in the comparison cell. By this method, the changes in transmission at all 4 analytical wavelengths were found to be of the order of 0·1 % per °C. This corresponded to a change of about 1 % of the total signal and indicated that during the absorption measurements, the sample temperature must be controlled to within $\pm$ 1 °C.

Temperature effects were very much reduced when both sample and water comparison cells were held at the same temperature; no differences in band intensities were found when the temperatures of both cells were increased from 20 to 40 °C.

Effects due to changes in pH and to the addition of preservatives

Changes in pH within the range 6–8 would not be expected to cause errors at any of the 4 analytical wavelengths, since the bands concerned arise from groups which are unaffected by such pH changes. Natural souring of milk produces carboxylic acids whose pK values are less than 5, so that even at a pH of 6, such acids will exist predominantly as the carboxylate anions whose two most intense absorption bands are close to 6·5 μm (1540 cm^{-1}) and 7·1 μm (1410 cm^{-1}). Since additional absorption near 6·5 μm increases the optical density at 6·46 μm, the first effects of natural souring will be an increase in apparent protein content, coupled with a decrease in lactose content as the latter is converted into lactic acid by bacterial action.

A fresh milk sample originally at pH 6·7 was stored at room temperature for 3 days until the pH had fallen to 6·5 and the milk had developed a pronounced bad odour. At the end of this period, the IRMA protein reading (%) had increased by 0·03, whilst the lactose reading (%) had fallen by 0·08. These changes are hardly greater than the experimental errors.

During transit to Milk Marketing Board Laboratories, milk samples are normally preserved by the addition of tablets containing 0·01 g mercuric chloride, 0·05 g potassium dichromate, 0·01 g boric acid and 0·02 g sodium chloride; 1 tablet being added to a 50 ml milk sample. The dichromate ion shows no strong absorption bands in this region of the spectrum (Miller & Wilkins, 1952). Although boric acid shows some absorption at 7·9 μm it can be calculated that at the concentration produced by addition of these tablets, boric acid contributes less than 1 % of the total optical density at this wavelength and can therefore be neglected. Addition of twice the normal preservative concentration produced no errors in the IRMA readings for any of the components, thus confirming these calculations. The IRMA readings on a milk sample preserved at room temperature with these tablets remained unchanged for at least 10 days.

Correction of protein reading for changes in fat content

As already shown, it is possible to obtain values of the protein correction in terms of the fat content. The validity of this procedure is confirmed by the results in Fig. 5, where the correlation between errors in uncorrected IRMA protein readings and fat content is demonstrated for milk samples from 50 different cows. The slope of the line in Fig. 5 is $-0{\cdot}16$, as calculated from the least squares regression of the protein error upon the fat %. This slope is of the same order as $-0{\cdot}22$ predicted from the relative extinction coefficients recorded in Table 2.

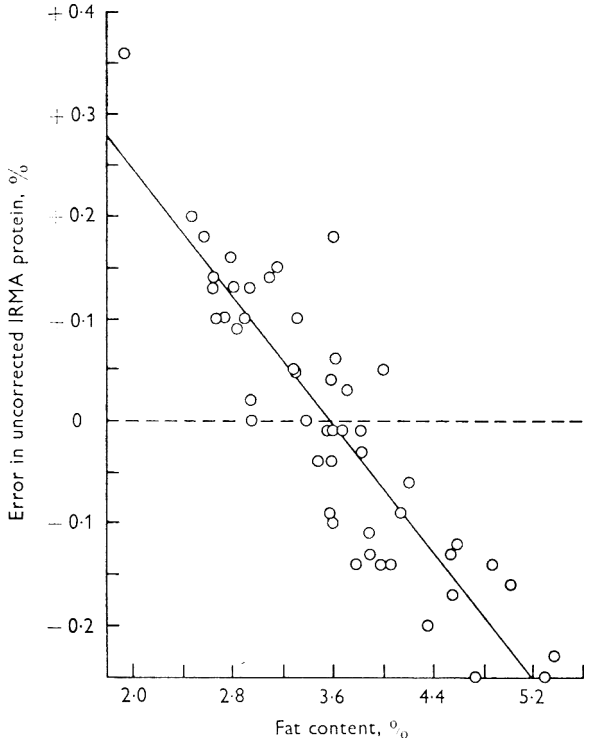

Fig. 5. Relationship between errors in uncorrected IRMA protein readings and fat content.

CALIBRATION OF IRMA

Since the concentration of each component has been shown to be proportional to the optical density (Fig. 4), IRMA has been designed to produce an output signal which is proportional to the appropriate optical density. In order to obtain a direct reading in percentage of each component, the output signal is fed into the appropriate resistor network to provide variable scale-expansion and backing-off. Within the limits of adjustment, both the slope and intercept of each calibration line can be set as required. Calibration is effected from the results of a least squares regression analysis on the IRMA and chemical analyses of a representative population of milk samples. With the aid of suitable optical filters, the backing-off and scale expansion are adjusted so that each component follows the appropriate calibration line.

A calibration method using aqueous solutions of pure chemicals is now under development to enable calibration data to be transferred from one instrument to another and thus avoid the need to calibrate with chemically analysed milk samples.

TESTS OF ACCURACY

The results of preliminary tests carried out at Shinfield are recorded in Table 5. In expt. 3, the protein/lactose ratio varied from 0·56 to 0·94 about a mean value of 0·68. Instrument A was a two-component version whilst instrument B was a four-component analyser which determined protein and lactose in addition to fat and SNF. A further set of 45 milk samples from individual cows was used to test a prototype four-component IRMA and this instrument gave results almost as good as those obtained with the improved instrument B. Since digital voltmeters were not available at the time of these tests, strip chart potentiometric recorders were used.

Table 5. *Correlations between IRMA readings and chemical analyses of milk*

Experiment	Instrument	Number of milk samples	Type of sample	Fat		Protein		SNF		Lactose	
				s	c.v.	*s*	c.v.	*s*	c.v.	*s*	c.v.
1	A	211	I.	0·095	2·36	—	—	0·21	2·44	—	—
2	B	60	F.B.	0·081	2·07	—	—	0·122	1·43	—	—
3	B	50	I.	0·102	2·85	0·068	2·13	0·186	2·16	0·071	1·58

I., individual cows, including Friesian, Ayrshire and Channel Island breeds.
F.B., farm bulk samples, as received by a commercial dairy. *s*, standard deviation.
c.v., coefficient of variation (%) = $100 \times s/\text{mean}$.

These results show that c.v.'s of better than 3 % can be obtained for all components. It is hoped that more detailed tests of accuracy will be carried out by other laboratories on larger numbers of samples.

Thanks are due to Mr D. J. Manning for assistance in the development of this method, to Mr A. Wagstaff and other members of the N.I.R.D. Chemistry Department for carrying out chemical analyses and to Mrs Joyce Conway and Mrs Barbara Harris for assistance in the operation of IRMA and calculation of results.

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The cysteine content of casein micelles

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Summary. Following enzymic digestion with pronase, masked SH groups in micellar casein became available for titration in disaggregating media with mercurial reagents. The content of cystine and cysteine was also estimated after reduction with (*a*) sulphite, and (*b*) borohydride, and also by reaction in alkaline conditions with $Cd(OH)_2$. The results show that the micelles contain mainly cysteine, and that it is likely that cystine is not present.

The question as to whether cysteine in addition to cystine is present in casein has been a subject of investigation for many years. As early as 1938 Kassell & Brand attempted to detect cysteine in casein but failed, and later Gordon, Semmett, Cable & Morris (1949), Zweig & Block (1953), Hipp, Basch & Gordon (1961), Yoshino, Wilson & Herreid (1962) and others have reported similar results for casein or casein components. Christ (1956) suggested that SH groups were involved in the syneresis of the rennet-casein gel, but like the other workers could not detect any by amperometric titration with organic mercurial reagents. In this laboratory attempts to detect cysteine in casein using mono-functional mercurials such as phenylmercury acetate and methylmercuric iodide also yielded negative results even in strongly disaggregating conditions. However Beeby (1964) has shown that freshly prepared κ-casein contains cysteine that can be estimated with phenylmercury reagent in strongly disaggregating conditions at pH 9 provided the last traces of calcium have been removed with EDTA or oxalate. This result cast doubt on all previous work and raised the question as to how much of the cystine previously reported in whole casein was in fact cysteine. However, attempts to detect cysteine in freshly prepared micellar casein using methods similar to those used by Beeby for κ-casein were completely unsuccessful, even though it could be detected in κ-casein made from the same milk. This paper gives a description of the method eventually used to establish the presence of this strongly masked cysteine.

MATERIALS AND METHODS

The milk was fresh raw milk from which the fat had been removed as described by Hill (1963). Bovine serum albumin, insulin, and defibrinated sheep's blood were supplied by the Commonwealth Serum Laboratories, Melbourne. Pronase was a product of Kaken Chemical Co., Tokyo, Japan. Sephadex was supplied by Pharmacia, Uppsala, Sweden.

The casein micelles were separated from the serum proteins, non-protein nitrogen and other low-molecular-weight components of the milk by the technique of gel filtration on Sephadex G 100 in 0·03 N sodium acetate buffer at pH 6·25 (Porath & Flodin, 1959; Hill & Hansen, 1964). Disulphide was estimated by amperometric titration with phenyl mercury acetate at pH 9 in 8–10 M urea, after Allison & Cecil (1958) or as described in this paper. Nitrogen was determined by semi-micro Kjeldahl distillation, using a factor of 6·4 to convert nitrogen to casein.

For the estimation of cysteine and cystine 3 methods were used. The method used initially depended upon digestion of the protein at pH 8–8·5 by pronase (1 % by weight of protein) in the presence of excess phenylmercury acetate for 2·5–3 h at 40 °C. The solution was then made 8–10 M in urea, adjusted to pH 9 with NH_4OH–NH_4CL buffer and deoxygenated in the polarograph cell with oxygen-free nitrogen. To this was added 2 ml of a deoxygenated solution of sheep haemoglobin in the carboxy form. The amount of haemoglobin was greater than that required to react with excess phenylmercury acetate. The residual SH in the haemoglobin was then estimated by amperometric titration with phenylmercury acetate. Knowing the SH content of the haemoglobin, the uptake of phenylmercury by the SH in the casein could be estimated. SS + SH content was determined by performing the amperometric titration in the presence of sulphite (Allison & Cecil, 1958) on undigested protein.

Sheep haemoglobin was used in this method because it contains 4 reactive SH groups with no slowly reacting SH nor disulphide (Snow, 1962), and was converted to the more stable carboxy form (Cecil & Snow, 1962) to reduce the possibility of errors caused by denaturation of the haemoglobin and oxidation of the SH groups. As Cecil & Loening (1960) have indicated that mercurial reagents may react with the SS in small peptides control tests with cystine in the pronase digests were undertaken. These indicated that the method should be reliable, but later tests using as control a pronase digest of insulin, which contains disulphide but no SH (Cecil & Loening, 1960), showed that at pH 9 it was possible to obtain extensive reaction with the S–S groups in the digest in the conditions of the above test. Even though this result may depend on the presence of specially sensitive S–S bonds in the insulin digest, it showed that the method could not be applied to proteins in general, and that confirmation by other methods of the result obtained with casein was needed. As this confirmation was obtained, the results are included for their quantitative value.

A second method of estimating the SH was the digestion of the protein in the presence of excess methylmercury iodide (10×10^{-2} ml of 0·008 N-CH_3HgI in 50 % propanol) at pH 8 in NH_4OH–NH_4Cl buffer ($\simeq$ 0·1 N). The digestion was otherwise as previously described. Propanol and KCl were then added to give final concentrations of 44 % (v/v) and 0·1 N, respectively. Propanol was used because of its purity and effectiveness as a disaggregating agent. The solution was then deoxygenated in the polarograph cell at 30 °C with oxygen-free nitrogen and the height of the first reduction wave of the unreacted methylmercury iodide was measured. An aliquot of CH_3HgI (usually 4×10^{-4} ml) was added and the wave height again measured. From the 2 wave heights the amount of unreacted CH_3HgI and hence the uptake of CH_3HgI by the protein could be estimated. This gave a measure of the reactive SH

in the digested protein in these conditions. Sulphite (1 ml, 10%, w/v) was then added and the uptake of CH_3HgI by the protein again estimated as above. The total uptake (designated V_1) in the presence of sulphite represents one mole of mercurial/mole S–S + 1 mole mercurial/mole SH. This uptake was compared with that obtained when the pronase digested protein was reduced with sodium borohydride in 44% propanol, following the method of Brown (1960). After destruction of the borohydride the SH in the reduced protein was estimated amperometrically at 52 °C and $-0{\cdot}75$ *V*. This uptake (V_2) represents 2 moles mercurial/mole SS plus 1 mole mercurial/mole SH. The SS content is then estimated from $V_2 - V_1$ and the SH from $2V_1 - V_2$. If $V_1 = V_2$, no S–S can be present.

Because borohydride has been reported to reduce methionine in casein, producing methyl mercaptan (D. A. Forss, personal communication) which if not removed completely by the bubbling with nitrogen may combine with mercurial reagents, a further estimate of the amount of SS and SH in the undigested protein was obtained using the method of Robbins & Fioriti (1963). This method relies on the reaction of SS and SH groups with alkaline cadmium hydroxide to yield ultimately 1 mole of methylene blue for every mole of sulphur in cystine and cysteine, and therefore gives an estimate similar to that from the borohydride reduction method. The authors reported that there was no reaction with methionine sulphur after 110 h digestion. The method is not convenient for routine use as it requires digestions of up to 120 h for complete reaction but it is useful for obtaining an independent estimate of cysteine + cystine sulphur.

RESULTS AND DISCUSSION

The results of the titrations in which haemoglobin was used are given in Table 1. The SH content of the casein micelles (as estimated by this method) is in all cases quite similar to the SS + SH content, which would mean that the micelles contain SH and not SS. On the other hand, the results for the 2 skim-milk samples indicate that they contain both SH and SS, as would be expected because of the presence of serum proteins. The inference that cysteine only is present in the micelles receives support from the tests using complete reduction with sodium borohydride, or reaction with alkaline $Cd(OH)_2$. The results (Table 2) show that the uptake of mercurial in the absence of reducing agents at pH 8 by the digested protein is usually considerably less than the uptake of the reduced protein. Control tests showed that there was little or no reaction between the methylmercury iodide and disulphide groups in small peptides at pH 8. For example, pronase-digested bovine serum albumin, which contains 0·7 SH and 17 S–S groups per molecular weight of 65000 g (Cecil & McPhee, 1959), showed an uptake of 1 mole of mercurial/65000 g. In addition control tests in which insulin and cystine were included in the digests showed no evidence at all of reaction between the methylmercury reagent and S–S groups at pH 8 in non-reducing conditions. The uptake of mercurial at pH 8 can therefore be taken as showing that SH groups are present but the results obtained with the reduced proteins show that not all the SH in the digested casein had reacted under these conditions.

If the uptake of mercurial by the casein reduced with sulphite is compared with the uptake following reduction with borohydride, it can be seen that in four of the

five examples given in Table 2 the two uptakes are closely similar (samples 1, 4, 5, 6) while sample 7 showed a 25 % greater uptake after reduction by borohydride. This means that SH only is present in the first mentioned 4 samples, and that the maximum amount of S–S possible in sample 7 is 1 mole/200000 g protein. Similar conclusions are derived when the results from the $Cd(OH)_2$ treatments (column 5, Table 2) are compared with those in column 3, Table 2. If the micelles contain cystine, and no cysteine, the estimate in column 5 should be twice that in column 3. If cysteine alone is present, then the 2 estimates should be the same. For the 7 samples which can be compared in this way the 2 estimates are closely similar, the mean of the 7 results from column 3 being 2·54 moles/100000 g and the mean of those in column 5 being 2·46 moles/100000 g. The results in columns 3, 4 and 5, Table 2, therefore, show that in most of the samples cysteine only is present, and suggest rather strongly that this is so in all of them.

Table 1. *Uptake of phenylmercury by casein micelles and skim-milk*

	Mole mercurial/100000 g protein	
Sample no.	Pronase digest	Sulphite reduced, not digested
1 (Micelles)	1·8 (BSA)*	1·7
2 (Micelles)	1·7 (HbCo)**	1·8
3 (Micelles)	2·2 (HbCo)	2·0
4 (Skim-milk)	2·6 (HbCo)	3·3
5 (Micelles)	1·5 (HbCo)	1·4
6 (Skim-milk)	3·3 (HbCo)	4·4

Excess phenylmercury acetate added to test solution and the excess phenylmercury estimated by addition of bovine serum albumin (BSA*) or sheep carboxyhaemoglobin (HbCo**).

Table 2. *Reaction of casein micelles with CH_3HgI or $Cd(OH)_2$*

	Uptake of CH_3HgI (moles/100000 g protein)			$Cd(OH)_2$ treatment,† moles S in cystine + cysteine/ 100000 g protein
Sample no.*	Pronase digest, pH 8	Pronase digest, reduced with sulphite	Pronase digest, reduced with $NaBH_4$	
1	1·8	2·3	2·4	—
2	2·3	3·0	—	3·2
3	—	2·6	—	2·3
4	0·66	2·8	2·8	2·3
5	0·83	2·0	2·1	1·6
6	0·83	2·7	2·9	2·9
7	0·98	2·0	2·5	1·8
8	1·5	2·7	—	3·1

* These samples do not correspond to those in Table 1.
† These tests were performed on undigested protein.

It is apparent from these results that the SH groups in casein are strongly masked, and that this is the reason for the failure of earlier attempts to detect them. The strength of this masking can be further judged from the fact that SH groups could

not be detected when intact micellar casein was titrated with phenylmercury acetate in the presence of:

(1) 10 M urea, pH 9 in 0·2 N-NH_4OH–NH_4Cl (or $Na_2B_4O_7$),

(2) 10 M urea, pH 10·5 in 0·5 N-NH_4OH–NH_4Cl,

(3) 40 % formamide, pH 9 in 0·2 N-NH_4OH–NH_4Cl,

(4) 44 % propanol, pH 9 in 0·2 N-NH_4OH–NH_4Cl,

(5) 44 % propanol, pH 9 in 0·2 N-NH_4OH–NH_4Cl + 0·2 M–NaCl,

nor could they be detected when the titrations were performed at 3 °C, instead of 37 °C, nor when Ca sequestering reagents were added. The only method which could be relied upon to unmask at least some of the SH groups was the preliminary digestion with pronase, followed by dispersion in disaggregating media. The use of pronase for a similar purpose has been discussed by Hird (1963) who reported that after extensive proteolysis all four of the disulphide bonds of ribonuclease became reducible. In the present case, digestions were performed in the presence of mercurial reagents to avoid loss of SH groups by oxidation as they became accessible.

Although the digestion with pronase at pH 8 at times rendered most of the SH groups available for reaction (samples 1 and 2, Table 2), the usual effect was that strong masking of some of the SH persisted in spite of the digestion (samples 4–8). The masked SH becomes available for reaction when the protein is treated with sulphite at pH 9—an effect similar to the one reported by Beeby (1964) for κ-casein. The most reliable method of assessing the SH content of the casein is therefore considered to be the comparison of the uptake of methyl mercury reagent by the pronase-digested protein in the presence of sulphite with the uptake by the digested protein reduced by borohydride. This avoids the possibility of error due either to the masking of the SH or to scission of sensitive disulphide bonds in non-reducing alkaline conditions.

As Beeby (1964) has shown that freshly prepared κ-casein contains SH but no SS, the SH content of the micelles is of interest for it is possible that all the SH in the micelles is in the form of κ-casein. If this is so the SH content as well as the sialic acid content may be a useful indication of the κ-casein content of the micelles. The fact that the SH content of milk is considerably higher than has been reported to date may also be important in understanding the behaviour of processed milks.

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With 2 plates

The separation of milk protein on dextran gel

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Summary. A method was devised for separating proteins of skim-milk on Sephadex G100 dextran gel. Four fractions were obtained, the first 3 corresponding to casein micelle, lactoglobulin, and lactalbumin fractions while the last contained no protein. The fractions were examined using starch gel electrophoresis, and results are also given for the average composition of the fractions. Differences between the composition of the casein micelle fraction and that of sodium caseinate made from the same milks are reported.

Since the technique for separating proteins of different molecular sizes by filtration through dextran gel was introduced by Porath & Flodin (1959) it has been applied to the separation of proteins in many natural systems. The gels originally available could resolve particles having molecular weights up to 25000, but more recently gels were developed which could resolve at molecular weight levels of 100000 (Sephadex G100) and 200000 (Sephadex G200).

As the protein particles in (skim)-milk have weights which range from millions for the casein micelles through 36000 for lactoglobulin to 16000 for lactalbumin (Haurowitz, 1963), it was apparent that the G100 gel might be used to separate the casein micelles from the smaller molecules. In early trials it was established that the lactoglobulin and lactalbumin could also be separated from each other and from the smaller molecules such as lactose, milk salts, etc. Morr, Kenkare & Gould (1963) reported a similar separation of milk into 4 fractions, but gave no information as to the nature of the proteins in each fraction. The method has been used in this laboratory since 1962 as part of a study of seasonal changes in the composition of milk, and has proved useful as a means of estimating changes in relative proportion of the various milk proteins. In this paper, details of the method, averaged composition of the fractions, and the results of examination of the fractions by means of starch gel electrophoresis are given.

EXPERIMENTAL

The dextran gel was Sephadex G100 supplied by Pharmacia, Uppsala. Twenty-five grammes of dry gel were stirred into M/30 sodium acetate buffer, pH 6·25, allowed to settle and the fines decanted. The gel was poured into a clear cylindrical extruded PVC column 5 cm in diam., having a hemispherical bottom cap containing glass micro-beads to a depth of 5 mm. The gel was allowed to settle, washed through with 1 l. of buffer, and aged for 24 h. The depth of the bed was about 23 cm.

Fresh raw bulk milk was warmed to 37 °C and spun in the Servall SS34 rotor at 8000 rev/min for 15 min at 37 °C. The temperature control was set to 3 °C and spinning continued for a further 15 min. The frozen fat plug was carefully removed, the milk decanted and any sedimented casein redispersed in the milk by stirring at 37 °C for approximately ½ h.

A layer of approximately 5 cm of buffer was placed on the top of the Sephadex gel bed, and a 20 ml sample of the milk carefully layered on to the gel surface beneath the supernatant buffer layer. The sample plus about 10 ml of the supernatant buffer was allowed to flow into the bed; at this stage the buffer above the bed was replenished and the flow rate kept at 2–2·5 ml/min. This rate was achieved with the outflow some 20 cm below the inlet level. Fractions were collected every 4 min on a Radi-Rac LKB fraction collector having a Uvicord (LKB) absorptiometer recording transmission at 253 mμm. The separations were done at room temperature (21 °C).

The fractions were collected, the contents of each peak pooled, and analysed for nitrogen, sialic acid, and cystine + cysteine contents. Nitrogen was determined by semi-micro Kjeldahl method and sialic acid by the procedure of Warren (1959). Cystine + cysteine was estimated by a method similar to that of F. J. Hird (unpublished), a description of which is in course of publication. A portion of each pooled fraction was freeze-dried, and these samples later compared by means of starch gel electrophoresis.

The starch gel apparatus was a cooled plate type similar to that described by Graham (1963) except that the gel bed was only 3·5 mm thick, and was used in a horizontal position. In addition, the cooled brass plate was insulated with a removeable layer of Saran wrap* 0·0015 in thick. This layer was stuck smoothly to the brass plate with a thin layer of heavy grease and was held at the edges by a rectangular bakelite frame surrounding the gel. When electrophoresis was completed the gel was sliced through the centre, the upper layer discarded and the lower lifted to the staining bath by the removeable Saran wrap. By this means weak and thinly sliced gels could be handled without damage. The samples for electrophoresis were dissolved in 1 ml of the cooled liquid gel at the same time as the gel bed was poured, and allowed to set under the same conditions as the gel bed itself—viz 4 h at room temperature followed by 20 h at 3 °C. Slices of the sample gel sufficient to fill the sample slots were then cut and placed in position. Conditions of electrophoresis were: initial potential 200 V (8 V/cm); initial current 33 mA falling to 12–15 mA after several hours; duration of run 20 h; temperature of cooling water 4 °C. The potential was, when needed, raised to 400 V toward the end of the run without apparent loss of resolution. The buffers and gel composition were as described in Wake & Baldwin (1961), except that the starch content was 10·8 %. Nigrosine water-soluble stain (Gurr) was used. Lactoglobulin and lactalbumin were prepared by the method of Aschaffenburg & Drewry (1957), except that the final crystallization step was omitted for lactalbumin.

