JOURNAL OF DAIRY RESEARCH

Volume 33 Number 1 February 1966

CAMBRIDGE UNIVERSITY PRESS

THE JOURNAL OF DAIRY RESEARCH

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

The annual subscription to the *Journal* is 80*s*. (U.S.A. $13.50) for a volume of three parts. Single copies may be purchased at 30*s*. (U.S.A. $5.00) each, plus postage. Subscriptions may be sent to any bookseller or to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Subscribers in the U.S.A. or Canada should send their orders to Cambridge University Press, American Branch, 32 East 57th Street, New York, N.Y. 10022.

Back Volumes. Enquiries about the price and availability of back volumes should be sent to the publishers.

Claims for the replacement of journals believed to have been lost in transmission will only be entertained if made within six months of publication.

JOURNAL OF DAIRY RESEARCH

VOLUME 33, 1966

CAMBRIDGE UNIVERSITY PRESS

CORRECTION

Journal of Dairy Research, **32**, 1

A method for studying the factors in milk which influence the deposition of milk solids on a heated surface

H. BURTON

Page 78. The last equation should read:

$$\theta = \left(\theta_m + \frac{C}{k_m}\right)\left(1 + \frac{CD}{k_d}t\right).$$

The effect of concentrates on the voluntary intake of roughages by cows

By R. C. CAMPLING and J. C. MURDOCH
National Institute for Research in Dairying, Shinfield, Reading

(*Received* 25 *August* 1965)

Summary. Seven changeover experiments were conducted to examine the effect of giving restricted amounts of concentrates on the voluntary intake of different roughages by non-lactating dairy cows. The addition of up to 6 kg concentrates daily to the diet of cows receiving roughage *ad lib.* caused little change in the intake of hay and a small increase in the intake of barley straw. Larger amounts of concentrates of 6 and 8 kg daily reduced hay intake by between 0·2 and 0·4 kg dry matter/kg concentrate dry matter given. When concentrates were given to the cows the rate of decline in intake of hay tended to be greatest with the hays of highest digestibility. The daily addition of up to 8 kg concentrates to the diet of cows offered silage *ad lib.* caused only small depressions in the intake of silage. The results are discussed in relation to recent work on this subject with cattle and sheep.

In farming practice in the United Kingdom, concentrates are frequently given in restricted amounts to cattle offered roughage *ad lib.* In spite of the common occurrence of this practice there appear to be few reports of experiments examining the effect of giving concentrates on the voluntary intake of roughage by cows. Also, there is considerable variation among the results reported from the few British experiments on this subject. For example, Danasoury (1954) found in 2 experiments with lactating dairy cows offered silage *ad lib.* and restricted amounts of concentrates that voluntary intake of roughage dry matter declined by about 0·6 lb/lb concentrate dry matter given. Holmes, Arnold & Provan (1960), also working with lactating cows, observed that increasing the daily intake of concentrates from 4 to 16 lb caused no significant depression in the intake of silage by the cows and only a slight depression in the intake of hay. Other similar examples were discussed by Corbett (1961). Reid (1956), reviewing American experiments, concluded that the reduction in the intake of roughage when concentrates were given to cows seemed to be less marked with high-quality than with low-quality roughages. More recently, Blaxter & Wilson (1963) found that, in the sheep, the voluntary intake of hay increased with increasing digestibility, although when concentrates were given in restricted amounts the depression in intake of hay was related inversely to the digestibility of the hay; the more digestible the hay the greater the depression of hay intake. With hay of low digestibility (45 %) small additions of concentrates increased the voluntary intake of hay and Blaxter & Wilson (1963) suggested that this was due to the protein contained

in the concentrate since it is well known that supplements containing nitrogen often increase the voluntary intake of low-digestibility roughage by ruminants (Morris, 1958; Campling, Freer & Balch, 1962).

In the present investigation further information was obtained on the effect of giving restricted amounts of concentrates on the voluntary intake of different roughages by cows.

EXPERIMENTAL

Seven changeover experiments with non-lactating dairy cows were done.

Expt. 1. The experiment was designed to test the suitability of changeover designs for measuring the effect of a standard concentrate on the the voluntary intake of roughage by cows. In the analysis of changeover experiments where each animal receives consecutively 2 or more treatments, it is usually assumed that no significant interaction exists between treatments and periods, and this experiment was designed to verify this assumption. Two treatments were compared, hay *ad lib.* (X) and hay *ad lib.* with a daily supplement of 4 kg/cow of a standard concentrate (Y). A reversal design was used with four 3-week periods and 2 groups of 6 cows (A and B) as follows:

	Period			
	I	II	III	IV
Group A	X	Y	X	Y
Group B	Y	X	Y	X

Expt. 2. This was a preliminary experiment to study the effect of 0, 2, 4, 6 and 8 kg concentrate/cow daily on the voluntary intake of hay, silage and barley straw. A randomized block experimental design was used with 4 blocks each of 3 cows and 5 periods each of 3-weeks duration. The cows were allocated to blocks on the basis of liveweight and within blocks to roughages at random.

Expts. 3, 4 *and* 5. These experiments were conducted to confirm the results observed in expt. 2. A 6×6 Latin square experimental design was used for expts. 3 and 4, using 6 cows and 12 cows, respectively. The periods were of 3-weeks duration. The following treatments were compared:

Expt. 3
- i Hay *ad lib.*
- ii Hay *ad lib.* + 3 kg concentrate daily
- iii Hay *ad lib.* + 6 kg concentrate daily
- iv Barley straw *ad lib.*
- v Barley straw *ad lib.* + 3 kg concentrate daily
- vi Barley straw *ad lib.* + 6 kg concentrate daily

Expt. 4
- i Hay *ad lib.*
- ii Hay *ad lib.* + 4 kg concentrate daily
- iii Hay *ad lib.* + 8 kg concentrate daily
- iv Barley straw *ad lib.*
- v Barley straw *ad lib.* + 4 kg concentrate daily
- vi Barley straw *ad lib.* + 8 kg concentrate daily.

Expt. 5 consisted of a 5 × 5 Latin square design with 5 cows and periods of 3-weeks duration and was used to compare the following treatments:

Expt. 5
- i Silage *ad lib.*
- ii Silage *ad lib.* + 4·5 kg concentrate daily
- iii Hay *ad lib.* + 4·5 kg concentrate daily
- iv Late-cut hay *ad lib.*
- v Late-cut hay *ad lib.* + 4·5 kg concentrate daily.

The silage and hay (treatments i, ii and iii) were prepared from similar herbage and the late-cut hay (treatments iv and v) from the same sward cut about 3 weeks later, and cured and baled in the field in good weather. The silage was made from unwilted herbage with the addition of 2 gal molasses/ton of herbage.

Expt. 6. The experiment was designed to measure the effect on the voluntary intake of hay by cows of giving large amounts of the standard concentrate. A 4 × 4 Latin square design with 8 cows and periods of 3-weeks duration was used to compare the following treatments:

Expt. 6
- i Hay *ad lib.*
- ii Hay *ad lib.* + 8 kg concentrates daily
- iii Hay *ad lib.* + 11 kg concentrates daily
- iv Hay *ad lib.* + 14 kg concentrates daily.

Expt. 7. An examination was made of the effect on voluntary intake of hay of the type of concentrate mixture given. A 4 × 4 Latin square design with 8 cows and periods of 27-days duration was used to compare:

Expt. 7
- i Hay *ad lib.*
- ii Hay *ad lib.* + 6 kg daily of a mixture of oats and decorticated groundnut meal
- iii Hay *ad lib.* + 6 kg daily of a mixture of flaked maize and decorticated groundnut meal
- iv Hay *ad lib.* + 6 kg standard concentrate daily.

Cows and housing

A total of 40 non-lactating, non-pregnant adult cows were used, the majority of the cows were Friesian and the rest Ayrshire, Shorthorn and a few crossbreds between these breeds. The cows were weighed at weekly intervals and the mean liveweight of the cows on each experiment was within the range 591–643 kg. The cows were housed in individual standings in a cowshed and sawdust and wood shavings were used as bedding material. Water was available from drinking bowls at all times.

Foods

The proximate chemical composition of the roughages is given in Table 1 and where available the apparent digestibility coefficients of the dry matter of the roughages. The digestibility of some of the roughages was determined in unrelated experiments in the usual way by the total collection of faeces from 3 cows or sheep

over 10-day periods. During the digestibility trials the cattle were given daily about 4 kg roughage dry matter and the sheep were offered roughage *ad lib.* With the exception of those used in expt. 5 the roughages were not prepared especially for the experiments. Most of the hays used contained in the dry matter about 8–10 % crude protein, 28–39 % crude fibre and the dry matter varied in digestibility from 58 to 65 %; however in expt. 5 the silage and one of the hays were of considerably higher quality. The content of dry matter in the silage used in expt. 2 was determined by drying samples at 100 °C for 42 h in a Unitherm oven. In expt. 5 the content of dry matter in the silage was determined by a toluene distillation method (Minson & Lancaster, 1963). The 3 batches of barley straw used were similar and contained about 3·5 % crude protein and 44 % crude fibre in the dry matter, with a mean digestibility of the dry matter of 51 %.

Table 1. *Chemical composition and apparent digestibility of the dry matter of the roughages used*

			% of dry matter					
Expt.	Roughage	Dry matter, %	Crude protein	Ether extract	Crude fibre	Nitrogen-free extract	Ash	Apparent digestibility of dry matter, %
1	Hay	82·4	8·1	2·0	38·6	45·3	6·0	—
2	Hay	83·4	7·2	1·2	31·1	54·3	6·2	65·2*
	Barley straw	83·2	3·4	1·5	43·4	44·3	7·4	50·5*
	Silage	18·6	16·7	3·8	31·7	38·0	9·8	—
3	Hay	81·5	9·8	1·8	34·6	47·1	6·7	59·2†
	Barley straw	80·9	3·9	1·4	46·1	42·8	5·8	52·4†
4	Hay	84·7	7·9	1·5	36·8	48·3	5·5	57·9*
	Barley straw	85·5	3·0	1·8	43·6	44·4	7·2	49·9*
5	Silage	21·9	12·6	2·9	33·2	39·0	12·3	77·1*
	Hay	83·1	12·5	1·8	29·4	46·7	9·6	77·7*
	Hay, late-cut	82·7	9·1	1·4	37·5	44·5	7·5	58·6*
6	Hay	80·0	9·9	2·3	27·8	51·0	9·0	63·2*
7	Hay	83·5	7·1	1·5	36·9	48·8	5·7	57·9†

* Digestibility determined with cattle.
† Digestibility determined with sheep.

In all experiments a concentrate mixture of standard composition and in the form of pellets was used, its mean proximate chemical composition is given in Table 3; the mixture contained (pts by wt.) barley 17, maize 20, wheat bran 20, decorticated groundnut meal 15, copra cake 10, palm kernel cake 5, molasses 10, dicalcium phosphate 1, calcium carbonate 1 and salt 1, with 5×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D added per ton. The variation between different batches of the same standard concentrate mixture was small, for example, the content of crude protein in the dry matter ranged only from 19·5 to 21·1 % and throughout this report mean values are used. The mean digestibility of the dry matter of the standard concentrate was 81 % when given to 3 non-lactating cows as the only food in amounts of 8·5 kg daily (Freer & Campling, 1963). In expt. 7 the standard concentrate was compared with mixtures composed of 82·5 % rolled oats and 17·5 % decorticated groundnut meal

and of 82·5 % flaked maize and 17·5 % decorticated groundnut meal both with added minerals and vitamins A and D. The latter 2 mixtures were not pelleted; their chemical composition is given in Table 2. Although in expt. 7 it was planned to make the 3 concentrate mixtures of similar crude protein content, analysis showed that the mixtures composed mainly of flaked maize and of rolled oats contained about 16 % crude protein and the standard concentrate contained 19·5 %.

Determination of voluntary food intake

The cows were given food in equal amounts in 2 meals daily at 09.00 and 16.00 h and concentrates were given before the roughage. The amount of roughage offered to each cow was adjusted daily so that about 15 % remained uneaten at the end of a meal. The duration of each meal was $2\frac{1}{2}$ h and at the end of a meal the uneaten food was removed and weighed.

In calculating the mean results of each experiment only values for the last week of each period have been used; those from the initial 2 weeks were ignored because of possible carry-over effects from one treatment to another.

Table 2. *Chemical composition of the concentrate mixtures used*

			% of dry matter				
Expt.	Food	Dry matter, %	Crude protein	Ether extract	Crude fibre	Nitrogen-free extract	Ash
1–7	Standard concentrate	85·9	19·5	1·9	5·7	65·5	7·4
7	Oats and groundnut meal	84·2	16·1	2·7	8·8	68·9	3·5
7	Flaked maize and groundnut	83·8	15·5	1·0	2·0	79·2	2·3

Table 3. *Interaction between treatments and periods with cows fed on hay or hay + concentrates*

	Mean daily voluntary intake of hay, kg dry matter		
Treatment	Periods I and II	Periods III and IV	Periods I–IV
Hay *ad lib.*	7·91	7·94	7·92
Hay *ad lib.* + 4 kg concentrates	7·42	7·58	7·50
Treatment differences	0·49	0·36	0·42
S.E. of difference			±0·135

RESULTS

Expt. 1. The mean daily voluntary intake of hay with each treatment during the last week of each period (Table 3) was 7·92 kg dry matter with hay alone, and was significantly depressed ($P < 0·05$) to 7·5 kg by the addition of 4 kg concentrates. During periods I and II the mean daily depression in hay intake caused by 4 kg concentrates was 0·49 kg dry matter and in periods III and IV 0·36 kg. Thus, the interaction between treatments and periods was small (0·13 kg dry matter) and statistically not significant ($P > 0·1$). The absence of a significant interaction between treatments and periods

suggests that in this respect changeover designs could be used in similar experiments investigating the voluntary intake of roughages by cows.

Expt. 2. This experiment was a preliminary study of the effect on voluntary intake of hay, silage and barley straw of different amounts of concentrates (Table 4).

The differences between the voluntary intakes of the 3 roughages by the cows were marked. On a dry-matter basis the mean intake of hay (8·8 kg) was nearly twice that of the barley straw (4·8 kg) and in spite of the low content of crude fibre and high protein content in the silage the intake of this roughage (5·9 kg) was considerably below that of the hay. It was noticeable that the voluntary intakes of hay and silage by the cows tended to decline when concentrates were given but the addition of 2, 4 and 6 kg concentrates increased the intake of straw. Although there were no significant differences ($P > 0{\cdot}05$) between the intakes of hay with different amounts of concentrates, there was a marked tendency for the voluntary intake of hay to decrease when the cows were given 6 and 8 kg concentrates. The decrease in the mean intake of hay observed when 2 kg of concentrates were given was due to a difference in intake in 1 cow; with the other 3 cows hay intake tended to increase slightly. On average, the daily addition of 6 and 8 kg concentrates to the diet decreased hay dry-matter intake by 2·1 and 2·5 kg, respectively, or by about 0·38 kg hay dry matter/kg of concentrate dry matter.

Table 4. *Expt.* 2. *Effect of concentrates on the mean daily voluntary intake of roughage by* 12 *cows*

	Mean daily intake of roughage (kg dry matter) when given concentrates at daily rate (kg) of					S.E. of a difference between two means
	0	2	4	6	8	
Hay	8·75	7·52	8·28	6·68	6·29	±0·875
Silage*	5·93	6·03	5·10	4·72	4·40	±0·326
Barley straw	4·75	5·44	6·01	5·12	4·50	±0·254

* One missing value was calculated.

With silage, the addition of 4, 6 and 8 kg concentrates significantly depressed ($P < 0{\cdot}01$) the intake of silage dry matter below that observed when silage was offered alone or with 2 kg concentrates daily. There was no significant difference ($P > 0{\cdot}05$) between the voluntary intake of silage dry matter when the cows were given 4, 6 and 8 kg concentrates daily. However, intake of silage dry matter decreased by 0·83 kg daily with 4 kg, by 1·21 kg with 6 kg and by 1·53 kg with 8 kg concentrates, and thus the depression in intake of silage dry matter/kg of concentrate dry matter given was only about 0·23 kg.

The voluntary intake of straw dry matter tended to increase when the cows were given 2, 4 and 6 kg concentrates and to decrease slightly with 8 kg. The intake of straw was greater with 4 kg concentrates than with any other treatment.

Expts. 3 *and* 4. These experiments were conducted to confirm the effects observed in expt. 2 on the voluntary intake of hay and straw of giving different amounts of concentrates (Table 5). In expt. 3, only 5 of the 6 periods were completed because insufficient hay was available; the experiment was analysed as an incomplete Latin square design. Two cows had to be removed from expt. 4 in period III because they

were pregnant and the results of this experiment were analysed as a 10 × 6 randomized block design ignoring the effect due to periods. However, in expt. 4 it was possible to test for the effect of periods in one square of 6 cows and the effect was found to be non-significant ($P > 0{\cdot}05$).

The results of these 2 experiments confirmed those found in expt. 2. The addition of 3 and 6 kg concentrates (expt. 3) to cows offered a medium quality hay caused no significant change ($P > 0{\cdot}05$) in voluntary intake of hay. Similarly, in expt. 4 with hay of about the same digestibility (58 %) the addition of 4 and 8 kg concentrates did not affect significantly ($P > 0{\cdot}05$) the voluntary intake of hay. However, in both experiments there was a tendency for the lower amounts of concentrates (3 and 4 kg) to cause slight increases of 0·3 and 0·6 kg in the daily intake of hay dry matter, and at the higher levels of concentrate supplementation (6 and 8 kg) slight decreases in intake of hay were observed. In both experiments the voluntary intakes of barley straw by the cows (3·8 and 4·6 kg dry matter) were considerably less than those of hay which were 8·9 and 7·2 kg dry matter in expt. 3 and 4, respectively, and in each experiment the difference was significant ($P < 0{\cdot}01$). The maximum voluntary intake of straw, 4·7 kg dry matter, in expt. 3 was with the addition of 6 kg concentrates and in expt. 4 the maximum intake of 5·6 kg straw dry matter occurred when the cows were given 4 kg concentrates.

Table 5. *Expts.* 3 *and* 4. *Effect of concentrates on the daily voluntary intake of roughage by the cows*

Expt. no.		Mean daily intake of roughage (kg dry matter) when given concentrates at daily rate (kg) of 0	3	4	6	8	S.E. of a difference between two means
3	Hay	8·88	9·48	—	8·86	—	±0·558
(6 cows)	Barley straw	3·67	4·25	—	4·71	—	
4*	Hay	7·23	—	7·53	—	6·31	±0·549
(10 cows)	Barley straw	4·59	—	5·58	—	5·52	

* In expt. 4 one missing value was calculated.

Expt. 5. In this experiment the effect of intake of roughages by cows was studied by adding 4·5 kg concentrates to a diet of silage of high digestibility (77 %), and to hay conserved from the same sward later in the season and of much lower digestibility (59 %). The fifth treatment measured the voluntary intake of highly digestible hay (78 %) when the cows received 4·5 kg concentrates daily. The mean voluntary intakes of roughage dry matter are given in Table 6. When given silage or late-cut hay as the only food the cows ate slightly more dry matter in the form of silage than as hay, but the difference was not significant ($P > 0{\cdot}05$). The daily addition of 4·5 kg concentrates did not alter significantly ($P > 0{\cdot}05$) the voluntary intake of dry matter as silage and late-cut hay by the cows, although the daily intake of silage dry matter declined by 0·63 kg and of hay dry matter by 0·45 kg. The voluntary intake of highly digestible hay by cows given 4·5 kg concentrates was markedly higher at 9·8 kg dry matter than that of any of the other diets. In a similar experiment which will be reported separately (Campling, unpublished), the addition of 4·5 kg concentrates to cows receiving highly digestible silage and hay from the same batches used in expt. 5

and prepared from similar herbage, caused a mean daily depression of 0·55 kg dry matter of silage and of 1·36 kg dry matter of hay. The results of these 2 experiments confirm those observed in expt. 2 that the addition of concentrates to the diet of cows offered silage caused only a small depression in voluntary intake of silage by the cows.

Table 6. *Expt.* 5. *Effect of concentrates on the voluntary intake of roughage by* 5 *cows*

	Mean daily intake of roughage (kg dry matter) when given concentrates at daily rate (kg) of		s.e. of a difference between two means
	0	4·5	
Silage	7·79	7·16	—
Late-cut hay	8·39	7·94	±0·372
Hay	*	9·84	—

* Not determined.

Table 7. *Expt.* 6. *Effect of concentrates on the voluntary intake of hay by* 8 *cows*

	Mean daily intake of hay (kg dry matter) when given concentrates at daily rate (kg) of				s.e. of a difference between two means
	0	8	11*	14†	
Hay	6·35	4·90	3·18	2·54	±0·268

* 10·7 kg eaten. † 13·6 kg eaten.

Table 8. *Expt.* 7. *Effect of different concentrate mixtures on the mean daily voluntary intake of hay by* 8 *cows*

Type of concentrate	Intake of hay dry matter, kg
None	8·30
Standard concentrate	6·30
Oats and groundnut meal	6·45
Flaked maize and groundnut meal	6·05
s.e. of a difference between two means	±0·232

Expt. 6. The effect on intake of hay of large amounts of concentrates was studied in this experiment. There were small refusals of concentrates by some of the cows when offered 11 and 14 kg/concentrates day. The mean amounts of concentrates eaten were 8, 10·7 and 13·6 kg. The addition of concentrates affected hay intake significantly, and with each level of concentrates the voluntary intake of hay dry matter was depressed below that observed when hay was offered alone (Table 7). The addition of 8 kg concentrates depressed the intake of hay dry matter by 1·45 kg, and 10·7 and 13·6 kg concentrates depressed the mean intake by 3·17 and 3·81 kg, respectively. On a dry-matter basis the addition of 1 kg of concentrates at each level of supplementation depressed the intake of hay by 0·21, 0·34 and 0·33 kg dry matter, respectively.

Expt. 7. The experiment was designed to compare the effect on voluntary intake of hay of 3 concentrate mixtures: the standard concentrates used in expts. 1–6, a

mixture of rolled oats and groundnut meal, and a mixture of flaked maize and groundnut meal. The addition of 6 kg of each of the concentrates daily caused a significant depression in voluntary intake of hay compared with that found with hay alone (Table 8). There were no significant differences ($P > 0{\cdot}05$) between the amounts of hay eaten by the cows when they received 6 kg of any one concentrate. However, the addition of 1 kg of dry matter in the form of the standard concentrate, rolled oats and flaked maize depressed intake of hay by 0·29, 0·37 and 0·45 kg dry matter, respectively, and there were large variations between cows in the extent of the depression in intake of hay. For example, the depression in intake of hay dry matter/kg standard concentrate dry matter varied from 0·08 kg in one cow to 0·64 kg in another. There were similar variations between the cows with each of the concentrate mixtures.

DISCUSSION

Changeover experimental designs provide a convenient method of examining the effect of supplements of concentrates on the voluntary intake of roughages by cows because there are considerable differences between individual cows in the voluntary intake of food. Although the results of expt. 1 showed that there was no significant interaction between treatments and periods the addition of 4 kg concentrates caused only a slight depression in intake of hay. There is a need to repeat this experiment using treatments which affect hay intake to a greater extent and also to study the effect of the length of the period on the response to different treatments.

The results of the 7 experiments show that the feeding of about 4 kg concentrates to cows receiving roughage *ad lib.* caused little change in the intake of hay and a small increase in the intake of barley straw. Larger amounts of concentrates of 6 and 8 kg daily reduced hay intake by between 0·2 and 0·4 kg dry matter/kg of concentrate dry matter given, but with barley straw a much smaller decrease in intake was observed with these larger amounts of concentrates. In this respect, our results agree with the conclusion drawn by Stone, Spadling, Merrill & Reid (1964) on the basis of a review of American experiments by Reid (1956), that with up to 6·4 kg/day concentrates roughage intake is not affected. However, with more than 6·4 kg daily Stone *et al.* (1964) found that the consumption of roughage declined 0·5–0·7 kg/kg of concentrates eaten by cows, this is a considerably greater reduction in hay intake than was observed in our experiments.

The relationship between the digestibility of the roughages and the depression in voluntary intake of roughage dry matter/kg concentrate dry matter given is shown in Fig. 1. In general, the results of the present experiments agree with those of Blaxter & Wilson (1963) that when restricted amounts of concentrates are given to ruminants the rate of decline in intake of hay is greatest with the highest-quality hay. However, in our experiments with cattle the decline in the intake of hay with increasing digestibility appeared to be less for a given amount of concentrates than was observed in sheep by Blaxter & Wilson (1963) but it agrees with the results of Murdoch (1964*b*) with sheep.

Comparisons of our results with cattle with those of Blaxter & Wilson (1963) with sheep suggest that per kg metabolic body weight ($W^{0{\cdot}73}$) the decline in hay intake/kg concentrate eaten was less with cattle than with sheep.

The results from all experiments show a large variation in the depression in the intake of roughage/kg concentrate for any one roughage (Fig. 1). This variation in the decline in intake of a given roughage/kg concentrate was due mainly to the effects of different amounts of concentrates. Large amounts of concentrates caused greater depressions in intake of hay/kg concentrate dry matter than smaller amounts, and with straw large amounts of concentrates occasionally depressed the intake of straw whereas small amounts invariably increased straw intake.

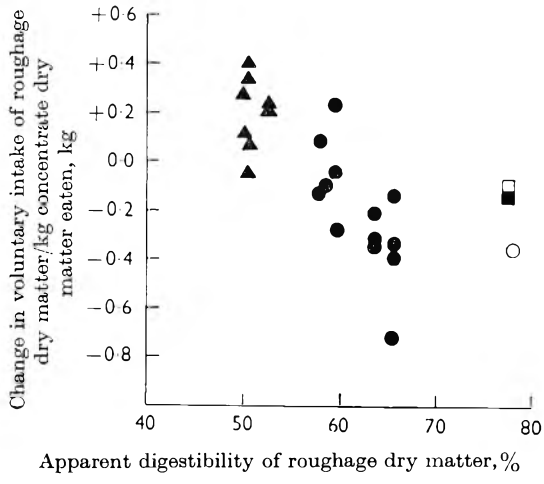


Fig. 1. Relationship between the apparent digestibility of the roughages and the change in voluntary intake of roughage dry matter/kg concentrate dry matter eaten by cows offered hay ●, barley straw ▲, silage ■. Values shown □ silage and ○ hay, are from the unpublished experiment referred to on page 7.

The daily addition of 2–8 kg concentrates to the food of cows offered silage *ad lib.* caused small depressions in silage intake so that on a dry-matter basis the depression in intake of silage by concentrates was 0·23 and 0·14 kg/kg concentrates eaten in expts. 2 and 5, respectively. It is very probable that the higher value found in expt. 2 than in expt. 5 was due, in part, to the underestimation of the content of dry matter of the silage, because this was determined by drying samples of silage in an oven at 100 °C. Considerable losses of volatile constituents can occur during drying at this temperature, and in expt. 5 the dry-matter content of the silage was estimated more accurately by a toluene distillation method (Minson & Lancaster, 1963). It is well known that cattle and sheep, offered grass silage containing about 20 % dry matter, eat considerably less dry matter than when offered hay conserved from similar herbage (Murdoch, 1964*a*). However, the present results and those of Holmes *et al.* (1960) and Murdoch (1964*b*) showed that when restricted amounts of concentrates were given the depression in intake of dry matter as silage was markedly less than occurred with hay and it is important to determine if these results are typical. Silage, prepared from wilted herbage and containing about 30 % dry matter, is eaten in considerably greater amounts than silage of lower dry-matter content (Brown, 1960; Murdoch, 1962), and in at least one experiment (Brown, 1960) the addition of concentrates to high dry-matter silage caused a greater depression in intake of dry matter than when

a similar quantity of concentrates was added to the diet of cows receiving low dry-matter silage.

The decrease in hay intake that occurred when 4–8 kg concentrates were given to the cows may have been due to decreased cellulolytic activity of the rumen micro-organisms and a depression in the rate of disappearance of digesta from the digestive tract (Campling, 1966).

The increase in the voluntary intake of a poor-quality roughage (barley straw) by the cows when given concentrates was probably due mainly to an increase in the rate of disappearance of digesta from the gut which was, in turn, due to the nitrogenous constituents of the concentrates stimulating the cellulolytic activity of rumen micro-organisms (see Campling *et al.* 1962; Hemsley & Moir, 1963). However, Egan (1965) has shown that an improvement in the protein status of sheep increased the voluntary intake of a low-protein roughage without altering the digestibility and the time of retention of food residues in the digestive tract.

The cause of the considerable variation between cows in their response in intake of roughage to addition of concentrates is not known. It may have been due to differences between the cows in the extent to which the rate of disappearance of digesta from the alimentary tract was altered by the addition of concentrates (see Campling, 1966).

The results of expt. 7 provide some evidence that the type of cereal used in the concentrates did not affect markedly the voluntary intake of hay. If the results from this experiment are confirmed and extended it would appear possible to substitute one cereal for another to a considerable extent without regard to its effect on voluntary intake of the roughage portion of the diet.

We are grateful to Dr R. N. Curnow and Mr D. R. Westgarth for advice and help on statistical matters and to members of the Chemistry, Dairy Husbandry and Feeding and Metabolism departments for their help.

REFERENCES

Blaxter, K. L. & Wilson, R. S. (1963). *Anim. Prod.* **5**, 27.
Brown, S. M. (1960). *Res. exp. Rec. Minist. Agric. Nth Ire.* **10**, 9.
Campling, R. C. (1966). *J. Dairy Res.* **33**, 13.
Campling, R. C., Freer, M. & Balch, C. C. (1962). *Br. J. Nutr.* **16**, 115.
Corbett, J. L. (1961). *Jl R. agric. Soc.* **122**, 175.
Danasoury, M. S. El- (1954). Thesis, University of London.
Egan, A. R. (1965). *Aust. J. agric. Res.* **16**, 451.
Freer, M. & Campling, R. C. (1963). *Br. J. Nutr.* **17**, 79.
Hemsley, J. A. & Moir, R. J. (1963). *Aust. J. agric. Res.* **14**, 509.
Holmes, W., Arnold, G. W. & Provan, A. L. (1960). *J. Dairy Res.* **27**, 191.
Morris, J. G. (1958). *Qd J. agric. Sci.* **15**, 161.
Minson, D. J. & Lancaster, R. J. (1963). *N.Z. Jl agric. Res.* **6**, 140.
Murdoch, J. C. (1962). *J. Br. Grassld Soc.* **17**, 133.
Murdoch, J. C. (1964*a*). *Proc. Nutr. Soc.* **23**, 99.
Murdoch, J. C. (1964*b*). *J. Br. Grassld Soc.* **19**, 316.
Reid, J. T. (1956). *Mem. Cornell Univ. agric. Exp. Stn* no. 344.
Stone, J. B., Spadling, R. W., Merrill, W. G. & Reid, J. T. (1964). *Proc. Cornell Nutr. Conf. Feed Manufacturers*, p. 82.

Printed in Great Britain

The effect of concentrates on the rate of disappearance of digesta from the alimentary tract of cows given hay

By R. C. CAMPLING

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 25 *August* 1965)

Summary. Two experiments were conducted with non-lactating cows to examine the effect of adding concentrates to a diet of hay on the rate of disappearance of digesta from the alimentary tract. The addition of large amounts of concentrates to the diet of cows offered hay *ad lib.* decreased the voluntary intake of hay, increased slightly the digestibility of the organic matter and decreased markedly the digestibility of the crude fibre of the diet. Also, the addition of concentrates increased the mean time of retention of stained hay residues in the alimentary tract and increased the time/kg hay the cows spent ruminating. At the end of a meal the amount of digesta in the reticulo-rumen of the cows offered hay *ad lib.* with restricted amounts of concentrates was about the same as that found when offered hay *ad lib.* as the only food. The results are discussed in relation to the regulation of the voluntary intake of hay by the cow.

Addition of restricted amounts of concentrates to the diet of ruminants, offered roughage *ad lib.*, often alters the voluntary intake of the roughage. The type and extent of the change in voluntary intake of roughage seems to depend largely on the quality of the roughage given. For example, with roughages containing small amounts of nitrogen such as cereal straws, marked increases in voluntary intake occurred when nitrogenous supplements were given (e.g. Morris, 1958, Campling, Freer & Balch, 1962; Coombe & Tribe, 1963; Hemsley & Moir, 1963). The increased intake of straw was probably due largely to an accelerated rate of disappearance of digesta from the reticulo-rumen (Campling *et al.* 1962; Coombe & Tribe, 1963; Hemsley & Moir, 1963), although part of the increase may have been caused by an improvement in the protein status of the animal (Egan, 1965). It has been shown that the voluntary intake of roughages by ruminants is determined mainly by the amount of digesta in, and its rate of disappearance from, the reticulo-rumen (Blaxter, Wainman & Wilson, 1961; Campling *et al.* 1962). With better-quality roughages, such as hay and silage, a depression in voluntary intake of roughage has often been observed when concentrates were given (Holmes, Arnold & Provan, 1960; Blaxter & Wilson, 1963; Campling & Murdoch, 1966). It seems likely that the depressing effect is due to a depression in the rate of disappearance from the reticulo-rumen of digesta derived from roughage. Therefore, a study was made of the changes in the rate of disappearance of digesta when concentrates were added to a diet of hay.

Two experiments were conducted in which measurements were made of the amount

of digesta in the reticulo-rumen and their rate of disappearance from the digestive tract together with certain factors related to the rate of disappearance: digestibility, the time of retention of roughage residues in the gut, and the eating and ruminating behaviour of the cows. In the first experiment the effect of adding a large amount of concentrates to the diet of cows receiving a restricted amount of hay was studied, and in the second experiment 3 different amounts of concentrates were given to cows offered hay *ad lib.* A brief account of some of the results of expt. 1 was published recently (Balch & Campling, 1965).

EXPERIMENTAL

Expt. 1

A single changeover design with 3 non-pregnant, non-lactating cows (A, B and C) was used to compare 2 treatments: 4·5 kg hay daily, and 4·5 kg hay with 6 kg concentrates daily. Each period consisted of 29 days, a 14-day preliminary feeding period, a 10-day collection period for the measurement of digestibility and the time of retention of hay residues in the gut, 3 days for the recording of the eating and ruminating behaviour of the cows and 2 days during which the amount of digesta in the reticulo-rumen was measured.

Expt. 2

A 4 × 4 Latin-square design with 4 non-pregnant, non-lactating cows (F, G, H and J) was used to compare the following treatments: hay *ad lib.*, hay *ad lib.* + 2·5 kg concentrates daily, hay *ad lib.* + 5·0 kg concentrates daily, and hay *ad lib.* + 7·5 kg concentrates daily.

Each period was of 33 days duration and comprised 18 days preliminary feeding, a 10-day collection period, 3 days for the recording of eating and ruminating behaviour and 2 days in which the amount of digesta in the reticulo-rumen was measured.

Cows and housing

Three of the cows were Shorthorn and 4 Friesian. The mean liveweight of the cows was 620 kg. Each cow had a permanent rumen fistula closed by a rubber cannula and bung. The cows were kept in individual standings with water and salt licks containing trace minerals available at all times.

Foods

The hay (A) used in expt. 1 was mainly perennial ryegrass and that used in expt. 2 (hay B) was predominantly timothy. The concentrates used in expts. 1 and 2 were similar. Their composition was (in parts): barley 17, maize 20, wheat bran 20, decorticated groundnut meal 15, copra cake 10, palm-kernel cake 5, molasses 10, dicalcium phosphate 1, calcium carbonate 1. The salt-content was 1 % and 5×10^6 i.u. vitamin A and 1×10^6 i.u. vitamin D were added per ton. The chemical compositions of the hays and concentrates are given in Table 1; the concentrates were pelleted.

Determination of voluntary intake

The total daily allowance of each food was given in one meal, concentrates were given first at 10 a.m. and immediately this food was eaten hay was given. When hay was offered *ad lib.* the uneaten hay was removed and weighed after 5 h. The amount of hay offered was adjusted daily so that the uneaten portion was about 10 % of the amount offered.

Table 1. *Chemical composition of the foods used*

			% of dry matter					
Expt.	Food	Dry matter, %	Organic matter	Crude protein	Ether extract	Crude fibre	Nitrogen-free extract	Ash
1	Hay	83·0	93·8	7·2	1·6	30·2	54·8	6·2
	Concentrates	85·3	92·7	19·0	1·9	5·8	66·0	7·3
2	Hay	83·3	94·7	7·9	1·2	31·2	54·4	5·3
	Concentrates	86·1	92·8	20·0	2·1	5·9	64·8	7·2

Digestibility

The digestibility of the diets was determined by collecting the faeces with the harness and equipment described by Balch, Bartlett & Johnson (1951).

Digestibility in the reticulo-rumen

The extent of digestion of food in the reticulo-rumen was estimated by the application of the lignin-ratio technique to bulked samples of digesta taken from close to the reticulo-omasal orifice. Details of this method and of the analyses used were given by Campling, Freer & Balch (1961).

Rate of disappearance of cotton thread in the rumen

The cotton thread technique described by Campling *et al.* (1961) was used to obtain an index of the rate of digestion of cellulose in the rumen.

Mean time of retention of food residues in the alimentary tract

On the second day of each collection period about 4 % of the daily intake of food was given as food stained with magenta and the numbers of stained particles in subsequent samples of faeces were counted (Balch, 1950). The mean time of retention of stained particles in the gut was calculated by the method of Castle (1956).

Amount of digesta in the reticulo-rumen

The weight of digesta in the reticulo-rumen was measured by manually emptying the reticulo-rumen and weighing, sampling and returning the contents before and after feeding on 2 successive days at the end of each treatment period.

Recording of eating and ruminating behaviour

The apparatus used was that described by Freer, Campling & Balch (1962) and consisted of a small, lightly inflated balloon placed under a side strap on a leather head-stall, and connected to a tambour in an Evershed and Vignoles recorder. The

tambour operated a pen and provided a continuous record of jaw movements on a chart moving at 0·5 in./min. The record was started immediately before a meal at 10 a.m. and continued for 72 h. The records were analysed to determine the time the cows spent eating, ruminating and resting; the resting period was the time during which the cows were neither eating nor ruminating. It was assumed that any rumination observed was due to the hay since the diet of concentrates alone did not induce rumination in cows in another experiment (Freer & Campling, 1965).

RESULTS

Voluntary intake (expt. 2)

The mean daily voluntary intake of hay and the amount of water drunk daily is shown in Table 2. The mean daily intake of hay decreased significantly ($P < 0·05$) from 10·5 kg with hay alone to 8·5 and 7·4 kg when 5 and 7·5 kg concentrates, respectively, were given, but the small decrease that occurred when 2·5 kg concentrates were given was not significant ($P > 0·05$). The total daily intake of food dry matter increased from 8·8 kg with hay alone to 12·6 kg when 7·5 kg concentrates were given. On a dry-matter basis the voluntary intake of hay declined by about 0·1, 0·4 and 0·4 kg/kg concentrates given over the range 0–2·5, 0–5, and 0–7·5 kg, respectively. There was some individual variation between the cows in intake of hay, cow H ate slightly less hay with 2·5 kg concentrates than when offered hay alone, and cow J ate slightly more hay with 7·5 kg than with 5 kg concentrates. The increases in the daily amount of water drunk when concentrates were added to the diet of hay were small and only approached significance at the 5 % level at the highest level of concentrates.

Digestibility

Expt. 1. The effect of supplementing a diet of hay with 6 kg concentrates was to increase significantly the digestibility of the proximate constituents of the diet except crude fibre and ash (Table 3). The mean digestibility of the organic matter increased from 72·2 to 78·1 % and digestibility of the crude fibre fell from 65·1 % with hay alone to 53·5 % with the addition of concentrates. Estimates of the apparent digestibility of the diet in the reticulo-rumen by the lignin-ratio technique showed that with hay about 48 % of the organic matter was digested in the rumen and when concentrates were added the digestibility fell to 40 %. The change in digestibility of the crude fibre in the rumen was more marked, being 45 % with hay alone and only 31 % with the addition of concentrates. The digestibility of the nitrogen-free extract in the rumen was about the same with both diets at 43 and 45 %. Thus, considerably more organic matter was digested in the hind gut when concentrates were given than with hay alone.

Expt. 2. The digestibility of the dry matter, organic matter and nitrogen-free extract of the diet increased slightly but significantly as the amount of concentrates given was increased to 5 kg, but there was little change with the further increase to 7·5 kg (Table 3). The digestibility of the crude fibre of the diet increased slightly when 2·5 kg concentrates were given and then decreased considerably as greater amounts were given. The digestibility of organic matter increased from 67 % with hay alone to 71 % with 7·5 kg concentrates while the crude fibre digestibility fell from 70 to 54 %.

Expt.	Diet	daily intake dry matter kg	Daily intake of hay, kg	Amount of water drunk, kg/day	breakdown of cotton thread. Time for 25% loss in weight, h	time of retention of hay residues, h
1	Hay	3·7	4·5	17·9	27	72
(3 cows)	Hay + 6 kg concentrates	8·8	4·5	32·1	35	90
	S.E. of difference	—	—	±3·58	±3·5	±5·1
2*	Hay	8·8	10·5	33·2	32	60
(4 cows)	Hay + 2·5 kg concentrates	10·7	10·3	35·1	31	61
	Hay + 5 kg concentrates	11·4	8·5	36·5	32	68
	Hay + 7·5 kg concentrates	12·6	7·4	42·1	34	70
	S.E. of difference between two means	—	±0·28	±2·65	±2·8	±5·2

* Hay in expt. 2 was offered *ad lib.*

Table 3. *Mean apparent digestibility of the foods in the alimentary tract and in the reticulo-rumen of cows receiving diets of hay or hay + concentrates*

Expt.	Diet	Digestibility in the alimentary tract, %							Digestibility in the reticulo-rumen, %		
		Dry matter	Organic matter	Crude protein	Ether extract	Crude fibre	N-free extract	Ash	Organic matter	Crude fibre	N-free extract
1	4·5 kg hay	65·5	72·2	48·8	57·1	65·1	70·5	51·6	48·2	44·6	43·3
3 cows)	4·5 kg hay + 6 kg concentrates	71·9	78·1	69·3	67·5	53·5	80·5	43·6	40·2	31·0	44·6
	S.E. of difference	±0·93	±0·24	±1·31	±3·93	±4·13	±0·74	±9·82	±4·93	±1·74	±4·60
2*	10·5 kg hay	65·9	66·9	49·4	24·6	69·9	71·0	45·6	44·6	50·4	51·2
4 cows)	10·3 kg hay + 2·5 kg concentrates	67·2	68·5	57·4	50·0	72·1	72·6	56·8	44·2	57·9	51·7
	8·5 kg hay + 5·0 kg concentrates	69·6	71·8	63·0	55·7	60·3	77·3	44·3	46·1	38·4	55·4
	7·4 kg hay + 7·5 kg concentrates	69·3	71·1	67·2	64·7	54·1	76·7	53·0	44·7	30·2	54·2

Estimates of the digestibility of the diets in the reticulo-rumen showed results very similar to those observed in the whole gut (Table 3). The digestibility of the organic matter and the nitrogen-free extract increased slightly but not significantly ($P > 0{\cdot}05$) as the amount of concentrates eaten increased, while the digestibility of the crude fibre increased with 2·5 kg concentrates and then declined significantly ($P < 0{\cdot}001$) to 38 and 30% with 5 and 7·5 kg concentrates, respectively. Each cow showed a similar pattern of changes in digestibility in the rumen except cow H, in which the digestibility of crude fibre remained about the same with hay alone and when 2·5 kg concentrates were given; in the other 3 cows an appreciable increase in digestibility of crude fibre was observed. When 7·5 kg concentrates were fed instead of 5 kg the digestibility of crude fibre did not change for cow J, but in the 3 other cows it decreased from 39 to 28%.

The rate of breakdown of cotton thread

Expt. 1. The rate of breakdown of cotton thread in the ventral sac of the rumen decreased when concentrates were added to the diet of hay (Table 2), but the difference was not significant ($P > 0{\cdot}05$).

Expt. 2. There were no significant differences between diets in the time taken for cotton thread to lose 25% of its weight ($P > 0{\cdot}05$). Differences between individual cows in the rate of breakdown of cotton thread were small. Balch & Johnson (1950) found similar rates of breakdown of cotton thread in the rumen of cows given hay and hay supplemented with concentrates.

The mean time of retention of hay residues

Expt. 1. The mean time of retention of stained hay residues in the gut was 72 h with hay alone and 90 h when concentrates were added (Table 2). The marked increase in the retention time of hay that occurred when concentrates were given was observed in all 3 cows.

Expt. 2. The mean time of retention of stained hay residues increased as the amount of concentrates was increased, from 60 h with hay alone to 70 h with 7·5 kg concentrates (Table 2). However, there was considerable variation between cows in the changes in retention time in response to concentrates. Although the time of retention decreased in cows F, G and J when 2·5 kg concentrates were added to the diet of hay, in cow H the time of retention increased from 62 to 70 h. Increasing the amount of concentrates from 5 to 7·5 kg was associated with an increase in the time of retention of hay residues in cows F, G and H, but in cow J the retention time decreased from 74 to 58 h.

Amount of digesta in the reticulo-rumen

Expt. 1. The total amounts of digesta and digesta dry matter in the reticulo-rumen immediately after a meal were largest when concentrates were given (Table 4). However, before a meal there were more digesta in the rumen with the diet of hay than with hay and concentrates, but the amount of digesta dry matter was least with the diet of hay. From knowledge of the times of emptying, the intake of food, and the amount of digesta in the rumen, it was possible to estimate that the rate of disappearance of digesta dry matter from the reticulo-rumen during a meal was

1·15 kg/100 min with the diet of concentrates and hay and with the hay diet there was a slight gain (0·07 kg/100 min) which was probably due to the saliva. Between meals the difference between diets in the rate of disappearance of dry matter was much smaller, 0·53 kg/100 min with hay and concentrates and 0·29 kg/100 min with hay.

Table 4. *Amount of digesta in the reticulo-rumen before and after feeding in cows receiving diets of hay or hay + concentrates*

		Digesta in reticulo-rumen			
		Total, kg		Dry matter, kg	
Expt.	Diet	Before feeding	After feeding	Before feeding	After feeding
1	4·5 kg hay	61·7	79·6	3·73	7·56
(3 cows)	4·5 kg hay + 6 kg concentrates	55·6	86·0	4·75	11·58
	S.E. of difference	±1·24	±1·68	±0·460	±0·436
2*	10·5 kg hay	68·4	103·7	7·15	14·04
(4 cows)	10·3 kg hay + 2·5 kg concentrates	67·9	100·6	7·02	14·46
	8·5 kg hay + 5·0 kg concentrates	61·8	101·3	6·67	13·83
	7·4 kg hay + 7·5 kg concentrates	62·7	94·4	7·16	14·27
	S.E. of difference between two means	±1·77	±2·12	±0·351	±0·280

* Hay in expt. 2 was offered *ad lib.*

Expt. 2. The amounts of digesta and digesta dry matter in the reticulo-rumen immediately after a meal varied little between the 4 diets. Although the amount of digesta was lowest when 7·5 kg concentrates were given, there was no significant difference between the amounts of digesta dry matter present. Immediately before a meal the amounts of digesta were significantly lower ($P < 0·05$) with the diets containing 5 and 7·5 kg concentrates than with the other diets, but there was no significant difference between the 4 diets in the amounts of dry matter ($P < 0·05$).

In a similar manner to that described in expt. 1 the mean rates of disappearance of dry matter from the reticulo-rumen were estimated. During a meal the mean rate of disappearance of dry matter increased from 0·56 kg/100 min with hay alone to 0·90, 1·60 and 1·60 kg/100 min with 2·5, 5·0 and 7·5 kg concentrates, respectively. Between meals the mean rates of disappearance were similar with all 4 diets: 0·61 kg/100 min with hay and 0·66, 0·61 and 0·61 kg/100 min with 2·5, 5 and 7·5 kg concentrates, respectively.

Eating and ruminating behaviour

Expt. 1. The daily time spent eating and ruminating was increased considerably by the addition of concentrates (Table 5). The mean time spent eating hay increased from 55 min or 12·1 min/kg hay on the hay diet to 81 min or 17·9 min/kg hay on the hay and concentrates diet. Similarly, the time spent ruminating increased from 221 min or 49·1 min/kg hay to 309 min or 68·7 min/kg hay. There was no significant change in number of boluses regurgitated/100 min when concentrates were added ($P < 0·05$). The number of periods spent ruminating each day tended to increase from 12 with hay alone to 16 with hay and concentrates. The frequency of reticular contractions tended to decrease during both eating and ruminating when concentrates were added to the diet of hay but the differences were not significant ($P < 0·05$).

Table 5. *Mean daily time spent eating, ruminating and resting per kg food and the frequency of contractions of the reticulum during each activity in cows receiving diets of hay + concentrates*

Expt.	Diet	Eating		Ruminating		Daily number of periods of rumination	Resting	
		Min/kg hay	Contractions/ 100 min	Min/kg hay	Contractions/ 100 min		Min/kg food	Contracti 100 mi
1	4·5 kg hay	12·1	132	49·1	102	12	258·7	91
3 cows)	4·5 kg hay + 6 kg concentrates	17·9	123	68·7	96	16	98·3	91
	s.e. of difference	±2·02	±11·0	±3·25	±4·5	±1·8	±2·26	±6·7
2*	10·2 kg hay	25·4	140	51·3	99	16	130	102
4 cows)	9·9 kg hay + 2·5 kg concentrates	26·1	140	51·6	95	16	108	102
	8·1 kg hay + 5·0 kg concentrates	25·2	142	58·2	91	15	116	99
	7·0 kg hay + 7·5 kg concentrates	29·3	144	71·8	91	16	100	104
	s.e. of difference between two means	±2·08	±1·9	±2·29	±2·6	±0·9	±2·4	±2·4

* Hay in expt. 2 was offered *ad lib.*

Expt. 2. The results given in Table 5 show that the eating behaviour of the cows on hay was only slightly altered by the amount of concentrates given but ruminating behaviour was altered appreciably. The time/kg spent eating hay increased slightly but not significantly ($P < 0{\cdot}05$) as the amount of concentrates was increased but the total daily time spent eating hay decreased. The frequency of reticular contractions during the eating of hay was similar with all diets. Concentrates were eaten very rapidly at a rate of 4·3 min/kg and the frequency of reticular contraction was 170/100 min and thus higher than when hay was eaten. The total daily time spent ruminating by the cows was 523 min or 51 min/kg hay with the diet of hay alone and when given 2·5 kg concentrates 511 min or 52 min/kg hay. When the concentrates

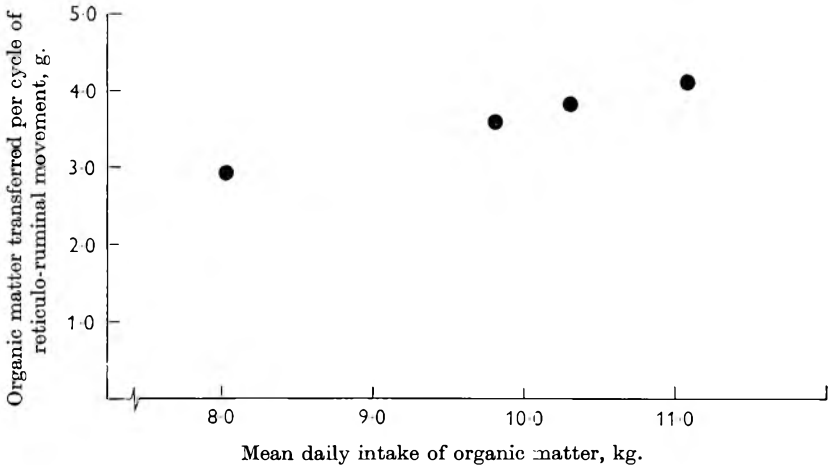


Fig. 1, expt. 2. Relationship between the mean daily intake of organic matter and the amount of organic matter estimated to have been transferred from the reticulo-rumen to the omasum during each cycle of reticulo-ruminal movement. Each value is the mean of the results for 4 cows.

were increased to 5 kg daily the mean time spent ruminating increased to 58 min/kg and a further marked increase to 71·8 min/kg hay occurred when 7·5 kg concentrates were given. However, the total daily time spent ruminating decreased slightly as increasing amounts of concentrates were given to the cows. Although the number of boluses regurgitated/100 min tended to decrease as the amount of concentrates increased this effect was not significant ($P < 0{\cdot}05$). The daily number of periods of rumination varied little between diets.

The amounts of organic matter transferred from the reticulo-rumen to the omasum per primary reticular contraction were estimated from knowledge of the intake of organic matter, the digestibility of the organic matter in the reticulo-rumen and the number of reticular contractions with each diet. In expt. 2 the mean values were 2·8, 3·6, 3·8 and 4·0 g/contraction with diets containing hay alone, 2·5, 5, and 7·5 kg concentrates, respectively. The amounts of organic matter estimated to have been transferred to the omasum per contraction were related directly to the mean daily intake of organic matter and are shown in Fig. 1. There was no evidence of curvilinearity of the relationship in Fig. 1, therefore it seems unlikely that the voluntary intake of hay was limited by the amount of organic matter that could be transferred per contraction.

DISCUSSION

That the addition of concentrates to a diet of hay in expt. 1 depressed the rate of disappearance from the digestive tract of digesta derived from hay was shown by the marked depression in the digestibility of the crude fibre of the diet and the increased time of retention of stained hay residues in the gut. Also, the increased time spent ruminating indicated that digesta derived from hay remained in the reticulo-rumen longer than when hay was the only food given. The results of expt. 2 confirm these findings and show, in addition, that when restricted amounts of concentrates were given to the cows offered hay *ad lib.* in one meal each day, the cows stopped eating hay when their reticulo-rumens contained about the same amounts of digesta and digesta dry matter as those found when only hay was offered. Thus, the control of the voluntary intake of hay when restricted amounts of concentrates were given was similar to that observed with diets of hay and dried grass (Freer & Campling, 1963). The limitation to the amount of hay eaten was probably set by the physical capacity of the reticulo-rumen and the rate of disappearance of digesta from this organ. However, there was a tendency for the amount of digesta in the reticulo-rumen immediately after a meal to be lower with the highest level of concentrates than with any of the other diets. Presumably giving the cows greater amounts of concentrates than 7·5 kg daily would give rise to a situation where the cows would stop eating hay before their reticulo-rumens contained an amount of digesta equal to that found with hay alone. Freer & Campling (1963) observed that when cows were offered concentrates *ad lib.* the amount of digesta in the reticulo-rumen at the end of a meal did not approach the limiting amount found when hay was offered *ad lib.*

The effect of supplements of carbohydrates in depressing the digestibility of the crude fibre of hay is well known (Hamilton, 1942; Watson, 1949; Head, 1953). The lowered digestibility of crude fibre was due to a reduction in the cellulolytic activity of the rumen micro-organisms, probably caused by competition between the cellulolytic and amylolytic groups of bacteria for nutrients (El-Shazly, Dehority & Johnson, 1961). The consequence of the low digestibility of crude fibre of the hay was an increase in the time of retention of hay residues in the gut and an increase in the time/kg spent ruminating hay. Eng, Riewe, Craig & Smith (1964) showed that the time of retention of hay increased when concentrates were added to hay, and Balch *et al.* (1955) observed very long times of retention of hay residues in cows given large amounts of concentrates. Also, it was possible in individual cows in expt. 2 to relate changes in hay intake to changes in the digestibility of the crude fibre of diet and the time of retention of hay residues in the gut. For example, in cow H voluntary intake of hay was not altered and the digestibility of crude fibre was unaffected by the addition of 2·5 kg concentrates, and retention time increased. In the other 3 cows hay intake increased, the digestibility of crude fibres increased and the time of retention of hay residues decreased. However, it was not possible to determine in expt. 2 whether there was a causal relationship between the changes in digestibility and retention time and voluntary intake of hay, and further detailed examination of this relationship is needed.