* A polyvinylidene–polyvinyl chloride copolymer.

RESULTS AND DISCUSSION

A typical elution pattern is shown in Fig. 1. The transmission of the casein fraction is greatly reduced because much of the casein is still visibly in micellar form. Average volumes of the fractions and their average protein contents are shown in Table 1. The nitrogen in fraction 4 did not represent protein since there was no material precipitable by 12% T.C.A. Of the material in the other fractions, fraction 1 contained 84%, fraction 2, 11·5% and fraction 3, 4·5% of the total nitrogen. All the nitrogen in these 3 fractions was taken to be protein nitrogen. These proportions differ from

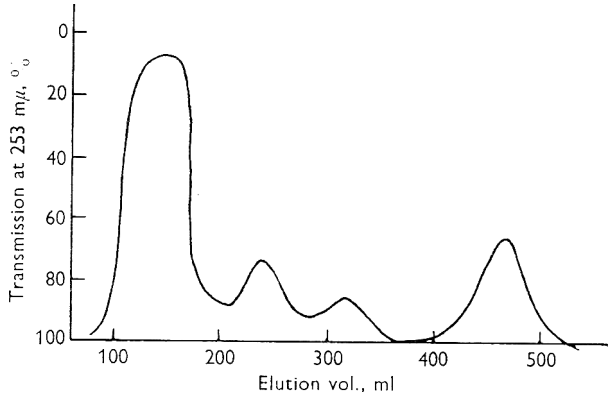


Figure 1. Chromatogram of fractions of skim-milk eluted from dextran gel.

Table 1. *Composition* of milk components eluted as fractions from Dextran gel (see Fig. 1)*

Fraction	Av. N; mg/ml original milk	Av. protein, mg/ml original milk	Av. sialic acid content, %	Av. vol. of fraction, ml
1	4·1	26·6	0·48	85
2	0·56	3·6	—	75
3	0·22	1·45	—	95
4	0·25	—	—	165
Na caseinate	—	23·4	0·35	—

* Average results from 12 samples of milk taken over a 6-month period.

those given by Morr *et al.* (1963), viz., 75%, 15% and 10% for the components separated by them in phosphate buffer at pH 7, and the differences would be consistent with a more complete separation by our procedure. If, for example, the time of elution is much increased, dissociation of the micelles may proceed to the extent that casein components elute in fractions 2 and 3. In particular some β-casein, which is known to be less firmly bound, might be expected to elute in fraction 2 if the micelles dissociate sufficiently. To check the extent to which dissociation occurred the compositions of the fractions were investigated by means of starch gel electrophoresis, a sensitive method for detecting minor components.

Plate 1 shows the electrophoresis patterns of fractions 1 and 2 from 2 different bulk milks from the same factory, along with those of a standard lactoglobulin and

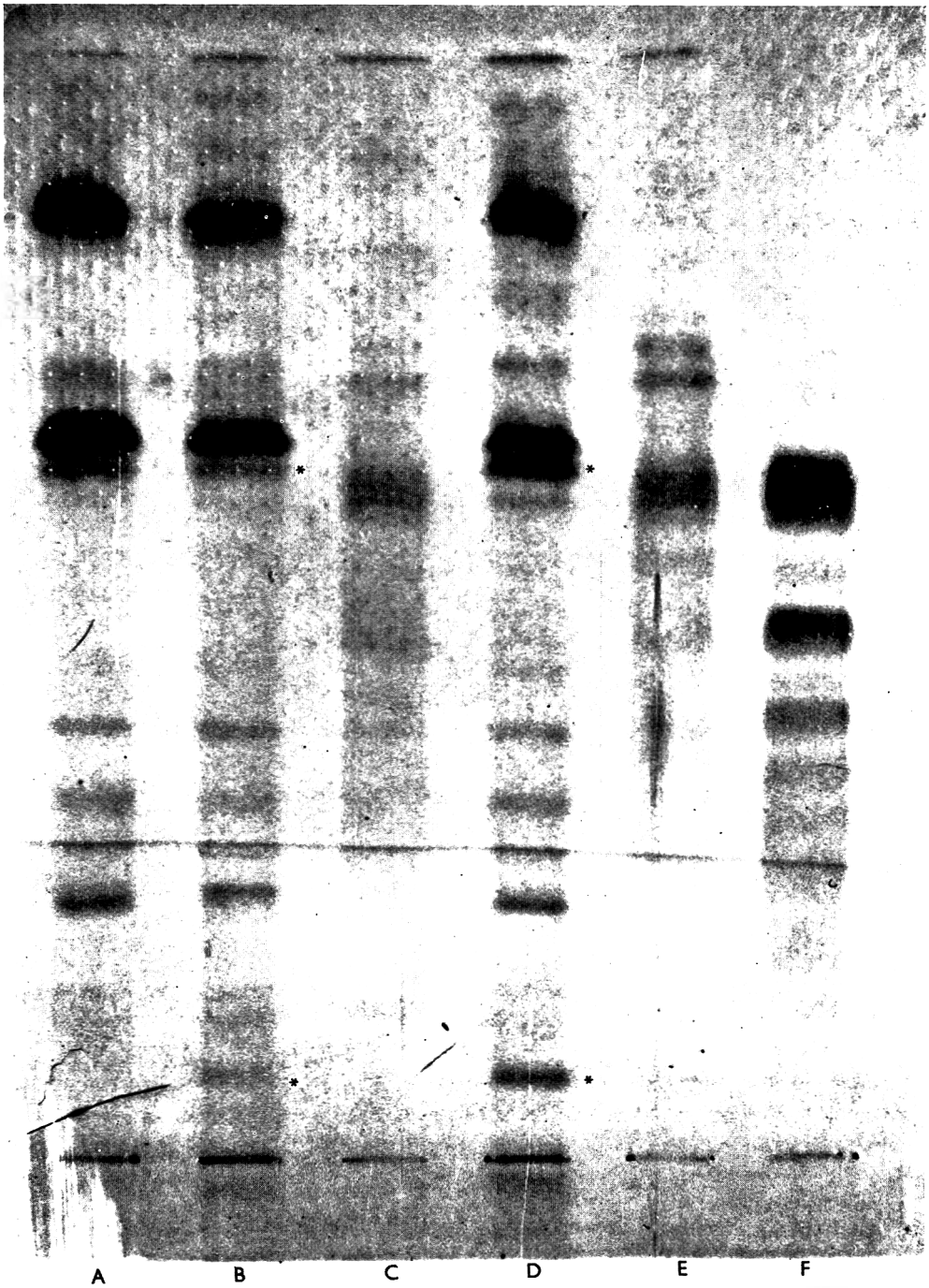
a standard sodium caseinate. The patterns of the fraction 1 samples are similar to those of the sodium caseinate. With the exception of a slow moving component in one of the fraction 1 samples (Plate 1 D), the components resolved are the same as those of the sodium caseinate. The starch-gel patterns show no evidence of contamination of fraction 1 with non-casein proteins. They do suggest however that there can be considerable variation in the proportions of some of the components of the casein micelle (compare starred bands in Plates 1 B, D).

The patterns of fraction 2 have no bands corresponding to the main α and β casein bands in fraction 1. In some of the patterns of fraction 2 there are weak bands from components which move faster than the main lactoglobulin band, but these do not appear to correlate with any casein components and the separation between fractions 1 and 2 must be almost complete, at least as judged by the starch gel patterns (see Plate 1 E). This is confirmed in Plate 2, in which the patterns of a set of fractions from one milk are shown. In a few samples, there was evidently a trace of fraction 3 protein in fraction 2 (Plate 2 C).

The fraction 3 patterns (Plate 2 D) reveal a predominant content of lactalbumin (see Plate 3) with some minor contamination. In one case the contamination appears to be caused by a slow-moving casein component. The shape of the fraction 3 peak in the elution curve is somewhat asymmetric, which suggests that there may be a difference in composition along the peak. To test this, the front and back halves of the peak were collected separately. The starch gel patterns of the two halves of the peak are shown in Plate 3, along with that of the standard lactalbumin. The patterns of the two halves are similar and there is no suggestion that other components are eluted in the trailing half of the peak. Starch gel patterns of fraction 4 showed only one weak band running with the front, and have therefore not been presented.

The starch gel patterns as a whole show that fractions 1–3 correspond to the casein, lactoglobulin and lactalbumin fractions, and suggest that there is little cross-contamination between fractions. This is supported by the results of analysis of fractions 2 and 3 for cystine + cysteine. The SS + SH content of fraction 2 was frequently found to be in the range 1 mole SS + SH/6000–7000 g protein, compared with a value of 1 mole/6000 g for the standard lactoglobulin. In such cases contamination with other components cannot be great. A number of the fraction 3 samples had SS + SH contents of about 1 mole/3300 g protein or about 5 moles/16000 g. In general the SS + SH content of this fraction seemed to be more variable than that of fraction 2, but even when the SS + SH analysis indicated the probable presence of other components, these could not be detected in the starch gel patterns in the expected proportion. The significance of these variations is not clear as genetic differences, oxidation of SH groups or undetected contamination may all contribute.

The caseins of fraction 1 had on the average a rather greater content of sialic acid than sodium caseinates made from the same milks (Table 1). That some components of the casein micelle are in fact lost when casein is precipitated at pH 4·6 was shown in a test in which a separated fraction 1 was precipitated at pH 4·6, centrifuged at 10000 g for 20 min and the clear supernatant analysed for nitrogen, SS + SH, and sialic acid. The supernatant contained 16 % of the original nitrogen, had a sialic acid content of 1·34 % and a SS + SH content of 1 mole/33000 g protein. This is in accord



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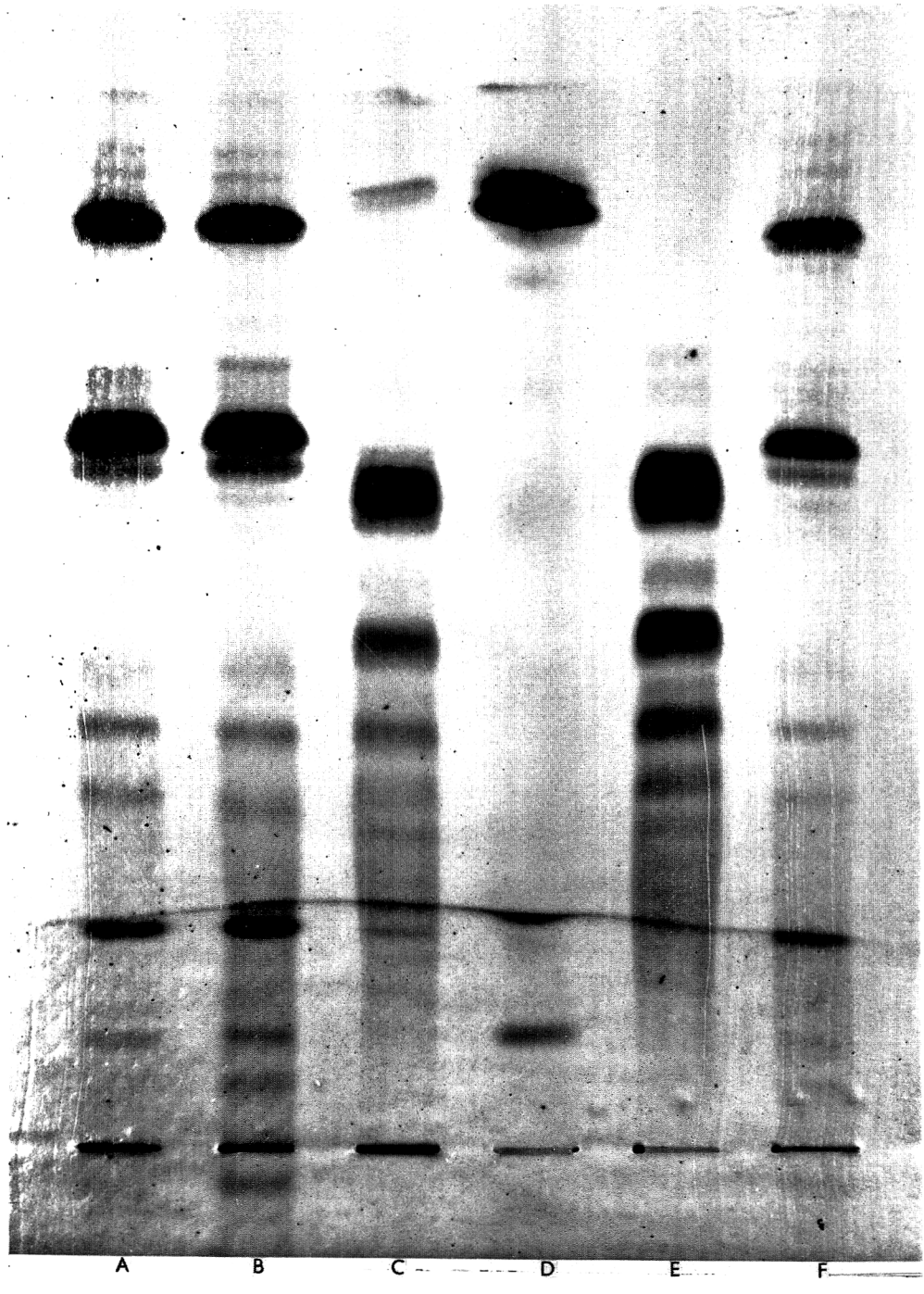
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with the results in Table 1 which shows that on the average some 25 % of the sialic acid content is lost by acid precipitation.

The gel filtration method therefore offers a simple means of separating the main groups of milk proteins from each other. It also has the advantage of isolating the casein fraction as it exists in the micelle, which may be important in systematic studies of variations in composition of this major component of milk.

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EXPLANATION OF PLATES 1–3

PLATE 1

Starch gel patterns of fractions from dextran gel. A, Standard Na caseinate; B, fraction 1 (August milk); C, fraction 2 (August milk); D, fraction 1 (November milk); E, fraction 2 (November milk); F, standard (crystalline) lactoglobulin.

PLATE 2

Starch gel patterns of fractions from dextran gel. A, Standard Na caseinate; B, fraction 1 (April milk); C, fraction 2 (April milk); D, fraction 3 (April milk); E, standard lactoglobulin; F, standard Na caseinate.

PLATE 3

Starch gel patterns of lactalbumins. A, standard lactalbumin; B, fraction 3 (1st half of peak); C, fraction 3 (2nd half of peak).

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A comparison of media for counting and isolating the bacteria from Cheddar cheese

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Summary. In an endeavour to find an ideal medium for the isolation of the bacteria from Cheddar cheese 10 media were compared. The initial criterion was of ability to yield maximal counts with each of 23 representatives of species common in Cheddar cheese. Subsequently the more promising media were compared for ability to give the highest counts for cheese emulsions without obvious distortion of the proportions of the various species present.

Since no one medium gave maximal counts with all strains, two complementary media of quite different composition are suggested for the bacteriological examination of cheese.

A variety of media have been used by different workers for the counting and isolation of the bacteria in Cheddar cheese, often without any apparent effort to establish that the medium used is suitable for the purpose. The essential characteristic of an ideal non-selective medium is that all viable organisms (chains and clumps of bacteria) should be capable of proliferating and giving rise to visible colonies. Although in practice this ideal is never completely obtained it is most important for enumeration purposes that the ratio of strains and species present should not be unduly distorted by the medium used.

The present paper gives details of a comparison of 10 media in which the problem of finding the most suitable medium was approached in two ways. In the first, representatives of the species most often reported in Cheddar cheese were plated on various media to find which medium, if any, consistently yielded the highest counts. In the second approach, emulsions prepared from several cheeses were tested to find which medium consistently yielded the highest counts without apparent distortion of the proportions of bacterial types present. Comparisons were also made of counts obtained by aerobic incubation and by anaerobic incubation in an atmosphere of 90% H_2 + 10% CO_2.

METHODS AND MATERIALS

Media

Four main types of media were tested: tomato dextrose agar (TDA) (Naylor & Sharpe, 1958); nutrient agar (NA); a solidified version (incorporating 1·25% Davis, N.Z. agar) of the liquid medium for lactobacilli developed by deMan, Rogosa & Sharpe (1960) (MRSA); and lactose yeast phosphate agar (LYPA) (Robertson,

1960*a*), a slightly modified version of the medium developed by Hunter (1946). In addition, a limited number of tests were made with yeast dextrose agar (YDA) (Naylor & Sharpe, 1958) but since this was not as satisfactory as the other medium, TDA, used by Naylor & Sharpe (1958) it was not examined exhaustively.

Apart from the above media the following variants of the original formulations were also tested: (*a*) modified TDA (TDA mod) in which the sodium chloride was omitted and 0·5 % (v/v) of mineral solution 'A' (Mabbit & Zielinska, 1956), 0·5 % (w/v) of $CH_3COONa.3H_2O$. 0·5 % (w/v) of KH_2PO_4, and 0·2 % (w/v) of diammonium citrate were substituted; (*b*) a triple sugar variant of MRSA (MRSAT) in which 5 g arabinose and 5 g sucrose were substituted for 15 of the 20 g glucose (deMan *et al.* 1960); (*c*) glucose lactose yeast phosphate agar (GLYPA) (Robertson, 1960*b*); (*d*) LYPA+; (*e*) GLYPA+. Medium c was the same as LYPA except that half the lactose was replaced with glucose and media (*d*) and (*e*) were prepared by adding 0·5 % (v/v) of mineral solution A (Mabbitt & Zielinska, 1956), 0·1 % (w/v) of $CH_3COONa.3H_2O$ and Tween 80 to the media LYPA and GLYPA, respectively. These latter additions were included because they have been shown to improve the growth of some strains of lactobacilli (Briggs, 1953; deMan *et al.* 1960; Evans & Niven, 1951; Mabbitt & Zielinska, 1956; MacLeod & Snell, 1947; Snell, Tatum & Peterson, 1937).

In addition to the above media which are not regarded as being selective for particular species, the selective medium, acetate agar (AcA) of Mabbitt & Zielinska (1956), was included in the test programme to check its ability to give high counts with all strains of lactobacilli. It was found to be essential to incubate AcA plates in small closed containers in order to avoid loss of moisture with consequent concentration of acetate which resulted in low counts of lactobacilli.

Trials using known strains of bacteria

Preliminary comparisons of media were made with 11 strains representing 4 genera. This was subsequently increased to 23 strains from 5 genera (Table 1). All strains were originally isolated from Cheddar cheese or milk. Some strains of *Lactobacillus brevis* have been shown to be more difficult to grow than others (Naylor & Sharpe, 1958); for this reason more representatives of this species were tested than of other species.

In accordance with the proposals of Shaw, Stitt & Cowan (1951), the genus name *Micrococcus* (strains 949 and 1222) has not been recognized in the present investigation; and following the suggestion of Stadhouders & Mulder (1958) the name of Staphylococcus (Micrococcus) has been used to indicate that representatives of the former genus *Micrococcus* are included.

Suitable dilutions of 2- or 3-day-old cultures in yeast dextrose litmus milk (YDLM) (Wheater, 1955), were used as inocula throughout. Plates were counted after 5 or 6 days incubation at 30 °C. in aerobic conditions or in an atmosphere of 90 % H_2 + 10 % CO_2.

Trials with cheese

In a comparative study of the bacteriology of Cheddar cheese to be reported later a total of 104 cheeses about 6 months old, 26 from each of the countries, Australia, Canada, Great Britain and New Zealand, were examined bacteriologically. Six of

these cheeses, at least one from each country, were used for the present comparison of media. The cheese was sampled by inserting a large sterile trier into the alcohol-swabbed surface of the cheese. The outermost inch of the plug was returned to the cheese and the remaining portion (30–40 g) weighed into a sterile 500-ml Monel metal blender jar containing a known weight (about 110 g) of 2% (w/v) sodium citrate solution. The cheese samples were blended (Ato-mix blender) for 0·5 min at slow speed, then for 2 min at full speed. To allow the foam to subside, but at the same time to avoid excessive separation of the cheesefat, the emulsion was allowed to stand for exactly 2 min before dilutions were prepared.

Cheese emulsions were plated in duplicate on TDA, TDA mod, LYPA, GLYPA and MRSA. In addition to a comparison of the total counts obtained, a comparison was also made of the proportion and actual numbers of the different bacterial types which developed on these media. This was done by picking 25 colonies from each medium into YDLM and comparing the resultant cultures microscopically, physiologically and biochemically. The colonies were picked from uncrowded plates (15–150 colonies). To avoid bias and to ensure that the ratios of the various bacterial types in the isolates were similar to those on the plates, an area just large enough to contain 25 well separated colonies was marked off. All colonies, except those which were so close that there was a risk of obtaining mixed cultures, were picked from that area. Methods for identification of cultures are to be described in detail in a subsequent paper.

RESULTS AND DISCUSSION

Initial comparative trials of aerobic and anaerobic incubation of known strains on a variety of media showed conclusively that anaerobic incubation was less satisfactory than aerobic. The H_2+CO_2 atmosphere substantially reduced the counts obtained with strains of some genera, and with others conferred no advantage apart from increased colony size. Aerobic incubation was therefore adopted throughout the entire subsequent investigation.

The more important results of the trials of 23 known strains on 9 different media are summarized in Table 1. No strain was tested on all media since it was impracticable to make all tests simultaneously and early trials showed some media to be quite unsuitable. For example, using identical inocula, colony counts on NA, TDA mod, LYPA+ and GLYPA+ were often much lower than on other media. With some unsatisfactory media the colonies which did develop were often as large as or larger than those which developed on media which gave higher counts, a phenomenon which appeared to be independent of crowding on the plates. It is interesting to note that three of these unsatisfactory media contained additional ingredients based on those present in AcA. An investigation of these additional components showed that the lowering of the counts was only associated with the presence of mineral solution 'A'. In particular, the Mn^{++} in this solution was responsible for the inhibition while the Fe^{++} present lessened the adverse effect of Mn^{++}. A fourfold increase in Fe^{++} concentration overcame the inhibitory effect of Mn^{++} in salt solution 'A', but the 3 media still had little or no advantage over their counterparts not containing ingredients based on AcA.

From Table 1 it may be seen that no one medium gave maximal counts with all species. The medium TDA gave low counts with 7 of 21 strains but gave higher

counts with strain 'V7' than were obtained on any other medium. LYPA gave low counts with 5 of 23 strains and was thus a better medium than TDA. It was found that replacement of part of the lactose of LYPA by glucose to give GLYPA resulted in maximal counts for 3 of the 5 strains which gave low counts on LYPA. Indeed, if it be assumed that the 9 strains which were not tried on GLYPA gave maximal counts on GLYPA as they did on every other medium tested, then GLYPA was the best medium tested. MRSA and MRSAT were almost as good as GLYPA, but with *L. brevis* strains 'V7' and 'D127' they have low and very low counts respectively; colony size was not impaired.

Table 1. *A comparison of the colony counts* on 9 media of 23 strains of 8 species of bacteria often found in Cheddar cheese*

(Incubations: aerobic 5–6 days at 30 °C.)

Species	Strain†	TDA	TDA mod	LYPA	LYPA+	GLYPA	GLYPA+	MRSA	MRSAT	AcA
Lactobacillus casei	148	+ + +	+ + +	+ + +	+ +	+ + +	N	+ + +	N	N
	A121	+ +	N	+ + +	N	+ + +	N	+ + +	+ + +	+ + +
	B142	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
L. plantarum	A164	+ + +	+ + +	+ + +	+ + +	+ + +	N	+ + +	N	N
	L50	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
	H10	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
L. brevis	X1	+ + +	+ + +	+ + +	+ + +	+ + +	N	+ + +	N	N
	V7	+ + +	+ + +	+ +	+	+ +	+ +	+	+	N
	D127	+ +	+	+ + +	+ + +	+ + +	N	±	±	N
	K46	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
	T21	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
	D210	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
L. unclassified	K44	+ +	N	+ + +	N	+ + +	N	+ + +	+ + +	+ + +
	AC10	+ + +	N	+ + +	N	N	N	+ + +	+ + +	+ + +
Streptococcus lactis	763 (ML_3)	N	N	+ + +	N	N	N	+ + +	+ + +	N
Str. cremoris	607 (HP)	N	N	+ + +	N	N	N	+ + +	+ + +	N
	924	+ + +	N	+	N	+ +	N	+ + +	+ + +	N
Pediococcus sp.	CH 159	+ + +	+ + +	+ + +	+ + +	+ + +	N	+ + +	N	N
	991	+ + +	+ + +	+ + +	+	+ + +	+ + +	N	N	N
Leuconostoc sp.	517	+ +	+ + +	+ + +	N	+ + +	+	N	N	N
	523	+ +	+ +	+ +	+ +	+ + +	N	+ + +	N	N
Staphylococcus	949	+	±	+ +	±	+ + +	±	+ + +	N	N
(Micrococcus) sp.	1222	±	+	+ +	N	+ + +	N	+ + +	N	N

* + + +, Within 80–100 % of highest count: + +, within 40–80 % of highest count; +, within 10–40 % of highest count; ±, within 0–10 % of highest count: N, not determined.