I am grateful to Dr C. C. Balch for his advice and help, and to Mr V. W. Johnson and his colleagues for their care of the experimental animals.

REFERENCES

Balch, C. C. (1950). *Br. J. Nutr.* **4**, 361.
Balch, C. C., Balch, D. A., Bartlett, S., Bartrum, P., Johnson, V. W., Rowland, S. J. & Turner, J. (1955). *J. Dairy Res.* **22**, 270.
Balch, C. C., Bartlett, S. & Johnson, V. W. (1951). *J. agric. Sci., Camb.*, **41**, 98.
Balch, C. C. & Campling, R. C. (1965). *Proc. 2nd Int. Symp. Physiology of Digestion in the Ruminant, Iowa.*
Balch, C. C. & Johnson, V. W. (1950). *Br. J. Nutr.* **4**, 389.
Blaxter, K. L., Wainman, F. W. & Wilson, R. S. (1961). *Anim. Prod.* **3**, 51.
Blaxter, K. L. & Wilson, R. S. (1963). *Anim. Prod.* **5**, 27.
Campling, R. C., Freer, M. & Balch, C. C. (1961). *Br. J. Nutr.* **15**, 531.
Campling, R. C., Freer, M. & Balch, C. C. (1962). *Br. J. Nutr.* **16**, 115.
Campling, R. C. & Murdoch, J. C. (1966). *J. Dairy Res.* **33**, 1.
Castle, E. J. (1956). *Br. J. Nutr.* **10**, 15.
Coombe, J. B. & Tribe, D. E. (1963). *Aust. J. agric. Res.* **14**, 70.
Egan, A. R. (1965). *Aust. J. agric. Res.* **16**, 451.
El-Shazly, K., Dehority, B. A. & Johnson, R. R. (1961). *J. Anim. Sci.* **20**, 268.
Eng, K. S. Jr., Riewe, M. E., Craig, J. H. Jr. & Smith, J. C. (1964). *J. Anim. Sci.* **23**, 1129.
Freer, M. & Campling, R. C. (1963). *Br. J. Nutr.* **17**, 79.
Freer, M. & Campling, R. C. (1965). *Br. J. Nutr.* **19**, 195.
Freer, M., Campling, R. C. & Balch, C. C. (1962). *Br. J. Nutr.* **16**, 279.
Hamilton, T. S. (1942). *J. Nutr.* **23**, 101.
Head, M. J. (1953). *J. agric. Sci., Camb.*, **43**, 281.
Hemsley, J. A. & Moir, R. J. (1963). *Aust. J. agric. Res.* **14**, 509.
Holmes, W., Arnold, G. W., Provan, A. L. (1960). *J. Dairy Res.* **27**, 191.
Morris, J. G. (1958). *Qd J. agric. Sci.* **15**, 161.
Watson, J. (1949) *Proc. 5th Congr Int. Zootechnie, Paris*, p. 1ϱ.

Printed in Great Britain

Greenish discoloration of butterfat during an extended period of cold storage

By H. LÜCK

Animal Husbandry and Dairy Research Institute, Irene, Transvaal, South Africa

(*Received* 3 *September* 1965)

Summary. Dry butterfat occasionally develops a greenish colour when stored for at least $1\frac{1}{2}$ years at -12 °C whereas samples of the same fat kept for 2 years at $+2$ or $+15$ °C do not show this colour defect. Spectrophotometrical measurements have shown that the defect is accompanied by a chemical change of the carotene. Its absorption maxima are shifted towards the shorter wavelengths, and a new absorption is observed at 400–410, 430–435 and 450–460 mm. The possible involvement of oxidation products of carotene, such as isomeric diepoxides or furanoid oxides is discussed. The colour defect can be observed before any change in flavour is noticed.

INTRODUCTION

Dry butterfat or 'rendered' butter with a low moisture content (below 0·05 %) has an excellent keeping quality (approximately 2 years at 4 °C) which is better than that of any other butter. Interest in this type of butter is growing as it is increasingly being used for cooking, frying and baking purposes, as well as for the chocolate and ice-cream industries.

A bleaching of the yellow carotene of dry butterfat is common during storage at room temperature and is usually accompanied by fat oxidation (Holman, 1949). However, during an extended period of cold storage (e.g. -18 °C) a greenish discoloration is occasionally observed (Godel, 1956). It is unlikely that the latter defect is microbial in origin because of the low moisture content of rendered butter. There is in fact some evidence that carotene is involved in the colour change (Godel, 1956).

In this paper the results of further investigation into the mechanism of formation of the green colour defect are presented.

METHODS

The dry butterfat used was manufactured on an industrial scale in the normal way which, in essence, consists of melting the butter, vacuum and heat treatment of the liquid fat at 90 °C, and separation and cooling. For the purpose of this investigation the butterfat was dried further by the addition of approximately 2 % anhydrous sodium sulphate at 50 °C. Different samples of the fat were stored for 2 years at -12, $+2$ and $+15$ °C, respectively.

Spectrophotometric measurements were carried out on a solution of 10 g fat in

10 ml benzene or on the unsaponifiable extract of 10 g fat in 5 ml n-hexane (using a 1-cm cuvette and taking benzene or hexane as the reference solution). The unsaponifiable extract was obtained by saponifying 10 g fat with a *N*-solution of alcoholic potassium hydroxide, for a period of 24 h at 40 °C. After extraction with light petroleum, the extract was evaporated and the residue dissolved in 5 ml hexane. The carotene fraction was separated from the other unsaponifiable substances by chromatography on a column of aluminium oxide eluted by 1 % acetone in light petroleum.

The peroxide oxygen values calculated as m equiv./kg fat were determined by Lea's method (1931).

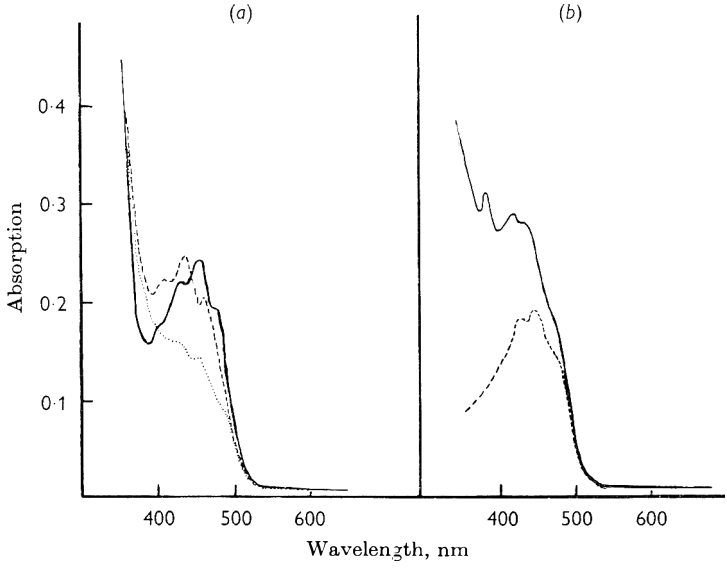


Fig. 1. (*a*) Absorption spectrum of butterfat in a benzene solution after storage of the dry butterfat for 2 years at various temperatures. - - -, −12 °C; ——, +2 °C; . . ., +15 °C. (*b*) Absorption spectrum in hexane of the carotene (- - -) separated from the unsaponifiable matter of a greenish butterfat by means of column chromatography, and the absorption spectrum of the total unsaponifiable extract of this butterfat (——).

RESULTS

The absorption curves of the solutions of the butterfat stored at the 3 different temperatures are presented in Fig. 1(*a*). They represent the absorption of total carotene; bleached butterfat does not absorb in this region. The greenish discoloured fat is characterized by a shift of the whole spectrum of the total carotenoids of approximately 30 nm towards shorter wavelengths, whilst the pattern near the absorption maximum remains nearly unchanged. This indicates strongly that a chemical alteration of the carotenoids is responsible for the colour defect. After 2-years storage the yellow butterfat had absorption maxima at 435, 460 and 485–490 nm which is identical with that of a fresh butterfat, whilst the maxima of the greenish fat were at 405–410, 435 and 460 nm and in another sample at 400, 430 and 450 nm. Only the fat that was stored at − 12 °C, showed this colour change whilst that kept at + 2 °C remained yellow. At a storage temperature of + 15 °C the well-known bleaching of the carotene could be observed; the absorption in the visible region

decreased and the curve lost its shape. Further experiments, using beef tallow to which synthetic β-carotene was added, confirmed that carotene was involved in the discoloration. The tallow became greenish after approximately 12 months at -18 °C.

After 2-years storage at -12, $+2$ and $+15$ °C the peroxide values of the stored butterfats were 0·60, 1·04, 1·64 m equiv./kg, respectively, compared with 0·2 m equiv./kg for the fresh butterfat. The discoloration occurred before any oxidation flavour or any other off-flavours had developed; the only remarks on flavour which could be made after 2-years storage were: 'slightly old'.

An attempt was made to separate the greenish colour from the butterfat by column chromatography. Different absorption materials such as Al_2O_3, silica gel, diatomaceous earth, were tried, but with limited success. The quantity of butterfat which had to be used was relatively large and the columns were, therefore, easily overloaded.

In further experiments the butterfat was saponified and the unsaponifiable matter was examined spectrophotometrically. The results are presented in Fig. 1(*b*). Comparison of Fig. 1(*a*) and Fig. 1(*b*) shows that the green colour is more sensitive to alkali than the yellow one. Further alteration of the green colour took place during longer treatment with the alkali but the spectrum of the unsaponifiable matter of the yellow butterfat was still similar to that of the original butterfat.

The unchanged carotene was easily separated from the other unsaponifiable matter by means of column chromatography. The fat, treated according to this method, showed that the greenish butterfat still contained unchanged carotene. The percentage of unchanged carotene varied from 15 to 30% depending on the degree of discoloration.

The heat stability of the greenish component in butterfat is similar to that of the unchanged carotene under the same conditions. The absorption at 400 nm of a greenish unheated sample was 0·22 and after heating for 1 or 2 h at 105 °C, 0·23 and 0·20, respectively. The corresponding figures for the yellow butterfat sample at 460 nm were 0·23, 0·24 and 0·25, respectively.

DISCUSSION

The results obtained are consistent with the hypothesis that the green discoloration which develops in the rendered butter after storage for long periods at -12 °C is derived from the carotene fraction.

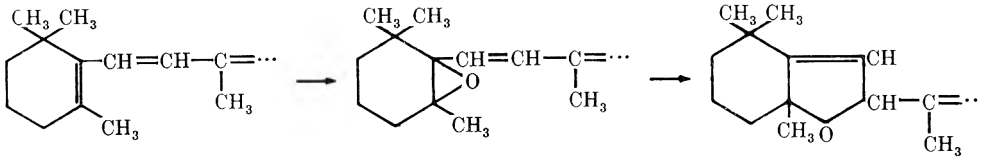
Spectra, similar to that observed in the discoloured butterfat, have been obtained by Tsukida & Zechmeister (1958) for stereo isomers of β-carotene epoxide. Absorption maxima in hexane were found as follows:

neo-β-carotene diepoxide A, 456, 428, 405·5 nm;
neo-β-carotene diepoxide B, (458), 429, (406) nm;
neo-β-carotene diepoxide T, 453, 426, 402·5 nm;
neo-β-carotene diepoxide V, 459·5, 431, 407·5 nm;
all-trans-luteochrome, 448, 422, 397·5 nm;
all-trans-mutatochrome, 453, 427, 401 nm;
all-trans-aurochrome, 426, 401, 380 nm;

From the spectra of the greenish butterfat it cannot be deduced whether oxidaton products with 2 oxygen atoms such as carotene diepoxides, or carotene monoepoxide-monofuranoid (luteochrome) or the corresponding difuranoid (aurochrome), or substance with 1 oxygen atom such as carotene monofuranoid oxides (mutatochrome have been formed. A deduction is more difficult because mixtures of the oxidation products and unchanged total carotene are present.

Except for the aurochrome all these substances have absorption maxima more or less in the region of the greenish butterfat, after making allowance for the fact that the wavelengths of the maxima in benzene solution are approximately 6 nm higher than in a hexane solution.

Reduction of the carotene molecule also results in a shift of absorption maxima to lower wavelengths but no reduction occurs in butterfat during prolonged storage. Therefore, although the increase in peroxide value during the $1\frac{1}{2}$–2-year storage at -12 °C was small, it is, nevertheless, suggested that the green pigment is an oxidation product of carotene probably of the epoxide type derived as follows:



Carotene — Carotene epoxide — Carotene furanoid oxide

Holman (1949) found that nearly all the carotene in a carotene-linoleate mixture was oxidized (bleached) after absorption of 0·1 moles O_2/mole linoleate. Otaka (1962) observed that the absorbance maxima for fresh butterfat in the visible region, produced by carotene pigments, disappeared before the onset of fat oxidation.

The green discoloration described in this paper was mostly not confined to the surface but occurred uniformly throughout the block of butter. Its formation is, therefore, not very dependent on the oxygen concentration.

The oxidation products of carotene (epoxide type) are not harmful and are widespread in the vegetable kindgom as demonstrated by Karrer & Jucker (1950). When administered to mammals they are strongly active as pro-vitamins. In the presence of traces of acids the epoxides are easily converted to furanoid oxides.

It is not clear why the greenish discoloration is observed after storage at -12 to -18 °C but not at $+2$ °C. It is possible that the production of the green pigment is dependent on the formation of free radicals formed in the early stages of fat oxidation. Experiments in which butterfat was irradiated with γ-rays (Lück, Deffner & Kohn, 1964) or ultra-violet light (Deffner, Lück & Kohn, 1964) have shown that the free radicals produced are only relatively stable at low temperatures.

An alternative hypothesis would be that the green pigment is formed at the higher temperatures of storage and is rapidly oxidized further. This possibility is supported by the finding of Hunter & Krakenberger (1947) that when β-carotene is oxidized in arachis oil at 50 °C small quantities of oxidized carotenoids having the spectroscopic characteristics of β-carotene epoxide, luteochrome or mutachrome can be detected.

REFERENCES

DEFFNER, C. U., LÜCK, H. & KOHN, R. (1964). *Z. Lebensmittelunters. u. -Forsch.* **125**, 281.
GODEL, A. (1956). *Moloch. Prom.* **17** (2), 38. (*Dairy Sci. Abstr.* (1956). **18**, 522.)
HOLMAN, R. T. (1949). *Archs Biochem.* **21**, 51.
HUNTER, R. F. & KRAKENBERGER, R. M. (1947). *J. chem. Soc.* p. 1.
KARRER, P. & JUCKER, E. (1950). *Carotenoids*, p. 60. New York: Elsevier Publishing Co.
LEA, C. H. (1931). *Proc. R. Soc.* B, **108**, 175.
LÜCK, H., DEFFNER, C. U. & KOHN, R. (1964). *Fette Seifen AnstrMittel*, **66**, 249.
OTAKA, Y. (1962). *Nippon Chikusangaku Kaiho*, **33**, 65.
TSUKIDA, K. & ZECHMEISTER, L. (1958). *Archs Biochem. Biophys.* **74**, 408.

Printed in Great Britain

n-Alkan-1-ols in oxidized butter

By W. STARK and D. A. FORSS

Division of Dairy Research, C.S.I.R.O., Melbourne, Australia

(*Received* 9 *September* 1965)

Summary. The $C_{1,2,5-8}$ n-alkan-1-ols were identified from oxidized butter prepared from cream treated with cupric chloride and ascorbic acid. The six n-alkan-1-ols occurred in approximately equimolar amounts and in concentrations of approximately 1 μmole/kg, the same order of magnitude as that of the C_{6-9} n-alkanals. The amounts increased rapidly during the first 4 weeks of storage, but only slowly during the succeeding 4–20 weeks. Only insignificant amounts of the C_{3-8} alkanols formed in the untreated samples. It is suggested that C_{1-8} alkanols are formed by the oxidation of unsaturated fatty acids according to the theory of Farmer, Koch & Sutton (1943) from primary alkoxy radicals resulting from the decomposition of lipid hydroperoxides.

In an investigation of oct-1-en-3-ol as a compound responsible for a mushroom flavour in oxidized dairy products (Stark & Forss, 1964), n-heptan-1-ol was always associated with the gas chromatographic fraction containing oct-1-en-3-ol. The presence of three n-alkanols in oxidized butter was reported in the *Annual Report of Division of Dairy Research*, 1963–64, and the presence of the $C_{1,2,5-8}$ n-alkan-1-ols was reported by Stark & Forss (1965). Evans (1961) obtained gas chromatographic and mass spectral evidence for the presence of ethanol and a C_4 hydrocarbon as a result of decomposition of 16-hydroperoxy linolenate.

According to Bell, Raley, Rust, Seubold & Vaughan (1951) primary and secondary alkoxy radicals decompose to form the corresponding alkanols. However, the formation of primary (terminal) hydroperoxides from radicals formed during the decomposition of lipid hydroperoxides has received little attention but recently Klöpfer, Esterbauer & Schauenstein (1965) have isolated n-amyl hydroperoxide from aqueous oxidized methyl linoleate.

This paper reports the identification of six n-alkan-1-ols in butter prepared from oxidized cream, and discusses the formation of the primary alkoxy radicals necessary for the formation of the n-alkan-1-ols.

EXPERIMENTAL

Cream was separated from pasteurized milk. Half the cream was churned on the following day while the other half was treated with 5 ppm. of copper as cupric chloride and 50 ppm. ascorbic acid, and held at 2 °C for 6 days and then churned. Both control and experimental lots of butter were stored in sealed 1-gal cans at − 12 °C and examined at 1, 2, 4, 8 and 20 weeks. Six-kg quantities of butter were

steam-distilled as previously described (Stark & Forss, 1964), and in order to obtain a comparison of the relative amounts of the alcohols and carbonyls formed the distillates were divided into 2 parts: one part (1500 ml) was treated with 2,4-dinitrophenylhydrazine in dilute H_2SO_4 and held for 24 h at 2 °C; the other part (500 ml) was also held at 2 °C but was otherwise left untreated. Both parts were redistilled at 2–8 mm Hg through a vertical condenser held at 0 °C until 40 ml of distillate was collected in a liquid-nitrogen trap. The 40 ml was redistilled in the same apparatus until 4–8 ml was collected (Forss & Ramshaw, unpublished). A 10-μl sample of the aqueous distillate was analysed in a gas chromatographic apparatus with a flame ionization detector. Two 10 ft × $\frac{3}{16}$ in. O.D. columns were used; one containing 5 % Carbowax 20 M' on 60–80 mesh 'Chromosorb G' was programmed from 72 to 154 °C at 2 deg C/min and the other containing 20 % 'Apiezon M' on 60–80 mesh 'Embacel' was programmed from 40 to 195 °C at 4 deg C/min.

Distillations of aqueous solutions containing known amounts of alkanol in water (2 distillations through the condenser at 0 °C) resulted in approximately 90 % recovery of all the C_{1-9} n-alkan-1-ols and higher for n-alkanals, n-alk-2-enals and n-alk-1-en-3-ols. However, from deodorized control butter or deodorized commercial butterfat the recoveries of the alkan-1-ols were: C_{1-6}, 50–65 %; C_7, 40 %; C_8, 30 % and C_9 less than 5 %. This was taken into account in estimating the total amounts of the alkanols in butterfat.

The alkan-1-ols were identified by comparing their retention times with those of authentic compounds on the 2 gas chromatographic columns, by mass spectrometry, and by their infra-red spectra in carbon tetrachloride solution. To obtain sufficient amounts for mass and infra-red analyses, the concentrated distillate (4–8 ml) from the carbonyl-free distillate was degassed on a vacuum manifold to less than 10^{-3} mm Hg and allowed to distil through a trap immersed in a mixture of acetone and dry ice at −55 °C into a U-trap immersed in liquid nitrogen. The C_{1-3} alkan-1-ols were quantitatively collected almost free of water in the U-trap, while the water and the remaining alkanols condensed in the trap held at −55 °C (Forss & Ramshaw, unpublished). The water fraction was extracted with 2 ml diethyl ether and this extract and the contents of the U-trap were fractionated separately on a 6 ft × $\frac{1}{4}$ in. column containing 20 % 'Carbowax 20M' on 60–80 mesh 'Embacel'. The column was programmed non-linearly from 50 to 150 °C, and a thermistor detector was used.

RESULTS

Fig. 1 shows typical gas chromatograms of the carbonyl-free distillates of control and oxidized butter after 20-weeks storage. Insignificant amounts of n-alkan-1-ols apart from methanol and ethanol were observed in the control butters whereas appreciable amounts (approximately 1 μmole/kg) of the $C_{1,2,5-8}$ n-alkan-1-ols were observed in the oxidized butter even after 4 weeks storage although they increased only slowly in amount thereafter. Traces of n-propan-1-ol and n-butan-1-ol according to retention times were observed but the amounts were insufficient for mass and infra-red analyses.

A compound occurring in large amount (*X* in Fig. 1) and with a retention time similar to that of pent-1-en-3-ol was also observed. The concentration of n-alkanals

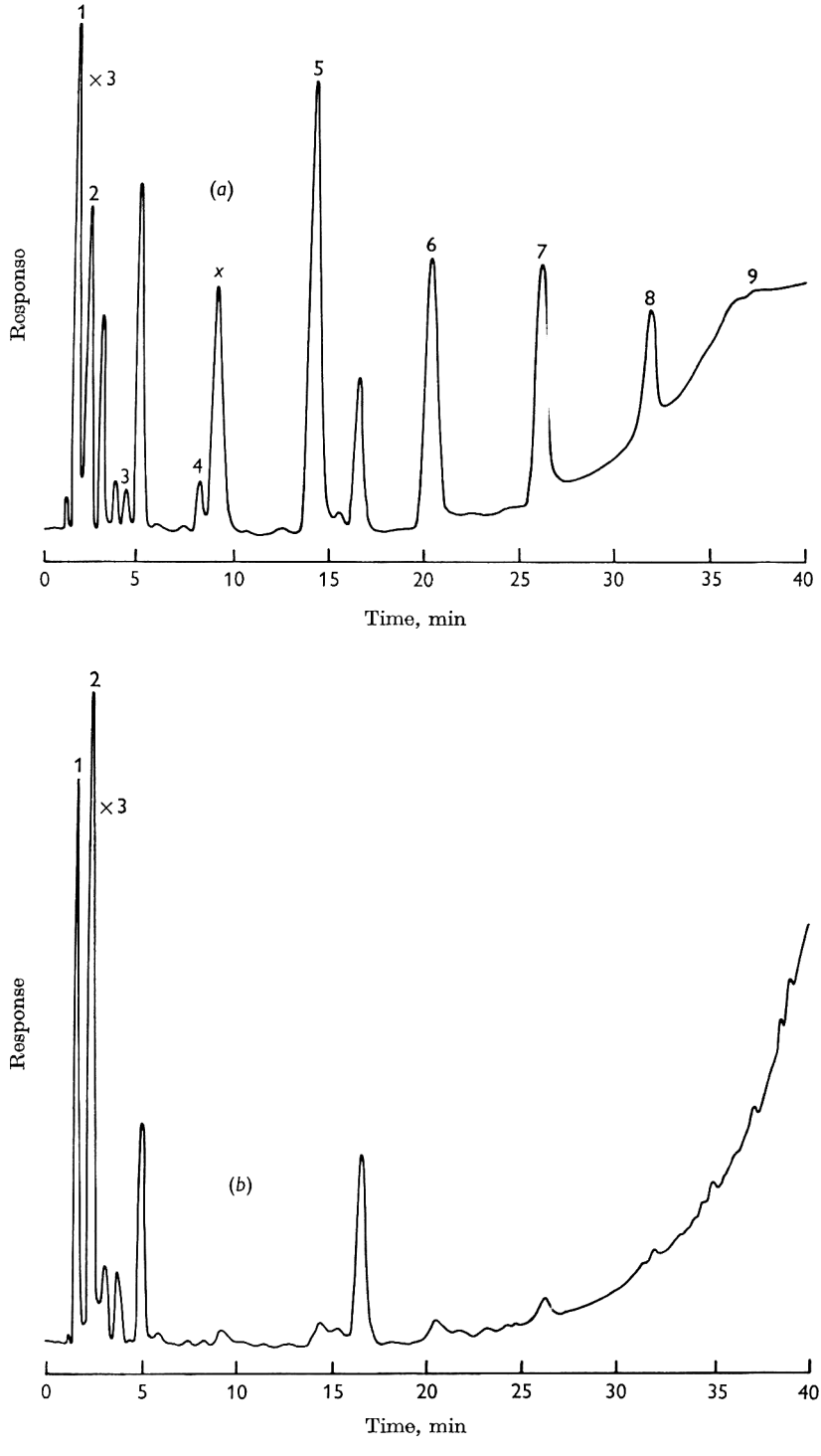


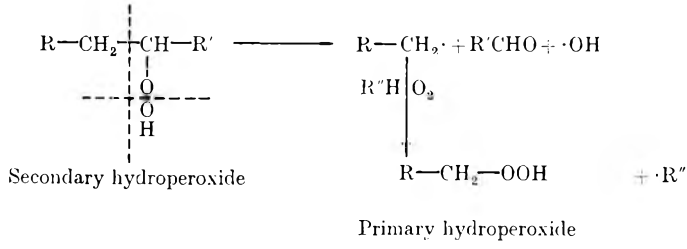
Fig. 1. Gas chromatograms of 10 μl samples of aqueous solutions of concentrated carbonyl-free distillates of (*a*) oxidized, (*b*) control butter after 20 weeks storage; Apiezon M column; numbers indicate number of carbon atoms in n-alkan-1-ols.

was similar to that of the n-alkan-1-ols while the n-alk-2-enals were present in approximately one tenth the amount of that of the n-alkanals (Stark & Forss, unpublished).

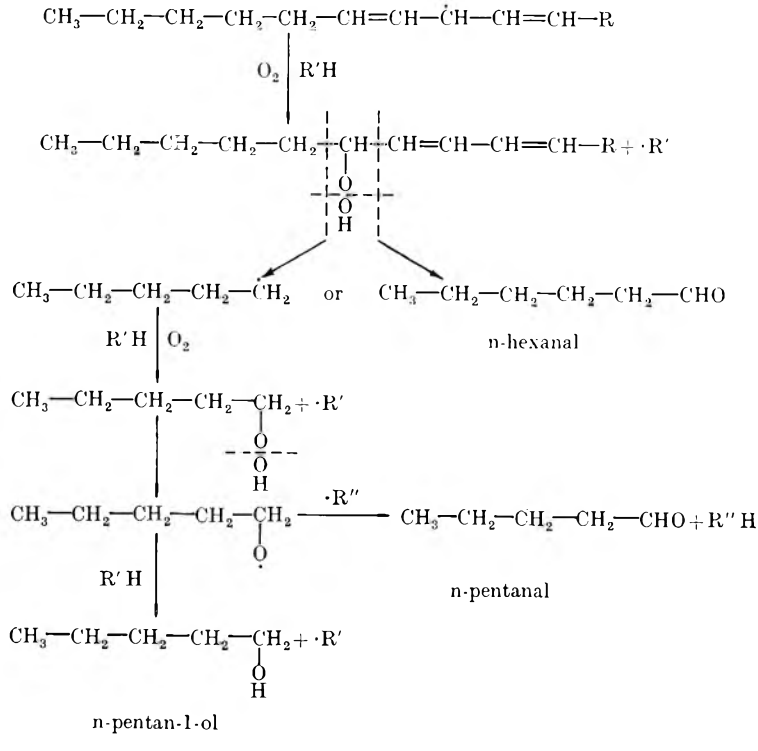
Infra-red and mass spectra of the isolated compounds were identical with those of the corresponding authentic n-alkan-1-ols.

DISCUSSION

The formation of primary terminal hydroperoxides as a result of decomposition of lipid hydroperoxides (secondary) appears to occur at an early stage of oxidation:



These may then form n-alkan-1-ols and/or n-alkanals via the alkoxy radical (Bell *et al.* 1951). The following mechanism depicts the formation of n-hexanal, n-pentanal and n-pentan-1-ol from the 13-hydroperoxide of linoleic ester:



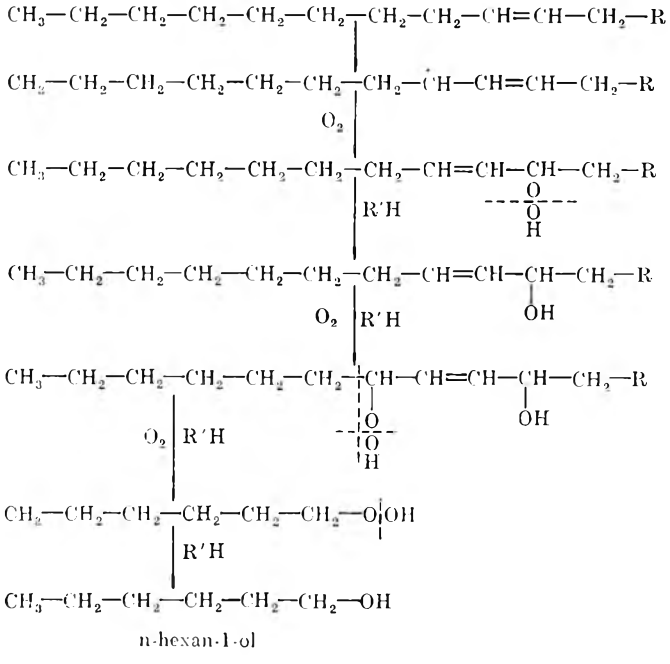
Similary, the 3 compounds can be formed from the 11-hydroperoxy-9-ene of palmitoleic ester and the 15-hydroperoxy-5,8,11,13-tetraene of arachidonic ester.

Table 1 lists the n-alkan-1-ols isolated from oxidized butter, together with the probable precursors and the percentage of the parent fatty acids found in butterfat. Our work shows that n-alkanals and n-alkan-1-ols are produced simultaneously even in the early stages of oxidation (Stark & Forss, unpublished), the major n-alkanal and n-alkan-1-ol being n-hexanal and n-pentan-1-ol, respectively.

Large amounts of n-heptanal are found in oxidized dairy products (Forss, Dunstone & Stark, 1960), but its formation from the more common unsaturated acids of butter has been difficult to explain. Lillard & Day (1964) have suggested that non-2-enal could be the precursor of n-heptanal. However, it is not necessary to consider subsequent oxidation of relatively stable unsaturated aldehydes to explain the formation of n-heptanal in oxidized butterfat. The 11-hydroperoxide of oleic acid, cleaved between carbon atoms 11 and 12 would yield a 7-carbon radical which, as shown in the above mechanism would produce n-heptanal.

The formation of only minor amounts of n-propan-1-ol and n-butan-1-ol is to be expected as their precursor (myristoleic ester) is present in only minor amounts in butter. The minor amounts of the $C_{3,4}$ n-alkan-1-ols also indicated that the formation of methanal and a homologous series of n-alkanals by stepwise degradation of the primary alkoxy radical as outlined by Bell *et al.* (1951) occurs only to a limited extent in oxidized butter.

While n-hexan-1-ol may be formed from the 10-hydroperoxide of palmitoleic ester, the relatively large amounts of this alkanol formed suggest that it may also be derived by the following mechanism from oleic acid:



Theory predicts the formation of minor amounts of C_{9-12} alkan-1-ols from the $C_{20,22}$ mono-unsaturated acids of oxidized butter, but the recent work on recoveries

in this laboratory has shown that steam distillation at reduced pressure is unsuitable for the isolation of alkan-1-ols above C_8. Distillation at lower pressure will be used in an attempt to isolate higher alkan-1-ols and for quantitative studies.

Table 1. *n-Alkanols identified from oxidized butter*

Alkanol	Probable precursor	Parent ester	Acid in butterfat, %: Herb *et al.* (1962)	Acid in butterfat, %: Maeno *et al.* (1964)
Methanol	17-Hydroperoxy-12-hydroxy-octadeca-9,13,15-trienoate	Linolenate	0·5	2·0
Ethanol	16-Hydroperoxy-octadeca-9,12,14-trienoate	Linolenate	0·5	2·0
n-Propan-1-ol	11-Hydroperoxy-tetradec-9-enoate	Myristoleate	0·8	1·9
n-Butan-1-ol	10-Hydroperoxy-tetradec-8-enoate	Myristoleate	0·8	1·9
n-Pentan-1-ol	11-Hydroperoxy-hexadec-9-enoate	Palmitoleate	1·8	0·8
	13-Hydroperoxy-octadeca-9,11-dienoate	Linoleate	2·1	2·7
	15-Hydroperoxy-eicosa-5,8,11,13-tetra-enoate	Araehidonate	0·14	Trace
n-Hexan-1-ol	10-Hydroperoxy-hexadec-8-enoate	Palmitoleate	1·8	0·8
	12-Hydroperoxy-9-hydroxy-octadec-10-enoate	Oleate	30	30
n-Heptan-1-ol	11-Hydroperoxy-octadec-9-enoate	Oleate	30	30
n-Octan-1-ol	10-Hydroperoxy-octadec-8-enoate	Oleate	30	30

The authors thank their colleagues Mr J. F. Smith for the mass spectrometry and Mr R. A. Wilkinson for helpful criticism.

REFERENCES

BELL, E. R., RALEY, J. H., RUST, F. F., SEUBOLD, F. H. & VAUGHAN, W. E. (1951). *Disc. Faraday Soc.* No. 10, 242.

EVANS, C. D. (1961). *Proc. Flavor Chem. Symp. Camden, New Jersey*, p. 123. Organized by the Campbell Soup Co.

FARMER, E. H., KOCH, H. P. & SUTTON, D. A. (1943). *J. chem. Soc.* p. 541.

FORSS, D. A., DUNSTONE, E. A. & STARK, W. (1960). *J. Dairy Res.* **27**, 373.

HERB, S. F., MAGIDMAN, P., LUDDY, F. E. & RIEMENSCHNEIDER, R. W. (1962). *J. Am. Oil Chem. Soc.* **39**, 142.

KLÖPFER, W., ESTERBAUER, H. & SCHAUENSTEIN, E. (1965). *Fette Seifen, Anstrichmittel*, **67**, 198.

LILLARD, D. A. & DAY, E. A. (1964). *J. Am. Oil Chem. Soc.* **41**, 549.

MAENO, M., RYOKI, T. & KUDO, T. (1964). *J. Jap. Soc. Fd Nutr.* **16**, 41.

STARK, W. & FORSS, D. A. (1964). *J. Dairy Res.* **31**, 253.

STARK, W. & FORSS, D. A. (1965). *Nature, Lond.*, **208**, 190.

Printed in Great Britain

Lactose in the blood and urine of cows

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

(*Received* 16 *September* 1965)

Summary. Lactose could not be detected in the urine of non-lactating cows but was invariably found, though usually in low concentration, in the urine of lactating cows; the highest values were observed with cows of high lactation number. Towards the end of an extended milking interval of 39 h, the lactose concentration in the blood and the excretion of lactose in urine increased markedly, but on milking there was a rapid return to original values. Lactosuria was observed in pregnant cows from about 20 days before parturition, and at parturition there was a temporary, marked increase in the excretion of lactose in urine.

Colostrum and milk which is removed from the udder after a long milking interval or after a period of incomplete milking are characterized by a low lactose content (Rook & Campling, 1965; Wheelock, Rook & Dodd, 1965), and the question arises whether the altered composition is the result of a change in the composition of the mammary secretion or of a preferential resorption of the secreted lactose. Lactosuria in women in the early stages of lactation, or even in the latter part of pregnancy, is not uncommon (Sjollema, 1927*a*; Watkins, 1928; Brock & Hubbard, 1935): only traces of lactose are detected in the blood since lactose, which cannot be utilized by body tissues, is excreted rapidly. Lactosuria and lactosaemia have been reported also in lactating cows and goats (Suzuki & Umezu, 1957, 1958, 1960; Hayashi, 1960) and a marked increase in the lactose content of the blood and in the loss of lactose in the urine has been found after inflation of the udder (Sjollema, 1927*b*). The present investigation was made to determine the quantitative importance in cows of the loss of lactose in the urine under normal management conditions, after an extended milking interval, and at parturition.

EXPERIMENTAL

In one experiment samples of urine were collected from 44 Friesian cows of the Institute herd at or about the time of the afternoon milking. In a second experiment with 5 Friesian cows (1–5), quantitative collections of urine were made during (1) an initial control period of 33 or 57 h, when the cows were milked at the usual 9-h day and 15-h night intervals, (2) a period of 39 or 24 h when the cows were left unmilked, and (3) a final control period of 33 h when normal milking was reintroduced. In a third experiment with 2 cows (6 and 7), quantitative collections of

* Present address: Department of Agriculture, The University, Leeds.

urine were made at intervals throughout the last month of pregnancy and the first week of lactation. Quantitative collection of urine was made with the equipment of Balch, Bartlett & Johnson (1951), with the exception of cows 4 and 5 in the second experiment when collection was made through a catheter. In the second experiment, blood samples were taken from all the cows, except cow 4, from the jugular vein through an indwelling polythene cannula, at intervals throughout the control and experimental periods. At each milking the yield of milk was recorded and a sample taken.

Lactose in samples of blood plasma or urine was detected by thin-layer chromatography on Kieselguhr G (Stahl & Kaltenbach, 1961): samples of urine or of an ultrafiltrate of blood plasma were freeze-dried and an appropriate quantity (where possible containing at least 0·5 μg lactose) of a pyridine extract of the residue was applied to the layer. For the estimation of glucose and lactose, samples of plasma or urine were deproteinized by dilution (1:10) with 0·16% (w/v) uranyl acetate in 0·9% (w/v) saline. Glucose was estimated by the glucose oxidase method of Huggett & Nixon (1957). Lactose was estimated as the increase in glucose concentration after acid hydrolysis; 0·5 ml deproteinized filtrates + 0·5 ml 2N-HCl in a stoppered test tube was incubated at 95°C. for 18 h. Lactose in samples of milk was determined by a modification of the method of Hinton & Macara (1927).

RESULTS

Excretion of lactose in the urine of cows under normal management conditions. The concentration of lactose in samples of urine of 4 non-lactating cows and of 40 lactating cows in the middle part of their lactation are shown in Fig. 1. The cows varied in udder health and the current lactation numbers ranged from 1 to 7. Lactose could not be detected (< 2 mg/100 ml) in the urine of the non-lactating cows, whereas invariably it was present in the urine of the lactating cows, but usually in low concentration. All the cows with lactation numbers of 1–3 had concentrations lower than 20 mg/100 ml but in the cows of higher lactation number concentrations of up to 70 mg/100 ml were observed.

Effect of an extended milking interval on excretion of lactose in the urine. The results obtained with cows 1–3 are given in Table 1. As observed previously (Wheelock *et al.* 1965), an extended milking interval was associated with a marked decrease in the yield and concentration of milk lactose. A large part of this was accounted for by the excretion of lactose in the urine in association with a measurable increase in the lactose content of the blood plasma. There was additionally, however, a temporary loss of ability to secrete lactose as evidenced by the reduced rate of secretion of lactose in the final control period. The detailed changes in the concentration of lactose in blood and in the excretion of lactose in urine are shown for cow 5 in Fig. 2. There was a rapid rise in the excretion of lactose which commenced when milk had been accumulating in the udder for 21 h. The maximum excretion rate of about 18 g/h was reached 8 h later and was maintained for the remainder of the extended interval, except for a decrease shortly before milking. After milking, the excretion decreased rapidly and values similar to those found during the initial control period were observed within 4 h. Parallel changes were observed for the concentration of lactose in blood.

The changes in lactose excretion in cow 4 were similar to those in cow 5, but since the extended milking interval was only 24 h the rate of excretion was still at a maximum when the cow was milked. Shortly after the end of the interval there was a rapid fall in the excretion rate.

The lactose excreted in the urine during the first hours after a milking which followed an extended milking interval would be largely lactose already present in the blood and urine at that time.

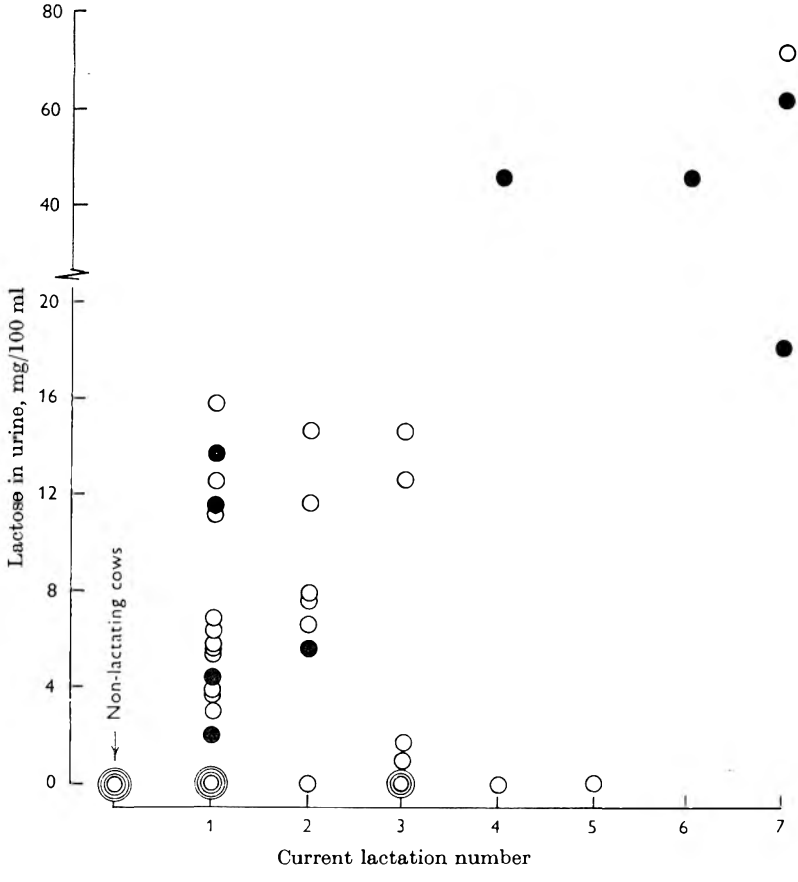


Fig. 1. The concentration of lactose in the urine of 4 non-lactating cows, and of 40 lactating cows in the middle part of their lactation but varying in lactation number and udder health. Single samples of urine were collected at about the time of the afternoon milking. ○, Animals free of infection in the current lactation; ●, animals in which clinical signs of udder infection had been reported in the current lactation.

Excretion of lactose in the urine of cows in late pregnancy and the first days of lactation. The results for cows 6 and 7 were similar and those for cow 7 are given in Fig. 3. Lactose was detectable (> 2 mg/100 ml) in urine as early as 20 days before parturition but the rate of excretion was low until the 8th or 6th day. There was a sharp increase in the rate of excretion at parturition but the rate then fell rapidly to low values similar to those for the main lactation period.

Table 1. *Effect in the cow of an extended milking interval on the excretion of lactose in urine, the concentration of lactose in blood, the yield of lactose in milk and the concentration of lactose in milk*

(During initial and final control periods the cows were milked routinely at 6 a.m. and 3 p.m. and for an intervening period of 39 h they were left unmilked. In calculating the increased urinary loss of lactose due to the extended milking interval allowance was made for the carry-over into the final control period.)

	Lactose excretion in urine, g/24 h		Lactose concentration in blood plasma, mg/100 ml		
Cow	Initial control period	Increase due to extended milking interval	Mean value during initial control period	Maximum value during experimental period	Final control value
1	1	284	1	35	3
2	0	256	0	21	6
3	1	111	10	44	11

	Yield of milk lactose, g/24 h			Lactose concentration in milk, % (w/v)	
	Initial control period	Period of extended milking interval	Final control period	Mean value during initial control period	Value at the end of extended interval
1	887	432	431	4·58	3·80
2	563	267	462	4·68	3·86
3	827	402	501	4·94	4·04

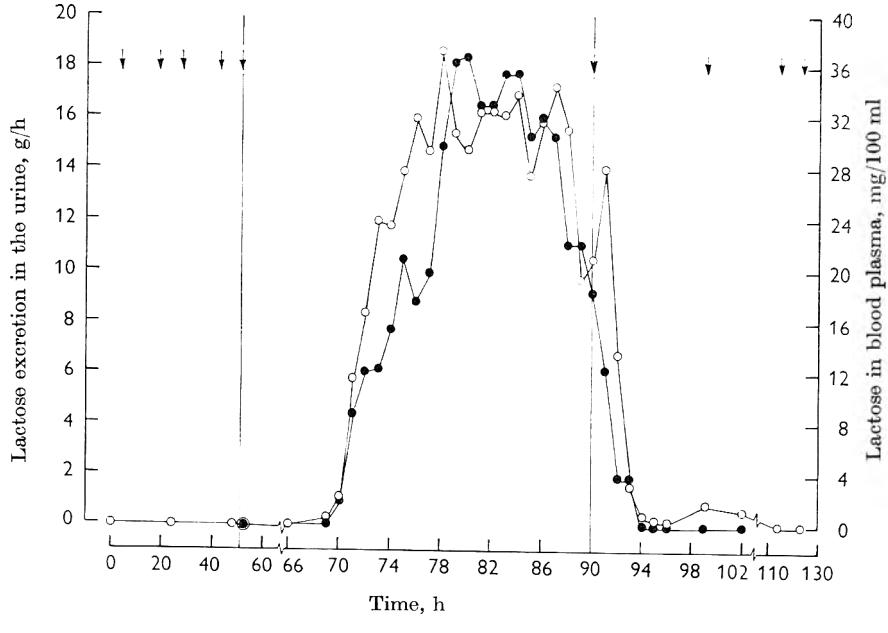


Fig. 2 (cow 5). The effect of an extended milking interval on the concentration of lactose in the blood plasma and on the excretion of lactose in the urine. ○, Lactose in urine; ●, lactose in blood; ↓, cow milked out.

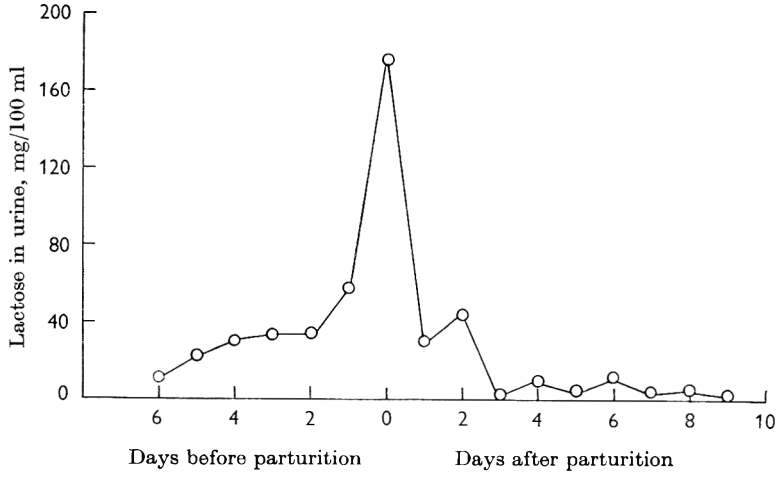


Fig. 3 (cow 7). The excretion of lactose in late pregnancy and during the first days of lactation. The cow was milked immediately after parturition and twice daily thereafter.

DISCUSSION

The absence of lactose from the blood and urine of non-lactating cows, and its invariable presence in the urine of lactating animals, is strong circumstantial evidence of a continuous slight loss of lactose into the blood from the actively secreting mammary gland. The generally high values observed with cows of high lactation number suggest that the extent of the loss is increased by physiological changes within the udder associated with age, which are possibly in part the result of damage caused by bacterial infection. Also, the evidence is that the accumulation of milk within the udder, as will occur towards the end of an extended milking interval and in the period immediately before parturition, increases considerably the rate of movement of lactose out of the gland.

The decrease in the excretion rate of lactose, observed 2 h before the end of the extended interval in cow 5, is consistent with other work which has shown that the rate of lactose production is depressed when milk accumulates in the udder (Wheelock *et al.* 1965). On the other hand, resorption is unlikely to cease completely while there is a large volume of milk present in the udder, as it has been shown that after drying off resorption continues until there are relatively small amounts of lactose remaining in the mammary gland (A. Smith, personal communication). Since at the end of the extended interval there was a substantial amount of lactose obtained at milking, which was followed by a rapid fall in the excretion of lactose in the urine, it is concluded that resorption of lactose ceases when milking is resumed.

Our results show that when the usual system of milking twice a day is practised the total loss of lactose in the urine is negligible in comparison with the amount secreted by the mammary gland, but that when the milking interval is extended, as much as 50% of the secreted lactose can be excreted in the urine. The decrease in lactose concentration and in lactose/casein ratio found in milk observed after an extended milking interval (Wheelock *et al.* 1965) could thus be accounted for by a

preferential resorption of lactose. The low lactose content of milk during the first days of lactation may also be due to lactose resorption.

We are grateful to Mr V. W. Johnston for care of the experimental animals and to Mrs D. J. Knight, Mr A. F. Hamnett, Miss M. Weston and Miss S. Futcher for skilled technical assistance.

REFERENCES

BALCH, C. C., BARTLETT, S. & JOHNSON, V. W. (1951). *J. agric. Sci., Camb.*, **41**, 98.
BROCK, H. J. & HUBBARD, R. S. (1935). *Am. J. dig. Dis.* **2**, 27.
HAYASHI, M. (1960). *Bull. natn. Inst. Anim. Hlth, Tokyo*, **38**, 143. (*Dairy Sci. Abstr.* (1961). **23**, 1967.)
HINTON, C. L. & MACARA, T. (1927). *Analyst, Lond.*, **52**, 668.
HUGGETT, A. St. G. & NIXON, D. A. (1957). *Biochem. J.* **66**, 12*P*.
ROOK, J. A. F. & CAMPLING, R. C. (1965). *J. Dairy Res.* **32**, 45.
SJOLLEMA, B. (1927*a*). *Biochem. Z.* **182**, 453.
SJOLLEMA, B. (1927*b*). *Biochem. Z.* **185**, 355.
STAHL, E. & KALTENBACH, U. (1961). *J. Chromat.* **5**, 351.
SUZUKI, J. & UMEZU, M. (1957). *Med. & Biol., Japan*, **42**, 47. (*Dairy Sci. Abstr.* (1961). **23**, 1969.)
SUZUKI, J. & UMEZU, M. (1958). *Med. & Biol., Japan*, **47**, 43. (*Dairy Sci. Abstr.* (1961). **23**, 1969.)
SUZUKI, J. & UMEZU, M. (1960). *Med. & Biol., Japan*, **56**, 115. (*Dairy Sci. Abstr.* (1961). **23**, 1969.)
WATKINS, O. (1928). *J. biol. Chem.* **80**, 33.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965). *J. Dairy Res.* **32**, 237.

Printed in Great Britain

The effect of mammary gland denervation on the fatty acid composition of goat's milk

By P. F. V. WARD and N. S. HUSKISSON
Agricultural Reseach Council, Institute of Animal Physiology, Babraham, Cambridge

(*Received* 10 *October* 1965)

Summary. Milk was analysed from twin Saanen goats. In one of the goats one mammary gland was transplanted to the neck. The milk yields and fat content from each gland were recorded and the composition of the fat examined using gas–liquid chromatography. The yield and fat content of the milk from all the glands fell within the normal range. With the operated goat, the milk from the transplanted gland differed little in fatty acid composition from that from the non-transplanted gland. Milk from either gland differed little in this respect from milk from the normal goat.

The transplantation of a goat mammary gland from its normal position to the ventral surface of the neck of the same animal has been successfully accomplished by Linzell (1960, 1963). A gland transplanted in this way appears to function quite normally yet contains no active nerve tissues. By leaving one mammary gland of a goat in the normal position and transplanting the other, the effect of transplantation on the gland may be examined in the presence of a control. This provides a system which lends itself readily to biochemical examination. It has long been considered that the function of the mammary gland is largely independent of nervous action and that it is controlled mainly by hormonal influence. There is, however, a large group of workers in Russia (Zaks, 1958) who have collected evidence to show the great importance of nerves on the mechanism of milk production. Linzell (1963) has shown that the milk yield of transplanted glands falls within the normal range and in the present work the fat content and composition have been analysed.

EXPERIMENTAL

Milk samples

Milk samples were obtained from twin, 20-month old, non-pregnant Saanen goats that had been fed on a pellet diet of known composition. The left mammary gland of one of the goats had been transplanted to the ventral surface of the neck 4 months previously (Linzell, 1960, 1963). Samples were collected each morning and evening for 3 days. The total contents of each gland were milked into a glass container and stirred with a glass rod until well mixed. Portions (50 ml) were removed and stored in a refrigerator at 1 °C. At the end of the 3-days' milking, the 6 portions from each gland were shaken well and mixed to form a single representative milk sample. The final samples were constituted by mixing the portions from each milking session in the ratio (by volume) of the corresponding milk yields.

Determination of fat

The fat contents of 10 ml milk samples from each gland were measured in duplicate using the Röse–Gottlieb method as published by the British Standards Institution (B.S. 1741: 1951).

Extraction of fatty acids

The fats from 10-ml milk were saponified for 4 h by refluxing with 5% w/v methanolic KOH (20 ml). Afterwards the methanol was removed at 40 °C using a rotary evaporator at reduced pressure (waterpump). All further manipulations were carried out in a cold room at 1 °C using cooled materials. The flask contents were acidified with 5 N-HCl (15–20 ml) and transferred to a separating funnel. The aqueous mixture was extracted 3 times by shaking for 1 min with 15 ml ether. The combined ether extracts were dried over anhydrous Na_2SO_4 and diazomethane gas from a micro-generator (Roper & Ma, 1957) was passed through the ethereal solution at room temperature until a yellow colour persisted. The ethereal solution of methyl esters was stored in a deep freeze until required for analysis.

Measurement of fatty acid composition

This was measured using a Pye Panchromatograph gas–liquid chromatograph (W. G. Pye & Co. Ltd., Cambridge). A column (0·5 cm × 5 ft) was packed with 10% (w/w) Apiezon L grease on Gas Chrom Z (Applied Science Laboratories, State College, Pa., U.S.A.). The temperature was maintained at 100 °C for 4 min after sample injection, then raised at 12 degC/min until 200 °C was reached. The argon carrier gas flow was maintained throughout at 40 ml/min using a Pye gas-flow regulator. Sample injections of about 70 μg fatty-acid methyl esters in 1–5 μl ether were made using a Hamilton syringe (Hamilton Co. Inc., Whittier, California, U.S.A.). A flame-ionization detector was employed. The results were presented simultaneously on 2 synchronized pen-recorders, one showing normal peaks measuring instantaneous concentration of fatty-acid esters in the column effluent, and the other integrating these peaks to give a step-like response. We found the use of an electronic integrating amplifier for peak area measurement, as described, superior in accuracy to either planimetry or triangulation. For the very tall, narrow peaks produced during temperature-programmed chromatography it is difficult to measure peak widths for triangulation purposes with accuracy, and for small peaks the areas approach the limit of sensitivity of a planimeter.