† Strains designated by number only were selected from National Collection of Dairy Organisms, Shinfield and those with a letter prefix were from the collection of Dr M. E. Sharpe (isolated by Naylor & Sharpe, 1958).

All comparisons were repeated at least twice; several were repeated up to 7 times.

In the light of the results of trials with known strains of bacteria a limited number of comparisons were made using TDA, TDA mod, LYPA, GLYPA and MRSA for plating emulsions of cheese. The total counts and proportion of different bacterial types isolated were in general agreement with what was to be expected from the trials using known strains; TDA, GLYPA and MRSA were the most satisfactory media.

Because of the large difference in bacteriological composition of different cheeses and because no one medium was entirely satisfactory it was decided that for studies of the bacteriology of Cheddar cheese each cheese should be plated on 2 media. TDA and GLYPA were chosen because on the basis of the results obtained with pure cultures they seemed to be complementary in that for the strains tested, one medium was satisfactory when the other was unsatisfactory. The wisdom of this choice was found in the main study, since although the mean counts on the 2 media were similar (Table 2) the counts for individual cheese were sometimes quite different. With 6 of

the 104 cheeses there was at least a twofold difference between the plate counts obtained on TDA and GLYPA. However, it is apparent from Table 3 that there is a difference even in the mean proportion of different species isolated on the 2 media. More *Str. thermophilus* and corynebacteria were isolated on GLYPA than on TDA, and conversely *L. brevis* strains were most frequently isolated on TDA. There was a tendency for more *L. casei* to be isolated on TDA. With individual cheese the differences were even more pronounced. An extreme example was the finding that for one cheese only 8% of the isolates from TDA were *L. plantarum* whereas this species represented 56% of the isolates from GLYPA. In this cheese *Str. lactis* strains represented 70 and 12% of the isolates on 2 media, respectively. In this instance the total counts on TDA and GLYPA were 162×10^7 and 57×10^7, respectively.

Table 2. *The mean results of plate counts on TDA, GLYPA and AcA for 26 cheeses from each of 4 countries*

	Organisms/g $\times 10^{-6}$		
Cheese from	TDA	GLYPA	AcA*
Australia	46·1	37·7	30·7
Canada	120·0	126·0	45·1
Great Britain	53·1	50·5	39·4
New Zealand	66·7	55·0	19·8

* AcA counts were only determined for about half the cheeses from each country.

Table 3. *The mean proportion (percentage) of different species isolated from 104 Cheddar cheeses on the media TDA and GLYPA.*

(Equal numbers of cheeses from Australia, Canada, Great Britain and New Zealand; about 2500 isolates from each medium.)

	TDA	GLYPA
Streptococcus lactis	8·7	8·2
Str. cremoris	1·7	0·5
Str. thermophilus	2·0	3·3
Leuconostoc sp.	1·8	1·8
Pediococcus sp.	14·2	13·9
Lactobacillus casei	37·4	32·7
L. plantarum	13·1	14·5
L. brevis	10·7	5·9
Corynebacterium sp.	0·9	6·1
Serological Gp. D streptococci	1·9	2·1
Staphylococcus (Micrococcus) sp.	1·1	1·6
Other, including unclassified	6·5	9·4

The counts on the selective medium AcA are included in Table 2 for comparison with the total counts on the 2 'general-purpose' media. AcA grows lactobacilli and some pediococci (Mabbitt & Zielinska, 1956; Naylor & Sharpe, 1958) and as lactobacilli comprise about 60% of the isolates from all cheese (Table 3) the AcA counts are in reasonable agreement with those on TDA and GLYPA but tend to be relatively low for Canadian and New Zealand cheese (Table 2).

Used alone, none of the 'general purpose' media tested was ideal for the enumeration and isolation of the bacteria in all cheese. With a particular cheese and a particular medium, even a good one, the distortion of the bacteriological picture could be

considerable. The disadvantages of the individual media could be largely overcome by plating each cheese on 2 complementary media. This procedure appears to avoid gross distortion of the total count and of the proportions of species isolated although it is impossible to know for certain the true proportions of the various species in a natural product like cheese. Some aspects of many earlier studies of cheese flora must be treated with caution because of an apparent disregard for the suitability of the isolation media employed.

I wish to acknowledge the interest shown by the late Dr A. T. R. Mattick and Drs M. E. Sharpe and M. Rogosa, the technical assistance of Mrs S. L. Ruby and Miss S. Boon, and the excellent facilities extended to me at the National Institute for Research in Dairying, Shinfield, Reading, where this comparison was carried out.

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Mechanics of machine milking

I. Pressures in the teatcup assembly and liner wall movement

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Summary. A method is described of measuring pressures in a teatcup assembly using strain gauge transducers and simultaneously following movement of the liner wall by means of a cine camera. In preliminary experiments with a narrow bore type liner it was found that pressures below the teat could vary during a single pulsation cycle from a few inches of mercury below atmospheric pressure (inHg vacuum) to as high as 25 inHg vacuum in the absence of an airbleed. Bleeding air into the barrel of the liner or into the clawpiece considerably reduced fluctuation in pressure, and the vacuum barely rose above the nominal milking vacuum of 15 inHg. Reducing the rate of change of pressure in the pulsation chamber did not greatly affect the maximum vacuum obtained. Opening and closing of the liner by pressure change in the pulsation chamber was under some conditions considerably delayed by the pressure conditions existing inside the liner.

It is suggested that inertia effects of milk in the cluster and the natural frequency of the system are largely responsible for the observed pressure changes under the teat.

Although a good deal of attention has been given to pressure changes in the pulsation chamber of teatcups (e.g. Hupfauer, 1956; Neild, Clark, Hoffman & Olney, 1962; Noorlander & Schalm, 1958; Noorlander, 1960; Phillips, 1963; Whittlestone & Olney, 1962), records of pressures measured inside the liner under the teat have received little attention in the literature. Noorlander (1960) made reference to such pressure fluctuations, which were measured with strain gauge equipment, but few data are quoted. We have used a multichannel instrument employing strain gauge transducers to measure pressures at various points in the teatcup during milking. It enabled phase relationships to be followed easily, and had a sufficiently high response rate to record any transient pressures likely to have occurred. In addition the opening and closing of the liner mounted in a glass teatcup has been followed by the technique mentioned, but not described, by Ardran, Kemp, Clough & Dodd (1958). In the present paper some preliminary observations are made on factors affecting pressure changes and liner movement in the teatcup using 3 first-calf Ayrshires in mid- and late lactation.

METHODS

Pressure measurement

Apparatus. Bell & Howell unbonded strain gauge pressure transducers (Type 4-326, range 0–25 lb/in² absolute) were used in conjunction with their Type 5-124 ultraviolet light oscillograph equipped with Type 7-342 galvanometers having a flat (within 5 %) frequency response range of 0–135 c/sec to a sine wave input (Bell & Howell Ltd., Consolidated Electrodynamics, 14 Commercial Road, Woking, Surrey). With this combination of transducers and galvanometers no amplifiers were required. Each transducer was connected to a galvanometer through a resistance network so that the output of the bridge circuit could be adjusted to zero when the transducer was subject to prevailing atmospheric pressure. Also a variable resistance was fitted in the input circuit of each transducer so that the deflexion of the galvanometer for a known pressure change could be matched to the grid lines on the chart. A convenient chart speed was 3·25 in/sec with timing lines at intervals of 0·1 sec.

The transducers, being somewhat bulky and weighing 4 oz, could not be conveniently mounted direct on the teatcup assembly. They were therefore mounted 1·5–2 ft away from the clawpiece along the line of the long milk tube. Short pieces of 0·125-in. bore thin-walled stainless steel tubing, ending inside the liner at the positions selected for pressure measurement were sealed through the base of the liner and shell so as not to interfere with the milk outlet. These pressure probes were connected to the transducers by high density polythene tubes 0·110 in. internal diam. × 0·161 in. external diam. (Portland Plastics Ltd., Bassett House, Hythe, Kent). The inlet pressure cavity of each transducer and its connecting tube and pressure probe were filled with liquid. This was to maintain the frequency response of the system and to reduce inertia effects that would inevitably have occurred when a nominally air-filled system became partly filled with milk. Due to a leaking joint a liquid-filled system did on one occasion contain several large air bubbles. The pressure traces at once showed large amplitude damped natural frequencies—the pressure transmission system was 'ringing'.

Air-free water is a convenient filling liquid, but because the pressure probes led downwards from the liner the denser milk penetrated into the connecting tubes, sometimes as far as the transducer, making cleaning necessary on each occasion. However, penetration by the milk was much reduced by using an aqueous solution denser than milk, containing $Na_2B_4O_7.10H_2O$, 2·5 % (w/v) and Na_2SO_4, 3·5 % (w/v) and having a density of about 1·04. When the connecting tube was flushed with this solution (using a side connexion at the entrance to the transducer) at intervals of about a week, no difficulties were experienced.

Accuracy of pressure measurement. When steady pressures over the range 0–20 inHg vacuum were applied to the transducers the pressure values read from the record charts agreed to within 0·2 inHg with those measured with a mercury manometer. No direct measurements of frequency response were made, but under conditions of pressure change similar to those shown in Fig. 2, a transducer mounted on the clawpiece with an 8-in. polythene connecting tube gave curves identical in amplitude and phasing with those of a transducer with a 36-in. connecting tube, the 2 pressure probes ending symmetrically inside the liner about 1·5 in. below the teat. The highest

frequency recorded was about 70 c/sec. Open ended probes would give false results if the momentum of milk impinging on the probe resulted in pressure. In comparisons of an open-ended probe and a symmetrically placed capped probe with 4 holes 0·078 in. diam. just behind the cap, the 2 curves were indistinguishable, but as a precaution capped probes were used for all measurements inside the liner.

A much larger error arises, when the probe is mechanically vibrated, owing to the inertia of the filling liquid. To measure the amplitude of such errors and to compare their phasing with pressure changes inside the liner and the pulsation chamber, a teatcup was equipped with a pressure connexion to the pulsation chamber and 2 identical probes ending inside the liner, one being sealed at the outer end. The polythene tube attached to the sealed probe had 4 holes 0·078 in. diam. in the wall just beyond the point of attachment so that in effect atmospheric pressure was being measured. The single teatcup was connected direct to a large pulsator to give fast change of pressure in the pulsation chamber and hence the maximum vibration of the assembly. Typical results with the teatcup held upright in the milking position by a thumb inserted in the liner, and also when milking a cow, are shown in Fig. 6. Inertia effects of the liquid filling the connecting tube attached to the dummy probe resulted in errors of measurement of atmospheric pressure, occurring at the same time as violent pressure changes in the pulsation chamber, as high as $\pm$ 1·5 inHg. These unwanted pressure changes necessarily have fairly high frequency as their occurrence is related to acceleration of the confined filling liquid. While errors of this magnitude do not affect the main conclusions of this paper, it is important to remember them when interpreting the graphical data given.

Cine camera technique

A diagram of a glass teatcup assembly equipped with probes for measuring pressures in the pulsation chamber and at 3 heights below the teat position on the axis of the liner is given in Fig. 1. A piece of white paper was cemented to the internal surface of the rear half of the teatcup to improve contrast. The assembly was so arranged that when in place on the teat there was a clear view of the edge of the collapsed liner seen from a position at right angles to the side of the cow.

A 16 mm Vinten camera operating at 100 frames/sec with a 7·6 cm lens and an exposure time of 0·002 sec (W. Vinten Ltd., 715 North Circular Road, Cricklewood, London, N.W. 2) was mounted on a metal frame, to which was also attached a 1 kW spotlight. The frame tilted and rotated easily and with the aid of a view-finder attached to the frame the camera and spotlight could be directed simultaneously. The processed film was projected in a film reader to give a full-sized image for making measurements of width of liner and position of the teat in the collapsed liner.

Synchronization of the film and oscillograph records

A 4-in. diam. dial with an engraved scale evenly dividing the circumference into 100 parts was attached to a synchronous motor which rotated a pointer at 1 rev/sec over the scale. Every fifth mark of the scale was elongated and every tenth figured so that time could be easily read from the cine film to 0·01 sec. As the hand passed the zero mark it contacted a light leaf spring, thus energizing an event marking galvanometer in the oscillograph. The timer was held in the field of view of the

camera close to the milking cluster during recording. Both the camera motor and the chart drive motor of the oscillograph were started simultaneously so that the position of the first deflexion of the event marker shown on the chart corresponded to the first frame of the film sequence which showed the timer hand at zero.

Milking equipment

The cows were milked in a 2-level tandem parlour. When the glass teatcup was one of four attached to a clawpiece in the usual way, a long milk tube (0·5 in. bore × 39 in. long) elevated the milk to a weigh jar. The connexion to the weigh jar was about 14 in. above the tips of the teats. A magnetic pulsator operated all 4 pulsation chambers simultaneously. In some experiments the glass teatcup assembly was attached independently to a magnetic pulsator (4 ft of 0·31-in. bore tubing), the milk being conveyed by 3 ft of 0·31-in. bore tubing to one volumetric jar of a quarter milker. The entrance to the volumetric jar was about level with the tip of the teat. With both arrangements the amount of milk in the jar was recorded at 15 sec intervals so that accurate estimates of flowrate when pressure measurements were made could be obtained graphically.

An extruded natural rubber liner with a mouthpiece formed by inserting a nylon ring was used. Its main dimensions when under tension in the teat cup are given in Fig. 1.

The conditions under which the various experiments were conducted are given in Table 1.

RESULTS

Preliminary measurements showed that pressures measured inside the teatcup liner at a position 1–1·5 in. below the teat fluctuated widely during each pulsation cycle. It seemed possible that the whole of the space under the teat might not, at any particular time, be at the same pressure. This aspect was therefore first explored and subsequently the relationship of pressure change and liner wall movement investigated.

Pressure probes at 3 levels inside the liner (expt. 1)

A teatcup assembly was equipped with 3 capped side entry pressure probes close to the axis of the liner (Fig. 1). The lowest one opened about 0·5 in. above the milk outlet hole and in this position remained out of contact with the liner even when there was a pressure difference of 25 inHg closing the liner. The second probe was 1·25 in. higher than the first. The third was adjustable and was set during milking with the opening 0·25–0·5 in. below the tip of the teat. The distance from the tip of the teat to the bottom of the liner varied from 4·25 to 3 in. with the 3 cows. The pressure records obtained were similar to those in Fig. 4*a*. Only barely discernible differences in phase and amplitude were shown by the sets of 3 pressure traces, the 2 upper probes not, apparently, being occluded by the closed liner. Subsequent measurements were made with probes at the middle position (about 1–1·5 in. below the teat).

Pressures in the teatcup assembly under conditions likely to be found in practice (expt. 2)

The chief characteristics of the milking conditions used were no leakage of air past the teat or into the clawpiece, little elevation of the milk (about 1·5 ft into the weigh

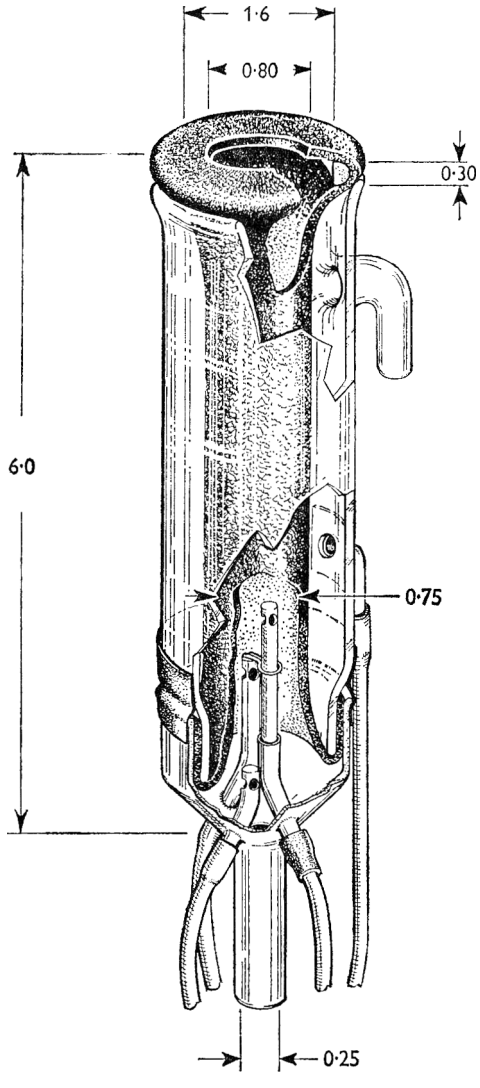

Fig. 1. Diagram of a liner assembled in a glass teatcup with probes for pressure measurement at 3 levels under the teat and in the pulsation chamber. The lower end of the stretched liner was folded over the end of the glass teatcup and retained by binding with thin cord. The metal end piece carrying the central probes fitted snugly over the rubber and was retained in place by adhesive tape. The longest of the 3 probes consisted of polythene tubing thrust through a metal guide tube with a short length of rubber tubing used as a seal. This probe could be adjusted during milking, guide marks at 0·5 in. intervals on the visible part indicating the height within the liner. Dimensions are given in inches.

jar), a pulsation rate of 54 c/min, fairly slow pressure change in the pulsation chamber (0·15–0·2 sec for the pressure change to be substantially completed) and a wide ratio. The results for the 3 cows were similar, the data shown for one animal in Fig. 2 being typical. Pressure changes inside the liner were as expected when measurements were made before milking began with the mouths of the liners closed with bungs, and also when the cluster was replaced on the cow after milking, the system having been cleared of milk by admitting air through the liners. Opening and closing of the liner

Table 1. *Experimental conditions when pressures in the teatcup assembly and movement of the liner wall were recorded*

(Extruded narrow bore liner under tension; milking vacuum 15·3 inHg.)

Experimental conditions	Expt. 1. Probes at 3 levels under teat			Expt. 2. Common milking conditions	Expt. 3. Fast change of P.C. pressure	Expt. 4. Slow and fast pulsation and P.C. pressure change	Expt. 5. Airbleeds	Expt. 6. Pulsator stopped open and closed
Cow no.	Y64	Y65	Y67	Y64	Y65	Y67	Y67	Y65
Single teatcup or whole cluster	Whole cluster			Whole cluster	Single cup	Whole cluster	Whole cluster	Single cup
Weight of assembly suspended in milking position, lb	5·6			5·6	0·7	5·6	5·6	0·6
Length and bore of milk tube, in.	39 × 0·5			39 × 0·5	36 × 0·31	39 × 0·5	39 × 0·5	36 × 0·31
Length and bore of pulsator tube, in.	56 × 0·375			48 × 0·375	48 × 0·31	48 × 0·375*	54 × 0·375	48 × 0·31
Pulsation rate, c/min	65			64	64	64 & 32	64	64
Ratiometer reading % liner open time†	49			57‡	53	49	49	48
Yield at milking:								
Quarter, lb	—	—	—	—	4·8	—	—	5·2
Whole udder, lb	9·1	18·5	19·0	10·0	18·5	16·5	17·6	18·3
Flowrate during recording:								
Quarter, lb/min	—	—	—	—	2·9	—	—	—
Whole udder, lb/min	2·8	9·3	7·0	3·4	—	9·5	7·3	—

P.C. = Pulsation chamber.
* An 0·125 in. diam. restrictor was inserted when required into the pulse tube close to the pulsator.
† Method described by Thiel & Akam (1964).
‡ The pulsation control system consisted of an electric master pulsator controlling a pulse relay line with a pneumatic relay at the milking unit.

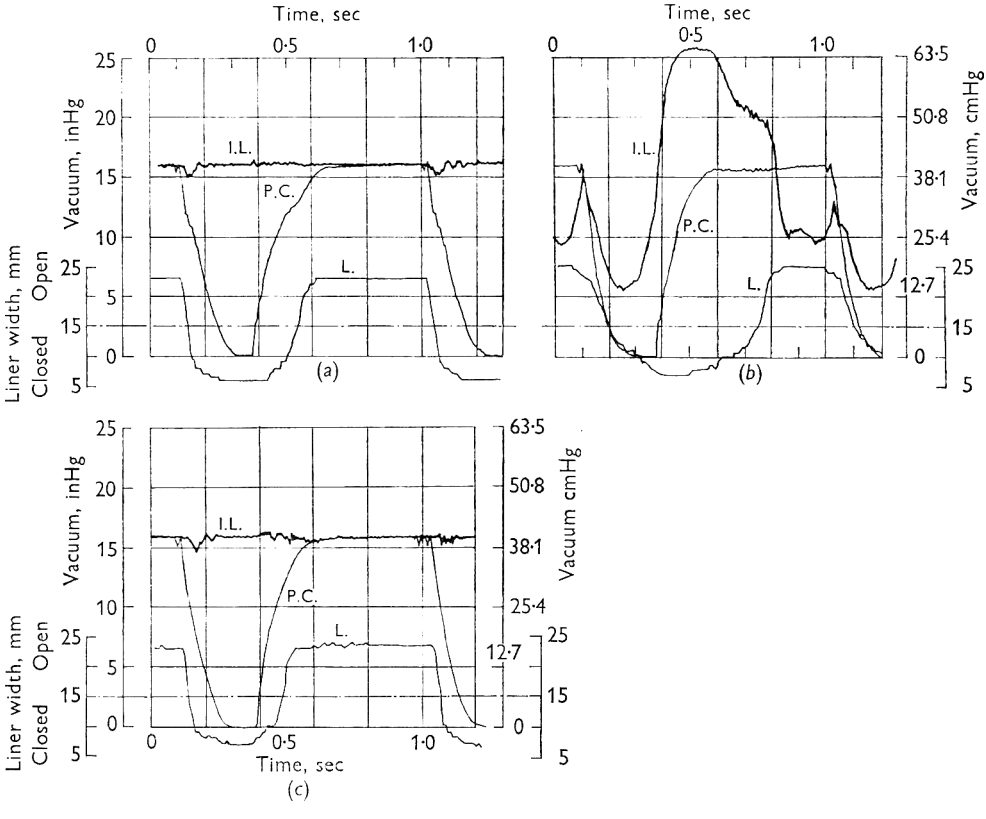

Fig. 2. Pressures measured in the pulsation chamber (P.C.) and inside the liner (I.L.), and liner wall movement (L.) measured in one teatcup assembly of a cluster: (*a*) liner mouths closed with rubber bungs; (*b*) during peak milk flow; (*c*) after clearing the cluster of milk and replacing it on the cow after milking. Vacuum, 16·7 inHg; pulsation rate, 64 c/min; ratiometer reading, 57 %; % of cycle time that liner was more than half open, (*a*) 56 %, (*b*) 39 %, (*c*) 63 %.

followed accurately the pressure change in the pulsation chamber, the half open position occurring at a pressure difference across the wall of the liner of 3–5 inHg. However, measurements made during the period of maximum milk flow (Fig. 2*b*) showed wide pressure fluctuations inside the liner. Closing of the liner was somewhat delayed, but its opening was much delayed. The half open positions again corresponded to a pressure difference across the liner wall of 3–5 inHg for all 3 cows. Pulsation ratio defined as percentage of the pulsation cycle that the liner was more than half open changed from about 60 % with no milk present to 38 % at full flow.