Fig. 1 illustrates the method used for calculating peak areas. An extreme case has been chosen in which a component is eluted before the solvent peak has entirely emerged. The direction of pen travel is from right to left on both records. The 2 records were lined up as shown and lines AA_1 and BB_1 were drawn perpendicular to the zero response lines through points judged to represent the start and finish, respectively, of the peak. This gave a more accurate indication of the start and finish of the peak on the integrated record than observing its change of slope. A third line CC_1 for each peak was drawn midway between AA_1 and BB_1. Lines XX_1 and YY_1 tangential to the integrated record were constructed at the start and finish of each peak and pro-

duced until they cut CC_1 at P and Q. That the length $PR+RS+SQ$ is, to a good approximation, proportional to the area of the peak on the normal record can be seen from the following explanation.

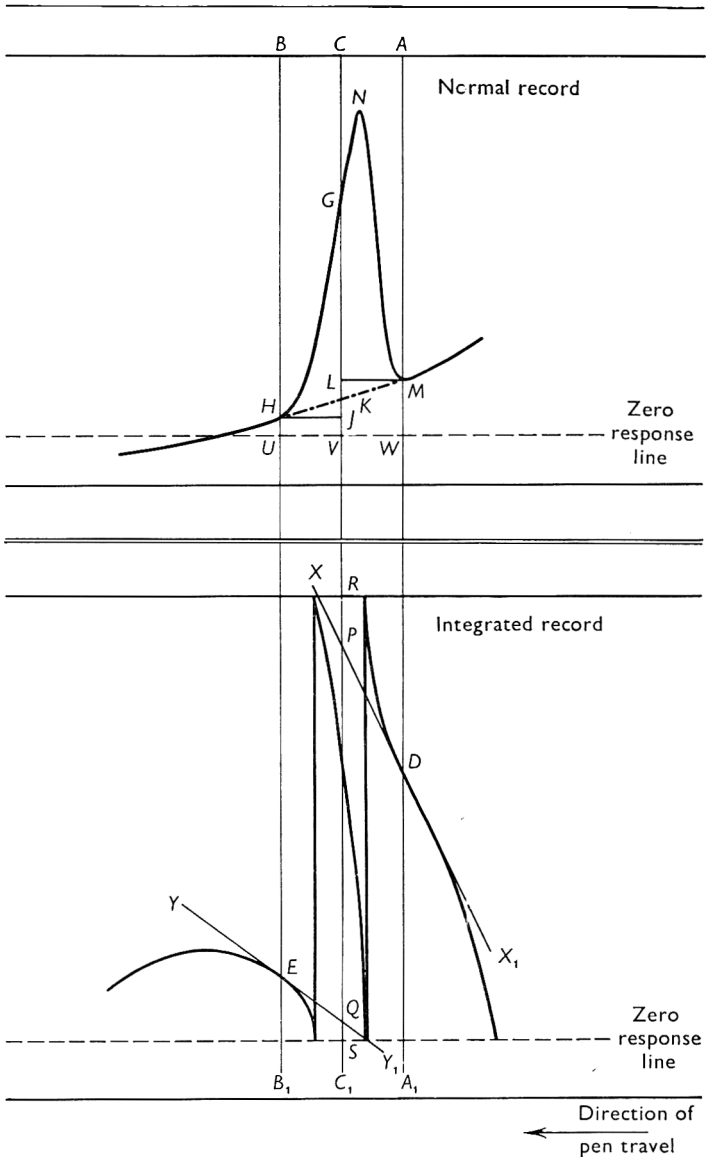


Fig. 1

At any instant the rate of rise on the integrated record is proportional to the height of the pen above the zero response line on the normal record. Therefore, considering the part of the peak between AA_1 and CC_1, as the pen travels from M to G on the normal record, the rise on the integrated record is proportional to the area $MNGVWM$. If during the same time the pen had remained at the height of M it would now be at L, and the pen on the integrated record, if it had started at D, would have

risen to P. If these two rises are subtracted from one another a rise proportional to the area $LMNGL$ is obtained. By a similar argument, considering the part of the peak between CC_1 and BB_1, the area integrated is proportional to the area $HJGH$.

The sum of the two halves of the peak integrated by this method is the area $HJKLMNH$, where HJ and LM are parallel to the chart zero response line and HKM is a straight line. Since the triangles HJK and KLM are congruent, the area integrated is equal to the area of the peak $HKMNH$. This assumes that during the time in which the peak is being eluted, the rate of change of base line is constant. Under the conditions used this was very nearly so, since when the variations in base line are greatest, the peaks are very narrow, and later, when the peaks are wider, the variations in base line are correspondingly less.

The fatty acids from each milk sample were chromatographed 3 times and 8 peaks chosen for measurement. Milk samples were compared by percentage composition tables in which each peak was represented by

$$\frac{\text{peak step height on integrated record}}{\text{total peak step height for the eight chosen peaks}} \times 100.$$

RESULTS AND DISCUSSION

In our experiments the transplantation of a mammary gland to the neck of a goat had no detectable effect on the milk yield (Table 1). This gland not only produced as much milk as its non-transplanted counterpart, but also the yield from both glands was well within the normal range. These results confirm those of Linzell (1963). The milk fat content from the transplanted gland also fell within the normal range (Table 2).

We have used gas–liquid chromatography under carefully controlled conditions to detect any changes in fatty acid composition following transplantation of the mammary gland. The 7 largest acid peaks and the composite peak formed by the unsaturated 18-carbon atom acids were chosen for measurement. These represent approx. 89% of the total acids in the milk fat. None of the other acids was present in a concentration greater than 1%. Figures for the percentage composition of the acids (Table 3) are not values for the percentage composition in the milk fat as no account has been taken of the other acids present.

Although gas chromatography is often used for the quantitative analysis of fatty acids, all too often these analyses are based on the area measurement of peaks from a single chromatogram. In our experience surprisingly different analyses can be obtained from 2 chromatograms of the same substance and this is substantiated by the survey of Horning *et al.* (1964). For this reason 3 chromatograms were run for every milk fatty acid sample. The volume of every sample injected was adjusted to give peaks of similar size to aid in the accurate measurement of areas.

The gas chromatographic results show very little difference in the combined fatty acid composition of the milk from all 4 glands (Table 4). In goat 1, the only difference in the mean percentage composition between the transplanted and control glands that was greater than the sum of the 2 standard deviations was represented by the composite peak 18:1 + 18:2 + 18:3 and this was only 0·05%. In goat 2, the differences between the left and right glands were slightly greater. The peaks 8:0, 10:0 and

18:1+18:2+18:3 gave values 0·01, 0·58 and 0·22, respectively, for the difference between the 2 mean percentage compositions minus the sum of the standard deviations but none of these differences in composition are significant ($P > 0{\cdot}01$). These greater differences may be due to the wider variation in the milk yield and its fat content between these 2 glands (Tables 1 and 2). Whereas the transplanted and control glands of goat 1 gave yields differing by only 80 ml in 3 days, the difference in yields between the left and right glands of goat 2 was as much as 1450 ml over the same period.

Table 1. *Milk yield from twin Saanen goats*

	Goat 1		Goat 2	
Mammary glands ...	Transplant, ml	Control, ml	Left, ml	Right, ml
Day 1 a.m.	870	950	670	980
p.m.	340	320	270	420
Day 2 a.m.	700	710	610	960
p.m.	270	260	280	420
Day 3 a.m.	700	710	640	1000
p.m.	350	360	270	410
Total	3230	3310	2740	4190

Goat 1 had the left mammary gland transplanted to the ventral surface of the neck.

Table 2. *Fat content of milk from twin Saanen goats*

Goat	Mammary gland	Sample	Fat content, % (w/v)	Average fat content, % (w/v)
1	Transplanted	1	4·60	4·63
		2	4·65	
	Control	1	4·74	4·78
		2	4·82	
2	Left	1	3·66	3·65
		2	3·63	
	Right	1	4·00	4·01
		2	4·01	

The results from goat 2 give an indication of the variation that can be expected both of milk yield, fat content and composition of milk fatty acids in the normal animal. It may, therefore, be concluded that the transplantation and consequent denervation of a goat mammary gland has no effect on the relative amounts of the major combined fatty acids in the milk, the yield of the milk or its fat content.

These results are in contrast to those obtained by a group of Russian workers under Baryshnikov (Zaks, 1958) who uphold the view that the nervous system is essential for the normal function of the mammary gland. In particular Zotikova (1955) has reported the extrusion of cell contents, including fat, into the aveolar lumen following nerve stimulation of mouse mammary gland though her views were somewhat modified later (Zotikova, 1962). Pavlov & Markaryan (1957) also found an increased milk fat concentration in the cow following stimulation of the lumbar spinal cord. In a later paper, Tverskoy (1962) was unable to show that efferent nerves in the goat udder influenced fat secretion, but believed they exerted an effect on the chemical composition of the fat secreted.

	Goat 1								Goat 2							
	Transplanted gland				Control gland				Left gland				Right gland			
	Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2		Sample 1		Sample 2	
Acid*	%†	Range‡	%	Range	%	Range	%	Range	%	Range	%	Range	%	Range	%	Ra
8:0	0·54	0·06	0·62	0·21	0·61	0·14	0·57	0·05	0·60	0·28	0·55	0·12	0·41	0·07	0·44	0·
10:0	7·95	0·56	8·08	0·91	7·57	0·81	7·94	0·80	8·49	2·02	8·57	1·29	6·79	1·05	6·99	0·
12:0	4·33	0·05	4·37	0·26	4·08	0·20	4·28	0·14	5·03	0·92	5·40	0·76	5·21	0·08	5·23	0·
14:0	10·35	0·38	10·64	1·18	10·03	0·05	10·13	0·18	10·48	0·84	10·83	0·44	10·65	0·17	10·65	0·
16:1	1·48	0·08	1·54	0·06	1·49	0·11	1·65	0·25	1·81	0·14	1·64	0·29	1·73	0·25	1·86	0·
16:0	28·78	0·42	27·59	0·30	28·11	0·22	28·36	0·42	27·08	1·05	27·23	0·45	27·33	0·40	27·09	0·
18:1+18:2+18:3	33·44	0·92	34·37	0·59	34·83	0·35	34·54	0·71	33·82	1·33	33·39	0·75	34·56	1·32	34·87	0·
18:0	13·11	0·79	12·83	0·29	13·27	0·97	12·58	0·65	12·69	0·53	12·40	0·90	13·33	0·40	12·87	0·

* Acid nomenclature is that of Farquhar *et al.* (1959).
† These figures give the amount of an acid expressed as a percentage of the sum of the amo
of all the acids determined and is the mean of 3 analyses.
‡ Difference between highest and lowest values.

Table 4. *Fatty acid composition of milk samples from denervated and normal goat mammary glands*

	Goat 1					Goat 2				
	Transplanted gland		Control gland			Left gland		Right gland		
Acid*	%†	σ‡	%	σ	Variation, %§	%	σ	%	σ	Variatio
8:0	0·58	0·07	0·59	0·05	1·7	0·57	0·09	0·42	0·05	30·0
10:0	8·03	0·33	7·78	0·39	3·2	8·56	0·75	6·89	0·34	21·6
12:0	4·35	0·08	4·16	0·11	4·5	5·23	0·42	5·21	0·13	0·4
14:0	10·46	0 42	10·08	0·07	3·7	10·71	0·34	10·65	0·08	0·6
16:1	1·51	0·42	1·57	0·11	3·9	1·70	0·13	1·77	0·11	4·0
16:0	28·24	0·62	28·23	0·19	0·04	27·19	0·34	27·22	0·17	0·1
18:1+18:2+18:3	33·80	0·55	34·67	0·27	2·5	33·53	0·50	34·70	0·45	3·4

The authors wish to thank Dr J. L. Linzell for making available to them his experimental goats and for helpful suggestions.

REFERENCES

FARQUHAR, J. W., INSULL, W., ROSEN, P., STOFFEL, W. & AHRENS, E. H. (1959). *Nutr. Rev.* **17**, suppl. 18, part 2.
HORNING, E. C., AHRENS, E. H., LIPSKY, S. R., MATTSON, F. H., MEAD, J. F., TURNER, D. A. & GOLDWATER, W. H. (1964). *J. Lipid Res.* **5**, 20.
LINZELL, J. L. (1960). *Nature, Lond.*, **188**, 596.
LINZELL, J. L. (1963). *Q. Jl exp. Physiol.* **48**, 34.
PAVLOV, E. F. & MARKARYAN, A. K. (1957). *Izv. Akad. Nauk. armyan. SSSR. Biol. selskokhoz. Nauki*, **10**, 23.
ROPER, R. & MA, T. S. (1957). *Microchem. J.* **1**, 245.
TVERSKOY, G. B. (1962). *16th Int. Dairy Congr.*, Copenhagen, p. 119.
ZAKS, M. G. (1958). *The Motor Apparatus of the Mammary Gland*, Moscow U.R.S.S. Acad. Sci., English translation, 1962. London and Edinburgh: Oliver & Boyd.
ZOTIKOVA, I. N. (1955). *Trudy Inst. Fiziol. I. P. Pavlova*, **4**, 63.
ZOTIKOVA, I. N. (1962). *Dokl. Akad. Nauk. S.S.S.R.* **142**, 204.

Printed in Great Britain

Determination of leucocyte concentrations in cow's milk with a Coulter counter

By L. W. PHIPPS

National Institute for Research in Dairying, Shinfield, Reading

AND F. H. S. NEWBOULD

Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

(*Received* 21 *October* 1965)

SUMMARY. A technique for determining the concentration of leucocytes in cow's milk is described which is more rapid and accurate as well as much less tedious than conventional microscope methods. The leucocytes are isolated from the fat globules of comparable size by a novel centrifuging procedure. Sizing of the leucocytes with a Coulter electronic counter shows that their volumes range from about 45 to 1770 μm^3 but a partially overlapping distribution of other particulate matter prevents the accurate determination of the total number of cells present. The numbers counted in truncated leucocyte distributions, however, correlate linearly with total cell counts determined with an improved microscope method up to 6×10^6 cells/ml of milk.

INTRODUCTION

The method of determining the concentration of leucocytes in cow's milk with the microscope using the technique of Prescott & Breed (1910) is well known and in widespread use. The process is slow and tedious, however, and ordinarily the accuracy is low (Paape, Hafs & Snyder, 1963). Accordingly, the application of the Coulter electronic counter (Coulter, 1956) to this particular problem has been studied. This has resulted in a suitably accurate procedure which has now been in routine use for some months in one of our laboratories (Ontario Veterinary College). The method has been outlined in a previous communication (Phipps & Newbould, 1965); the present paper describes the work in detail.

The electronic counter offers the possibility of rapid, accurate counting if a procedure can be devised for discriminating between leucocytes and other material, particularly the fat globules which are in superior numbers and whose size range overlaps that of the cells. Since direct discrimination with the instrument is not possible a preliminary separation of the cells and the interfering fat globules is necessary. An appreciation of the nature and the size distribution of the recovered cellular material then enables estimates of leucocyte numbers to be made from counts taken with the instrument at suitable threshold size levels.

Chemical methods of treating milk to enable the cells to be differentiated from the fat globules are quite unpromising. Thus, counts on milks done before and after

chemical destruction of the cells are not likely to lead, by difference, to accurate estimates of cell numbers owing to the preponderance of the numbers of fat globules of sizes comparable with the leucocytes and to their variable clumping behaviour. Chemical dissolution of the fat globules to leave intact cells would seem a possible approach, but most chemicals can be expected to alter the size of the leucocytes and it is doubtful whether repeatable conditions or accurate counting could be achieved.

After much experiment a separation of cells and fat globules under special centrifuging conditions was devised. When centrifuging whole milk at laboratory temperatures progressive creaming of the fat carries some of the cells, which would otherwise sediment, to the milk surface. For adequate separation it was found necessary to dilute the milk and convenient to use 0·85 % saline, a suitable medium in which finally to suspend cells for counting. Reliable and complete physical removal of the creamed fat in the centrifuge tube was achieved by a novel, simple procedure.

A model A Coulter counter was used incorporating a manometer metering a 50-μl suspension volume through a 70-μm diam. orifice. With this orifice size, suspensions were metered rapidly and blocking of the orifice by debris was not troublesome. Since the 'drop-out' threshold size for the smallest leucocytes is almost always ill defined owing to an overlapping distribution of other particulate matter, counts were taken at thresholds which minimize the influence of this material. This led to a measure of about 70 % of the leucocytes present per ml of milk and it is these data that have been correlated with careful counts obtained with an improved microscope method. A single operator was able to analyse about 50 samples/day by the electronic method without the use of automatic sampling and dispensing devices.

MATERIALS AND METHODS

Leucocyte counting with a microscope

With micro-pipettes, four 0·01 ml samples of each well-shaken milk were deposited on a glass microscope slide and spread as evenly as possible with a fine wire. Each sample occupied an area of nominally 1 cm^2 defined by a square etched on the slide with a diamond point. The dimensions of each square were measured and subsequent counts corrected for any small differences in film areas. After drying, the films were de-fatted in xylene, fixed in ethanol-acetic acid and stained (Moats, 1964) with toluidine blue (pH 4·0).

Since in most films the cells can be expected to be unevenly distributed (Hanks & James, 1940) a system of stratified sampling of each film area was used. Cells were counted in strips across a film. The magnification produced by the combination of a $\times$ 10 ocular and a $\times$ 60 oil immersion objective was such that the width of each strip, defined by the length of a graticule line in the ocular, was 0·078 mm. Two strips from each of the five 2-mm wide bands into which a film can be divided were selected by a random method and the number of leucocytes in these 10 strips counted. An estimate of the leucocyte concentration was made from each of the 4 films of a milk and the mean of these estimates taken to be the absolute count.

A pneumatic, vibration-free slide-moving device was designed especially for this work to increase the speed and ease of counting. The film scanning rate could be

adjusted to one appropriate to the concentration of cells in a film. All counting was done by the same observer. The coefficient of variation of estimated cell concentrations was about 10 % for all concentrations down to about 300000/ml but rising rather rapidly to about 19 % at 50000. The data indicate that the main source of error arises from the 'between-film' component.

Isolation of cells for electronic counting

Centrifuging apparatus. A Magnum (MSE) refrigerated centrifuge was used with an 8-place swing-out head equipped with 4-tube trunnions and buckets (International) of 15 ml size. The special centrifuge tube assembly used is shown in Fig. 1.

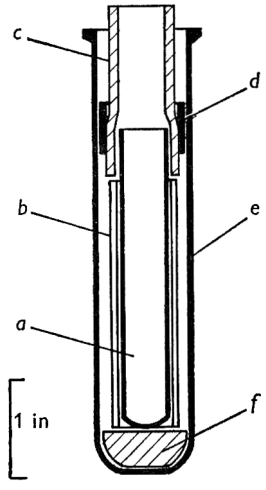


Fig. 1. Centrifuge tube assembly. *a*, glass Kahn tube; *b*, Nalgene sleeve; *c*, rubber tube; *d*, copper collar; *e*, centrifuge bucket; *f*, rubber cushion.

Siliconed, glass, Kahn tubes (length, 75 mm; outer diam., 12 mm) are each provided with a rubber tube extension (outer diam., 15 mm; wall thickness, 2 mm) approximately 4 cm long. The slight necking of the rubber tube at the glass rim is not enough to constrict the cross-section and impede the passage of particles up or down the tube. A 4-ml graduation mark is etched on each glass tube. A close-fitting copper collar 12 mm long seals the rubber to the glass tube and prevents liquid leaking during centrifuging. Collars were conveniently made from copper pipe-joint couplings whose outside diameters were turned down sufficiently to make each a sliding fit in the buckets. Each Kahn tube slides loosely into a plastic sleeve (length, 64 mm; outside diam., 17 mm; inside diam. approximately 14 mm) contained in each bucket which serves to centralize the tube as well as helping to prevent the rubber slipping down the glass under centrifugal action. Sleeves were cut from Nalgene (Nalge Co., Inc., Rochester, N.Y.) centrifuge tubes (13 ml capacity). A hard rubber cushion at the bottom of each bucket completes the assembly.

It was found worthwhile to adjust all bucket assemblies to the same weight ($\pm$ 0·2 g) and thereafter avoid interchange of components. Balancing was best done by appropriate trimming of the rubber cushions. After addition of the standard

volume of diluted milk, no attention to balancing was generally needed other than perhaps a weight check of each trunnion load.

Preparation of milk dilutions. For all routine work, milk samples (about 15 ml) were held in 25-ml capacity screw-capped glass bottles. The importance of thorough shaking of bottles to disperse the contents before sampling cannot be overstressed. After allowing samples to reach room temperature, bottles are shaken vigorously for 15 sec, left standing for about 5 min to allow air bubbles and foam to clear and finally agitated gently for a few seconds just prior to sampling.

One ml of milk and 7·25 ml of 0·85 % NaCl are dispensed into a centrifuge tube, previously rinsed in the saline. If not using an auto-dilutor, dilutions are mixed by inverting the tubes 2 or 3 times. The surface level of the dilute milk (8·25 ml) now lies about $\frac{1}{4}$ in. below the top of the rubber tube.

Removal of the fat. The experimental data leading to the appropriate centrifuging conditions are discussed in a later section. A satisfactory treatment is 2700 ***g*** (maximum radius) for 15 min at a temperature of 23–25 °C. This brings about sedimentation of the cells while all fat globules larger than about 3 μm in diameter are brought to the liquid surface. Globules remaining in suspension are not detected at the size thresholds selected for counting the leucocytes nor are they in sufficient numbers to form clumps large enough to interfere. Each batch of tubes in its trunnion is removed carefully from the centrifuge to the bench so as not to disturb the fat layers. Taking each tube in turn, the copper collar is loosened and lifted slightly and the rubber tube clamped off with haemostatic forceps so as to isolate the fat layer, the rubber tube and fat being then bodily removed. Further supernatant is aspirated so as to leave 4 ml of liquid in each centrifuge tube. Tubes are then closed securely with rubber bungs preparatory to redispersion of the cells.

Redispersion of the cells. The cells, with occasionally some proteinaceous material, form a well-packed, visible clump at the bottom of the tube. Redispersion is effected by gentle movement of the tubes on a rocker-type mixer (Lab-Tek) with a 3–4 sec rocking cycle until all visible deposit has disappeared. The final treatment to break up remaining cell clumps consists of 1$\frac{1}{2}$–2 min vigorous agitation on a Vortex test-tube mixer (Scientific Industries Inc.)

For analysis with the counter a suspension is now decanted into a cylindrical glass beaker (about $\frac{3}{4}$ in. diam. × 1$\frac{1}{2}$ in.) previously rinsed in saline and shaken to remove superfluous liquid. The centrifuge tube is rinsed twice (finger closure) with 3 ml quantities of saline which are also decanted into the beaker giving, finally, 10 ml of suspension. The beaker is closed with a rubber bung and the contents are briefly mixed. Before counting, 15–30 sec are allowed to elapse for air bubbles to rise clear.

To obviate the carry-over from one sample to the next of small quantities of a suspension left adhering to the orifice tube of the counter, the tube is dipped in clean saline between sample tests.

RESULTS AND DISCUSSION

Nature and size distribution of recovered particulate material

Examination of many milks with the Coulter counter indicated that the size distribution of recovered particles was typically as shown in Fig. 2, which was prepared from measurements on a milk of fairly high leucocyte content (4×10^6/ml).

The apparent diameters of the particles (that is, the diameters of the spheres of equivalent volume) have been plotted. An apparently fairly sharp 'drop-out' threshold for the smallest leucocytes around 5·25 μm apparent diam. (76 μm^3) can be observed. The distribution extends to about 15 μm diam. (1767 μm^3). Since the cells are irregularly shaped and, under the microscope, only the stained nuclei are easily seen in milk films, there is considerable difficulty in directly matching cell sizes estimated with the microscope and the volume-sensing electronic method. Comparisons of the electronic counts of many milks with careful estimates obtained with the microscope indicated that 4·5 μm

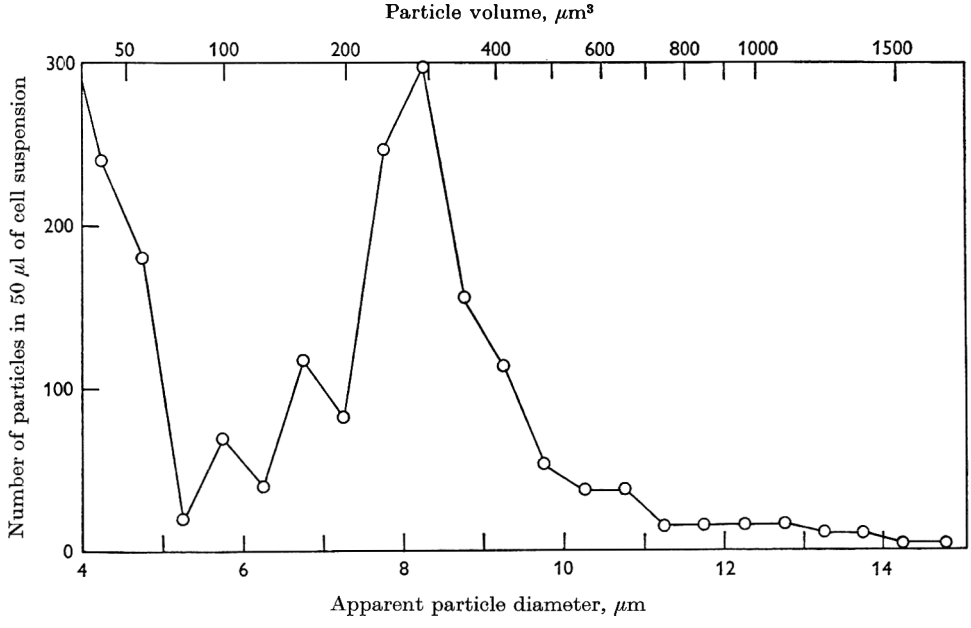


Fig. 2. Typical size distribution of particles recovered from a cow's milk of fairly high leucocyte content (4×10^6 cells/ml).

was nearer the 'drop-out' threshold. Only with milks of high cell content, however, was it possible to estimate cell concentrations at 4·5 μm which were roughly the same as those obtained with the microscope. In general, too, the smaller the cell concentration the higher the threshold setting needed to produce a matching count. It appeared that a distribution of other particulate matter was usually present which overlapped the distribution of leucocytes. This, mainly cellular, material was taken to include broken, large (e.g. epithelial) cells, red blood cells, bacterial clumps and some debris not easily identifiable under the microscope.

The approximate distributions of particles deduced from the data are shown schematically in Fig. 3. The shape of the leucocyte distribution curve is variable, but a peak is commonly found between 7 and 9 μm. The position of the 'debris' curve is also uncertain, particularly below 6 μm, but its influence extends to about 10–12 μm although with diminishing effect. Cognizance is taken of the fact that blood in varying amounts, although generally small and negligible, occurs in milks from mastitis-infected cows. Measurements showed the red cells to range in size from 3·5 to about 7·5 μm apparent diam.

Electronic counting of leucocytes

Direct counting of all the leucocytes at their drop-out threshold of around 4·5 μm is clearly not possible and the following procedure was devised which gives cell counts bearing an easy relation to microscope estimates. To avoid counting red blood cells and to minimize the influence of the other material, a count is obtained at a threshold equivalent to an apparent diam. of 7·5 μm (221 μm^3). The count at a threshold of 15 μm, the contribution of the irrelevant large particulate matter, is subtracted from

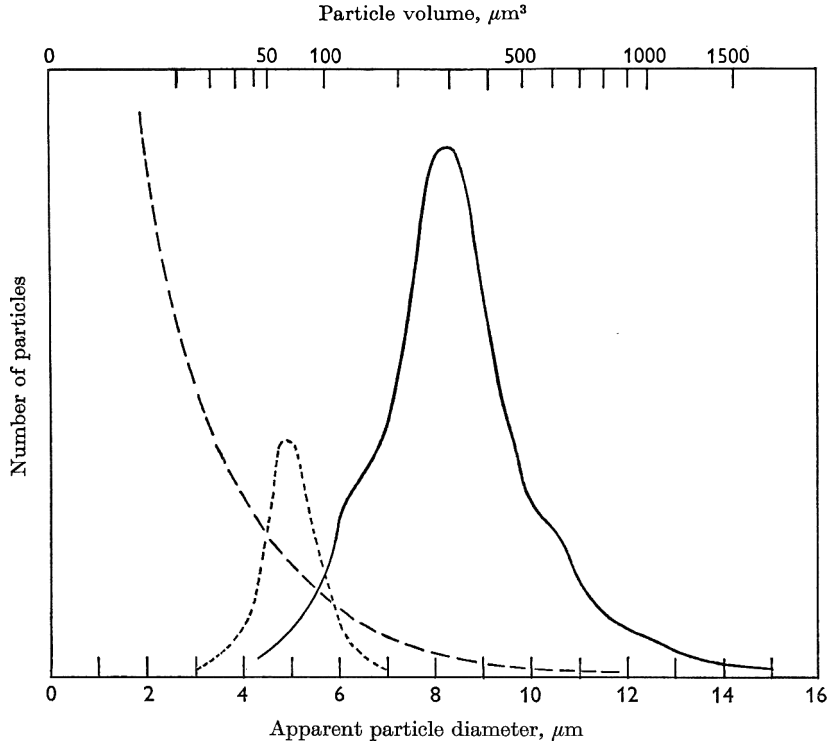


Fig. 3. Schematic diagram of the approximate size distributions of the particulate cellular material recovered from cow's milk. ——, Leucocytes; - - - - -, red blood cells; — — —, 'debris', including cell fragments, bacterial clumps and foreign matter.

this figure to give a number, say N. Since, finally, cells from 1 ml of milk are suspended in 10 ml of saline and the metered suspension volume is 50 μl, the electronic cell count is given by $0{\cdot}2N \times 10^3$/ml. Counts at the 15 μm threshold are ordinarily less than 10 % of the corresponding counts at 7·5 μm.

The milk serum undergoes, routinely, a final dilution with saline of roughly 1:20. Hence the maximum likely variations encountered in the electrical conductivity of different milks (Pinkerton & Peters, 1958) will have a negligible influence ($< 0{\cdot}5$ %) on the conductivity of the final cell suspensions and hence on the accuracy of the cell counts.

Correlation of microscope and electronic counts

In Fig. 4, the leucocyte concentrations of 60 fresh milks determined with the microscope have been plotted against corresponding electronic counts, the latter being the mean values of duplicate determinations. Corrections for coincidence counts (that is, the simultaneous passage through the orifice of more than one particle at a time) were made to the electronic data in the usual way.

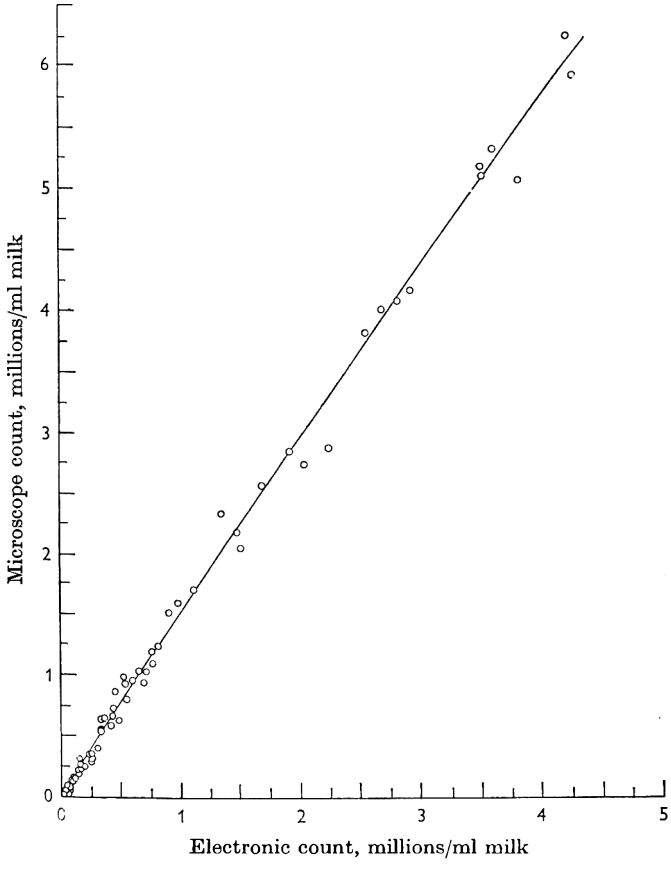


Fig. 4. Relation between microscope and electronic leucocyte counts in milk. Coincidence corrections applied.

The maximum cell concentration measurable at the customary dilution (1:10) is determined by the magnitude of the coincidence correction applied to the count at the 7·5 μm threshold. For reasonable accuracy this should not exceed 15 % of the count. The computed maximum is then about 5×10^6 cells/ml of milk. Higher cell concentrations can be determined by increasing the dilution rate, but the correlation has not been extended in this way owing to the increased difficulties of accurate counting which arise in both methods from cell clumping and general messiness of many of the milks. Moreover, the usefulness of accurate cell counts above 5×10^6/ml is questionable.

It would seem that a line through the points (Fig. 4) is slightly concave to the abscissa but could be taken as linear in conversions of electronic to microscope counts. However, the advantage of a simplification of the procedure is gained while still retaining sensible linearity of the correlation, by the omission of the customary coincidence corrections to the electronic counts. The data are therefore shown replotted with this modification in Fig. 5. Giving equal weight to each point a straight line through the origin has been fitted which has a slope of 1·617 (S.E. $\pm$ 0·012).

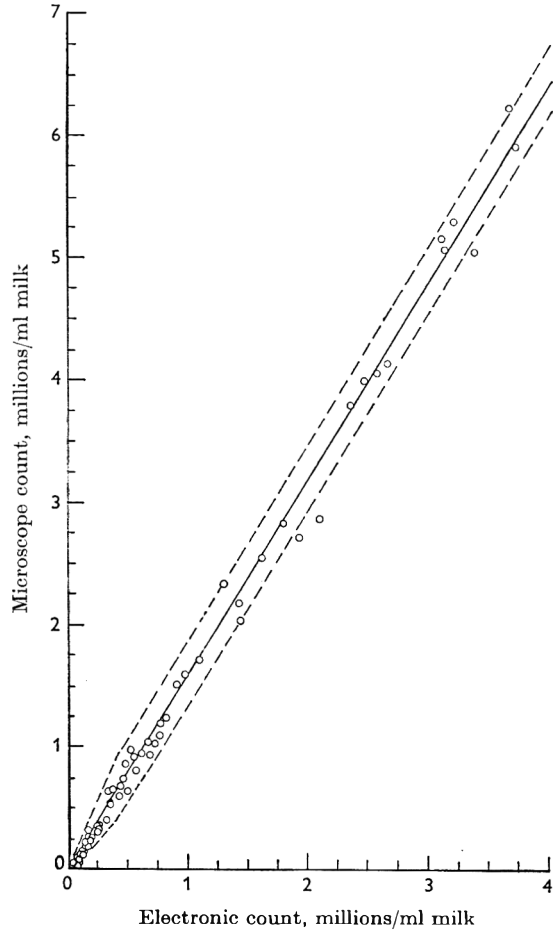


Fig. 5. Relation between microscope and electronic leucocyte counts in milk. Coincidence corrections not applied.

To convert electronic to microscope counts (M), therefore, the following relation is finally used:

$$M = 1{\cdot}617 \times 0{\cdot}2N \times 10^3,$$

i.e.

$$M = 0{\cdot}3234N \times 10^3 \text{ leucocytes/ml milk,}$$

where N is the difference between the 7·5 and 15 μm threshold counts as before but uncorrected for coincidence losses.

In milk of low cell content a greater proportion of the electronic count tends to

arise from 'debris' particles and there is a possibility of the line making a small positive intercept on the abscissa. However, the choice of a line through the origin is justified because no significant reduction in residual variation is obtained when fitting a line of the form $y = a + bx$.

The possibility of this last form of equation for low cell counts (< 200000/ml) was also examined but again a line through the origin proved more satisfactory.

The dotted lines in the figure represent the 95 % confidence limits of sample mean microscope counts corresponding to mean electronic counts. The limits vary from $\pm 266 \times 10^3$ to $\pm 284 \times 10^3$ counts/ml over the range shown. The variation about the fitted line appears fairly uniform throughout most of the range of counts, but there is indication that for electronic counts less than 400000/ml the confidence limits are overestimated and are more realistically represented by lines converging on the origin as shown.

The within-sample standard deviation from duplicate electronic counts on the 60 samples is $\pm 58 \times 10^3$ counts/ml. If each single electronic count is converted to an estimated microscope count and these estimates compared with the observed mean microscope count for each sample, then the distribution of all such differences has a standard deviation of $\pm 148 \times 10^3$ counts/ml.

Factors relating to results obtained with the electronic counter

Speed and time of centrifuging. The extent to which the creaming globules would interfere with the sedimentation of the cells was unpredictable and experiments were therefore carried out to determine suitable centrifuging conditions. It was desirable to minimize the time taken, so relatively high centrifuge speeds were employed. Preliminary work had shown that the rates of fat creaming and cell sedimentation were not too different and that centrifuging times which were adequate to cream all fat particles of about 3 μm in diameter and larger would also be satisfactory for concomitant cell recovery.

Following the procedure just outlined, though with a different final suspension volume. samples of a milk containing about 2×10^6 cells/ml were centrifuged for different times at 23–25 °C and 3250 rev/min. At the bucket tip ($r = 23$ cm) this speed corresponds to about 2700 g. Fig. 6 shows the mean counts obtained at different threshold size-levels. At the smaller thresholds (< 4 μm) the course of each curve is at first determined mainly by the behaviour of the fat globules. As these rapidly cream off the counts decrease until, after removal of the fat and sedimenting of the cells, a constant value is reached. At the higher threshold levels (6 and 7 μm) fewer fat globules are detected and the curves initially show a slight increase reflecting progressive sedimenting of the cells. The higher the threshold the more quickly the counts settle to a sensibly constant value.

The dotted line on the graph joins points on the threshold curves which indicate the minimum times for separation. It may be seen that for the 3 μm threshold level, 15–16 min is required to complete the separation. As would be expected from theoretical considerations, a plot of 1/(threshold diam.)^2 against minimum centrifuging time is linear. These results were obtained with a partly loaded centrifuge head. Heavier loads reduced the maximum speeds attainable, necessitating increased spinning times. Experiment showed, however, that equivalent combinations of speed

and time calculated in the usual way led to final cell counts which differed by no more than the experimental error of the method. Thus, typically, 7% difference only in the mean cell counts was obtained when the same milk was centrifuged at

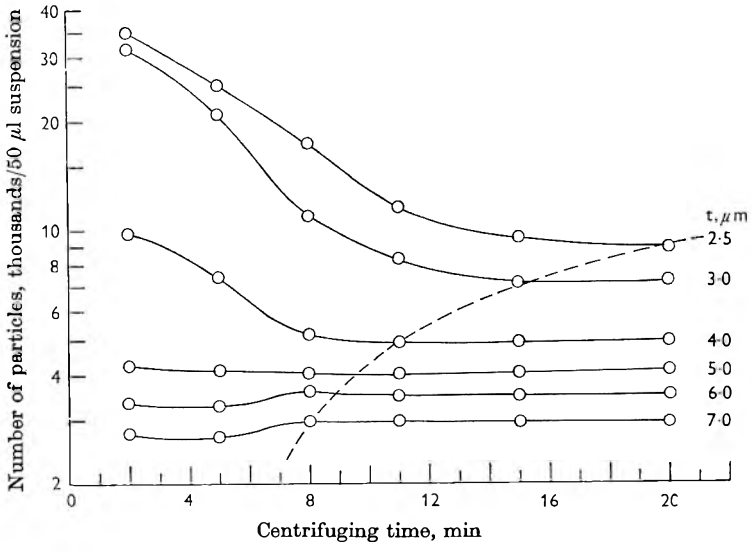


Fig. 6. Variation of particle counts at different threshold size levels, t (apparent diam. in μm), with centrifuging time. Centrifuge speed, 3250 rev/min (2700 g, max. radius). Temperature, 23–25 °C.

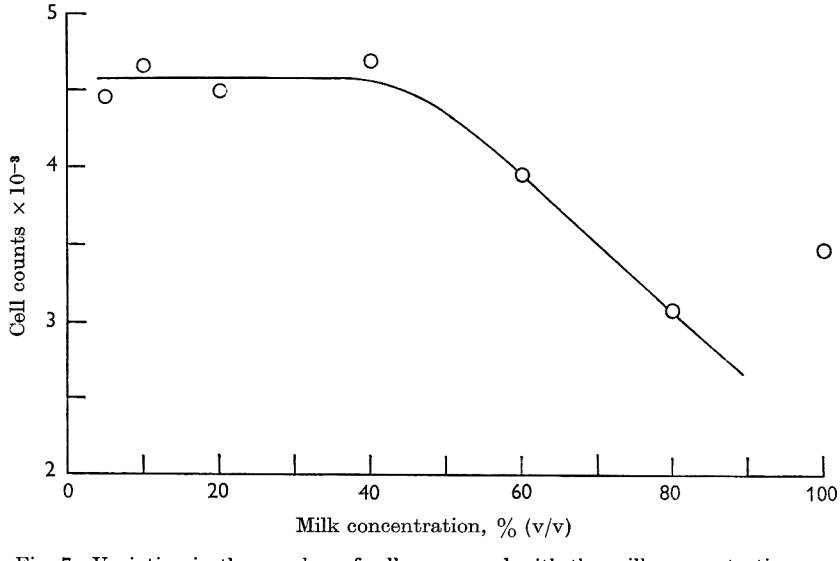


Fig. 7. Variation in the number of cells recovered with the milk concentration used in centrifuging. Cell counts are adjusted to refer to the same volume of milk.

2700 g (r = 23 cm) for 15 min and 1300 g (2250 rev/min) for 31 min. The variances of replicate determinations at the two g values were not significantly different.

Speeds less than about 2900 rev/min were not normally used.

Dilution of sample for centrifuging. A milk sample of 1 ml is a convenient volume for routine work and large enough to be representative of its source provided this is previously well stirred. In addition, a useful range of cell concentrations can be covered using an economical counting volume of 10 ml. The choice of this sample size was governed initially, however, by the results of experiments designed to test the effect on the cell recovery of centrifuging the same milk at different dilutions. Fig. 7 shows that, within the experimental error, the cell numbers recovered remain constant as the milk:saline ratio increases to 4:6. Thereafter, cell counts diminish and results are irregular; it is presumed that the increased numbers of creaming fat globules now hinder the cell movements and prevent them from fully sedimenting.

Milk volumes up to 3·3 ml diluted to 8·25 ml could therefore safely be used. No advantage such as increased precision would appear to be gained, however, by taking the larger samples. Indeed, more difficulties are experienced and more time spent in satisfactorily redispersing the larger clumps of sedimented cells.

Temperature of centrifuging. Without temperature control of the centrifuge used, samples initially at an ambient of 22 °C attained temperatures of about 40 °C after a 15-min run; even higher temperatures would be expected after immediate, successive runs. It was undesirable to risk damage to the cells by exposure to high temperatures. In addition, excessive temperature gradients which might produce thermal convection in the milk and impair clean separation were to be avoided. A few experiments indicated that, compared with results obtained using constant temperature conditions, cell counts were lower when the temperature was allowed to vary although the replication error was low enough to be acceptable. Nevertheless, more consistent results were to be expected by adopting a temperature of 23–25 °C for centrifuging and other conditions were not investigated.

Silicone treatment of centrifuge tubes. The replication error is no greater when unsiliconed rather than siliconed tubes are used but mean counts tend to be higher by a few percent, particularly in milks of low cell content. This may arise from the adherence of fat globules to the tube walls, so this possibility and that of the cells themselves adhering are minimized by treating the tubes with silicone. Treatment is repeated every 2 or 3 weeks.

Redispersion of cellular deposits. Observations with the microscope show that after gentle re-suspension of deposits with the rocker-type mixer, the dispersed material generally consists of a mixture of individual as well as small aggregates of cells. The time taken to reach this stage varies from a few minutes to about half an hour and sometimes longer, depending on the state of the milk. Deposits from some milks contain proteinaceous as well as cellular material and these often take the longer times. This time delay is normally of no hindrance when treating a number of different milks together, since some deposits will usually disperse easily and quickly and these tubes may be removed for the next treatment while the remainder continue to rock.

Subsequent agitation on the Vortex mixer was examined in several experiments by following the changes in counts at the usual threshold size levels with increasing agitation time. Samples of the suspension were taken periodically and the cells examined under the microscope. The general behaviour is shown by the results of the following experiment.

Cells from eight 1-ml samples of the same fresh milk were sedimented, redispersed on the rocker-type mixer as usual, pooled and 4-ml samples re-allocated to the centrifuge tubes. These were then treated for different times on the Vortex mixer. In Fig. 8 a reduction in the count at the 15 μm threshold following the break-up of cell clumps is apparent during the first minute. Thereafter, up to about 2 min agitation, the numbers remain roughly constant but then tend to diminish as cells are broken. The cell count in the interval of 7·5–15 μm increases at first but then changes very little ($< 2\%$) between 1 and 2 min. Treatment times of $1\frac{1}{2}$ min may thus be regarded as optimal.

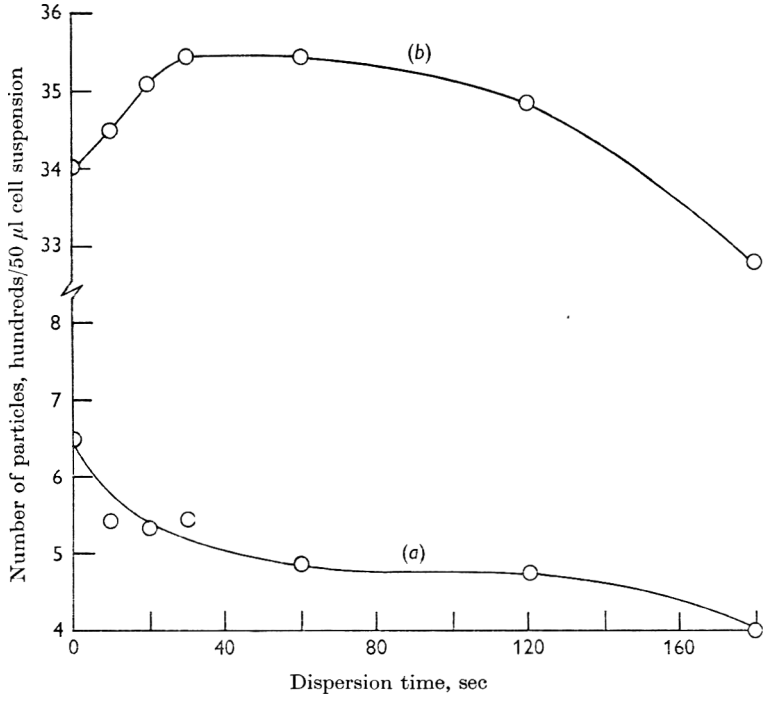


Fig. 8. Change in counts with time on the Vortex agitator: (*a*) at a threshold diameter of 15 μm; (*b*) between the threshold diameters of 7·5 and 15 μm.

The extent of cell clumping appears to be influenced by the age and/or storage temperature of the original milk, for after one or more days in the refrigerator, cell counts at the 15 μm level were lower than those shown, although the same optimum agitation time was needed for dispersion.

Attempts to shorten the time for redispersion by using the Vortex mixer alone were unsuccessful, the violent agitation breaking the cellular deposits away from the tube in relatively large lumps which were then difficult to disperse further.

Milk storage conditions. Very small quantities of the common milk preservatives (e.g. formalin) alter the cell volumes sufficiently to prevent the determination of accurate cell counts even as early as on the day following milking. An extensive study was not made of the use of chemical preservatives since experiment showed that milk samples may be safely kept for several days prior to cell counting by simply

storing them at ordinary refrigerator temperatures (40 °F). The results in Table 1 show the relatively small (< 10 %), gradual increase in the mean (8-fold replication) cell count of a milk stored for 7 days under these conditions.

Table 1. *Variations in cell counts with storage time at 40 °F*

Days after milking	0	1	2	5	7
Electronic cell counts (thousands/ml)	74·1	76·1	77·8	77·3	80·0
Standard deviation	5·3	4·9	3·9	3·3	2·5

The low standard deviations are to be noticed; the decrease with increasing storage time is curious.

Other experiments on milks of different cell concentrations stored for varying times up to 6 days confirmed that only small changes in count occur. These were not consistently in one direction, however, both increasing and decreasing values with time being observed.

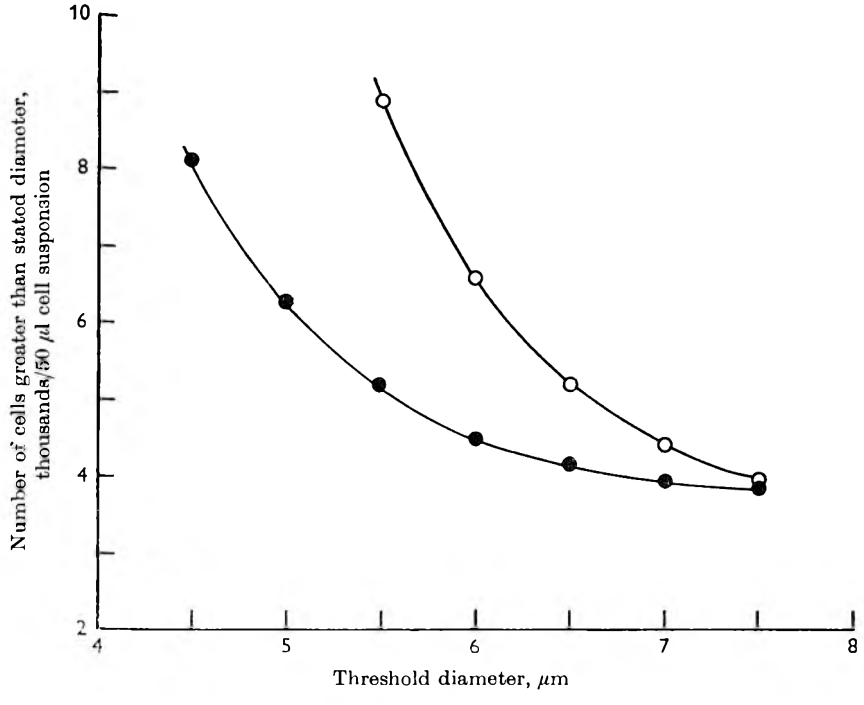


Fig. 9. The influence of blood cells in milk on the cell counts at different threshold size levels. Milk containing: ●, no blood; ○, 1000 ppm. cow's blood.

Blood in the milk. The addition of blood to milk up to 1000 ppm. (milk visibly pink) does not significantly increase the leucocyte count. At low thresholds the blood cells contribute markedly to the cell counts (Fig. 9) but add less than 3 % to the numbers at the 7·5 µm size level.

Quantities of 10 ppm. in the whole milk can usually be observed in centrifuged deposits from routine milk dilutions.

Volume changes of cells in saline. Cells appear to increase in volume gradually from the time of dilution with 0·85 % saline and after several hours begin to disintegrate. Keeping the milk diluted for excessive periods should be avoided but experiment showed that up to about $2\frac{1}{2}$ h is not detrimental to the accuracy of cell-count replication. The effect on the count at 7·5 μm during this time is small and variable, being influenced by the cell size distribution and the numbers present. No regular corrections for the effect are possible and the uncertainties introduced by cell size changes are part of the overall error of the method. Other suspending media (e.g. Eagles' solution) in which cells would be stable for longer periods might be considered but the possibility of a significant improvement in the precision of the electronic method seems unlikely.

A consequence, though not serious, of the time restriction is that the maximum number of samples at each centrifuging that a single operator can deal with is roughly 16–24. In some circumstances, therefore, other suspending media might be more suitable despite the cheapness and ready availability of saline.

Bacteria. It appears that the larger bacterial clumps are rather easily broken up by the agitation given by the Vortex mixer. The presence of bacteria in the milk in not unreasonable numbers does not affect the determination of leucocyte concentrations. Indeed, the bacterial content of a few of the milks tested for the correlation in Fig. 4 was noticeable. Even extensive growth may not prevent reasonable values (though somewhat overestimated) being obtained, but leucocyte degradation through long contact with bacteria could lead to inaccurate results.

Support for this work to one of us (L.W.P) by the National Research Council of Canada is gratefully acknowledged. We are indebted to Mr D. R. Westgarth and Miss Z. D. Hosking for their advice on and assistance with the statistical analysis and to Mr R. H. Johnston and Mr A. W. Walters for their technical assistance.

REFERENCES

COULTER, W. H. (1956). *Proc. natn. electron. Conf.* **12**, 1034.
HANKS, J. H. & JAMES, D. F. (1940). *J. Bact.* **39**, 297.
MOATS, W. A. (1964). *J. Milk Fd Technol.* **27**, 308.
PAAPE, M. J., HAFS, H. P. & SNYDER, W. W. (1963). *J. Dairy Sci.* **46**, 1211.
PHIPPS, L. W. & NEWBOULD, F. H. S. (1965). *Vet. Rec.* **77**, 1377.
PINKERTON, F. & PETERS, I. I. (1958). *J. Dairy Sci.* **41**, 392.
PRESCOTT, S. C. & BREED, R. S. (1910). *J. infect. Dis.* **7**, 632.

Printed in Great Britain

A note on the estimation of D(−) lactic acid using lactic dehydrogenase

By ELLEN I. GARVIE

National Institute for Research in Dairying, Shinfield, Reading

(*Received* 21 *October* 1965)

Van den Hamer & Elias (1958) described a method of estimating D(−) lactic acid using lactic dehydrogenase and a Warburg technique. The enzyme was obtained as an acetone preparation of *Escherichia coli* grown on a medium containing lactate as energy source.

The method was tried in this laboratory using a strain of *E. coli* (N.C.D.O. 1246) received from Dr Hamer and was found to be unsatisfactory. The organism did not grow well on the recommended medium and the acetone preparation oxidized L(+) as well as D(−) lactic acid. One preparation had an equal activity for both D(−) and L(+) lactic acid despite the observation of Haugaard (1959) that the L(+) lactic dehydrogenase was destroyed by acetone precipitation.

Table 1. *Estimated quantities of* D(−), L(+) *and total lactic acid in culture fluids of some lactic acid bacteria*

	Lactic acid, mg/ml			
	D(−)	L(+)	Total	D(−)+L(+)
Streptococcus cremoris	0·00	5·00	5·00	5·00
Leuconostoc mesenteroides	4·85	0·00	4·23	4·85
Lactobacillus jugurti	1·7	1·5	3·05	3·2
L. fermenti	3·65	3·6	7·45	7·25
L. helveticus	3·85	3·65	7·8	7·50
Control				
Potassium (DL) lactate	1·06	1·09	1·90	2·15

The difficulty was overcome by using further information given by Haugaard (1959) that *E. coli* grown on glucose produced only the D(−) lactic dehydrogenase (see also Kline & Mahler, 1965). The lactate in the medium used by Van den Hamer & Elias was therefore replaced with glucose (final concentration 0·2%, w/v) which was dissolved separately from the other ingredients and added aseptically after sterilization. Several acetone preparations of cells grown on this modified medium have been used and as predicted they all oxidized D(−) lactic acid but failed to oxidize the L(+) isomer.

A solution containing 1 mg/ml lactic acid was examined in duplicate at 2 levels (0·5 and 0·8 mg) using a different acetone preparation for each level of substrate. The estimated amounts of lactic acid were 98·2 and 107% of the expected value at the lower level and 101·5 and 99·5% at the higher level.