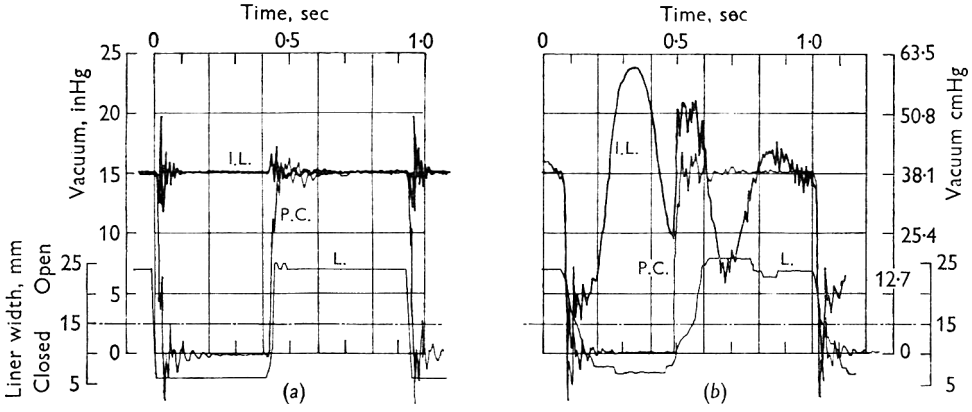

Fig. 3. Pressures measured in the pulsation chamber (P.C.) and inside the liner (I.L.), and liner wall movement (L.), with fast change of pulsation chamber pressure. A single teatcup assembly with a 0·31-in. bore milk tube was used: (*a*) liner closed with a bung; (*b*) during peak flow. Vacuum, 15·3 inHg; pulsation rate, 64 c/min; ratiometer reading, 53 %; % of cycle time that liner was more than half open, (*a*) 53 %, (*b*) 54 %.

The effect of rapid pressure change in the pulsation chamber (*expt.* 3)

When the rate of pressure change in the pulsation chamber was increased to 15 inHg in 0·02 sec or less during milking by using a single teat cup assembly, the pressure inside the liner attained atmospheric pressure or higher (Fig. 3, for confirmation see Fig. 6). The peak of high vacuum seen in Fig. 2 separated into 2 peaks, the first occurring when the liner was fully closed and the second as the pulsation chamber was evacuated. There was a very pronounced trough during the milking phase. The pressure graphs relating to the pulsation chamber and the space beneath the teat showed typical damped natural frequencies. When the cine film was projected at 24 frames/sec, fluttering of the liner could be seen. Opening and closing of the liner during milking were somewhat delayed compared with when the liner was empty, but the pulsation ratio (liner more than half open) was about the same (53 %, liner empty; 54 %, milking), the liner again passing through the half open position when the pressure difference across its wall was in the region of 3–5 inHg. Fig. 3*b* shows particularly well that liner wall movement reflected the extremes of pressure difference across it, closing very tightly with the peak vacuum inside the liner and ballooning during the milking phase when the pressure inside was greater than the pressure outside.

Pressure changes under the teat with pulsation rates of 32 *and* 64 *c/min and fast and slow rates of change of pressure in the pulsation chamber* (expt. 4)

A short length of pulse tube with an 0·75 in. long restrictor of 0·125 in. bore was connected into the long pulse tube near to the pulsator by 2 T-pieces. By manipulating 2 pinch clips controlling the alternate routes it was possible to alter quickly the rate of change of pressure in the pulsation chambers of the cluster from fast to very slow. As the pulsation rate could also be changed rapidly, it was possible to take records of all 4 combinations of pulsation rate and pulsation characteristic during the course of a single milking. Results for one cow are summarized in Fig. 4.

The minimum and maximum pressures measured inside the liner were not greatly affected by either pulsation rate or rate of change of pressure in the pulsation chamber. However, it is notable that there were 2 peaks of high vacuum at the slow rate of pulsation (cf. Fig. 3*b*). Also the proportion of the cycle time that the liner was more than half open increased from about 30 % at the fast pulsation rate to about 50 % at the slow rate.

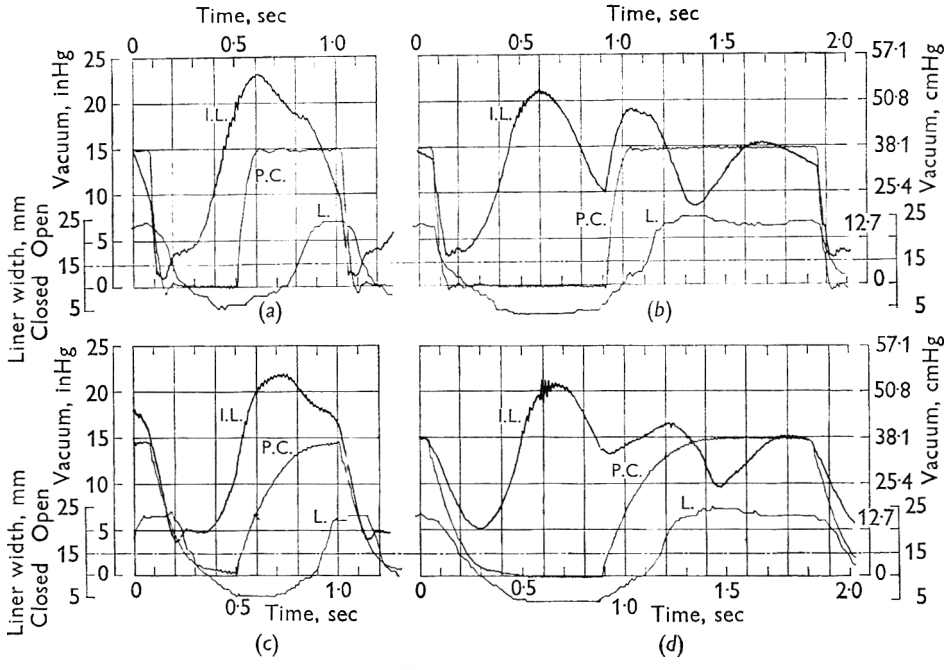

Fig. 4. Pressures measured in the pulsation chamber (P.C.) and inside the liner (I.L.), and liner wall movement (L.) at pulsation rates of 64 and 32 c/min, and with fast and slow pressure change in the pulsation chamber: (*a*) 64 c/min, fast pulsation pressure change; (*b*) 32 c/min, fast pulsation pressure change; (*c*) 64 c/min, slow pulsation pressure change: (*d*) 32 c/min, slow pulsation pressure change. Vacuum, 15·3 inHg; ratiometer reading, 49 %; % of cycle time that liner was more than half open: (*a*) 34 %, (*b*) 54 %, (*c*) 31 %, (*d*) 54 %.

The effect of bleeding air into the individual liners or into the clawpiece (*expt.* 5)

The 4 liners of a whole cluster were equipped with 0·018-in. diam. × 0·09-in. long air inlet nozzles carried on the inner ends of 0·125 in. outside diam. tubes inserted through the base of the liner. The nozzle inside the liner of the glass teatcup was

attached to a short piece of polythene tube inserted through the base of the liner as shown for the adjustable probe in Fig. 1. The height of the nozzle was adjusted for each cow to admit air about 0·5 in. below the tip of the teat. The rate of flow of air through each nozzle for a pressure difference of 15 inHg was 0·12 ft³/min measured at a pressure of 0·5 atm. These air inlets could be quickly closed by covering with adhesive tape and a single large airbleed hole in the clawpiece, 0·037 in. diam.. uncovered. It admitted 0·52ft³ of air/min measured at a pressure of 0·5 atm.

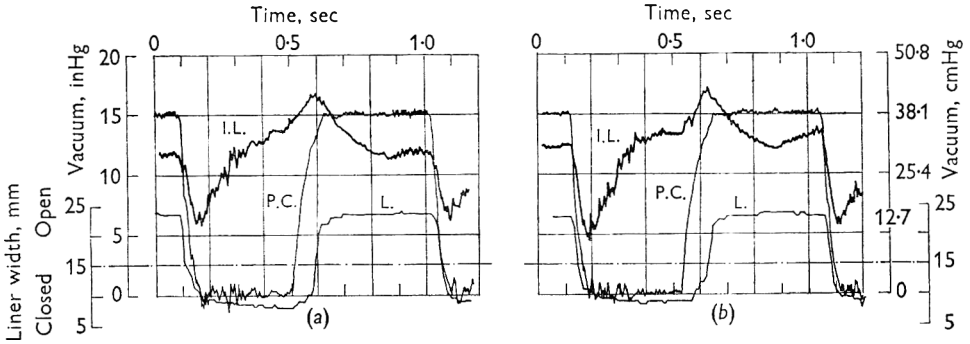

Fig. 5. Pressures measured in the pulsation chamber (P.C.) and inside the liner (I.L.) of one teatcup assembly of a cluster during milking, and liner wall movement (L.): (*a*) separate airbleeds to each liner each admitting 0·12 ft³ air/min measured at 0·5 atm; (*b*) single airbleed into the clawpiece admitting 0·52 ft³ air/min measured at 0·5 atm. Vacuum, 15·3 inHg; pulsation rate, 64 c/min; ratiometer reading, 49 %; % of cycle time that liner was more than half open, (*a*) 50 %, (*b*) 49 %.

The results in Fig. 5, compared with similar conditions in Fig. 4*a* without airbleeds, show a marked reduction in pressure fluctuation under the teat, and a marked improvement in response of the liner to pressure change in the pulsation chamber. The decline in vacuum under the teat as the liner closed was perhaps a little greater with the clawpiece airbleed than when the airbleed holes into the liner were open, but the maximum vacuum recorded was about the same.

The demonstration of resonance in the milking equipment (expt. 6)

It appeared that the most likely cause of the severe fluctuations in pressure inside the liner was movement of milk to and fro at a natural frequency dependent on the mass of milk and the elasticity of some of the components of the system. The originating impetus would presumably be the force exerted on the milk through the wall of the liner as air entered the pulsation chamber.

It was felt that this hypothesis would be strengthened if it could be demonstrated that pressure changes in the pattern of a damped natural frequency occurred when interfering forces were removed. The most favourable experimental conditions appeared to be the single teatcup assembly with a rapid change of pressure in the pulsation chamber, since this combination had given the steepest pressure changes inside the liner (Fig. 3). Expt. 3 was therefore repeated with the difference that during peak flow the pulsator was stopped first with the liner closed, and a little later in milking, with the liner open. The results obtained with one cow are given in Fig. 6.

With the liner held closed the presence of a resonant frequency at about 2·5 c/sec is clear. With the liner held open the resonance was less obvious, presumably because of interference by milk flowing from the teat. The frequency was then about 2 c/sec.

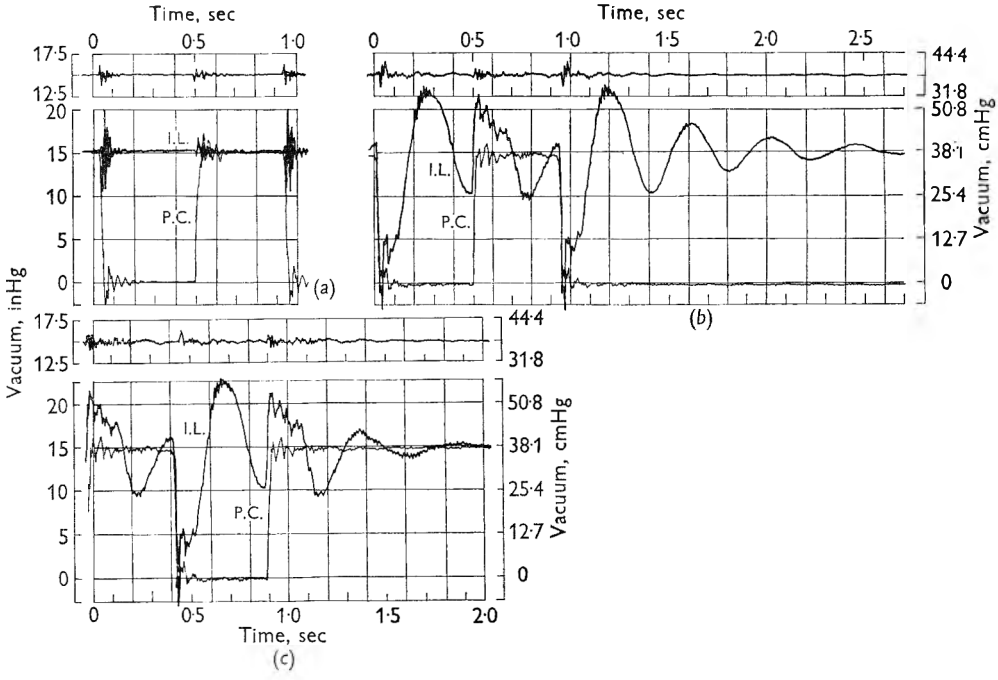

Fig. 6. Pressure records of the dummy probe indicating errors due to vibration (upper curve), and pressures measured in the pulsation chamber (P.C.) and inside the liner (I.L.) of a single teat-cup assembly attached to a 0·31-in. bore milk tube: (*a*) liner closed with a thumb; (*b*) pulsator stopped during milking when the liner was closed; (*c*) pulsator stopped during milking when the liner was open. Vacuum, 15·3 inHg; pulsation rate, 64 c/min; ratiometer reading, 47 %.

DISCUSSION

The fluctuations in pressure inside the liner beneath the teat were larger than expected. It seems, however, that they were not artifacts imposed by the measuring equipment, the best indication of this being the movement of the liner wall in accordance with the pressure difference across it, both when closing and opening. As the deviations from the nominal milking vacuum persisted for periods which were long compared with the duration of each pulsation cycle, it followed that the percentage liner open time was affected.

A striking feature, apart from amplitude, of the pressure variations under the teat is the length of time that a vacuum greater than the steady milking vacuum of the recorder jar could endure. Fig. 2, for example, shows a time longer than 0·4 sec. Even when the liner was, from the cine film, closed and apparently stationary the vacuum could remain above 15 inHg for as long as 0·35 sec (Fig. 4*b*). It was also noted that for any particular experimental arrangement of the milking equipment the pressure curves were remarkably similar for the 3 cows although their peak milk flow rates varied from about 3 to over 9 lb/min. Thus the amplitude and duration

of particular parts of the pressure fluctuations seemed to be much more related to the milking equipment than to the cow being milked. Finally, the pattern of pressure change, apart from the initial decline in vacuum as the liner was closing, almost disappeared when airbleeds were used.

The simplest explanation of these observations is that kinetic energy acquired by the milk as the liner closes is partly transformed to potential energy in the elastic parts of the system (gas bubbles and rubber), the minimum (or maximum) pressure measured corresponding to the moment when the milk becomes stationary. It would then be expected that the stored potential energy would give rise to motion of the milk in the reverse direction, the frequency of the cyclical energy transformation (and hence static pressure change) being the natural frequency of the system. In Figs. 2–5 the frequencies of such pressure changes are masked by the regularly occurring pressure changes in the pulsation chamber. When, however, the pulsator was stopped during milking with the liner closed (Fig. 6*b*) the pattern of pressure change clearly indicated the damped natural frequency of the system. When the pulsator was stopped with the liner open the pattern was less clear, presumably because of interference by milk added to the system from the cow.

As the natural frequencies of the milking equipment used were apparently not much higher than the pulsation frequency, it is not surprising that the pressure differences controlling liner wall movement were seriously interfered with. Adding substantial amounts of gas to the system by means of airbleeds slowed down the rate of recovery of vacuum inside the liner after it had collapsed. This might be interpreted as the expected decrease in natural frequency with increase in 'softness' of the system.

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Reviews of the progress of dairy science

Section E. Diseases of dairy cattle. Brucellosis

(*Received* 16 *May* 1964)

This review covers the period 1960–63 inclusive.

BRUCELLA PHAGE

Source and isolation of phage. Much of the recent work on brucella phage has been done using the Tbilisi (or Tb) phage isolated from liquid manure of a cowshed in the U.S.S.R. (Popkhadze & Abashidze, 1955). Since then, reports, especially from the U.S.S.R., have appeared on the isolation of phage from sewage, faeces and urine of cows, blood, urine, cerebro-spinal fluid, foetuses and internal organs of man and animals (Drozevkina, 1963), urine of infected goats (Lazuga & Renoux, 1960) and spleen of an infected mouse (Parnas, 1963*a*).

The isolation of phage from old laboratory, as well as from recently isolated brucella cultures, has been widely reported in Soviet and European literature. Ostrovskaya (1961*a*) demonstrated phage in 126 of 197 brucella cultures but pointed out that the phages were only weakly virulent and their lytic effect could only be seen on solid media; not all the phages could be passaged. Similar phage isolations have been reported by Lazuga & Renoux (1960), Parnas (1963*a*), Lazuga (1962) and Renoux & Suire (1963). Renoux & Suire showed that 60 unselected brucella cultures (representing *Brucella abortus, melitensis suis* and *intermedia*) and containing cell types

which gave rise to several morphologically distinct colonial types, were all naturally infected with phage. They concluded that smooth (S) and smooth-intermediate (SI) colonies do not carry phage and are phage susceptible. Rough (R) colonies do not carry phage and are phage resistant. Butyrous or sticky-white (P) colonies develop from carrier cells, carry phage and are phage resistant. The presence of phage could be demonstrated when plates were flooded with cultures but not when cotton-wool swabs were used for making lawns. However, in no case was phage propagated by these workers—the difficulty in doing so has already been pointed out (Ostrovskaya, 1961*a*). However, the isolation of virulent phage from lysogenic or carrier cultures has been reported by Drozevkina (1963) and Parnas (1963*a*).

Phage morphology. Morgan, Kay & Bradley (1960) showed that the Tb phage particles were polyhedral, 65 mμm across, hexagonal in plan view and bore a wedge-shaped tail 14 mμm long attached to one corner. They were indistinguishable morphologically from *coli* phage T3 and were believed to be the first of this morphological type outside the *coli-typhoid* group of phages. This morphology was confirmed by McDuff, Jones & Wilson (1962) and Parnas & Chiozotto (1962). Ostrovskaya & Solov'ev (1962) reported that the phage had a diverse morphology which was believed to be dependent on the stage of phage development. Many phage particles had no tail and others had a tail which, from their photographs, was longer than the head.

Plaque morphology. Morgan *et al.* (1960) showed that phage Tb gave rise to a mixture of large and small plaques, using *Br. abortus* Strain 544 as propagating culture. Subculture of single, small or large plaques always gave rise to a mixture of large and small plaques. This was confirmed by McDuff *et al.* (1962) and Edlinger (1962). McDuff *et al.* (1962) further showed that, by limiting the period of adsorption to 10 min by neutralizing free phage with phage antiserum, plaques of uniform size were obtained. The diversity of plaque-size was, therefore, a reflexion of the slow rate of adsorption of phage, phages adsorbed early producing large plaques and those adsorbing later producing small plaques. Jablonski (1962), working with Polish phages, described 3 plaque variants; neutralization tests with an antiphage serum gave identical K values against all 3 variants. Variations in plaque morphology were described by Jones, McDuff & Wilson (1962); the variations depended on the colonial morphology of the culture used as lawn.

Replication of phage. McDuff *et al.* (1962) showed that phage had a slow rate of adsorption on to brucella cells ($3{\cdot}6 \times 10^{-11}$ ml/min) with a latent period of 100 min and a burst size of 121 particles. They also showed that the latent period lengthened with the age of the bacterial culture. Edlinger (1962), using brucella phages 212 and 371, assessed the latent period to be at least 120 min, a burst size of 23 particles and a slow rate of adsorption. The changes occurring in bacterial cells after the addition of phage Tb have been studied by Ostrovskaya (1961*b*), and Ostrovskaya & Solov'ev (1960, 1962) and consisted of swelling, differentiation of protoplasm, formation of protuberances, granularity and complete disintegration. They confirmed the slow rate of adsorption. A phenotypic change in the colonial morphology of a culture of *Br. abortus* of intermediate colonial morphology due to a phage carrier stage was reported by Jones *et al.* (1962). Whereas phage produced complete lysis (at a 10^{-4} dilution) and clear plaques on cultures of smooth or smooth-intermediate colonial form, no lysis at 10^{-4} dilution occurred on a culture of intermediate colonial morphology

after 24 h of incubation. After 48 h, sticky white growth occurred in the area of the phage drop. Evidence was brought forward to suggest that the sticky white colonies were carrier colonies in which lysis was delayed until after cell division resulting in the establishment of a colony containing some phage-free progeny. The relationship between phage susceptibility and colonial phenotype of *Br. abortus* was studied by Kessel & Braun (1961). Mucoid colonies of genotypically smooth, penicillin-resistant cells of *Br. abortus* on penicillin agar were lysed by phage suggesting that the changes responsible for phenotypic changes in colonial morphology had no effect on sites responsible for phage adsorption. Changes in biochemical properties, metabolism and virulence of cultures after the addition of phage have been reported by Ostrovskaya (1960, 1961*b*), Dubrovskaya & Ostrovskaya (1960), Parnas (1963*a*) and Ciuča, Dimitriu, Cerbu & Pop (1962). [See also reviews by Drozevkina (1963) and Parnas (1963*a*).] Kessel, Aronson & Braun (1960) showed that untreated or ultra-violet-treated phage inhibited the multiplication of *Br. abortus* either under extra-cellular conditions or after phagocytosis by guineapig mononuclear phagocytes; heat-treated phage had no effect. They further showed that the effect on intracellular bacteria could occur when exposure to phage was delayed 2 h after phagocytosis of the brucellae.

Stability of phage. Brucella phage appears to be quite stable over a wide range of temperature and pH values (Ostrovskaya, 1961*b*; McDuff *et al.* 1962). Chloroform destroys the phage (McDuff *et al.* 1962) but toluene has no effect on it (Morgan, 1961, unpublished). McDuff *et al.* also showed that at 60 °C, for 1 h, 18 % of the phage was inactivated, and a 100 % was inactivated at 70 °C for 1 h; it can be lyophilized without loss of titre. At 4 °C the phage is stable for long periods (Ostrovskaya, 1961*b*; McDuff *et al.* 1962; Morgan, 1961, unpublished).

Use of phage in typing and classification. A large number of reports has appeared during the period under review on the susceptibility of brucella cultures to lysis by phage (van Drimmelen, 1960*a*; Drozevkina, 1963; Jones, 1960; Lazuga & Renoux, 1960; Lazuga, 1962; Morgan *et al.* 1960; Morgan, 1963; Münter, 1963; Meyer, 1961*a*, *b*; Picket & Calderone, 1963; Parnas, 1960, 1962, 1963*a*; Parnas & Burdzy 1963; Parnas, Burdzy & Münter, 1963; Wundt, 1962; Ulbrich & Weinhold, 1962; Ostrovskaya, 1961*b*). Many workers have used different phages and without reference to their titration. At the Round Table discussion on brucella phages, held in 1961 as part of the 4th International Congress for Biological Standardization, the phage Tb, as well as phages 212/XV, 371/XXIX, and P were recommended to be used for routine typing.*

Morgan *et al.* (1960), using phage Tb, and Jones (1960), using phage type *abortus* Strain 3, showed that at routine test dilution (R.T.D.), the phage lysed cultures of *Br. abortus* including dye-sensitive (type 2) Strains, and cultures that are *Br. abortus* culturally and biochemically and *Br. melitensis* serologically but did not lyse cultures of *Br. suis*. However, the phage lysed some cultures of *Br. melitensis* whereas other cultures of *Br. melitensis* were not lysed. It was found that cultures with the biochemical and serological properties of *Br. melitensis* isolated from cattle in Britain (British *melitensis*) were lysed by the phage, thereby distinguishing them from cultures of *Br. melitensis* isolated from sheep and goats—the classical *Br. melitensis*.

* These phages, with their propagating cultures, can be obtained from the FAO/WHO Brucellosis Centre, M.A.F.F. Central Veterinary Laboratory, Weybridge.

Jones also showed that cultures of *Br. melitensis* isolated from cattle in Malta were not lysed by phage. Whereas the majority of cultures of *Br. melitensis* isolated from cattle, which had so far been studied, were lysed by phage, not all cultures of *Br. melitensis* isolated from cattle were lysed. Recently, Payne (personal communication) isolated *Br. melitensis* from human blood as well as from bovine milk samples from the supplying farm. All these cultures were lysed by phage (and, in the author's laboratory were also shown to have the oxidative metabolic pattern of *Br. abortus*). Therefore, cattle and humans can be infected with both the British and Mediterranean strains of *Br. melitensis*; this fact in no way invalidates the usefulness of phage typing of *Brucella* as suggested by Parnas & Burdzy (1963). Ulbrich & Weinhold (1962), using phage F1 and F25 from South Africa, examined 143 strains of brucella and also found that cultures of *Br. abortus* were lysed, *Br. suis* were not lysed and some *Br. melitensis* were lysed and some were not.