Table 1 shows typical results when the method was applied to the estimation of

lactic acid in bacterial cultures and also in a solution of potassium (DL) lactate containing the equivalent of 2 mg lactic acid/ml. L(+) lactic acid was determined with muscle lactic dehydrogenase and nicotinamide-adenine dinucleotide (Gercken, 1960) and total lactic acid by the method of Barker & Summerson (1941).

The results indicate that the method for D(−) lactic acid can be used to determine the quantity of this isomer formed by bacterial cultures and that the degree of accuracy is similar to that claimed in the original paper (Van den Hamer & Elias, 1958).

Publication of this note has been prompted by verbal indications that other workers have experienced difficulty in estimating D(−) lactic acid by the lactic dehydrogenase method.

REFERENCES

BARKER, J. B. & SUMMERSON, W. H. (1941). *J. biol. Chem.* **138**, 535.
GERCKEN, G. (1960). *Hoppe-Seyler's Z. physiol. Chem.* **320**, 180.
HAUGAARD, N. (1959). *Biochim. biophys. Acta*, **31**, 66.
KLINE, E. S. & MAHLER, H. R. (1965). *Ann. N.Y. Acad. Sci.* **119**, (3), 905.
VAN DEN HAMER, C. J. A. & ELIAS, R. W. (1958). *Biochim. biophys. Acta*, **29**, 556.

Printed in Great Britain

J. Dairy Res. (1966), **33**, 67
With 2 plates

The stability of milk protein to heat

I. Subjective measurement of heat stability of milk

By D. T. DAVIES and J. C. D. WHITE
The Hannah Dairy Research Institute, Ayr, Scotland

(*Received* 30 *September* 1965)

Summary. A subjective test for the determination of the stability of milk protein to heat is described. In the test, the time required for particles of coagulated protein to become visible throughout a 2·5-ml sample of separated milk maintained at 135 °C in a glass tube rocking at 8 c/min is taken as a measure of stability. The precision of the test was such that single determinations were generally adequate.

Coagulation time decreased by about 12% as rocking speed was increased over the range 4–12 c/min and increased by a factor of about 3 for a decrease in heating temperature of 10 degC over the range 140–105 °C; with some milks the $Q_{10\ °C}$ value increased to 5–8 as temperature decreased. As sample volume was increased over the range 1–3 ml coagulation time increased, especially with milks whose coagulation was poor (initial clots small). This volume effect appeared to be a consequence of the accompanying decrease in the proportion of headspace oxygen to volume of milk.

In their pioneer investigations on the heat coagulation of milk, Sommer & Hart (1919, 1922, 1926) devised a test to measure the heat stability of milk protein. In this test, milk samples were sealed in small glass tubes, the tubes attached to a rack that could be tilted, and the rack inserted into xylene vapour at a temperature of about 137 °C; the time from the beginning of heating to the appearance of moving particles of protein in the milk was taken as a measure of the stability of a milk to heat. Subsequently, tests similar in principle have been widely used to assess the heat stability of milk (e.g. Webb & Holm, 1932; Cole & Tarassuk, 1946; Pyne & McHenry, 1955; White & Davies, 1958; Davies, 1959; Rose & Tessier, 1959; Belec & Jenness, 1962). All these tests are empirical and subjective, and apart from limited studies by Cole & Tarassuk (1946) little information has been published on the effect on the heat stability values so obtained of varying the experimental conditions used in the tests. There also appears to be no information on whether the coagulation times obtained for a series of milks by one particular test adequately characterize the heat stability of the milks, and hence whether they are strictly comparable, or on how heat stability may alter during the ageing of milk under different methods of storage.

To provide the desired information, a critical examination was made of a typical, subjective heat stability test and the effect on coagulation time, obtained by this

test, of ageing milk at different temperatures was also examined (Davies & White, 1966). In addition, a comparative study was made of an objective method of measuring heat stability (White & Davies, 1966).

Materials and methods

Subjective heat stability test

The milk sample is placed in a glass tube of 4·0-ml capacity and 12·2 cm in length made from precision-bore Pyrex tubing (6·50 ± 0·02 mm bore, 8·75 ± 0·25 mm outside diam.). The tube is closed with a silicone-rubber stopper and clamped crosswise on a brass carriage so that the stopper is held in position (Plate 1). The carriage is held horizontal and immersed to a depth of about 4 cm in a bath of hot liquid paraffin, the ends of the carriage fitting into slots in brass bearings fixed on the front and rear walls of the bath. The rear bearing is linked by 2 brass connecting rods in a vertical plane to an eccentric (3·2 cm effective diam.) driven by a variable-speed (4–12 rev/min), geared electric motor (Plate 2). The movement of the rotating eccentric causes the sample tube to rock in a vertical plane about its centre through an arc of about 36° and the milk sample to flow gently from one end of the tube to the other. The tube is illuminated from above by a 150-W clear lamp and viewed at its centre through a stereoscopic microscope with a working distance of approximately 20 cm and a magnification of 8·4.

The bath (Plate 2) is made of copper sheet ($\frac{1}{16}$ in.), is well insulated, and has the following internal dimensions: length 38 cm, breadth 23 cm, depth 23 cm. Two 1-kW immersion heaters are fitted horizontally, using neoprene gaskets, in the right-hand side of the bath 4 cm above the bottom and are covered by a removable copper sheet (27 × 23 cm) 7 cm above the bottom. A stirrer centrally placed on the left-hand side of the bath circulates the 16·5 l. of liquid paraffin (colourless, density 0·830–0·870 g/ml at 20 °C) downwards below the copper sheet, over the heaters and then upwards into the upper part of the bath. Both heaters are used to raise the temperature of the liquid paraffin to the desired level, one heater is then switched off and a variable voltage transformer, set to provide a voltage that will minimize temperature fluctuation and periodic time (Bruyne, 1961; Finch, 1963), switched into the circuit of the control heater. The control heater is operated through an electronic relay by an adjustable mercury contact thermometer (100–170 °C) placed in the rear right-hand corner of the bath adjacent to the control heater. The temperature of the liquid paraffin is indicated by a mercury-in-glass thermometer (100–155 °C, graduated in 0·1 deg) and is controlled to within ± 0·15 degC. The bath is housed in a fume-cupboard and fumes removed by a strong flow of air over the top of the bath and away from the operator. This is achieved by fitting one end of a length of ducting (6 in. diam.) to the overhead exhaust outlet in the cupboard and fixing the other end about 5 cm above the rear wall of the bath.

Standard procedure

Pipette 2·5 ml of separated milk at 20 °C into the tube, close the tube with the silicone rubber stopper so that the bottom of the stopper is 3·5 cm above the surface of the milk, and clamp the tube on the carriage. Tilt the carriage so that the milk

wets all the inside surface of the tube and then, with the liquid paraffin at a temperature of 135 °C, place the carriage in the bath and simultaneously start a stop-watch and the rocking mechanism (8 c/min). View the milk through the microscope and note any changes in its physical appearance. As soon as moving particles or clots are seen throughout the milk, stop the watch. Continue to observe the milk for several minutes and note the manner in which coagulation proceeds, i.e. whether the particles increase rapidly in size or not. The time recorded on the watch (to the nearest 0·01 min) is taken as the 'coagulation time' or heat stability of the milk and the type of coagulation is recorded as 'very poor' or 'poor', 'very good' or 'good', or 'moderate', depending on whether the particles were initially small and slowly increased in size, were initially large and rapidly increased in size or were intermediate in both respects.

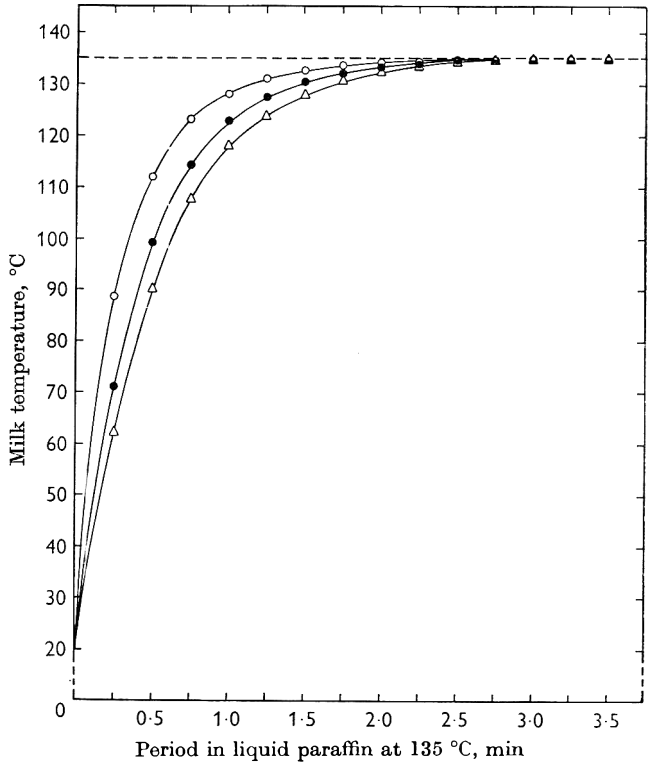


Fig. 1. Increase in temperature of different volumes of separated milk heated as in the standard procedure for the heat stability test. ○, 1·0 ml milk; ●, 2·5 ml milk; △, 3·0 ml milk.

Notes on standard procedure

(1) The coagulation time obtained by the standard procedure naturally includes the period required for the temperature of the milk to rise from 20 to 135 °C. To determine the extent of this period and how it varied with sample volume, heating-up times were measured using 1·0, 2·5 and 3·0 ml of milk. A copper-constantan thermocouple connected to a sensitive galvanometer was used to measure the temperature of the milk. One junction was placed in the bath at 135 °C and the other was immersed in the milk at 20 °C with the connecting wires threaded through the silicone-

rubber stopper. The tube of milk was then placed in the bath and rocked as in the standard procedure. Galvanometer readings were taken at intervals and later converted to degC (*Handbook of Chemistry and Physics*, 1947). No corrections were applied and the rates of increase in temperature of the milk samples (Fig. 1) are, therefore, more of relative than absolute significance. The curves show, however, that whether the sample volume was 1·0, 2·5 or 3·0 ml, the heating-up time was approximately 2·75 min. This fact and the similarity of the 3 curves mean that with different sample volumes errors in the relative coagulation times due to variation in the heating-up rate will be of little significance.

(2) The precision-bore tubes employed in the present heat stability test can be used repeatedly and are much more convenient than the once-used, heat-sealed ampoules employed in many other tests. Several methods of cleaning the tubes were found satisfactory and without influence on values obtained for coagulation time. The method adopted was first to remove the coagulated protein by rinsing with warm detergent solution, then to soak the tubes for at least 24 h in chromic acid and finally to rinse thoroughly with tap and distilled water and to dry at 100 °C. It was found that the formation of a deposit on the tubes, which is sometimes extensive before coagulation proper occurs, could be prevented by coating their inside surface with a silicone film. This had no effect on coagulation time, but did not aid its determination as expected because the coated surface became covered with minute water droplets. The use of silicone-coated tubes was, therefore, not adopted as a regular procedure.

Milk samples

Most of the milk samples were obtained from the Institute herd of Ayrshire cows and included herd bulk milk, milk from individual cows and milk from individual quarters. Individual quarter samples are designated by LF, LH, RF and RH as abbreviations for left fore, left hind, right fore and right hind, respectively; the term 'composite' is used for a sample prepared by mixing the milks obtained at 1 milking from the 4 quarters of a cow, according to the weight of milk from each quarter. As unpublished experiments by the present authors had shown that the addition of 1–2 ppm of copper to separated milks caused either a large increase or a small decrease in coagulation time, any parts of the milking equipment that contained copper were replaced by stainless steel parts. A few 'commercial bulk' samples were obtained at a local creamery from a tank containing 4500 gal of whole milk. Each milk was obtained in the late afternoon, conveyed immediately to the laboratory and its temperature adjusted to 20 °C. The milk was then centrifuged for 30 min at 1000 *g*, and the separated milk removed from beneath the fat layer by suction and its temperature adjusted to 20 °C.

All determinations were made on separated milks either when fresh or after storage at 20 or 4 °C. Many samples were analysed for lactose, total-N, casein-N, lact-albumin-N plus lactoglobulin-N and proteose-peptone-N plus non-protein-N, and their pH determined (White & Davies, 1958). To help in deciding whether a sample came from a cow with subclinical mastitis the differential cell count of the whole milks was determined (Blackburn, Laing & Malcolm, 1955).

The numbers used to identify the milk samples, unless indicated otherwise, apply

only to the particular table or figure in which they are used. Where 2 or more sets of results are given for milk from the same cow, the samples were taken at different stages of lactation.

RESULTS AND DISCUSSION

Precision of standard procedure for determining coagulation time

Milk was obtained from each of 23 cows, chosen at random, and the coagulation time of the samples determined in duplicate by the standard procedure. The individual values ranged from 2·66 to 48·55 min (mean value 19·67 min) and the type of coagulation varied from very poor to very good; the 23 milks thus showed a wide variety in their reaction to heat. The standard error of a single determination was found to be 0·26 min, the 99% fiducial limits ± 0·73 min and the coefficient of variation 1·32%; the corresponding values for the mean of duplicate determinations were 0·18 min, ± 0·51 min and 0·92%. It should be noted, however, that occasionally milks of the poorly coagulating type are encountered where the onset of coagulation is especially difficult to detect and in consequence the difference between duplicate coagulation times exceeds 1 min. Nevertheless, the precision of the standard procedure can be regarded as satisfactory and such that a single determination would usually be adequate for most purposes. The only other comparable information on the precision of a similar subjective heat stability test appears to be that of Rose (1963) who states that 'with normal milks at 140 °C this method is reproducible within ± 0·5 min, but with some abnormal and modified milks of various types prepared for experimental purposes the error increases to ± 1 or even ± 2 min.'

Effect of degree of agitation of sample

In the subjective heat stability test of Cole & Tarassuk (1946) the sample tubes are rocked so that the milk flows from one end to the other every 20–25 sec with a degree of agitation described as 'not extreme'. These authors state that the rate of rocking and the extent of tilt are factors that influence coagulation time and must therefore be kept constant. On the other hand, Whitney, Paulson & Murthy (1952) found that in their objective heat stability test, in which the tubes are given a pendulum motion, the results were not influenced by rocking speed over a range of 19–30 oscillations/min.

In view of the above results, the effect of varying the degree of agitation on values obtained by the present test was investigated. One herd bulk milk and 7 milks from individual cows, giving a wide range of coagulation time and type of coagulation, and 5 rocking speeds covering the range 4–12 c/min, were used; the conditions of the standard procedure were otherwise adhered to. The results (Fig. 2) show that as rocking speed increased coagulation time decreased in a slightly curvilinear way. The percentage decrease in coagulation time from minimum to maximum agitation was about 12% for most of the samples.

The two extreme rocking speeds used are at the limits of practicability for the test; at the highest speed viewing the tube soon becomes tiring and at the lowest speed the relatively long quiescent period favours deposit formation on the tube. A rocking speed of 8 c/min was considered the most suitable. It is clear from Fig. 2 that a small deviation from the standard rocking speed of 8 c/min will have little influence

on coagulation time; in practice, the motor driving the eccentric was found to maintain its set speed with negligible variation.

Several reasons for the slight decrease in coagulation time with increasing degree of agitation can be suggested, the most plausible probably being an increase in the rate of collision between incipiently aggregating protein particles. It should not be assumed that extrapolation of the lines in Fig. 2 to zero rocking speed would give the coagulation time of the milks if they were kept stationary. Under these conditions a gel forms, a process that is much slower and rather indeterminate compared with the coagulation that occurs when milk is agitated.

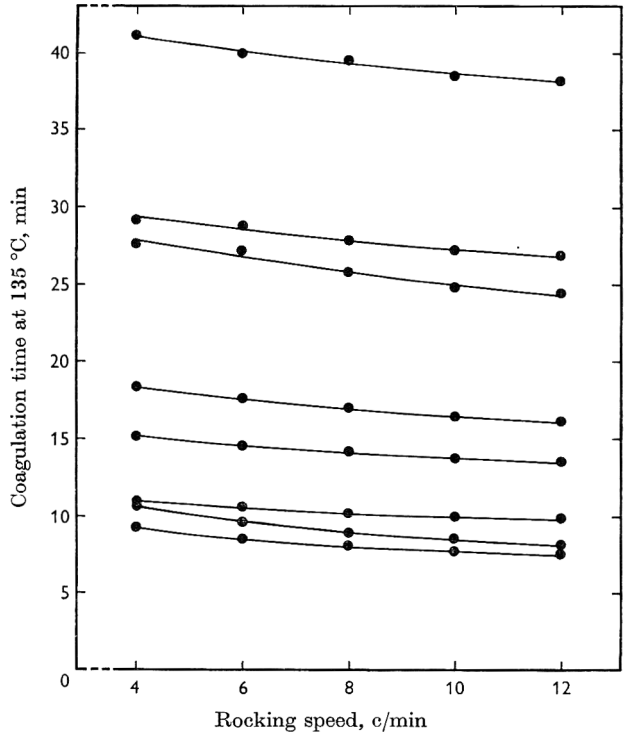


Fig. 2. The relation between coagulation time and rocking speed for 8 separated milks; standard rocking speed in the heat stability test is 8 c/min.

Effect of temperature

The temperature at which coagulation time is determined in subjective heat stability tests is usually in the range 120–140 °C, apparently so as to obtain coagulation times that are neither inconveniently long, nor too short. It is, however, desirable to know how coagulation time varies with temperature. Webb & Holm (1932) reported that the logarithm of coagulation time decreased linearly with increasing temperature but the much more extensive data of Cole & Tarassuk (1946) revealed that this was by no means a general rule for all milks and that the relative stability of a series of milks at one temperature could differ from their relative stability at another, in the range 108·6–160·0 °C. As this phenomenon does not seem to have been investigated further, the coagulation time of 6 milks, from widely differing sources,

was determined over the temperature range 105–150 °C at intervals of 5 deg; the conditions of the standard test were otherwise adhered to. At 145 and 150 °C, some of the milks coagulated before they reached bath temperature and therefore the coagulation times at these temperatures are not reported.

Table 1. *Coagulation time of separated milks at different temperatures*

Temperature, °C ...	105	110	115	120	125	130	135	140
Milk	Coagulation time, min							
(1) Cow, Bright Maid (LH)	>375	130	47·4	25·0	13·0	7·2	4·3	2·9
(2) Cow, Moonshine (RH)	435	212	78·0	32·4	17·5	10·6	6·3	4·0
(3) Herd bulk	345	140	67·3	39·5	21·5	14·4	8·0	5·0
(4) Cow, Doe (RH)	296	190	122	78·0	51·3	31·7	19·7	12·3
(5) Cow, Vesta (LH)	459	287	174	108	67·3	41·7	25·1	15·5
(6) Commercial bulk	368	208	125	93·5	68·2	43·5	28·6	16·0

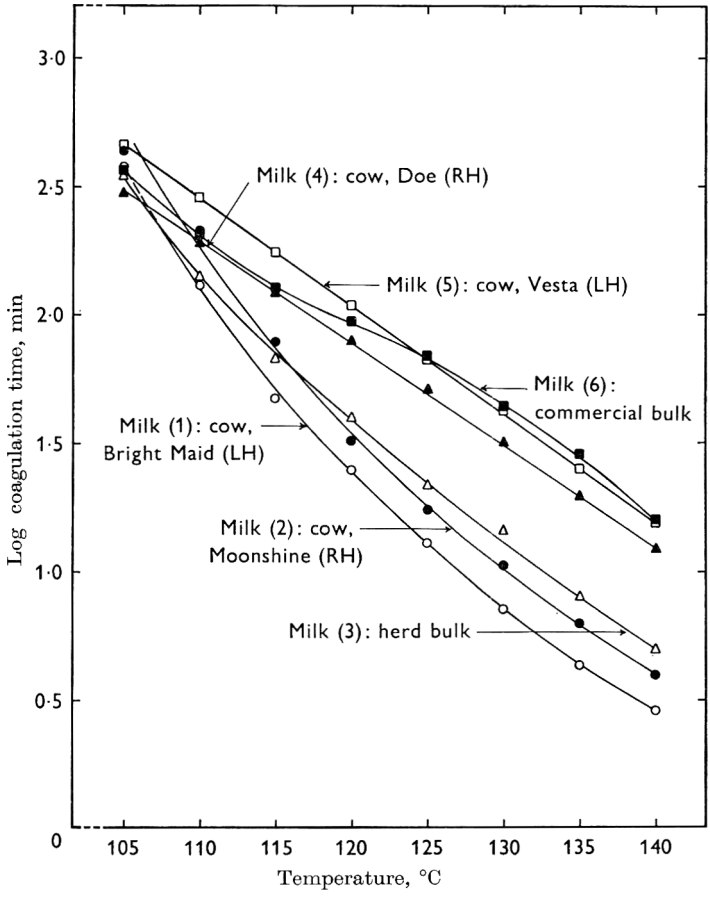


Fig. 3. The relation between logarithm of coagulation time and temperature for 6 separated milks; standard temperature in the heat stability test is 135 °C.

The coagulation times (Table 1) show that the stability of the samples relative to each other at the standard temperature (135 °C) was maintained from 120 to 140 °C except for transposition of the 2 most heat-stable milks at 120 °C. At the 3 tempera-

tures below 120 °C, however, the orders of stability differed from each other and also from the order at 120–140 °C. The relation between log. coagulation time and temperature for each milk is shown in Fig. 3. With the less heat-stable milks, milks (1), (2) and (3), the relation was curvilinear whereas with the more heat-stable milks, the relation was linear with milks (4) and (5) and slightly sigmoid with milk (6). Thus, the milks could be regarded as falling into 2 groups, namely, those less stable to heat and characterized by a progressively increasing change in log. coagulation time per unit decrease in temperature, and those more stable to heat and characterized by a fairly constant change in log. coagulation time. This characteristic difference between the 2 groups can be seen more clearly from the '$Q_{10\ °C}$ values' (Table 2), which are the ratios of coagulation time at one temperature to coagulation time at a temperature 10 degC higher. All the $Q_{10\ °C}$ values for milks (4), (5) and (6) are in the range 1·8–2·9 characteristic of homogeneous reactions whereas for milks (1), (2) and (3) this was not true below 120–125 °C.

Table 2. *Ratio of coagulation time of separated milks at one temperature to coagulation time at a temperature* 10 *degC higher*

Temperatures, °C ...	105 / 115	110 / 120	115 / 125	120 / 130	125 / 135	130 / 140
Milk	Ratio					
(1) Cow, Bright Maid (LH)	>7·9	5·2	3·6	3·5	3·0	2·5
(2) Cow, Moonshine (RH)	5·6	6·5	4·5	3·1	2·8	2·6
(3) Herd bulk	5·1	3·5	3·1	2·7	2·7	2·9
(4) Cow, Doe (RH)	2·4	2·4	2·4	2·5	2·6	2·6
(5) Cow, Vesta (LH)	2·6	2·7	2·6	2·6	2·7	2·7
(6) Commercial bulk	2·9	2·2	1·8	2·1	2·4	2·7

The coagulation of all the milks was classed as 'very good' or 'good' at the higher temperatures, but as temperature decreased, the initial clots were progressively smaller and their rate of increase in size progressively less, until at the temperatures below 120 °C all the milks had a coagulation classed as 'very poor'. This change in type of coagulation and the accompanying increase in difficulty in detecting the beginning of coagulation, was more marked with milks (1), (2) and (3). The curvilinear nature of their log. coagulation time-temperature relations and their differing relative stabilities over the range 105–115 °C may, therefore, have resulted to some extent from varying over-estimates of the coagulation time of these 3 milks at the lower temperatures. More results are required to establish whether milks can be classified according to the nature of their log. coagulation time-temperature relation and whether this relation is generally curvilinear for milks unstable to heat and linear for milks stable to heat.

It can be deduced from the results in Tables 1 and 2 that a difference of the order of 0·1 degC in mean bath temperature from the standard 135 °C would cause only small absolute errors and a small constant percentage error in coagulation time. This was confirmed by determining the coagulation times of 6 milks over a temperature range of 133–137 °C at 1 deg intervals; the milks were chosen to have a wide variation in coagulation time and type of coagulation. The results (Fig. 4) show that over this small temperature range the relation between coagulation time and temperature

was linear or almost linear for each milk. The slope of the lines for the least and most heat-stable milks indicate that an error in mean bath temperature of 0·1 degC, which is not likely to be exceeded, would cause errors in coagulation time of about 0·1 and 0·4 min, respectively, an error in each case of approximately 1 %.

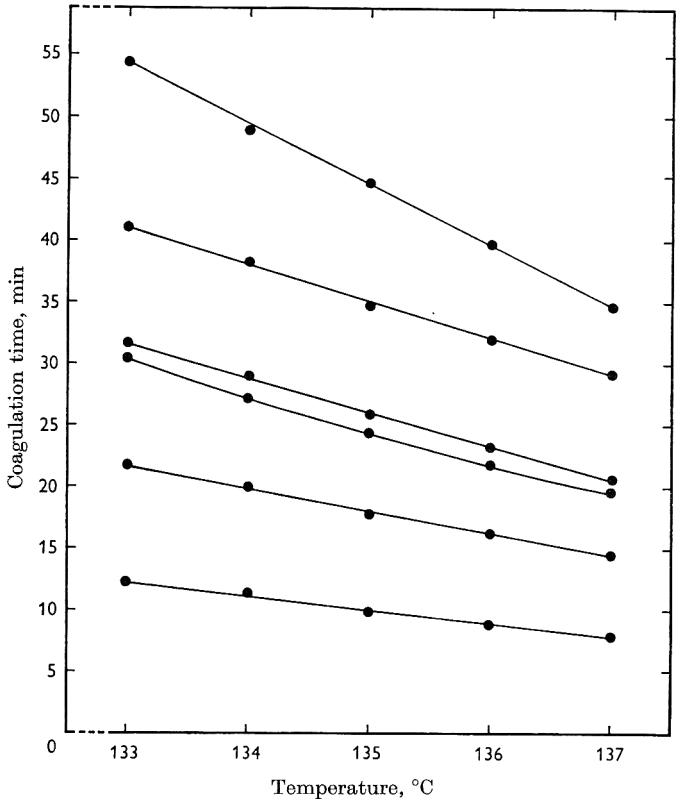


Fig. 4. The relation between coagulation time and temperature for 6 separated milks over a small temperature range; standard temperature in the heat stability test is 135 °C.

Effect of volume of sample

A survey of heat stability tests devised by various investigators reveals a considerable variation in volume of milk and capacity of tube used. However, only Whitney *et al.* (1952) appear to have investigated the influence of these factors. They found for their objective heat stability test, in which 1 ml of milk is heated in a Pyrex tube with a length of 12–13 cm and an inside diam. of approximately 6 mm, that the heat stability value obtained was governed by both sample volume and inside diam. of the tube, but that by controlling these factors to within ± 0·01 ml and ± 0·5 mm, respectively, little error would be introduced. To find what effect variation in sample volume would have on values obtained by the present subjective test, the coagulation time of 13 milks was determined using sample volumes ranging from 1·0 to 3·0 ml in increments of 0·5 ml; the milks were chosen to provide a wide variety of coagulation time and type of coagulation. The test was otherwise as in the standard procedure.