The position was considerably clarified by the work of Meyer (1961*a*) (see also below under 'Classification of Brucella') who showed quite clearly that cultures with the oxidative metabolic pattern characteristic of *Br. abortus*, irrespective of their properties as determined by conventional tests, were lysed by phage type *abortus* Strain 3 at R.T.D. Morgan (1963) examined nearly 4000 brucella cultures using phage Tb and 4 Polish phages at R.T.D. and 10000 × R.T.D. All cultures of *Br. abortus* examined were lysed by all 5 phages at both dilutions. In addition, cultures with the oxidative metabolic pattern characteristic of *Br. abortus*, irrespective of their properties as determined by conventional typing methods, were lysed by both phage dilutions. Twenty-two cultures of *Br. suis* were not lysed by phage at R.T.D. but all showed lysis by phage at 10000 × R.T.D. Cultures of *Br. melitensis* and those with the oxidative metabolic pattern characteristic of *Br. melitensis*, irrespective of their properties as determined by conventional typing methods, were not lysed by phages at either dilution. All the 5 phages used displayed an identical host range; this close similarity was confirmed by neutralization tests with phage antiserum. Wundt (1962), using 8 Polish phages, found that all cultures of *Br. abortus* were lysed by all the phages, whereas cultures of *Br. suis* were not lysed. Of 17 cultures of *Br. melitensis* isolated from cattle in Northern Germany, 13 were lysed whereas of 29 cultures of *Br. melitensis* isolated in south-western Germany, only one was lysed. Münter (1963) examined 133 cultures with 22 phages (from U.S.S.R., Poland and South Africa). All 22 phages had an identical host range. Cultures of *Br. abortus* and *Br. suis* showed complete lysis at 10000 × R.T.D. but, at R.T.D. only cultures of *Br. abortus*, cultures that were culturally and biochemically *Br. abortus* but *Br. melitensis* serologically and British *melitensis*, were lysed. Cultures of *Br. melitensis* were not lysed. Parnas & Burdzy (1964) found that only a minority of cultures of *Br. suis* were not lysed at R.T.D. but showed lysis at 10000 × R.T.D. Many cultures showed no lysis with either dilution or were lysed by both dilutions. This work was not supported by oxidative metabolism tests nor was it stated whether all the cultures—especially those not showing lysis with phage at 10000 × R.T.D., were in the smooth phase. Picket & Calderone (1963), examining strains of *Br. suis* including the *Br. suis* type 3 (the so-called American *melitensis*), also found that they showed lysis at 10000 × R.T.D. Parnas (1963*a*) claimed that *Br. suis* Strain 1330, was lysed by phage Tb but that this did not always happen.

Meyer (1962) also showed that cultures of *Br. melitensis* isolated from cattle, as well as cultures of *Br. melitensis* producing H2S (see Stableforth, 1959) and those agglutinating in both *abortus* and *melitensis* monospecific antisera, were lysed by phage at R.T.D. and had the oxidative metabolic pattern characteristic of *Br. abortus*. Meyer & Morgan (1962) examined 87 cultures of brucella that showed conflicting identity by biochemical and serological methods and confirmed the previous observation of Meyer (1961*a*) that cultures with the oxidative metabolic pattern of *Br. abortus* were lysed. It was further shown that some cultures of *Br. intermedia* had the oxidative metabolic pattern of *Br. abortus* and were lysed by phage, whereas other cultures of *Br. intermedia* had the oxidative metabolic pattern of *Br. melitensis* and were not lysed by phage.

Drozevkina (1963) claims that all freshly isolated strains of brucella are susceptible to phage lysis because of the presence of a Vi antigen. This antigen—and therefore susceptibility to phage—is easily lost on subculture. Parnas (1960, 1963*a*) reported that, of a large number of phages studied, none was species specific. Lazuga & Renoux (1960) and Lazuga (1962) found similar results when testing 4 phages which they had isolated. Van Drimmelen (1960*a*) reported that he had obtained a host range mutant of phage Tb that also lysed *Br. suis* 1330 as well as *Br. abortus* Strain 544. *Br. melitensis* 16M was not lysed by either phage.

Cultures of *Br. rangiferi tarandi* (sp.nov. Davydov, 1961*a*), isolated from reindeer in the far north of Russia, as well as cultures of brucella isolated from caribou and from humans in Alaska (Huntley, Philip & Maynard, 1963), have been examined in the writer's laboratory. They were not lysed by phage at R.T.D. but showed lysis by phage at 10000 × R.T.D. This was also confirmed by Parnas & Sarnecka (1963).

CLASSIFICATION AND NOMENCLATURE

The use and value of the conventional methods for the classification of members of the genus *Brucella* have been given in excellent reviews by Huddleson (1961), Biberstein & Cameron (1961) and Stableforth & Jones (1963). [For earlier reviews, see Huddleson (1943) and Stableforth (1959).] The incidence of the types of brucella commonly found in various countries has been given by Alton (1960, 1963), Anderson, Pietz, Nelson, Kimberling & Werring (1963), Chilev & Milanov (1962), van Drimmelen (1960*b*), Doğuer (1961), Gulasekharem & Cockburn (1961), Ivanov, Kirillov & Kolesnikova (1962), Jones (1960), Kamel & Abdel Fattah (1961). Mathur (1963*a*), Morgan (1963), Münter (1963), Nižnánsky (1962*a*, *b*), Picket & Calderone (1963), Robertson (1961), Report (1961), Stableforth (1960), Ulbrich & Weinhold (1962), Vershilova & Ostrovskaya (1960) and Wundt (1963). In Britain, Stableforth (1960) found that 78·3 % of 415 cultures isolated from all sources from cattle were typical of *Br. abortus*, 10·4 % were British *melitensis*, 9·4 % were dye-sensitive *Br. abortus* type 2 and 0·7 % were *Br. abortus* biochemically and *Br. melitensis* serologically. An interesting variant of this last type but which was dye sensitive *Br. abortus* biochemically and *Br. melitensis* serologically was reported by Morgan (1963). Alton (1960) reported the isolation of dissociated strains of *Br. melitensis* from goat milk in Malta.

Redfearn & Berman (1960), using extracts of brucella against positive brucella

antisera, found that only those cultures which were agglutinated with monospecific *Br. melitensis* antiserum, irrespective of their reactions in other differential tests, gave positive reactions in gel diffusion tests; precipitin lines were given with either anti *Br. abortus*, *Br. suis* or *Br. melitensis* sera. They suggested that this method could be used instead of agglutination with monospecific antisera for identification. The usefulness of the gel diffusion technique in identifying *Br. melitensis* was confirmed by Vivek (1961).

Moreira-Jacob (1963*a*) found that medium containing 1/10000 dilution of Safranin O did not support the growth of cultures of *Br. suis* whilst allowing the growth of cultures of *Br. abortus* and *Br. melitensis*. This has been confirmed in unpublished work at the writer's laboratory. Cultures of *Br. suis* types 1, 2 and 3 as well as cultures isolated from reindeer and caribou (referred to earlier) did not grow on Safranin O medium with the exception of 3 cultures of *Br. suis* type 1 that were dye-resistant (grew on basic fuchsin as well as on thionin and Safrarin O). Bühlmann, Vischer & Bruhin (1960) found that cultures of *Br. suis* type 2 were more sensitive to phenazine dyes than cultures of *Br. suis* type 1 and 5 other cultures of brucella. van Drimmelen (1962*a*) described a urease test recorded as 'pH threshold values' for characterizing brucella strains. Vivek (1961) confirmed the value of the urease test in detecting *Br. suis*. The importance of the catalase test in distinguishing *Br. suis* from *Br. abortus* and *Br. melitensis* was stressed by Huddleson (1961) who also showed, on the basis of the catalase test, that cultures of 'American *melitensis*' were in fact *Br. suis*. This was confirmed by Meyer & Cameron (1963) on the basis of oxidative metabolism tests. The high catalase activity of *Br. suis* was also confirmed by Reusse (1960). Levaditi, Hult & Kchouk (1962) showed that triphenyl methane derivatives allowed the differentiation of *Br. suis* from *Br. melitensis* and *Br. abortus* and that thiazine derivatives distinguished between *Br. abortus* and *Br. melitensis* and *Br. suis*. Renoux (1960*a*) divided 1086 cultures of brucella into 4 groups on the basis of growth on Janus green (1/80000) and erythromycin (15 μg/ml). The results did not agree with the conventional methods using basic fuchsin and thionin. He pointed out that, in any case, both methods were arbitrary.

The use of the thionin blue sensitivity test for distinguishing between *Br. abortus* Strain 19 and field strains of *Br. abortus* was reported by Morgan (1961) who showed that sensitivity to thionin blue was not pathognomonic for Strain 19. Zorawski (1962) found that Strain 19 was more sensitive to Furacin than were other cultures of brucella and Drazan & Nižnánsky (1962) confirmed the greater sensitivity of Strain 19 to penicillin.

Davydov (1961*a*) reported on the properties of cultures of brucella isolated from reindeer in the far north of Russia. All 38 strains were aerobic, did not produce H_2S, grew on medium containing basic fuchsin and thionin and were antigenically distinct from *Br. abortus* and *Br. melitensis*. They were virulent for guineapigs and more virulent for reindeer than *Br. abortus* and were also of low pathogenicity for man. Davydov proposed the creation of a new species for these: *Br. rangiferi tarandi*. Similar results were reported by Pinigin & Petukova (1962) and Cherchenko & Bakaeva (1962), who considered that they were stable variants of *Br. melitensis*. Huntley *et al.* (1963) isolated and described cultures of brucella isolated from caribou (*Rangifer tarandus*) and from the native population in Alaska. These cultures were

aerobic, did not produce H_2S, grew on media containing basic fuchsin and thionin and showed a closer relationship to *Br. melitensis* than to *Br. abortus* or *Br. suis*.

Strains with the cultural, biochemical and serological properties indistinguishable from *Br. melitensis* have been isolated from cattle in Great Britain (Stableforth, 1960) although such strains had a low pathogenicity for man. Morgan *et al.* (1960), Jones (1960) and Parnas (1962) showed that these cattle strains, often referred to as 'British *melitensis*', differed from the classical Mediterranean strains of *Br. melitensis* in that they were lysed by phage. Jones (1960) also showed that the British *Br. melitensis* strains were more sensitive to thionin than the Mediterranean strains when incubated in air.

Of considerable interest in this connexion is the work of Meyer & Cameron (1961*a*, *b*). By using a series of amino acids and carbohydrates as substrates, they showed that the type 1 of each of the species *Br. melitensis*, *Br. abortus* and *Br. suis* had a characteristic metabolic pattern which was not influenced by dissociation and appeared to be independent of any change in dye tolerance. It was also shown that biotypes of *Br. abortus* displayed a pattern identical with that of the type 1 of the species, irrespective of their properties as determined by dye tests. All the biotypes of *Br. suis* differed from the other 2 species by their ability to oxidize L-arginine, DL-citrulline and DL-ornithine. The biotypes of *Br. suis* could also be differentiated from each other by differences in the rate of oxidation of L-glutamic acid and L-lysine.

In an extension of this work, Meyer (1961*b*) examined cultures that were atypical by conventional tests, including strains described as *Br. intermedia* (Renoux, 1952). Of 83 strains examined, 44 of *Br. melitensis*—which did not produce H_2S or required added CO_2 for growth—displayed the metabolic pattern characteristic of *Br. melitensis*. No metabolic variety was found and it was concluded that *Br. melitensis* constituted a homogenous species. Of the 24 cultures of *Br. intermedia* studied, 10 had the oxidative metabolic pattern of *Br. melitensis*, 13 of *Br. abortus* and 1 of *Br. suis*. It was concluded that *Br. intermedia* did not constitute a new species. Meyer (1961*a*) further showed that cultures with the oxidative metabolic pattern characteristic of *Br. abortus*, irrespective of their properties as determined by conventional typing procedures, were lysed by phage type *abortus* strain 3; cultures with the oxidative metabolic pattern of *Br. melitensis* were not lysed by phage. She also showed that cultures of *Br. melitensis*—including the British *melitensis*, had the oxidative metabolic pattern of *Br. abortus* and were lysed by phage; such cultures were, therefore, biotypes of *Br. abortus* and not *Br. melitensis*. This observation on cultures of British *melitensis* was confirmed by Meyer (1962) who also showed that cultures described as *Br. melitensis* but producing H_2S, or requiring added CO_2 for growth or agglutinating in both *abortus* and *melitensis* monospecific antisera had the oxidative metabolic pattern of *Br. abortus* and were lysed by phage and those with the oxidative metabolism of *Br. melitensis* were not lysed. Meyer & Morgan (1962) confirmed the work of Meyer (1961*a*) that cultures having the oxidative metabolism of *Br. abortus* were lysed by phage at R.T.D. They concluded that neither serological methods, using monospecific antisera, nor biochemical tests were a reliable guide to *species* identification in *Brucella*.

Wundt (1963) examined 98 cultures of brucella for their oxidative metabolism by methods essentially similar to those described by Meyer & Cameron (1961*a*). The

cultures could be divided into 3 groups corresponding largely with the 3 classical species. He also confirmed that only cultures with the oxidative metabolism of *Br. abortus* were lysed by phage and that strains of *Br. melitensis* isolated mainly from cattle in North Germany were similar to the British *Br. melitensis* strains in their phage susceptibility and their metabolic pattern.

At the 7th International Congress for Microbiology (Stockholm, 1958) a Subcommittee on Taxonomy of Brucella was appointed to study the question of species designation in the genus *Brucella*. In its report (Stableforth & Jones, 1963). the Subcommittee recommended the retention of the 3 species—*Br. melitensis*, *Br. abortus* and *Br. suis*—speciation being confirmed by oxidative metabolism test and phage susceptibility. The Subcommittee also proposed a number of biotypes within each species, biotype differentiation being based on the results of conventional typing methods. Accordingly, cultures of British *Br. melitensis*—which have the oxidative metabolism of *Br. abortus* and are lysed by phage at R.T.D.—were redesignated *Br. abortus* biotype 5. The Subcommittee was not satisfied that *Br. ovis* (Buddle, 1956) was a member of the genus *Brucella*; with regard to *Br. neotomae* (Stoenner & Lackman, 1957), it was recommended that further studies should be made before a decision on its position in the genus could be made. Parnas disagreed with the proposals of the Subcommittee and published his views separately (Parnas, 1963*b*). He advocated the creation of 4 species as follows:

Br. melitensis with 2 subspecies (*Br. melitensis* and *Br. melitensis intermedia*).

Br. bovis with 1 subspecies (*Br. bovis intermedia*).

Br. suis with 2 subspecies (*Br. suis thomsen* and *Br. suis intermedia*).

Br. atypica—to include 'species in the stage of development' such as *Br. ovis* and *Br. neotomae*. *Br. rangiferi tarandi* was also placed in the species *Br. atypica*. by Parnas & Sarnecka (1963).

In the writer's laboratory (Morgan, unpublished) cultures of *Br. ovis* isolated in different parts of the world have been examined for their oxidative metabolic rate using the substrates recommended by Meyer & Cameron (1961*a*). They all utilized L-alanine, L-asparagine and L-glutamic acid, thereby giving the same pattern as *Br. melitensis*. Cultures of *Br. rangiferi tarandi*, as well as a few cultures isolated from caribou in Alaska (Huntley *et al.* 1963) have also been examined. All the cultures had similar properties: aerobic; no H_2S produced; growth inhibited by basic fuchsin but not by thionin; agglutinated by both *abortus* and *melitensis* monospecific antisera and showing lysis with phage at 10000 × R.T.D. only; growth inhibited by Safranin O. On oxidative metabolism tests, they gave a pattern similar to that given by *Br. suis* biotype 3.

GENERAL BACTERIOLOGY

Transformation and stability of the species. Closely related with the question of taxonomy and classification of *Brucella* is the stability of the species. Clearly, if transformation from one 'species' to another occurs, either *in vivo* or *in vitro*, then any system of classification that ignores this would be of limited value. The whole question was discussed in an excellent article by Huddleson (1961). Some confusion has been caused (e.g. Karsten, 1960), since brucella isolated from pigs in the U.S.A were named *Br. melitensis* or American *melitensis*. They have since been correctly

classified as *Br. suis* type 3. Todorov (1962), by making multiple reseedings on peptone agar with 10 % glycerine or on Lowenstein–Jensen egg medium in 10 % CO_2 found that some cultures of *Br. suis* formed variants which superficially resembled *Br. melitensis*, and Sprockhoff (1960) claimed that, after repeated subculture of 4 strains of *Br. melitensis*, one reacted with immune serum against *Br. abortus* and not against *Br. melitensis* and all 4 produced H_2S. Mutations from Smooth (S) to rough (R) and R & S, after prolonged storage in sealed ampoules at room temperature were recorded by Jacotot & Vallée (1962*a*).

Variation of *Br. abortus* from type 2 to type 1 was shown to occur by Shibata, Isayama & Shimizu (1962) who showed that the mutation rate per cell division of type 2 was $6{\cdot}9 \times 10^{-10}$ thionin fast and $2{\cdot}2 \times 10^{-10}$ fuchsin fast. Type 2 organisms were found in milk predominantly with type 1 organisms. Large-scale species transformation was reported by Moreira-Jacob (1963*b*) as well as type changes within the species *Br. abortus*. In the writer's laboratory, 9 cultures of *Br. abortus* (3 of type 1, 3 of type 4 and 3 of type 5) have been repeatedly subcultured on medium containing either brucella negative or a positive bovine serum and, over a period of 12 months, there has been no change in biochemical, phage or serological properties except that cultures grown on the brucella positive serum medium were autoagglutinable. After 1 subculture on a brucella negative serum medium, the cultures behaved normally.

Huddleson (1961) attempted to change the biochemical and antigenic characteristics of brucella cultures by laboratory procedures, exclusive of animal inoculations. Cells of the different species could be adapted to grow on dye media which normally inhibited their growth but this was not considered to be a mutation. The only enzyme system which showed alteration was the one which forms H_2S from sulphur-containing compounds. The other biochemical and antigenic properties remained unchanged.

Antigenic structure. Barber, Dimitriu, Vasilesco & Cerbu (1961, 1962), extracted M antigen from cultures of *Br. abortus*, *Br. melitensis* and *Br. suis* using 90 % phenol, which gave characteristic precipitation lines in gel diffusion tests with homologous and heterologous antisera. Similar extracts, but weak in M antigen, were prepared using sodium deoxycholate. Parnas, Burdzy & Cegielka (1963) found that extracts of *Br. melitensis* 16 M, *Br. abortus* 544 and *Br. suis* 1330 gave 6 precipitation lines with homologous and heterologous antisera, but that 2 atypical strains gave fewer lines. Studies on DNA-sensitive antigens have been reported (Plescia, Noval, Palczuk & Braun, 1961; Palczuk, Plescia & Braun, 1961; Palczuk, Braun, Plescia & Cora-Figueroa, 1963; Olitzki, 1960). The latter author found that DNA-conjugated proteins from *Br. abortus*, *Br. melitensis* and *Br. suis* were highly antigenic but DNA-protein was not specific for any single species. Roux & Serre (1963), working with cytoplasmic and cell wall fractions of the 3 species, could not find species-specific fractions and Glenchur, Briand & Renoux (1962) and Glenchur, Seal, Zinneman & Hall (1963) showed that insoluble cell wall fractions of *Br. melitensis* provoked the production of agglutinins, precipitins, blocking antibodies and skin sensitivity. The soluble antigens were capable of producing all the secondary immunological phenomena. Smith, Keppie, Pearce & Witt (1962), using *Br. abortus* 544, also found that cell wall preparations were immunogenic for mice and guinea-pigs but that the soluble cytoplasmic content, although containing a number of precipitating antigens, had negligible immunogenic activity although capable of

provoking delayed hypersensitivity in infected guineapigs. Fleck & Evenchik (1962) described a technique for detecting small quantities of brucella antigen in biological materials by the inhibition of latex agglutination, and Shimizu & Shibata (1962) were able to get stable suspensions of rough forms of brucella using Menzel's buffer at pH 9·1. Such suspensions did not agglutinate in anti S sera.

Media for the primary isolation of brucella. A selective medium, consisting of antibiotics incorporated in a serum dextrose agar basal medium, was reported by Morgan (1960) to be the only one capable of supporting the growth of all the cultures studied using small inocula. Although Tween 80 could replace serum for the growth of *Br. abortus* type 2 strains in an unselective medium, it could not support their growth when antibiotics were added to the medium. A 'semi-synthetic' solid medium but also containing chick embryo extract, tryptose and trypticase soy broth, antibiotics and ethyl violet, was reported by Hejzlar & Duniewicz (1962) to be superior to other media for the isolation of brucella. A liquid medium for the mass cultivation of *Br. ovis* was described by Lawrence & Jones (1961). [See also review by Lawrence (1961) for various media for the growth of *Br. ovis*.] Other media have been described by Nikolaeva & Voitsekhovskii (1962), Entel (1960*a*, 1962) and Korotich, Kucherova, Mol'chenko & Netrebko (1960), who added seitz-filtered bovine amniotic fluid, and Perzadaev & Zhirkova (1960) who added testicular and ovarian extracts to basal media. Many batches of medium seem to be variable and should be tested for their ability to support the growth of fastidious organisms (e.g. *Br. abortus* type 2) from small inocula. The presence of negative serum enhances the growth of such strains.

Fluorescent antibody. There are numerous reports on the use of fluorescent antibody for the detection of brucella. Biegeleisen, Moody, Marcus & Flynt (1962) labelled the globulin fraction of *Br. suis* antiserum with fluorescein isothiocyanate, which stained 58 cultures of *Br. melitensis*, *Br. abortus* and *Br. suis* but not heterologous Gram-negative and Gram-positive bacteria. It was also found that the technique could detect brucella or antigen in animal tissues. Janney & Berman (1962) also found that a brucella fluorescent antibody specifically stained brucella cells and antigens in guineapig peritoneal exudate cells in tissue culture. Fluorescent antibody stained smears containing as few as 2500 brucella organisms/ml (Moody, Biegeleisen & Taylor, 1961), and 5000 organisms/ml (Chistov, Pesina & Voronova, 1961). The latter authors also found that the fluorescent antibody did not stain dissociated strains and, of 33 species of other bacteria studied, *Staphylococcus aureus* fluoresced after treatment with the labelled serum. Ignat'eva (1961, 1962) used the fluorescent antibody technique to follow the distribution of brucella in the tissue of guineapigs after experimental inoculation with Strain 19 and Strain 54 of *Br. abortus*. Brucella could be detected in lymph nodes and spleen 20 h after injection and in almost all organs after 3–5 days. Axt & Jentzsch (1962) and Jentzsch & Axt (1963) used 1-dimethylamino-naphthalen-5-sulphonyl chloride for labelling brucella serum and confirmed its specificity. Stolbikov (1961) used Rivanol (6,9-diamino-ethoxyacridine lactate) for obtaining gamma globulins for labelling with fluorescein isocyanate and found this easier than using ammonium sulphate to obtain gamma globulins.

Virulence. The biochemical properties of virulent and avirulent strains of brucella were ably discussed by Wilson & Dasinger (1960) who noted that 3 known biochemical differences between smooth strains of high and low virulence had been described:

1, Huddleson & Stahl (1943) reported that the catalase activity of *Brucella* spp. was directly related to virulence but this relationship held only within the species as each species had a different range of catalase activity (see also Huddleson, 1961); 2, Levine (1949) showed that the oxidative rates of strains of high virulence were stimulated by capryl alcohol over the endogenous rate whilst strains of low virulence showed an initial stimulation followed by a subsequent depression to a level below that of the endogenous rate; 3, Wilson & Dasinger (1960) and Dasinger & Wilson (1962) showed that *Br. abortus* strains of low virulence oxidized glutamate at a high rate whilst strains of high virulence oxidized glutamate at a relatively low rate. The latter observation was shown to be unrelated to differences in the pathway of glutamate oxidation or to differences in total enzyme activity. Of special interest also is the work of Ralston & Elberg (1961) and Ralston, Baer & Elberg (1961) [see also Elberg, 1960], who showed that brucella cells exposed to sufficient amounts of glycine were rendered susceptible to lysis by a lysozyme-like enzyme extractable from rabbit monocytes. Rough bacterial cells were more susceptible than smooth cells and a virulent strain of *Br. melitensis* was more resistant than the less virulent Rev. 1 variant strain. Their results suggested that glycine was taken up by monocytes and altered the intracellular bacteria so that the lysozyme-like factor could inhibit bacterial growth. Vershilova & Grekova (1960) showed that the amount of glycogen in the protoplasm of macrophages was proportional to their phagocytic activity. After inoculation with Strain 19, phagocytic activity was greater during the phase of generalization than after inoculation with *Br. melitensis*. Inclusions which reacted to a stain for polysaccharides was present between 12 h and 8 months after inoculation.

The cytotoxicity of nucleoprotein fractions from brucella was studied by Heilman, Rice, Howard, Weimer & Carpenter (1960). The nucleoprotein had no effect on splenic cells from normal guineapigs but had a pronounced effect on splenic cells from brucella-infected guineapigs; brucella antibody had no effect on this specific cytotoxic activity. Nucleoproteins from the 3 species of *Brucella* did not display species specificity when tested in a heterologous system. Carpenter, Fukuda & Heiskell (1962) also showed that brucella antigen caused lysis of spleen cells from brucella-infected guineapigs but had no effect on spleen cells from normal guineapigs. Lysis of 10–30% of lymphocytes and neutrophils was also observed by Feeley & Pickett (1962) when brucella antigen was added to heparinized blood from infected guineapigs and rabbits. Freeman, Kross & Circo (1961) reported that smooth, virulent brucella had little or no cytopathogenic effect on guineapig macrophages whilst rough brucella were rapidly destroyed. They postulated that, *in vivo*, rough cells killed the macrophages and were then liberated and destroyed by the host defences whilst smooth cells, being intracellular, were not so destroyed. Stinebring (1962) found that *Br. abortus* grown within guineapig mononuclear phagocytes showed an enhanced resistance to bovine serum bactericidins and enhanced ability to multiply in animals and Braun, Kessel & Pomales-Lebrón (1962) found greater multiplication of *Br. abortus* in monocytes from guineapigs vaccinated with formalized organisms than in monocytes from guineapigs that had survived infection with virulent *Br. abortus*. Furthermore, Kessel, Boughton & Braun (1961) found that *Br. abortus* did not multiply in monocytes from guineapigs treated with meprobamate

and that it was due to an indirect action of the drug. Aronson & Elberg (1962) reported that rabbit peritoneal histiocytes labelled with titrated thymidine did not lose any thymidine to *Br. melitensis* Rev. 1 which they had ingested, although histiocytes and brucella multiplied. Of considerable interest also is the work of Kessel, De Petris & Karlsbad (1963) and Pearson, Freeman & Hines (1963) who found that both smooth and rough brucella, ingested by guineapig monocytes, were enclosed in a vacuole in the host cell. The progeny of phagocytosed bacteria remained within the original ingestion vacuole, and it was suggested that this spatial separation of brucella inside the cell could be responsible for the resistance of intracellular brucella to inimical agents.

The presence of a spreading factor in cultures of brucella, irrespective of their virulence, was described by Mamatsashvili (1960). A simple and accurate micro-colony technique for brucellicidal antibodies was described by Bienvenu, Rode & Schuhardt (1961) who also reported (Bienvenu, Jr. & Young, 1962; Bienvenu Jr., Hyde, Young, Gibson & Peery, 1963), that the brucellicidal mechanism in non-vaccinated virgin heifers depended on the serum level of ionized magnesium. A lowering of the renal threshold for magnesium occurred in early pregnancy with a lowering of serum magnesium to a level below that required for protective brucellicidal activity. Simon & Berman (1962) found that streptomycin-dependent mutants of *Br. abortus* and *Br. suis* were avirulent for guineapigs whether selected in the presence of streptomycin only or streptomycin plus normal or immune serum. The pathogenicity of *Br. neotomae* was studied by Cameron (1963) who found that large doses failed to produce acute brucellosis in sheep or abortion in goats.

Resistance of Brucella. The effect of X-rays (Beutel, 1962), beta irradiation (Ryaguzov & Litvinov, 1960), disinfectants (Boden, 1963) and antibiotics (Agius & Mifsud, 1962; Agius, 1963; Rozansky & Sulitzeanu, 1961; Dimitriu, Cerbu & Vasilesco, 1961) have been reported. The synergistic action of streptomycin with other antibiotics on intracellular *Br. abortus in vitro* was studied by Richardson & Holt (1962) who found that streptomycin alone did not prevent multiplication but was bactericidal when combined with penicillin and tetracycline; penicillin and tetracycline alone were bacteriostatic.

A number of reports has also appeared on the survival of brucella in meat, soil, hay, water and after pickling and salting (Kosilov & Seletskaya, 1962; Ivanova, 1960; Zhurnakova, 1961; Ogarkov, 1962; Lerche, Entel & Mossdorf, 1960; Cedro, Cisale, de Benedetti & Gil, 1962).

PATHOLOGY AND PATHOGENESIS

Cattle. Considerable advances in the knowledge of the pathogenesis of brucellosis in cattle have been made by Smith, Keppie, Pearce, Fuller & Williams (1961). [See also Williams, Pearce & Smith (1961); Williams, Keppie & Smith (1962); Pearce, Williams, Harris-Smith, Fitzgeorge & Smith (1962); Smith, Williams, Pearce, Keppie, Harris-Smith, Fitzgeorge & Witt (1962).] These workers showed that the fulminating infection, after experimental inoculation of pregnant cows, was confined almost entirely to the foetal cotyledons, fluids and chorion; over 90 % of the total yield of organisms in each pregnant cow ($0{\cdot}3$–$1{\cdot}5 \times 10^{14}$) were found in those tissues. From the foetal fluids, erythritol was isolated and was shown to be the compound

responsible for the ability of these fluids to stimulate the growth of *Br. abortus* in bovine phagocytes and also for the preferential growth of *Br. abortus* in foetal tissue. Furthermore, erythritol was found in normal bovine foetal fluids, in placental extracts from goats, sheep and pigs and extracts of bovine seminal vesicle and testis but not in significant amounts from human, rat, rabbit and guineapig placentas. Very small amounts of erythritol stimulated, *in vitro*, the growth of *Br. abortus*, *Br. melitensis* and, to a lesser extent *Br. suis*, and injections of erythritol to calves 1–5 days old enhanced infection with *Br. abortus* and also enhanced the degree of infection in guineapigs challenged with *Br. melitensis* and *Br. suis*. It was also shown that, whereas erythritol stimulated the growth of virulent strains of *Br. abortus*, it had little or no effect on the growth of attenuated or avirulent strains (Williams, 1963). Anderson & Smith (1963) showed that erythritol was used as an energy source by a virulent strain of *Br. abortus* and that the yield of the organism was increased twofold. In conclusion, erythritol, which is a growth stimulant for members of the *Brucella* species, is concentrated in those tissues of animal species which are most heavily infected with brucellosis and its presence in those tissues may explain the predilection of *Brucella* for those sites.

Wagener, Bisping & Schulz (1963) reported that, after previous specific sensitization, abortion and retained placentas occurred in cows by the reinjection of killed brucella and that the macro and microscopic changes in the placenta were similar to those occurring after abortion caused by living organisms. [See also Urbaschek, Bäurle & Kotowski (1961).] The histopathological changes in foetus and foetal membranes were described by Wenzel (1960), using serologically positive cattle and non-reactors as controls. Payne (1960*a*) working with virgin heifers, found that progesterone had no effect on the pathogenesis of the disease. In non-pregnant heifers, the disease was milder and less progressive than in pregnant animals. The isolation of brucella from placentas which were microscopically negative for brucella was reported by Bürki (1962) and from herds free from clinical brucellosis by Seelemann, Börger & Meyer (1961).

Data on the excretion of brucella in the milk of experimentally infected cows have been given by Morgan & McDiarmid (1960), Rosaschino & Corsico (1960) and Schwerdtfeger (1963), the last author also giving details of the clinical, serological and pathological changes found in the udder and milk. Schwerdtfeger reported that the colony count varied from a few to 15000/ml of milk and were higher in strippings and in milk from the posterior quarters. Lambert, Amerault, Manthei & Goode (1961) studied the distribution and persistence of *Br. abortus* in cattle with complete clinical, serological and bacteriological histories before and after exposure. Calfhood vaccinated and non-vaccinated cattle, which had been naturally or artificially exposed to virulent *Br. abortus*, were autopsied and cultured at intervals from 3 months to 11 years. The tissues in which *Br. abortus* localized and persisted more frequently were (1) supramammary lymph node, (2) udder, (3) iliac lymph node, (4) retropharyngeal lymph nodes. The incidence of isolations was constant for 4 years after exposure and no differences were observed between naturally or artificially infected cattle. In addition to the persistence of infection for years in the supramammary lymph node, they also stress that calves fed on infected milk excrete *Br. abortus* in the faeces. The persistence of brucella in the udder and its associated lymph node

was also found by Entel (1961). The pathology and pathogenesis of udder infection in brucella-infected cows were also discussed by Jacob (1960) and by Renk (1962).

A high incidence of hygromas in infected cattle was found by Thienpont, Vandervulden, Fagard & Mortelmans (1961); old (7–10 years) cattle were most commonly affected. Outbreaks of *Br. melitensis* infection in cattle which were traced to sheep were described by Moldavskaya, Lifshits-Vasil'chenko, Yanchenko, Polyakov & Uraleva (1960) and Salmakov (1962) and infection of sheep with *Br. abortus* was described by Bannatyne (1960) and Khristoforov & Sivovski (1962). Voroshilov (1960) reported that a high proportion of infected cows, when kept in isolation for up to 3 years, became negative to the agglutination and complement-fixation tests; some of these were vaccinated and returned to negative herds with no evidence of any subsequent spread to healthy cows.

Studies on the persistence, clinical picture and post-mortem findings of 2 naturally infected bulls were reported by Lambert, Manthei & Deyoe (1963). They found that the serum and semen plasma agglutination tests persisted at diagnostic levels throughout the period of observation (5 and $2\frac{1}{2}$ years) and *Br. abortus* was consistently recovered from the semen. When bred to one of the bulls, 7 of 15 cows conceived and 2 later aborted. Boyd & Reed (1960) reported that cows in infected herds had a poorer fertility than those in non-infected herds.

Goats. Detailed bacteriological and serological studies, over a period of $2\frac{1}{2}$ years, on *Br. melitensis* infected goats were made by Renoux (1961). Transmission from the experimentally infected to non-infected, in-contact goats occurred after 5 months. Eighteen of 59 infected goats made a spontaneous recovery and 33 became chronic carriers. Excretion in vaginal mucus often occurred before parturition and could persist for as long as 33 weeks; milk excretion was irregular and persisted for as long as 5 months. Further, Renoux (1962*a*) showed that reduced fertility in a goat herd suffering from acute brucellosis, was of greater economic importance than abortion. Also, infection of kids depended on the severity and stage of infection in the dam. About a third of apparently healthy kids were carriers without clinical or serological evidence.

Sheep. A detailed study of the placental lesions found in sheep experimentally infected with *Br. melitensis* and *Br. abortus* was given by Molello, Jensen, Flint & Collier (1963) and Molello, Jensen, Collier & Flint (1963). *Br. melitensis* infection was characterized by placental oedema with small amounts of brownish red exudate in the periplacentome and widespread necrosis and cellular infiltration of the septal tips. A minimal inflammatory reaction was seen. With *Br. abortus* infection, the pathological picture was indistinguishable from infection due to *Br. melitensis* except that the inflammatory reaction was more marked, and there was less placental oedema and more periplacentomal exudate.

Brucella ovis in sheep. Ovine brucellosis caused by *Br. ovis* has been reviewed by Lawrence (1961) and Nižnánsky (1961). The pathology of *Br. ovis* infection in the pregnant ewe has been described by Hartley (1961), McGowan, Biberstein, Harold & Robinson (1962) and Molello, Flint, Collier & Jensen (1963) and in young rams by Gdovin (1960), Gdovin, Vrzgula, Bogdan & Belák (1961), Bogdan (1960, 1961) and Tudoriu *et al.* (1961, 1963). In the pregnant ewe, infection was localized in the interplacentomes with abundant exudate and moderate inflammatory reaction.

This localization in the interplacentome created no serious interference with metabolic interchange and was the essential difference between infection with *Br. ovis*, and with *Br. melitensis* and *Br. abortus* (Molello *et al.* 1963). Further, Hartley (1961) found that 60 % of lambs born in association with *Brucella ovis* infected membranes were alive at birth and also made the interesting observation that 50 % of lambs had calcified plaques on the walls of the hooves and accessory digits. McGowan *et al.* (1962) observed that ewes were more susceptible to conjunctival challenge in the second than in the third or fourth months of pregnancy. Clapp, Keogh & Richards (1962) showed that rams could be infected by shearing with artificially infected shears. Bogdan (1961) found that *Br. ovis* had an affinity for spleen, liver, lymph nodes and the vascular tissues especially of the kidneys and genital organs in rams. Periorchitis and epididymitis were common. This was also confirmed by Gdovin (1960) and Gdovin *et al.* (1961).

Horses. The clinical aspects of equine brucellosis have been described by Cosgrove (1961), Langenegger & Szechy (1963), Millar (1961), Poberezkin (1961), Ritscher (1963), Fechner & Meyer (1960) and Vandeplassche & Devos (1960). Structures other than the ligamentum nuchae may be affected and symptoms similar to those of undulant fever in man may be seen. Ritscher considered that it was essentially an allergic disease.

Laboratory animals. A number of reports has appeared on the course of brucellosis in laboratory animals. Urbaschek (1961) found that a lipopolysaccharide fraction of *Br. abortus* caused contraction of the isolated guineapig uterus and abortion in some pregnant guineapigs and rabbits. The distribution of brucella in the tissues of guineapigs and mice has been described by Watanabe, Yamano, Onchi & Inui (1960), Gafni, Olitzki & Ickowicz (1960), Pini & Gambino (1961) and by Sulitzeanu, Bdolach & Sperling (1960). Harper & Hood (1962) studied the retention of *Br. suis* in the lungs of mice exposed to clouds of mainly single cell particles of P-32 labelled *Br. suis* and found a mean lung retention value of 6·8 ml/min representing about 27 % of the respiratory minute volume. Spink & Bradley (1960) found that tetracycline therapy suppressed the multiplication of brucella in infected mice but that the number of mice with persistent infection was the same as in the untreated controls and that the treatment had no effect on the pattern of agglutinination titres. Payne (1960*b*) found that the ability of *Br. abortus* to infect the placenta of the pregnant rat depended on the route of infection and on the stage of pregnancy. Petzoldt (1961) was able to passively transfer the allergy produced by the injection of live brucella by intraperitoneal injection of serum. Knyazeva (1962) showed that the simultaneous injection of guineapigs with brucella and Q fever did not cause any interference but that the presence of Q fever enhanced antibody production. Bakaeva & Ostrovskaya (1963), using the slow complement-fixation test, could detect the presence of antigen in sera of guineapigs as early as 12 h after infection with 100 or more cells of *Br. melitensis*.

Brucellosis in other animals. Brucellosis in camels was reviewed recently by Rutter & Mack (1963). Brucellosis in dogs and their importance as possible carriers have been stressed by Akhmedov, Mikailov & Bairamov (1960), Aleandri & Mannini (1962*a*, *b*). Cheremisin (1963) and Ehrlein, Schimmelpfennig & Bisping (1963). The importance of hares has been stressed by Bendtsen (1960), Rementsova & Levit

(1960), Pavlov, Tchilev, Mateev Milanov, Tatarov & Krastev (1960), Willinger (1960), Hay (1960), Kardevan & Kemenes (1961), Klähn (1962), Kauker & Zettl (1962) and Fenske (1963), in rats (Renoux, 1960*b*); in deer, (Davydov, 1961*b*); Cherchenko, 1961; Cherchenko & Samsonova, 1961; Orloff, 1963; Huntley *et al.* (1963); in Saiga antelopes (Shulyak & Borisovich, 1961); in mink (Bisping & Löliger, 1963); in deer in U.S.A. (Ferris, Hanson, Rhoades & Alberts, 1961); in rodents and East African game animals (Heisch, Cooke, Harvey & de Souza, 1963; Rollinson, 1962); and in fleas and ticks (Parnas, Zwolski, Burdzy & Koslak, 1960; Rementsova, 1962; Khristoforov, 1961; Ozsan, 1962). No serological reactor was found by Youatt & Fay (1961) in a survey of Michigan wild life. Sidorov & Gubina (1962) found that Strain 19 lived in ticks (*Ornithodorus laborensis*) for 22 months without any change in its properties. Reviews on brucellosis in wild animals by McDiarmid (1960*d*) and Rementsova (1962, 1963) are also available.

DIAGNOSIS

General. A number of reports has appeared on the properties of anti-brucella globulins in man and animals and on factors which influence antibody formation, and a brief review of these is pertinent here. Studies on the heat stability of brucella agglutinins have been made by Amerault, Lambert & Manthei (1962), White & Roepke (1962) and Lambert, Amerault & Rose (1963). Sera of calves vaccinated with Strain 19 contained both heat labile (macro) and a heat stable (micro) anti-*Br. abortus* globulins and could be differentiated by heat treatment at 65 °C for 15 min. The macro globulin was also found in the sera of cattle known to be free of brucellosis, in heifers with persistent post-vaccinal titres and in cattle recently exposed to *Br. abortus*. The microglobulin was found only in the sera of infected or exposed cattle and was an indication of past exposure to *Br. abortus*. The characteristics of a 12S brucella agglutinin present in the milk of 2 cows which showed reactions to the milk ring test but were culturally negative, were described by Kenyon, Anderson & Jenness (1963). Shibata, Suzuki, Isayama & Shimizu (1961) also found that treating sera to 56 or 65 °C for 15 min reduced the titre, and that low titred sera were more affected than those with high titres. The specificity of agglutinins in low titred sera was also discussed by Guerra & Aleandri (1961). Blocking antibodies and precipitins in sera of man and in experimental animals have been studied by Glenchur, Zinneman & Hall (1961), Glenchur, Seal, Zinneman & Hall (1962), Zinneman, Briggs, Hall & Glenchur (1960) and in rats by Wolf & Live (1961). In the rabbit, the results suggested that the presence of blocking antibody indicated active infection or continued antigenic stimulation. In the human, the blocking antibody consisted of protein molecules which were considerably smaller than the agglutinating antibodies. The transfer of brucella agglutinins across the gut of rats and mice has been studied by Brambell, Halliday & Hemmings (1961) and Morris (1963) who found that, in young mice, the rate of absorption of antibodies depended on the antigen used for their production rather than on the animal species in which they were produced.

Hochstein-Mintzel (1962*a*, *b*) found that young rabbits, calves and a lamb, inoculated immediately after birth with Strain 19, developed positive serological titres.

Contrary to what might be expected, he found that the titres persisted for nearly a year in the calves and the lamb. Nagy (1963) exposed young lambs daily to Strain 19 for the first 65 days of life. When challenged as young adults with *Br. abortus* 544, there was a strong suppression of agglutinin formation but not of complement-fixing or of non-agglutinating antibodies. Renoux (1962*b*) also found that antibodies were not demonstrable in kids or guineapigs born of infected dams and kept in an infected environment. Silverstein, Uhr, Kraner & Lukes (1963) found that the foetal lamb, *in utero*, was able to produce large amounts of specific antibody but that the response depended on the antigen used, e.g. it responded well to phage and horse ferritin but not at all to *Salmonella typhosa* or B.C.G.

Cattle. The serum-agglutination test, using either the tube or rapid plate techniques, is still the most widely used and generally the most accurate serological test for the routine diagnosis of brucellosis in man and animals. The antigen used must, however, be standardized against the International Standard anti-*B. abortus* serum and the antibody content of any serum can then be expressed in terms of International Units (i.u.) of anitbody/ml. Experience in countries where the incidence has been reduced to low levels has shown that the test, especially the rapid plate test, misses a proportion of infected animals (see Anderson, Pietz, Nelson, Kimberling & Werring, 1963; Mingle, 1963*b*) and the test does not differentiate between agglutinins produced as a result of infection and vaccination. Much work has been done recently on the properties of anti-*Br. abortus* macro and micro globulins in order to help to both differentiate infection from vaccination and to aid in diagnosis especially in areas of low incidence.

Heuner (1961*a*, *b*) found that the addition of various salts to the brucella antigen permanently affected its sensitivity. Ulbrich & Heuner (1960) found that a mixture of antigens of *Br. abortus*, *Br. melitensis* and *Br. suis* was no better than single antigen whilst Karkadinovskaya, Zubkov & Shirobokova (1961) found a higher proportion of positive reactors using 12% instead of 0·85% NaCl as diluent. The slow tube-agglutination test was also recommended by Neumann & Kalicke (1960). Entessar (1962*a*, *b*) used sera and whey absorbed on to filter-paper for agglutination tests and also the surface fixation test (Ruiz Castañeda, 1961) and, in each case, obtained identical results with the rapid plate-agglutination test. The surface-fixation test, when properly standardized, could be very useful for the large-scale screening of sera. The latex-agglutination test (Wiedermann, 1961; Fleck & Evenchik, 1962) has the advantage that usually results can be read in 2 h. A technique for agglutination testing of haemolysed sera by precipitating the haemoglobin with phenol was described by Ferreira de Abreu & Mário (1960). Panda, Misra & Acharya (1963) reported that inoculation of cattle with haemorrhagic septicaemia vaccine led to an increase in brucella titres, whereas King (1961) observed no such effect.

A heat-inactivation (HI) test for differentiating specific from non-specific agglutination reactions in cattle sera was described by Amerault, Manthei, Goode Jr. & Lambert (1961). [See also Amerault *et al.* (1962); Lambert & Amerault (1962*a*); Lambert, Amerault & Rose (1963).] Sera from artificially and naturally exposed cattle were compared using the HI and agglutination tests; positive HI reactions were obtained from all cattle with positive or suspicious agglutination titres from which *Br. abortus* was isolated; 74% of the sera with suspicious agglutination titres

were negative to the HI in cattle from which *Br. abortus* was not isolated. Lambert & Amerault (1962*a*) also compared the HI. agglutination and complement-fixation (CF) tests using sera from artificially infected cattle.

Reactions to the HI test developed sooner than to the CF test but all 16 infected cattle gave reactions to both tests 140 days after exposure. In the group of animals which were challenged but did not become infected, some gave reactions to both the HI and the serum agglutination test shortly after exposure but all the animals except one were negative within 80 days after exposure. All these cows, however, remained negative to the CF test. The HI test has also been reported upon by Hellmann & Hein (1960) who essentially confirmed the results of Amerault *et al.* quoted above and also by Beimhauer (1962). A modification of the HI test, where the serum–antigen mixture was tested to 56 °C for 24 h was reported by Westphal & Dickel (1960). The importance of non-specific titres was also discussed by Hellmann (1961).

The use of acidified antigens in the plate-agglutination test has been reported by Rodabaugh & Elder (1961) and by Lambert & Amerault (1962*b*) who found that the test was of little value as a supplemental test in cattle with 'suspect' titres.

Since the last review, several papers have been published on the use of the complement-fixation test (CF), first to differentiate vaccinal from infection titres, and secondly as a supplemental test in chronically infected cattle with low or suspicious titres to the agglutination test. Although it was known from the work of Blagoveschtschenskaya (1954) and Yuskovets (1956) that, in animals vaccinated before 1 year old, the CF titres became negative sooner than the agglutination test, much of the recent work has been concerned with the problems of standardization of the test, e.g. time and temperature of fixation of complement, the antigen to be used and the use of a reference serum for evaluating the significance of titres obtained by different workers. These problems have been discussed by Hill (1960, 1963*a*), Bürki (1961, 1963), Mackinnon (1963) and by Jones, Hendricks & Berman (1963), who also reviewed the earlier literature.

A description of the technique and the results obtained in the Netherlands were given by Hill (1960, 1963*a*, *b*) and also by Bertschinger (1961) who used Hill's method in Switzerland. Hill (1963*a*) considered that sera showing $\frac{1}{12}$th the CF antibody content of the International Standard Serum should be considered positive. Burki (1960*a*, *b*, 1961, 1963) recommended that sera showing $\frac{1}{24}$th the CF activity of the International Standard Serum should be considered suspicious and, if higher, as positive. Mackinnon (1963) used the CF and agglutination tests on cattle sera of known brucella status. Under the condition of his test, he recommended that calfhood vaccinated animals whose sera showed $\frac{1}{12}$th the CF antibody content of the International Standard Serum should be considered positive; for non-vaccinated animals or those of unknown status, it should be $\frac{1}{24}$th of the content of the International Standard Serum. Lambert & Amerault (1962*a*) found that cattle reacted sooner to the HI than to the CF test but all the infected cattle reacted to both tests by 140 days after exposure. Except for one test on one animal, none of the cattle that did not become infected reacted to the CF test. Jones *et al.* (1963) found that, in several herds with recent infection, cattle reacted to the CF test before the agglutination test. These workers, using cold fixation, obtained 50% fixation at 1:80

dilution of the International Standard Serum. The use of the CF test has also been reported by Andersen (1961), Kocowicz, Ratomski & Wisniowski (1960), Wisniowski & Drozdzynski (1961) and van der Schaaf & Jaartsveld (1962). Hill (1963*b*) used several tests for diagnosis in cattle and concluded that the agglutination test at 56 °C, the CF and Coombs tests were especially useful in distinguishing between infection, vaccination, non-specific antibody and chronic infection and gave diagrams to illustrate their use and the interpretations of the results.

A comparison of the opsonin and agglutination tests has been reported by Seelemann, Czerniki, Meyer & Klepp (1960*a*, *b*) and by Bisping (1960) who found that the opsonin test indicated recent infection earlier than the agglutination test.

Antigen extracts for the CF test have been used by Khristoforov & Sorokin (1961), Trbić (1960) and by Gargani & Aleandri (1960), Aleandri & Gargani (1963). Pashkovskii & Zinenko (1961) found that the CF antigen was specific after $2\frac{1}{2}$ years of storage.

The vaginal mucus agglutination test has been used by Boyd & Reed (1960), Roberts & Philip (1960), Dubanský (1962), Anzcykowski, Murat & Walkowski (1962), Hubrig, Kielstein & Wohanka (1962) and Hignett (1963). Roberts & Philip (1960) confirmed that vaccination with Strain 19 led to the appearance of agglutinins in vaginal mucus, especially if mucus was collected by the tampon method.

The Coombs (antiglobulin) test has been reported upon by Fritzsche & Kohl (1960), Buchmeiser (1961), Schüster (1961), Hajdu (1963*a*) and Hill (1963*b*). The test is specific and of considerable value in the assessment of non-specific reactions and for early diagnosis. Its use is not recommended in vaccinated herds.

The milk ring test (MRT) is still widely used especially as a presumptive or screening test for bulk or can milk samples in order to locate infected herds and also in the maintenance of progress towards eradication. Further details of the preparation and standardization of the stained antigen were given by Anczykowski (1960). It is important, of course, that the antigen should be standardized against the International Standard Serum. Improved sampling procedures in creameries were advocated (Anon, 1961).

Blum & Kuhlmann (1961) found that, if the milk-antigen mixtures were incubated at 10 or 4 °C, a high proportion of both false positive and false negative reactions was obtained. No difference was seen between incubation at 37 and 20 °C provided the latter tests were read after 2 h (and not after 1 h as for the 37 °C test). An excellent account both of the mechanism of the ring-test reaction and on the effect of time and storage of milk on the test was given by Pietz, Anderson & Werring (1963). The formation of a cream layer is dependent on a fat globule agglutinin to aggregate the fat globules in clusters. This globule agglutinin is a lipoprotein and is both heat and agitation labile. Both the fat globule agglutinin and brucella agglutinins are associated in the MRT reaction. Salts of metals (used for preservation of milk samples) decrease the creaming ability of milk and, hence, of the MRT reaction. Milk samples containing preservative (0·52 g $HgCl_2$) showed extensive loss of reactivity to the MRT when stored at room temperature, which was associated with loss of the fat globule and brucella agglutinins. There was a moderate loss of reactivity in samples stored at refrigerator temperature, which was associated with the loss of brucella agglutinins, but the samples were satisfactory for routine tests.

The MRT dilution test is recommended by Hill (1963*b*), Anderson *et al.* (1963) and Mingle (1963*a*, *b*). In an excellent account on the diagnosis of brucellosis in problem herds, Anderson *et al.* (1963) showed that 90 % of 42 infected cows in 'group 1 problem herds' had MRT titres of 1/25 or more and 78 % of 67 infected cows in 'group 2 problem herds' had MRT titres of 1/25 or more. Hill (1963*b*) considered MRT titres of 1/10 or over to be probably due to infection. Ferguson & Robertson (1960) made the interesting observation that infected cows with high MRT titres on composite milk showed considerable variations in the titres of milk of individual quarters. Recently vaccinated cows had low MRT titres on composite milk with no variation in the titres of milk of individual quarters (see also Hill, 1963*b*).

The effect of vaccination with Dessau-killed vaccine on the milk serological tests has been reported by Hubrig (1961), Weser (1962) and also by Wilkowa (1960) using Strain 19 vaccine. Ugorski & Strojna (1960) obtained identical results when comparing the standard ring test and a capillary tube method. Schönberg & Lochmann (1962) used the MRT stained antigen for detecting antibodies in meat.

An evaluation of the various milk serological tests and of factors influencing them has been made by Boyd & Reed (1960), Hahn (1961), Biberstein, Cameron & Meyer (1961), Mackinnon, Lawson, Morgan, Lapraik & Williams (1962), Brus & Jaarstveld (1963), Hignett (1963) and Panda & Mishra (1963). All the milk serological tests are affected to some degree, by adult vaccination, stage of gestation and presence of mastitis.

Close agreement between the results of biological and cultural examinations of foetal stomach contents was reported by Prati & Gualandi (1961). The isolation of brucella from placenta pre-treated with slaked lime has been reported by Bouvier (1960*a*, *b*) (see also section on 'Media').

The use of the allergic skin test for diagnosis in cattle has been reported by Pleva & Beninghaus (1960), Schaetz, Busch & Krüger (1960), Jeřábek (1962), Duniewicz (1962) and Woloszyn (1962). In general, it has been concluded that the test is unreliable because of the large number of doubtful reactions and the occurrence of positive reactions in cattle with tuberculosis but free of brucellosis. However, Duniewicz (1962) considered the allergic test superior to the other serological tests and Woloszyn (1962) recommended its use to differentiate between infection and vaccination. Renoux (1962*c*), using guineapigs, found that the use of adjuvant vaccines did not lead to the development of allergic reactions and found that allergic reactions were produced only in those guineapigs harbouring brucella organisms.

Sheep and goats. The effect of vaccination on the serological response of sheep has been reported. Gulyaev (1961) found that the serum-agglutination test became negative before the CF test (75 and 315 days, respectively), after vaccination with Strain 19 and that they did not give reactions to the intradermal test. Cheremisin (1960, 1962) found that the milk of sheep gave positive reactions to the MRT and whey tests for about 3 weeks after vaccination with Strain 19.

Cedro, Cisale & Maubecin (1960) and Cedro, Cisale, Maubecin, de Benedetti & Vizio (1962) obtained satisfactory results using a whole blood plate agglutination test for goat bloods and that the Coombs test was the most sensitive for individual diagnosis. Vukelić & Bastalić (1960) obtained more positive reactors using 0·85 % saline for the serum-agglutination test, compared with the use of a 15 % saline

solution, but Ugorski (1962) found that the use of 10 % saline gave better results than 0·85 % saline. He also found that the use of the CF test markedly increased the number of positives. The use of a *Br. melitensis* antigen, standardized against the International Standard Serum was advocated by Strauch (1960) for diagnosis in sheep. An analysis of cultural and serological tests in experimentally infected goats was given by Renoux (1961). Some goats, although culturally positive, never developed antibodies. Serum antibody levels of at least 50 i.u. or over, CF titres of 1 : 4 or over and incomplete antibody titres of 1 : 10 or over were considered significant and a combination of the 3 tests was diagnostic in 90 % of cases. He also made the interesting point that goats infected at, or soon after birth, gave negative serological reactions. The use of the CF test for diagnosis in goats was also advocated by Alton (1961*a*) and the Coombs test by Gaumont (1963). The use of allergic tests has been reported by Entel (1960*b*), Mayer (1961) and Ostertag, Bihr & Beck (1961). The importance of standardizing the brucellin used was stressed and the fact that some preparations were in themselves antigenic, leading to complications on re-testing.

The diagnosis of *Br. ovis* infection has been reviewed by Lawrence (1961). Methods employed are genital palpation of the ram, CF test, allergic test and the microscopic and cultural examination of semen and foetal membranes. No single method is completely reliable especially for individual animals.

Clapp (1961) found that the anticomplementary activity of *Br. ovis* antigens could be removed by using soluble antigens free of cells or cell debris and that such antigens were also more sensitive. Cedro, Cisale & de Benedetti (1963*a*, *b*) also used a soluble antigen both for the CF and allergic tests and obtained good correlation between the results of these tests and clinical symptoms. Ris & Te Punga (1963) described an indirect haemagglutination test and found that it was satisfactory and specific. Van Drimmelen, Botes, Claassen, Ross & Viljoen (1963) used the fluorescent antibody technique for demonstrating *Br. ovis* in semen smears and claimed that it was superior to any other staining technique.

Pigs. The serum agglutination test is satisfactory for herd diagnosis but not so for individual animals. Caldas (1962) recommended a minimum diagnostic titre of 1 : 100; lower titres should be regarded as non-specific in the absence of herd infection but as suspicious when other pigs in the herd have titres of 1 : 100 or more. The use of a 5 % saline solution for the serum-agglutination test has been recommended by Badnjević (1963) and Mateev & Krustev (1963). Mateev, Tsonev, Krustev & Lyutskanov (1963*a*, *b*) found the CF test gave the most positive reactions. Stirling-Mócsy (1961) also recommended the use of the CF and agglutination test. He also advocated the addition of zymosan to pig sera to neutralize the high concentration of the third component of complement normally present in pig sera. A modified Coombs test, using sera heated to 65 °C for 10 min, was recommended by Trumić, Majstorivić & Panjecić (1960). Krüger (1963) found 77 % of pigs positive by serological testing, compared with 11 % by microscopical examination, 7 % by cultural and 51 % by biological tests. The allergic test gave better results than serological tests (Ivanov, Pashkovskii & Kapustin, 1961; Ercegovac *et al.* (1963).

Man. The diagnosis of human brucellosis has been reviewed by Dalrymple-Champneys (1960) and Ruiz Castañeda (1961). The tests used depend on whether they

are to be applied for diagnosis in individual cases or for screening and mass surveys to determine the incidence in an area.

Other animals. Diagnosis in horses is based on clinical symptoms, serum-agglutination test and brucellin eye test (Ritscher, 1963; Poberezkin, 1961; Cosgrove, 1961). The CF and allergic tests were recommended for diagnosis in dogs (Nazar, 1961).

VACCINATION

General. An apparatus for the continuous growth of Strain 19 was described by Hauschild & Pivnick (1961). Denser growth was obtained when continuous growth was combined with continuous dialysis. Perreau (1962) obtained good growth by using a fermenter, confirming the results of other workers. A liquid medium for the preparation of vaccines for the treatment of human infection was described by Zaitseva (1961). Pivnick & Crawley (1962) found that Strain 19, grown by continuous culture, was as safe and immunogenic in guineapigs as the vaccine grown on solid medium.

Diluents for the freeze-drying of brucella vaccines have been described by Heckly, Faunce & Elberg (1960), Semcheva & Goryunova (1960), Ivanov & Kovsh (1961), Ciortea, Bîrzu, Ionescu & Tigăeru (1962). Generally, those containing sucrose gave best results. Renoux (1962*d*) obtained satisfactory results using a 5% solution of polyvinylpyrollidone. Hulse, Hopkins & Hebert (1961) obtained more consistent results using the graduated pipette technique than the dropping method of Miles & Misra (1938) for counting viable cells of Strain 19. A technique for enumerating the live brucella in Strain 19 vaccine, using the rate of reduction of methylene blue, was described by Ivanov & Datsevich (1962). The keeping qualities of liquid and freeze-dried Strain 19 were described by Utojo, Girindra & Soeroso (1961) and Tekliński, Kochánski, Tereszczukowa & Denis (1961).

Human infection with Strain 19, involving a bacteriologist, was described by Revich, Walker & Pivnick (1961) and the treatment following accidental inoculation of Strain 19 in man was discussed by McCullough (1963).

Experimental vaccination of laboratory animals. Vershilova & Chernysheva (1961) reported that the inflammatory lesion produced by subcutaneous injection of live tularaemia vaccine rendered guineapigs resistant to subcutaneous challenge with either *Br. melitensis* or *Br. abortus* 1 day later, but not at 5 and 15 days after immunization. Sulitzeanu, Bekierkunst, Groto & Loebel (1962) also found that mice vaccinated with living or killed BCG resisted challenge with *Br. abortus* and, similarly, guineapigs immunized with living *Listeria monocytogenes* showed increased resistance to challenge with *Br. abortus* (Hellmann, 1962). Orlov & Kas'yanov (1961) compared the degree of protection in guineapigs and sheep given by the American and Russian Strain 19 and a rough variant of the American Strain 19. Although the numbers of animals used were small, best protection was given by the American Strain 19 and the least protection by the Russian Strain 19. The rough variant occupied an intermediate position, although Jacotot & Vallée (1960) found that rough forms of Strain 19 and of the Russian BA variant did not protect rats. Tests on the residual virulence of Strain 19, Strain 19BA, and Strain M104 which are used for human immunization in the Soviet Union, using guineapigs and mice have been reported

by Braude (1961), Shmuter, Lopatukhina, Sosunova, Yastrebova (1960*a*, *b*), Taran, Polyakova, Nelyapin & Lunina (1963); Taran, Polyakova & Chernysleva (1963), Koturga (1963) and Kurdina (1963). Strain M104 has a greater residual virulence than Strain 19 or its variant. The origin of the 19BA variant of Strain 19 is given in an excellent article by Vershilova (1961). Ivanov, Ulasevich, Borisovich & Klochkov (1961) also reported that Strain 19 and *Br. suis* strain 61 were more immunogenic in guineapigs than *Br. abortus* strains 1, 68 and 112. The effect of multiple inoculations of Strain 19 in guineapigs was studied by Shmuter, Koturga, Lopatukhina, Sosunova, Uzbekova & Yastrebova (1962) and of streptomycin-dependent mutants of *Br. suis* and *Br. abortus* by Simon & Berman (1962). Boyakhchyan, Agababyan, Vardanyan & Melikyan (1960), using P32 labelled Strain 19, found that labelled antigen persisted longer after subcutaneous injection than after either intravenous or intradermal injections. Borod'ko & Sansonovich (1962) found that the immunity of guineapigs given a combined live vaccine against plague, tularaemia, anthrax and brucellosis was much weaker than in guineapigs given a single vaccine but no such difference was found by Silich & Shevtsova (1962) using a combined brucellosis and Q fever vaccine. The possibility of aerosol immunization has been studied by Aleksandrov *et al.* (1962), Aleksandrov, Gefen & Gapochko (1963) and Zhovanik, Maĭboroda, Serdyuk & Primak (1961); oral immunization by Korolev & Konstant (1960) and intrauterine immunization by Payne (1961). The possibility of using *Br. neotomae* as a vaccine was suggested in work by Stoenner (1963) and Cameron (1963).

The question of cross and species immunity in brucellosis was studied by Vershilova & Kurdina (1963). Guineapigs, vaccinated with either *Br. abortus* Strains M104, 19BA, or *Br. melitensis* Rev. 1, were challenged with *Br. melitensis*, *Br. abortus* and *Br. suis*. They found no evidence that cross immunity was weaker than the immunity to the homologous type. Similarly, Keppie, Witt & Smith (1963) vaccinated guineapigs with crude, cell-wall preparations from *Br. melitensis*, *Br. abortus* and *Br. suis* and found considerable cross protection against heterologous and homologous challenge. The cell-wall preparation from *Br. suis* was the most promising in protecting against any of the 3 species.

A number of papers has appeared on the use of extracts for immunization. In an extension of their work, Smith, Keppie, Pearce & Witt (1962) found that crude cell-wall preparations from *Br. abortus* were immunogenic for mice and guineapigs, in contrast to the cytoplasmic contents which were not immunogenic. Furthermore, appreciable amounts of the immunogen were shed into the external environment both *in vivo* and *in vitro*. The immunogen was not connected with any of the precipitinogens seen on gel-diffusion plates but appeared to be connected with the ability to inhibit the bactericidal effect of normal bovine serum against *Br. abortus*. Both the immunogen and non-immunogens provoked delayed hypersensitivity in infected guineapigs. The immunogenicity of cell-wall preparations has also been confirmed by Roux (1962), Markenson, Sulitzeanu & Olitzki (1962) and Foster & Ribi (1962), who also found that aqueous ether extracts of *Br. abortus* gave better protection than either whole-cell or cell-wall preparations. Aqueous chloroform extracts of *Br. abortus* and *melitensis* were used by Aleksandrov *et al.* (1961) and soluble immunogenic antigen, which was liberated into the medium during the log death phase of growth, by Allen (1961). Suire (1960) reported good protection in mice using *Br. abortus*

Strain B112 plus cell-wall extracts of *Br. melitensis* and by Olitzki, Markenson & Margalith (1960) using DNA conjugated protein extracts.

Continuing their work on the Rev. 1 vaccine strain of *Br. melitensis*, McCamish & Elberg (1962) described in detail the bacteriological, serological and histological response of the guineapig to graded doses, and its degree of attenuation in monkeys. goats and guineapigs (Elberg & Faunce, 1962). The Strain Rev. 1 was passaged in pregnant goats, using pooled material from goats for inoculating the next series of goats and for viable counts and virulence tests in guineapigs. At the fourth passage, no brucella was recovered from the goats. These authors also examined Strain Rev. 1 isolated from blood culture of humans vaccinated with Rev. 1 (Spink, Hall, Finstad & Mallet, 1962). Although they were dissociated, these primary isolates were still able to infect guineapigs although they showed no changes in characters used to differentiate Rev. 1 from virulent strains. The greater residual virulence of Strain Rev. 1 in guineapigs was also confirmed by Kurdina (1961). Hall, Spink, Finstad & Mallet (1961) and Spink *et al.* (1962) also studied Strain Rev. 1 and the Russian Strain 19BA in human volunteers; 12 of 16 men inoculated with Rev. 1 and 4 of 16 inoculated with with 19BA developed clinical brucellosis.

Studies on the histological and serological response of rats to injections of Strain 45/20 (McEwen) have been made by Jacotot & Vallée (1962*b*), Pilet, Labie & Goret (1962) and Petit (1962). Both Pilet *et al.* and Petit found no agglutinin production but a marked neutrophilia but Jacotot & Vallée, using living Strain 45/20, found specific agglutinin production in rabbits and rats but not in guineapigs and cattle. A comprehensive account of the properties of compounds required as adjuvants is given by Wilner, Evers, Trautman, Trader & McLean Jr. (1963).

Cattle. A summary of controlled research with Strain 19 was given by Manthei (1960) who stressed that the vaccine controls but does not eradicate brucellosis (see also Safford, 1960). As with any other vaccine, the degree of protection afforded is not absolute—65–70 % of vaccinated animals are completely protected whilst the remainder have varying degrees of protection. It was also estimated that, where the majority of heifer calves are vaccinated, animal infection is reduced by 80 %, and herd infection by 20 %. McDiarmid (1960*a*, 1961) also reported that 80 % of vaccinated animals are protected against light conjunctival challenge (15×10^6 cells of *Br. abortus* 544) but about 50 % against heavy (150×10^6) challenge. McDiarmid stressed the danger of vaccinating cattle under field conditions without taking steps to reduce the amount of exposure to infection. Stableforth (1960) reported that, in Britain, the use of Strain 19 had been followed by a reduction in the abortion rate to a little over 2 %, of which a sixth was due to brucellosis. Stuart, Bills, De Mattei & Mace (1960) reported that vaccination of calves had reduced the incidence of brucellosis in California from 17–18 % to 2 % in dairy cattle and from 7–9 % to 0·7 % in beef cattle.

The degree of protection given by Strain 19 in calves vaccinated at different ages was reported by King & Frank (1961) and Lambert, Amerault, Manthei & Goode (1962). King & Frank found no difference in calves vaccinated at 3, 6 or 9 months of age, although the numbers of animals used were small. Lambert *et al.* vaccinated calves at 4, 6 and 8 months of age and similarly found no difference in the degree of protection given. They made the interesting point that vaccination at 4 months of

age materially reduced the problem of persistent post-vaccinal titres. Zasedateleva *et al.* (1962) also found no difference in immunity whether calves were vaccinated at 6–11 or 17–22 months of age but found that Strain 19a gave better protection than Strain 19.

Van der Schaaf & Jaartsveld (1962) compared the degree of protection given by a mucoid strain of *Br. abortus* (Strain L) and Strain 19 in both guineapigs and cattle, and using a thionin-resistant strain of *Br. abortus* as the challenge strain. Strain L gave better protection than Strain 19 but it persisted longer in both guineapigs and in cattle and tended to be excreted in the milk of cattle. The avirulent Strain LM61 was used on small numbers of cattle by Lembke & Meyn (1962).

The haematological and serological changes following Strain 19 vaccination have been described by Teute (1960), Krishna Murty & Hajela (1962), Gorbunova, Islamov & Makarova (1961) and Woloszyn (1962). Hubrig, Kielstein & Wohanka (1962) found that uterine immunization with 20 ml of Strain 19 vaccine resulted in higher agglutinin titres in vaginal mucus than in the serum but it induced delayed conception. The effect of colostral immunity on the antibody response of calves to Strain 19 was studied by Bisping (1962): 2 of 4 calves which were still positive to the agglutination and Meinicke tests when 8–9 months of age did not develop an antibody response after injection with Strain 19; the 2 calves which were negative at the time of vaccination gave positive reactions. Death of 2 calves following vaccination with Strain 19 was reported by Roberts, Squire & Gilman (1962).

McDiarmid (1962) reviewed earlier work in guineapigs on the value of McEwen's Strain 45/20 incorporated in a water-on-oil adjuvant (Falba and liquid paraffin) and also reported that 2 doses of this vaccine, given at 6 weeks' interval in cattle, gave good protection against a low challenge (15×10^6) of *Br. abortus* 544; one dose of the vaccine gave little protection. This vaccine does not give rise to persistent antibody titres, but it did give rise to a severe and persistent reaction at the point of inoculation. Strain 45/20 in a mineral oil has also been used successfully in France (Goret, Pilet & Goudot (1962), Pilet & Goret (1963), Pilet & Garrec (1963) and Leymonie & Serre (1963)). Berthelon, Royal & Rampin (1962), however, obtained poor results using Strain 45/20 in an unspecified adjuvant ('NeoBrucel') using small numbers of animals. It must be stressed that this adjuvant was different from the ones used by McDiarmid and Goret *et al.* (see above), and it is obvious that a great deal depends on the nature of the adjuvant used. Unpublished work at Compton (McDiarmid, personal communication) has shown that the degree of protection given by Strain 45/20 is influenced greatly by the adjuvant used, e.g. when incorporated in Falba-liquid paraffin, 2 inoculations gave good protection in cattle but when incorporated in a commercial adjuvant, it gave poor protection. This has also been confirmed in unpublished work at this Laboratory in guineapigs, using killed *Br. melitensis* cells incorporated in a number of different adjuvants. Based on ID50 estimates, guineapigs vaccinated with cells incorporated in a commercial vehicle, in $AL(OH)_2$ (Alhydrogel) or in sodium alginate had poor immunity but good protection was afforded when the cells were incorporated in a water-in-oil adjuvant (Falba and liquid paraffin or Bayol 55 + Arlacel A).

The degree of protection given by a killed vaccine adsorbed on to $AL(OH)_2$ (as described by Frahm & Lembke (1955*a*, *b*)) and known as the Sielbeck adsorbed

vaccine has been reported by McDiarmid (1960*b*), Ulbrich & Wiegand (1960) and Meyn & Schrinner (1961*a*). The last-named authors, using older animals (9–15 months of age) and 2 injections of the vaccine, obtained protection in 9 of 13 animals. Ulbrich & Wiegand (1960) using young calves 3–4 months old and one inoculation of the vaccine, found that it gave appreciably less protection than Strain 19. McDiarmid found that one dose of the vaccine gave good protection against a low challenge (15×10^6) cells of *Br. abortus* 544 but, against a high challenge dose (150×10^6 cells) all 8 animals became infected although 7 living calves were born. Furthermore, as pointed out by McDiarmid, it has also the same disadvantage as Strain 19 in producing post-vaccinal agglutinins.

Comprehensive reviews on the vaccination of cattle against brucellosis have appeared by Seelemann (1960), Pilet (1961), Goret & Pilet (1962, 1963) and the Report of the Annual Meeting of the French Society of Microbiology (Report, 1962).

Goats. Vaccination experiments in both goats and sheep have been reviewed by Renoux (1960*c*, 1962*e*) Jones (1962) and Berthelon & Royal (1962). Most of the work on goats has involved the use of the living Strain Rev. 1 vaccine or killed cells incorporated in various adjuvants. Alton (1961*a*, *b*, 1962) reported on the use of both the Rev. 1 and adjuvant vaccines (using Bayol and Arlacel A as adjuvant) under natural conditions in goats in Malta. He found that the degree of protection given by both vaccines was about the same and that the degree of infection in both vaccinated groups was significantly less than in the control (non-vaccinated) group. He points out that antibodies persisted for much longer after vaccination with the adjuvant vaccine and that it also produced a significant but not necessarily unacceptable local lesion at the point of inoculation. On the other hand, the Strain Rev. 1 vaccine must be given to goats at least 1 month before mating because of the danger of its excretion in milk and it cannot be used in pregnant goats (see also Elberg & Faunce, 1962). Alton (1962) also reported that vaccination of infected goats with the Strain Rev. 1 vaccine had no adverse effect and, indeed, seemed to prevent abortion which frequently occurred in goats infected but not vaccinated. Szyfres, Blood, Cedro & Mendy (1962) conducted field trials, using Strain Rev. 1 vaccine in goats in Argentina and reported that it gave no apparent protection. However, it is possible that at least some of the animals were infected before vaccination and the results were based on serum agglutination tests; Strain Rev. 1 vaccine, in any case, causes the appearance of reactions to the agglutination test.

Renoux has continued his work on the vaccination of both sheep and goats (Renoux, 1960*c*, 1962*b*, *e*) based on ID50 estimates in vaccinates compared with controls. He found that a killed suspension of *Br. melitensis* Strain 53H38 incorporated in a water-in-oil adjuvant (Mayoline 2214 and Arlacel A) gave best protection which lasted for at least 18 and probably for 35 months. In order to minimize the local reaction, the vaccine is administered subcutaneously on the medial aspect of the thigh. Van Drimmelen (1962*b*) found that the antibody response of sheep was greater when cells—either killed or living (Strain Rev. 1)—were incorporated in an adjuvant. The adjuvant itself had no effect on the viability of the Strain Rev. 1. In unpublished work at this Laboratory, it was found that Strain 19 gave no protection in goats against experimental challenge with *Br. melitensis*. The Strain Rev. 1 vaccine gave appreciable protection, especially against generalized infection.

Sheep. Strain 19 has been widely used in the Soviet Union for vaccinating sheep (Orlov, 1962; Ivanov & Kirillov, 1962) but the immunity is relatively weak and short lived and annual revaccination is recommended (Ulasevitch, 1961; Ivanov, 1963*b*). This may be due to the fact that Strain 19 is more rapidly eliminated from the tissues of sheep than, for example, Strain 104M which also produces a more intense cellular reaction and better immunity than Strain 19 (Abakin, Zamakhaeva & Chernysheva, 1962). Ivanov (1961, 1963*b*) also found that the dose of Strain 19 used was important and advised the use of $30–60 \times 10^9$ cells/injection. The degree of protection given by a number of vaccines, including *Br. abortus* Strains 19, 104M, *Br. suis* Strain 61 and *Br. melitensis* Strain Rev. 1 in sheep, have been reported by Orlov, Ulasevich & Ivanov (1962) and Ivanov (1963*a*, *b*). Their results clearly showed that the Strain Rev. 1 vaccine, in a dose of 3×10^9 cells, gave the best protection. Ivanov (1963*b*) also made the interesting observation that there was some relationship between the allergic reaction and immunity but not between the serological response and immunity. Work at this laboratory in both guineapigs and goats has also indicated that such a relationship exists. Renoux (1960*c*) also found that the ID 50 in sheep vaccinated with Strain Rev. 1 was approximately a 100 times greater than in the controls.

Neeman (1963) exposed groups of sheep vaccinated with either Strain Rev. 1, Strain 19 or adjuvant vaccine (together with a control group) to contact with 15 infected sheep. No infection was found in the groups vaccinated with either Strain Rev. 1 or Strain 19 vaccines; 2 sheep given the adjuvant vaccine were infected but only 8 of the 24 controls became infected, which makes it difficult to evaluate fully these results.

Aerosol immunization of sheep with Strain 19 was described by Selivanov (1963), and good immunity was produced. Pankratov, Tret'yakova & Smirnov (1960) found that sheep vaccinated with a combined anthrax, brucella and pox vaccine had good protection against sheep pox but not against brucellosis and anthrax; Ivanov & Kirillov (1962) also found that simultaneous immunization against brucella and anthrax greatly reduced the efficacy of the brucella vaccine. *Br. suis* Strain 61 was used successfully in sheep by Manukyan & Melikyan (1960) and Yuskovets & Ulasevich (1960); Bulanov, Nauryzbaev & Myakushina (1961) also had good results using Strain A6 which had been attenuated by passage in snakes. This strain persisted in the tissues for at least 57 days after inoculation.

The serological response of sheep vaccinated with different vaccines has been reported by Pankratov, Egoshin & Tret'yakova (1961), Lafenêtre, Carrère, Cortez, Vollhardt & Quatrefages (1962*a*), Kosilov (1963) and the cytological response by Cheremisin, Ivanova & Rastorgueva (1962). Lafenêtre *et al.* (1961, 1962*b*) compared the degree of protection given by a number of vaccines; they confirmed that antibodies persisted for at least a year after vaccination with H38 adjuvant vaccine and claimed good results using Strain 45/20 in adjuvant ('Neobrucel').

Br. ovis *in sheep.* Buddle (1962) and Buddle, Calvertry & Boyes (1963) found that two 1-ml doses of killed *Br. ovis* cells in water-in-oil adjuvant gave as good protection in rams as the simultaneous injection of Strain 19 plus killed *Br. ovis* cells. Water-in-oil adjuvants were superior to Alhydrogel ($AL(OH)_2$). They also found that 1 ram inoculated with Strain 19 excreted Strain 19 in the semen for at least 38 weeks.

Biberstein, McGowan Jr., Robinson & Harrold (1962) also confirmed that combined vaccination with Strain 19 plus killed *Br. ovis* cells in $AL(OH)_2$ adjuvant produced a solid immunity in rams; 4 of 8 rams vaccinated with the adsorbed vaccine alone became infected.

Peterson (1960) found that rams injected with Strain 19 via the intratesticular or intra-epididymal routes excreted Strain 19 in the semen but not after intravenous or intrapreputial inoculation. Severe lameness and stunting of growth were reported by Kater & Hartley (1963) in rams vaccinated with the combined Strain 19 and *Br. ovis* adjuvant vaccine. An osteomyelitis, affecting the distal ends of the tibia and radius was found and Strain 19 was recovered in one case. Van Drimmelen (1960*c*) and van Heerden & van Rensburg (1962) found that Strain Rev. 1 given to young rams gave complete protection against challenge with *Br. ovis*, but much less protection was given when mature rams were vaccinated. They, therefore, recommended that young rams should be vaccinated with Strain Rev. 1 at weaning age.

Pigs. A reduced abortion rate was reported by Ionica, Tudoriu, Cambir, Predoiu, Garoiu & Amdrei (1961) in sows given a combined vaccine consisting of Strain 19, adsorbed *Br. suis* and *Br. suis* lysate. Similar results were reported by Cedro, Cisale & Barrantes (1961) using an avirulent strain of *Br. abortus* plus heat killed *Br. suis*. It is difficult to assess the significance of these results as reactors were culled before the vaccinations began.

Man. Vaccination of man against brucellosis is widely used in the U.S.S.R., using Strain 19 or its variant Strain 19BA. A comprehensive account is given by Vershilova (1961). Only the population at risk is vaccinated either by the subcutaneous or intra-cutaneous routes. This last route has been shown to be effective, especially for re-vaccination to avoid sensitization. Re-vaccination is done a year later and is so arranged that maximum immunity is obtained at the time of greatest risk, i.e. at the lambing season. The whole programme is strictly controlled and the dangers of vaccinating infected persons is stressed. It is also recognized that this is a temporary measure pending the eradication of animal brucellosis.

Treatment in animals. The use of 'Pecudin' (*N'*-dichloroacetyl-*N'*-phenylsemicarbazide) in the treatment of bovine brucellosis has been reported by Buri & Wupper (1960), Börger (1960), Kauker & Zettl (1960), Diernhofer (1961) and Meyn & Schrinner (1961*b*). Given over prolonged periods it seemed to reduce abortion but not infection. 'Menaphthone' was used by Rosaschino & Perini (1963) and luteinizing hormones and vitamin E by Bordogna (1960).

INCIDENCE

In an extension of their previous work Ferguson & Robertson (1960) found that 3·5, 5·3 and 9 % of herd milk samples in different areas in South Scotland contained *Br. abortus*. The Public Health Laboratory Service (Report, 1961) reported that 4·8 % of herd milk samples in England and Wales contained brucella, but it was believed that the real herd incidence was about 15 %. George & Payne (1961) found that 7·8 % of 361 milk samples were biologically positive for brucella and Robertson (1961) found that 18·7 % of herds in Lancashire were infected. Robertson also makes the point that, whereas most milk sold in towns is pasteurized, only between 3 and 50 % is pasteurized in some rural districts in Lancashire. Boyd &

Reed (1960) found an incidence of 5·4 and 4·7 % of MRT reactors in Devon and Somerset, respectively. McDiarmid (1960*a*, *c*) found that 4·4 % of farms in the Oxfordshire area were infected and 2·2 % in the Isle of Wight (see also Brodigan, McDiarmid, Mann & Skone, 1961). These results were based on the biological testing of samples which had been initially screened by the MRT and then subjected to the whey tube test, only samples reacting to the whey tube test being tested biologically. It was believed that the real incidence in these areas were probably nearer 8–10 %. In a survey of the incidence of the disease in dairy cattle carried out by the Ministry of Agriculture, Fisheries and Food in Britain in 1960–1961, it was estimated that about 25 % of herds were infected and 2 % of the cattle infected (Ritchie, 1962). The incidence of the disease in Northern Ireland is given in papers by Kerr (1960*a*, *b*).

In Canada (Anon, 1960), it was reported that 9·5 % of herds and 1·3 % of cattle were infected (see also Wells & Frank, 1963). Figures on the incidence in the U.S.A. are given in papers by Mingle (1963*a*, *b*). Brucellosis in sheep (due to *Br. melitensis*) was first recorded in Argentina by Ossola, Szyfres & Blood (1963), but is common in cattle (Morales, 1960; Cedro, Cisale, Cacchione & de Benedetti, 1961) and in goats (Cedro, Cisale, Maubecin & de Benedetti, 1962). The presence of *Br. ovis* infection in rams was also confirmed for the first time by Szyfres & Chappel (1961). Brucellosis in goats was eradicated by a slaughter policy in Chile in 1961 (Cornejo Merino, 1963), although the incidence of bovine brucellosis is still about 20 %. In Peru, Velasco (1961) reported an incidence of 20, 10 and 12 % in dairy cattle, pigs and goats, respectively. Estimates of the incidence in buffaloes and dogs in Brazil were given by Santa Rosa, Pestana de Castro & Troise (1961) and Batista & Hipóloto (1962); Villagomez (1962) estimated that 36·5 % of stock in Mexico are affected. Brucellosis was reported to be fairly common in sheep in Western Turkey (Alpar & Massie, 1962) and in Greece by Dragonas (1960). The disease was first recorded in sheep in Poland by Chodkowski, Ugorski & Kowalski (1960) and in cows in North Vietnam by Spinu & Vasilesco (1962). The first recorded case of *Br. abortus* infection in swine in Japan was reported by Akaike, Ueda, Isayama & Shibata (1963).

Reports on the incidence of the disease in animals in the U.A.R. were given by Kamel & Abdel-Fattah (1961), Alton (1963) and Hamada El-Hidik, Sherif, El-Sawah & Yousef (1963); in South Africa by van Drimmelen (1961); in the Sudan by el Nasri (1960) and Dafaalla (1962); in India by Das, Panda & Dutta (1961), Murthy & Gupta (1962), Anon (1962), Pargaonker & Raj (1962) and Mathur (1962, 1963*b*), and in Italy by Tasselli (1961), Rosati (1961) and Battelli (1962). Bovine brucellosis is common in France, variously estimated as at least 20 % (Berthelon, 1962; Pilet, Berthet & Goret, 1963). The incidence of brucellosis in cattle, sheep and goats was reported to be greatly reduced in the Soviet Union (Orlov, 1960; Korotich, Slesarenko, Isaenko & Shcherbak, 1960; Naimanov, 1962; Ivanov, 1963*b*). Brodauf & Krüger (1961) found that the incidence in Baden had been reduced from 0·44 % in 1956 to 0·16 % in 1960; 0·11 % infected herds was reported in Rhineland Palatinate in 1961 by Feils (1962). Brucella was reported to be the commonest cause of abortion in cows in Queensland by McTackett (1963). [See also Benning, 1960 and Mylrea, 1961.]

The prevalence of brucellosis in pigs and its importance in causing human infection have been stressed by a number of workers e.g. in the U.S.A. by Hendricks (1962),

Hendricks & Hausler (1962) Hendricks, Borts, Heeren, Hausler & Held (1962), Harris, Hendricks, Gorman & Held (1962), Stuart, Wixon & Vanderwagen (1963) and in other countries by de Keyser, Spincemaille & Brone (1962). Mateev *et al.* (1963*a*), Pavuna (1962), Wittig, Teichmann & Kunter (1962), Robin (1962), Neftyanova & Kunev (1963). Pilet, Perron, Candeillan, Julienne, Ollivier & Goret (1963) and Caldas (1963).

The incidence of *Br. ovis* infection was reviewed by Lawrence (1961), The incidence in Slovakia was given by Hajdu (1962) and in South Africa by van Drimmelen (1961).

The majority of cases of human brucellosis arise either directly or indirectly through contact with animals or their products. Exact figures on the incidence are not readily available since the disease is not notifiable in many countries and because of difficulties in establishing a positive diagnosis. These were well discussed by Dalrymple-Champneys (1960). The incidence and epidemiology of brucellosis in Britain have been studied by Bothwell (1960*a*, *b*, 1961, 1963) who found that the rural and urban ratio was 3·5:1. Figures on the number of cases reported to the Public Health Laboratory Service in England and Wales for the years 1945–1961 were given by Bothwell (1963). The majority of cases appear to be due to the ingestion of raw, infected milk.

In the U.S.A. it is now estimated that 60% of the 700 human cases that occur annually are attributable to pigs (Hendricks, 1962) and an outbreak affecting employees of a swine slaughtering plant was described by Harris *et al.* (1962) and Hendricks *et al.* (1962). Although most of the cases were due to the handling of infected carcasses, there was also evidence of airborne infection and *Br. suis* was recovered from the air. The percentage of cases contracted from cattle has decreased from 36·8 in 1957 to 15 in 1960 and, since 1947, there has been a steady decrease in the number of human cases reported—from 6321 in 1947 to 741 in 1960.

In France, Berthet (1963) found that 88% of human cases were due to direct contact with cattle, 5·5% to contact with sheep and goats and only 0·5% due to milk ingestion. Similarly, Pilet & Goret (1962) studying the epidemiology of brucellosis in French veterinarians found that cattle were the source of infection in 310 of the 349 cases. Brucellosis was also found to be common in Belgian veterinary surgeons (Lafontaine, Andre, van Oye, Thomas & de Berdt (1963) and in the Potsdam district by Schwartz (1962)).

Ter-Bartanov & Kozlov (1961) suggested that a fairer expression of the incidence of human brucellosis is in terms of the animal population and not of the human population. Brucellosis as an occupational disease was also discussed by Rozansky, Weber, Lehman & Bali (1961), Jindřichová (1962) and Suntych (1962). The role of meat in epidemiology was discussed by Leistner (1960) and Sadler (1960); the greatest danger is to people handling such material. Outbreaks of brucellosis due to *Br. melitensis* infection in cattle (and not sheep and goats) have been described by Moldavskaya *et al.* (1960) and Shafershtein, Feoktistov, Pokrovskaya & Likhonos (1961). Brucellosis is one of the most readily acquired of laboratory infections and accidental infections due to the vaccine Strain 19 have been reported by Revich *et al.* (1961) and McCullough (1963) who stressed that the damage was due primarily to an allergic response in subjects specifically and highly sensitized to brucella antigen.

Serious sequelae to human brucellosis were discussed by Dalrymple-Champneys (1960), Bothwell (1960*b*) and Zinneman, Glenchur & Hall (1961). Peery & Belter (1960) reviewed the literature on heart disease associated with brucellosis and presented data on 44 deaths. The incidence of endocarditis was highest in *Br. abortus* infections.

CONTROL AND ERADICATION

The need for the control and eventual eradication of animal brucellosis is based on the economic loss to the agricultural industry and consequent loss of valuable food for the human population—often in areas least able to afford such losses, and on the prevention of human infections which are invariably derived, directly or indirectly, from animal sources. Human to human spread of brucellosis is very rare. Forbes (1963) reported that the losses to the cattle industry, due to brucellosis in 1934 in the U.S.A., were 125 million dollars and, at present, are 25 million dollars annually. Furthermore, whereas in 1934, there were 10000 human cases, this has now been reduced to around 700 cases a year. Berthelon (1962) estimated that loss of calves and milk to the French dairy industry amounted to 64 million francs a year and in the Argentine, to $2\frac{1}{4}$ million dollars a year (Cedro, Cisale, Cacchione & de Benedetti (1961)). In Britain, McDiarmid (1960*a*) estimated the loss to be 16 million pounds sterling a year. In countries such as the U.S.A. and Scandinavia where brucellosis has been eradicated, or nearly so, there has been a marked decrease both in economic loss to the cattle industry and in the number of human cases of disease.

In Britain, the major brucellosis problem is in cattle (Stableforth, 1960). The control of the disease was reviewed recently by Ritchie (1962). Control is based on calfhood vaccination with Strain 19 with the object of building up a national herd well protected against the disease. On 1 May 1962 a free vaccination service was introduced, applying to female calves between the ages of 151 and 240 days; calves so vaccinated are identified by a tattoo mark. The statutory provisions were also reviewed by Ritchie (1962) and by Bothwell, McDiarmid, Bartram, MacKenzie-Wintle & Williamson (1962); suggestions for revising and simplifying the existing legislation were also made by Bothwell *et al.* (1962) as well as the establishment of pilot control schemes in areas of known incidence.

In Northern Ireland, brucellosis was made a notifiable disease in 1959 and, in that year, an eradication scheme was started, initially to cover herds selling raw milk (Grade 'A' milk) to the public (Kerr, 1960*a*, *b*, 1963; Kerr & Rankin, 1963). At the same time, a Register of Brucella-free herds was established to cover all types of herds. The need for controlling indiscriminate vaccination and the movement of animals was stressed.

Van Waveren (1960) reported that 82·5% of herds in the Netherlands were then free of brucellosis. The progress towards eradication in the U.S.A. has been reported by Mingle (1961, 1962, 1963*a*) and Forbes (1963). The programme is designed to have the whole country 'Modified Certified' by 1965 and 'Brucella-free' by 1975. Good progress in the swine eradication programme in Georgia and California was reported by Mingle (1963*a*) and experience had shown that it was best dealt with on a herd basis, the disposal of individual blood test reactors having a limited application.

No date had been fixed for the completion of eradication in Canada (Anon, 1960). The level of infection then was estimated as 8·5 % of herds and 1·3 % of animals infected.

A plan for the prevention of brucellosis in Europe was proposed by Wøldike Nielsen (1961) (see also Senthilie (1962) and Berthelon (1963)). Hess (1961) reported that a programme of eradication based on the slaughter of aborting cows and those excreting brucella in milk was started in the Canton of Zurich and had since been extended to the whole country. Strain 19 vaccination was used only exceptionally in infected herds. Although herds were screened regularly using the MRT, agglutination testing of individual cattle sera was done every 2–4 years. In the absence of vaccination, this period may be too long. Hajdu (1963*b*) reported the eradication of bovine brucellosis in 140 communities in Slovakia, based largely on the use of the Coombs test: calves reared in a disease-free environment were used as replacements. In Bulgaria, (Tchentchev *et al.* 1962) eradication of bovine brucellosis was based on the use of State isolation farms to which a selection of infected cows were sent and their progeny used for replacements, infected cows were then slaughtered. Rearing piglets in isolation and the slaughter of infected sows was also used to control porcine brucellosis (Dimitrov, 1962). In the German Federal Republic, vaccination of calves was recommended by Börger (1961). In the Ukraine Korotich, Slesarenko, Isaenko & Shcherbak, (1960) stated that brucellosis in sheep and goats might be eradicated by 1962 and that the incidence of bovine brucellosis would also be further reduced (see also Ivanov, 1963*a*, *b*).

Methods of the control of animal diseases—including brucellosis, which are in use in many countries, were presented recently at a meeting of the Office International des Epizooties (OIE) in Paris. The countries covered were Canada (Wells & Frank, 1963), Mexico (Ornelas, 1963), Chile (Cornejo Merino, 1963), U.S.S.R. (Ivanov, 1963*b*), Finland (Huhtala, 1963), Albania (Babameto, 1963), India (Sahai, 1963), Pakistan (Khan, 1963; Jabbar, 1963), Iran (Kaweh, 1963), Syria (Ladkany, 1963), Thailand (Subharngkasen, 1963); Angola (Dias & Nobre, 1963), Mozambique (Castro Amaro, 1963), Southern Rhodesia (May, 1963), and Senegal (N'Gom, 1963).

Br. ovis infection was eradicated from 13 flocks in Australia (Clapp *et al.* 1962) by the slaughter of infected rams. Control of the disease in Australia is based on such a 'test and slaughter' policy which is successful provided all rams, including vasectomized rams, are regularly tested (see also Lawrence, 1961). In New Zealand (Buddle, 1962) and in South Africa (van Drimmelen, 1960c; van Heerden & van Rensburg 1962) control of *Br. ovis* infection is by vaccination and this policy has been reported as being responsible for a marked reduction in ram wastage and perinatal losses in lambs.

In conclusion, experience with cattle has shown that vaccination alone will not lead to the eradication of the disease. A programme based on the vaccination of young stock and the simultaneous slaughter of infected animals is more likely to succeed. The dangers of re-introducing the disease across national boundaries and the spread from one species to another (e.g. sheep and goats to cattle) and from wild animals to domesticated animals, must also be borne in mind.

SUMMARY AND CONCLUSION

During the 4-year period under review, much knowledge has been accumulated on pathology and pathogenesis of brucellosis, on supplemental diagnostic tests and on methods of classification of members of the genus *Brucella* by the use of phage and oxidative metabolism tests. Even so, brucellosis is still a serious problem—both in man and animals, in various parts of the world. Reservoirs of infection in wild animals have been reported and these could have an important bearing on eradicating the disease, e.g. in pigs which acquire infection from hares. Countries, in which active eradication programmes are in existence for the eradication of bovine brucellosis, report considerable economic saving as well as a marked reduction in human cases. In countries where control measures are based on a vaccination policy, the abortion rate has been significantly reduced, but the infection rate, as judged by excretion in milk, has not been so greatly reduced.

Brucellosis in sheep and goats still remains a serious problem in many countries and a test and slaughter policy alone is unlikely to succeed in eradicating the disease. Vaccination, together with the segregation and elimination of reactors, would appear to be a more realistic approach. Any such programme must be done on a national scale in order to have any measure of success.

A 'test and slaughter' policy or vaccination alone has been successfully used in controlling *Br. ovis* infection in sheep.

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