With each milk, as sample volume was increased coagulation time increased (Fig. 5), the increase in coagulation time being especially marked with some of the milks giving a poor coagulation. To illustrate this more readily the curves in Fig. 5 have been grouped according to whether the coagulation was good or poor. In addition to the effect of sample volume on coagulation time, it was found that coagulation time could be most easily determined when the sample volume was

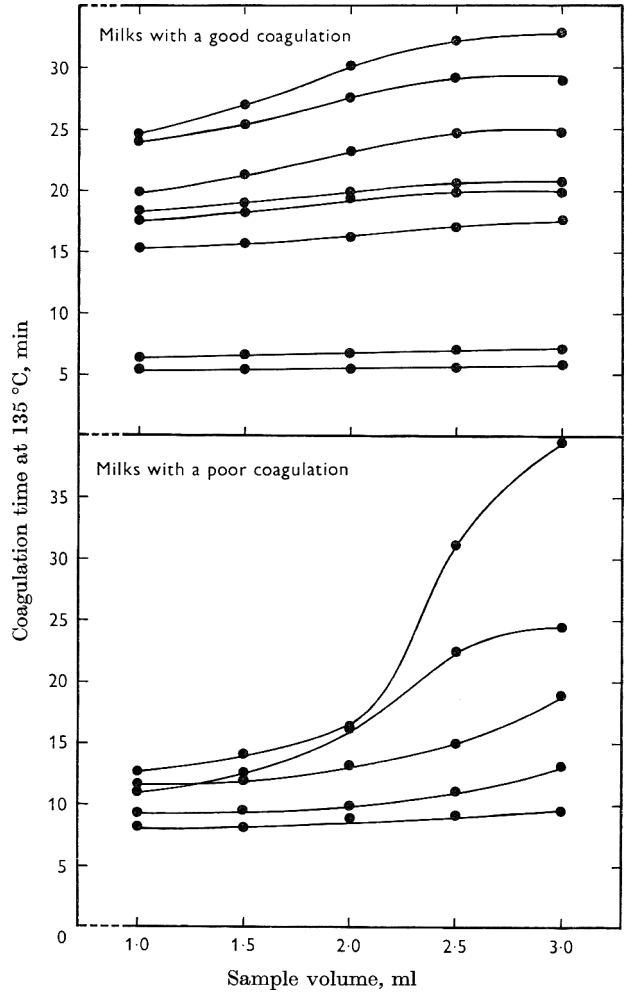


Fig. 5. The relation between coagulation time and sample volume for 8 separated milks with a good coagulation and for 5 separated milks with a poor coagulation; standard sample volume in the heat stability test is 2·5 ml.

2·5 or 3·0 ml. This was because the milk could be viewed best at the middle of the tube, where the rocking motion was least, and because less deposit formed on the walls of the tube than when smaller volumes of milk were used. For these reasons, in conjunction with the decision that a rocking speed of 8 c/min was the most suitable, a sample volume of 2·5 ml was adopted for the standard procedure. It can be concluded from Fig. 5 that even with a poorly coagulating milk any normal experi-

mental error in pipetting 2·5 ml of milk would cause a negligible error in coagulation time.

The influence of sample volume on coagulation time is such that neither the small differences in heating-up rates (see Fig. 1) nor any small differences in degree of agitation of the milk (see Fig. 2) could account for it. However, another variable

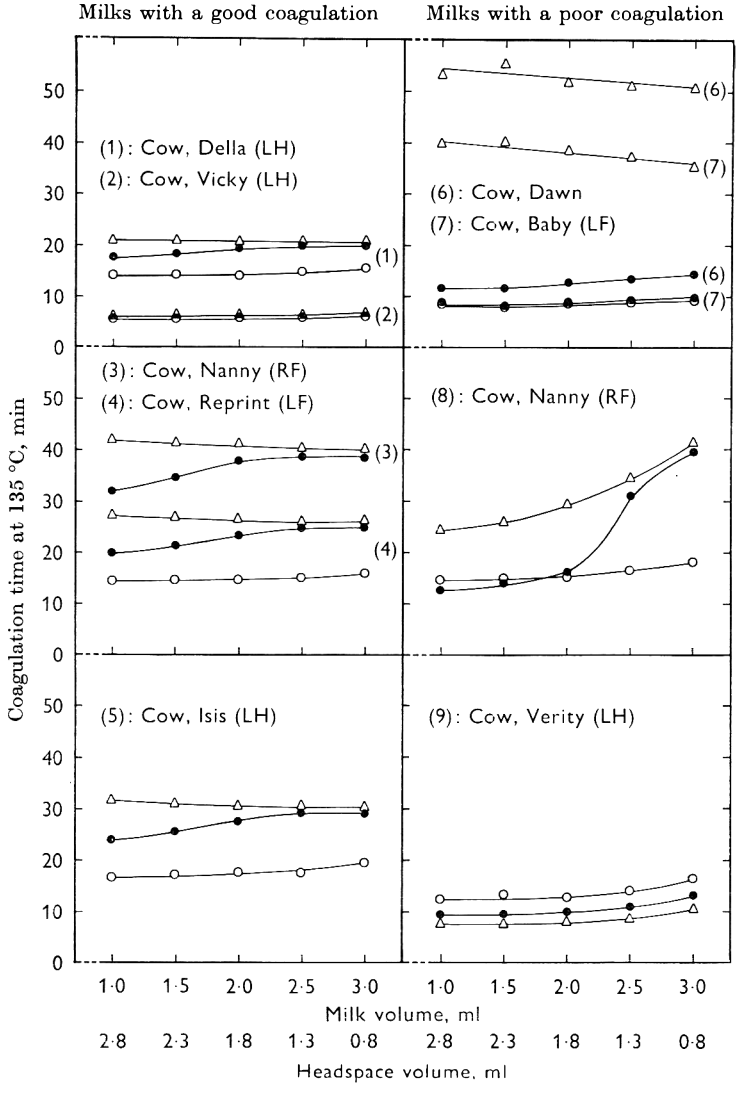


Fig. 6. Coagulation time of 5 separated milks with a good coagulation and of 4 separated milks with a poor coagulation, using different volumes of milk and different headspace gases in the sample tube. △, nitrogen in headspace; ●, air in headspace; ○, oxygen in headspace.

when different sample volumes are used, which might have some bearing on the sample volume-coagulation time interdependence, is the volume of headspace air in the sample tube. The capacity of the tube with the stopper inserted is about 3·8 ml and so milk samples of 1·0 and 3·0 ml leave headspace air volumes of about 2·8 and 0·8 ml, respectively, i.e. 74 and 21% of the capacity.

Effect of volume and composition of headspace gas in sample tube

On the assumption that oxygen would be the only component of air likely to have any direct influence on the mechanism of coagulation, and hence on coagulation time, the coagulation time of 9 milks was determined by the standard procedure but using sample volumes over the range 1·0–3·0 ml and using air, oxygen and nitrogen as headspace gas. A headspace atmosphere of oxygen or nitrogen, at a pressure of approximately 760 mm of mercury, was achieved by displacing the air above the

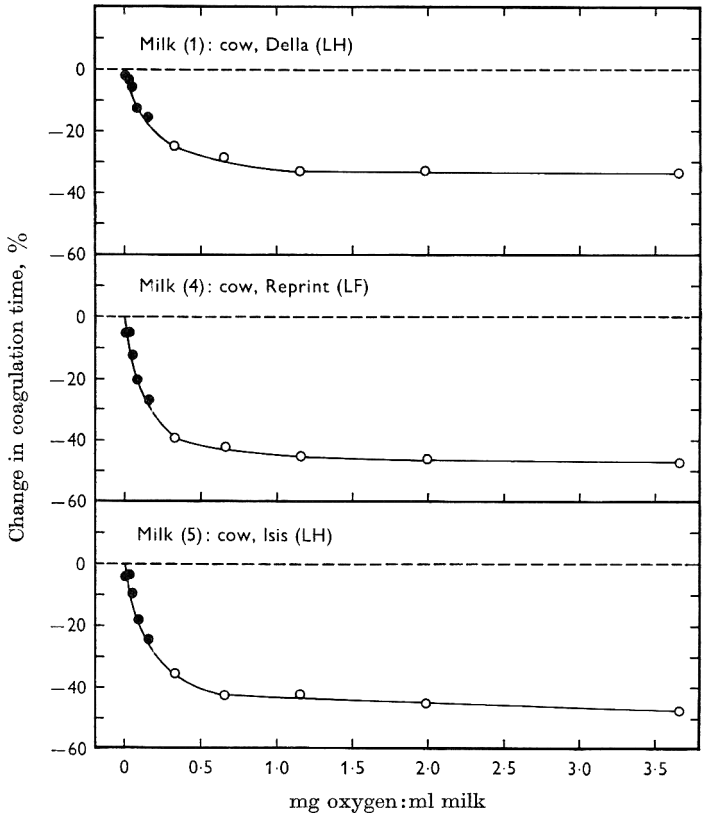


Fig. 7. Percentage change in coagulation time of 3 separated milks with a good coagulation with increasing proportion of headspace oxygen to volume of milk in sample tube; the corresponding coagulation time (min at 135 °C) with nitrogen as headspace gas is taken as reference. The proportion for the tubes with air as headspace gas are the calculated values multiplied by 159/760 (see p. 80). ●, air as headspace gas; ○, oxygen as headspace gas.

milk in the stoppered tube, by passing a gentle current of the appropriate gas through 1 of 2 hypodermic needles pushed through the self-sealing, silicone rubber stopper.

The results (Fig. 6) have been grouped according to the type of coagulation. In the group of 5 milks with a good coagulation, the coagulation time of the least heat-stable milk, milk (2), did not alter with changes in the volume or composition of the headspace gas. However, the other milks in this group showed a uniform pattern of behaviour: replacement of air with nitrogen caused an increase in coagulation time, and replacement of air with oxygen caused a decrease in coagulation time to levels

largely independent of the volume of headspace gas. With the 4 milks that had a poor coagulation, the effect on coagulation time of replacing air with nitrogen or oxygen was very variable. In the presence of oxygen the coagulation time of milks (6) and (7) was short and virtually unaffected by variations in headspace volume, but in the absence of oxygen, both milks were much more stable to heat and coagulation time decreased slightly with decreasing headspace volume. The coagulation time of milk (8) increased markedly with decreasing headspace volume, whether of air or nitrogen, and milk (9) differed from all the others in that its heat stability was slightly enhanced by the presence of oxygen in the headspace.

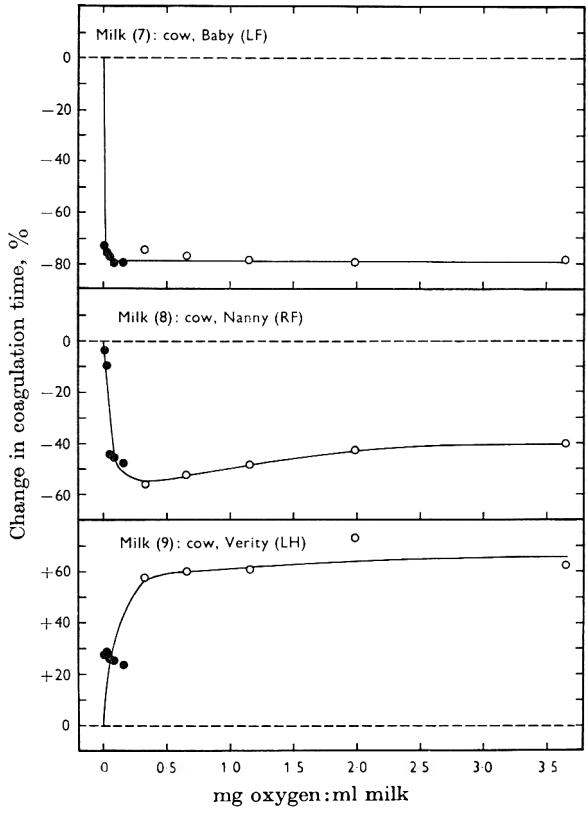


Fig. 8. Percentage change in coagulation time of 3 separated milks with a poor coagulation with increasing proportion of headspace oxygen to volume of milk in sample tube; the corresponding coagulation time (min at 135 °C) with nitrogen as headspace gas is taken as reference. The proportions for the tubes with air as headspace gas are the calculated values multiplied by 159/760 (see p. 80). ●, air as headspace gas; ○, oxygen as headspace gas.

These results suggest that the effect of sample volume on coagulation time (Fig. 5) could be attributed to the accompanying differences in the proportion of headspace oxygen to milk and show that with most of the milks, as this proportion increased, coagulation time decreased until constant minimum times were obtained when a critical value for the proportion was exceeded. The critical value for milks which reacted in this way was always exceeded when the headspace gas was oxygen, even at the minimum volume used. The sensitivity of some of the milks to the presence of

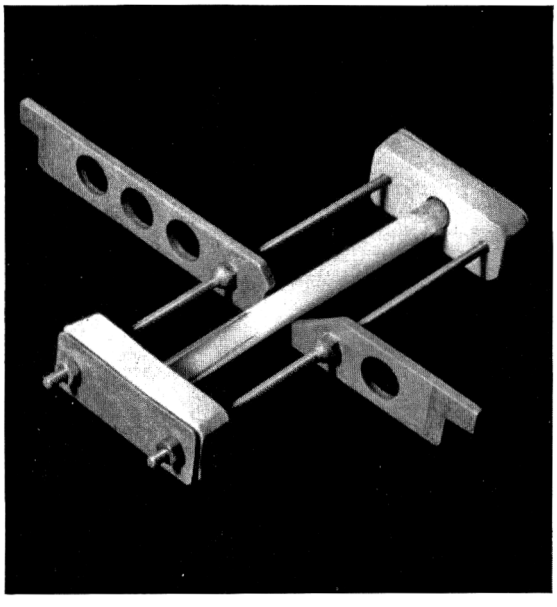
oxygen in the headspace was such that when the proportion of oxygen to milk was at the minimum (0·8 ml air, 3·0 ml milk), the critical value was already exceeded, e.g. milk (7) and probably milk (6) in Fig. 6. That the coagulation time of milk (2) was unaffected by changes in the volume and composition of the headspace gas (Fig. 6) suggests that the instability to heat of this milk was caused by other factors.

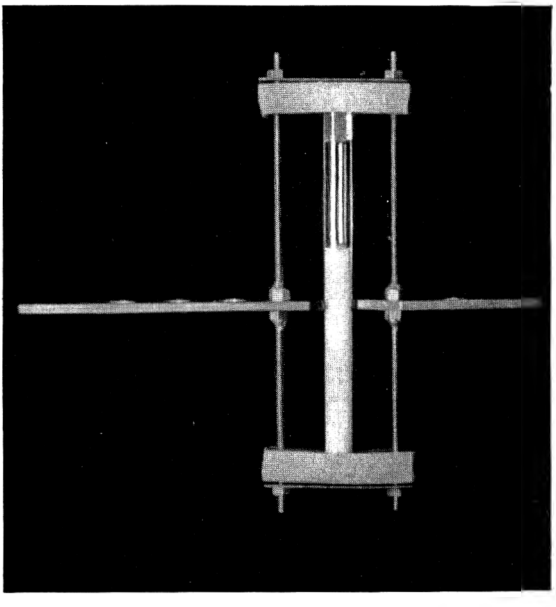
The influence of headspace oxygen on the coagulation time of 6 of the milks may be seen more clearly in Figs. 7 and 8. With the milks giving a good coagulation (Fig. 7), the relations between the percentage decrease in coagulation time and the proportion oxygen:milk, although not identical, were similar, the decrease in each case reaching a maximum when the headspace oxygen exceeded 1 mg/ml milk. Of the milks giving a poor coagulation (Fig. 8), milk (7) was so sensitive to the presence of headspace oxygen that only about 0·05 mg O_2/ml milk was required to cause the coagulation time to reach a minimum value that was 80% less than the coagulation time with only nitrogen in the headspace. Milk (8) was not quite so sensitive to the presence of oxygen, the maximum effect occurring when the proportion of oxygen to milk was about 0·3 mg/ml. Milk (9), as previously stated, was unique with stability being greater in the presence of oxygen; the effect seemed to reach a maximum when the proportion of oxygen to milk was about 0·5 mg/ml.

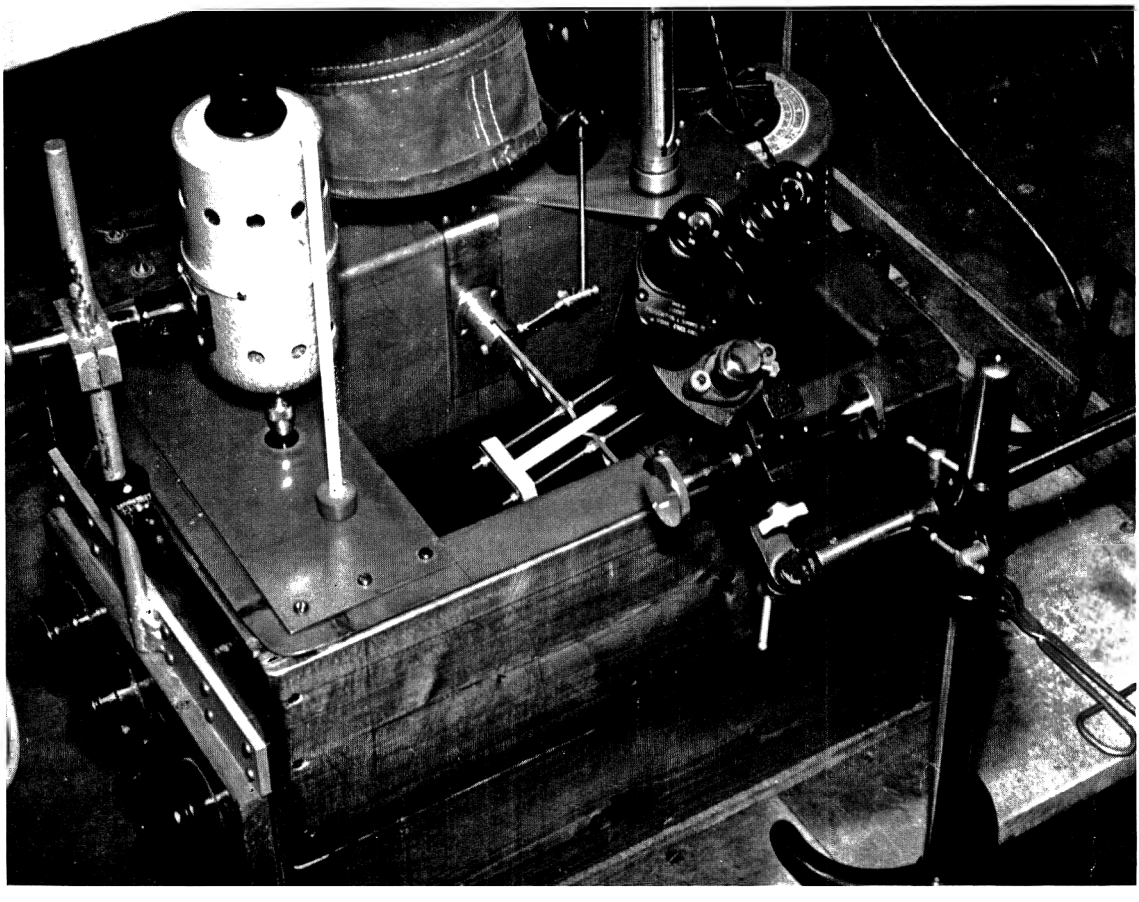
In preparing Figs. 7 and 8, the calculated oxygen:milk proportions for the tubes with air as headspace gas were multiplied by 159/760 to allow for the fact that the initial partial pressure of oxygen in these tubes was 159 mm of mercury compared with 760 mm in the tubes with oxygen as headspace gas. Only by so doing did the results for the 2 sets of tubes form the continuous curves shown in the figures. This indicates that whether headspace oxygen acts by replacing the initial, naturally occurring dissolved oxygen as it is used up, or through a milk-gas interface reaction, its influence on coagulation time is governed by its pressure in addition to its amount per unit volume of milk. Other observations made during these headspace gas experiments were, (1) a tendency for the type of coagulation to improve as the proportion of oxygen to milk increased, (2) a dependence of the rate of pH decrease in milk heated at 135 °C on the composition of the headspace gas, the rate being greatest with oxygen, least with nitrogen and intermediate with air, and (3) a more pronounced smell of hydrogen sulphide from milks heated in an atmosphere of nitrogen.

The involvement of oxygen in the heat-induced coagulation of milk protein appears to be a new observation that merits a much more extensive study than that reported here, for valuable information may thereby be obtained on the nature of the mechanism of coagulation. As the part played by oxygen is no doubt complex and varies according to the type of coagulation that occurs, any attempt to explain the present results from the limited information available would be premature. However, in future work, the oxidation of lactose and reactions directly or indirectly involving sulphydryl and disulphide groups will clearly need to be considered.

It now appears that in the heat stability test the response of milks to changes in the volume and composition of the headspace gas in the sample tube varies according to whether the coagulation of the milk is classed as 'good' or 'poor'. Further evidence of variable behaviour and the consequent desirability in heat stability studies of grouping milks into different classes is presented in the other parts of this investigation (Davies & White, 1966; White & Davies, 1966).







D. T. DAVIES AND J. C. D. WHITE

The authors thank Dr P. S. Blackburn for determining the differential cell counts and Miss V. D. Young for technical assistance.

REFERENCES

BELEC, J. & JENNESS, R. (1962). *J. Dairy Sci.* **45**, 20.
BLACKBURN, P. S., LAING, CONSTANCE M. & MALCOLM, D. F. (1955). *J. Dairy Res.* **22**, 37.
BRUYNE, N. A. DE (1961). *Res. Dev. Ind.* (1), 94.
COLE, W. C. & TARASSUK, N. P. (1946). *J. Dairy Sci.* **29**, 421.
DAVIES, J. M. (1959). *15th Int. Dairy Congr.* **3**, 1859.
DAVIES, D. T. & WHITE, J. C. D. (1966). *J. Dairy Res.* **33**, 83.
FINCH, A. C. M. (1963). *J. scient. Instrum.* **40**, 423.
Handbook of Chemistry and Physics (1947). 30th ed., p. 1978: Cleveland: Chemical Rubber Publishing Co.
PYNE, G. T. & MCHENRY, KATHLEEN A. (1955). *J. Dairy Res.* **22**, 60.
ROSE, D. (1963). *Review Article no.* 109. *Dairy Sci. Abstr.* **25**, 45.
ROSE, D. & TESSIER, H. (1959). *J. Dairy Sci.* **42**, 969.
SOMMER, H. H. & HART, E. B. (1919). *J. biol. Chem.* **40**, 137.
SOMMER, H. H. & HART, E. B. (1922). *J. Dairy Sci.* **5**, 525.
SOMMER, H. H. & HART, E. B. (1926). *Res. Bull. agric. Exp. Stn Univ. Wis.* no. 67.
WEBB, B. H. & HOLM, G. E. (1932). *J. Dairy Sci.* **15**, 345.
WHITE, J. C. D. & DAVIES, D. T. (1958). *J. Dairy Res.* **25**, 236.
WHITE, J. C. D. & DAVIES, D. T. (1966). *J. Dairy Res.* **33**, 93.
WHITNEY, R. MCL., PAULSON, KATHERINE & MURTHY, G. K. (1952). *J. Dairy Sci.* **35**, 97.

EXPLANATION OF PLATES

PLATE 1

Two views of carriage with attached sample tube as used in subjective test for determining the heat stability of separated milk.

PLATE 2

Thermostat bath used in subjective test for determining the heat stability of separated milk.

Printed in Great Britain

The stability of milk protein to heat

II. Effect on heat stability of ageing milk at different temperatures

By D. T. DAVIES and J. C. D. WHITE
The Hannah Dairy Research Institute, Ayr, Scotland

(*Received* 30 *September* 1965)

Summary. The effect on heat stability as measured by coagulation time, of storing separated milk at 20, 4 and −20 °C has been examined. Milk with a good coagulation (initial clots large) could be stored for at least 30 h at 20 °C, 1 week at 4 °C and 1 month at −20 °C with no significant change in coagulation time. With milks giving a poor coagulation (initial clots small), a common occurrence during storage at 20 °C was a marked progressive increase in coagulation time; the rate of increase was reduced by storage at 4 °C. The increase in coagulation time of these labile milks, which are usually obtained from cows with subclinical mastitis, may occur to the same extent in darkness as in light, may be enhanced by exposure to light or may occur only when the milk is exposed to light. From these results, together with others reported by Davies & White (1966) and White & Davies (1966) it is concluded that, in studying heat stability, milks giving a good coagulation should be regarded as in a different class from milks giving a poor coagulation.

In their investigation on heat stability, Cole & Tarassuk (1946) found that the refrigeration of milks for up to 2 days 'minimized the likelihood' of significant changes in coagulation time but Whitney, Paulson & Murthy (1952) on the other hand found that storage at 5 °C, preceded by the addition of toluene or chloroform or by heating at 148·5 °C for 2·1 sec, was not satisfactory for maintaining the initial heat stability of milk. In a previous investigation by the present authors (White & Davies, 1958), the coagulation time of milk was always determined about 6 h after collection from the cow, a procedure that assumed that the value so obtained would be the same as one obtained at any other time during the useful life of the milk. Because of this assumption, and because it is often desirable that a milk be used for a period longer than a day, together with the above contradictory results obtained with milks stored at a low temperature, it was necessary to try to establish how long milk could be kept, with or without a preservative, before its stability to heat altered.

With this aim in view, the coagulation time of separated milks when fresh and after various periods at 20, 4 and −20 °C was determined. A comparison was also made of storage in darkness and light, at 20 °C.

EXPERIMENTAL

The separated milks used in this part of the investigation were obtained from the same sources and were prepared, analysed and tested for coagulation time in the same way as described by Davies & White (1966). The colony count on 54 of the separated milks when fresh and after storage at 20 °C for 48 h was determined on Oxoid milk agar after incubation for 5 days at 22 °C. The differential cell count of all the whole milks was determined (Blackburn, Laing & Malcolm, 1955).

The numbers used to identify the milk samples apply only to the particular table or figure in which they are used. Where 2 or more sets of results are given for milk from the same cow, the samples were taken at different stages of lactation.

RESULTS AND DISCUSSION

Effect of ageing milk at 20 °C

As it was known (Davies & White, 1960) that when milk is stored at 3 °C a transfer of some calcium and phosphorus from the dispersed phase to the aqueous phase takes place, a change that might conceivably affect coagulation time, storage at 20 °C, with and without the addition of a preservative, was investigated first. In these experiments, 40-ml samples of milk were kept in glass-stoppered Pyrex conical flasks of 100-ml capacity, in the dark in an incubator, at 20 ± 1 °C.

Because potassium dichromate, formaldehyde and mercurial compounds, the commonly used milk preservatives, interact with protein they were not considered suitable for the present studies and only chloroform or a mixture of equal parts of penicillin, streptomycin and neomycin was tried. However, at concentrations likely to be effective, chloroform caused a decrease in coagulation time and the antibiotic mixture caused either a decrease or an increase. Accordingly, no further attempt was made to find a suitable preservative.

Experiments were then carried out to determine how the coagulation time of milk without preservative varied during storage at 20 °C. Seventeen milks were used, comprising samples from individual cows, individual quarters and herd bulk milk. The coagulation time of each milk was determined about 1·5, 2·5 and 3·5 h after it had been collected and thereafter at intervals of 2–3 h until there was detectable decomposition of the milk. A separate flask of milk was used for each determination of coagulation time and the pH of each stored sample was determined. Apart from an initial very slight upward or downward trend in coagulation time with a few of the milks, all the milks followed one or other of 2 distinctly different patterns of behaviour. The coagulation time of 10 of the milks remained virtually constant for periods of 30–70 h and then rapidly decreased to a very low value as the pH decreased (e.g. milks (1) and (2), Fig. 1); with milks of this group that had a coagulation time of 10 min or less, the final decrease in coagulation time was preceded by a marked transient increase (e.g. milks (3) and (4), Fig. 1). The coagulation time of the 7 other milks, on the other hand, gradually increased during storage (e.g. milks (5) and (6), Fig. 1), in some instances to more than treble the initial value, and then if storage was sufficiently prolonged coagulation time again finally decreased rapidly. With none of these 7 milks was the increase in coagulation time clearly associated with a concurrent

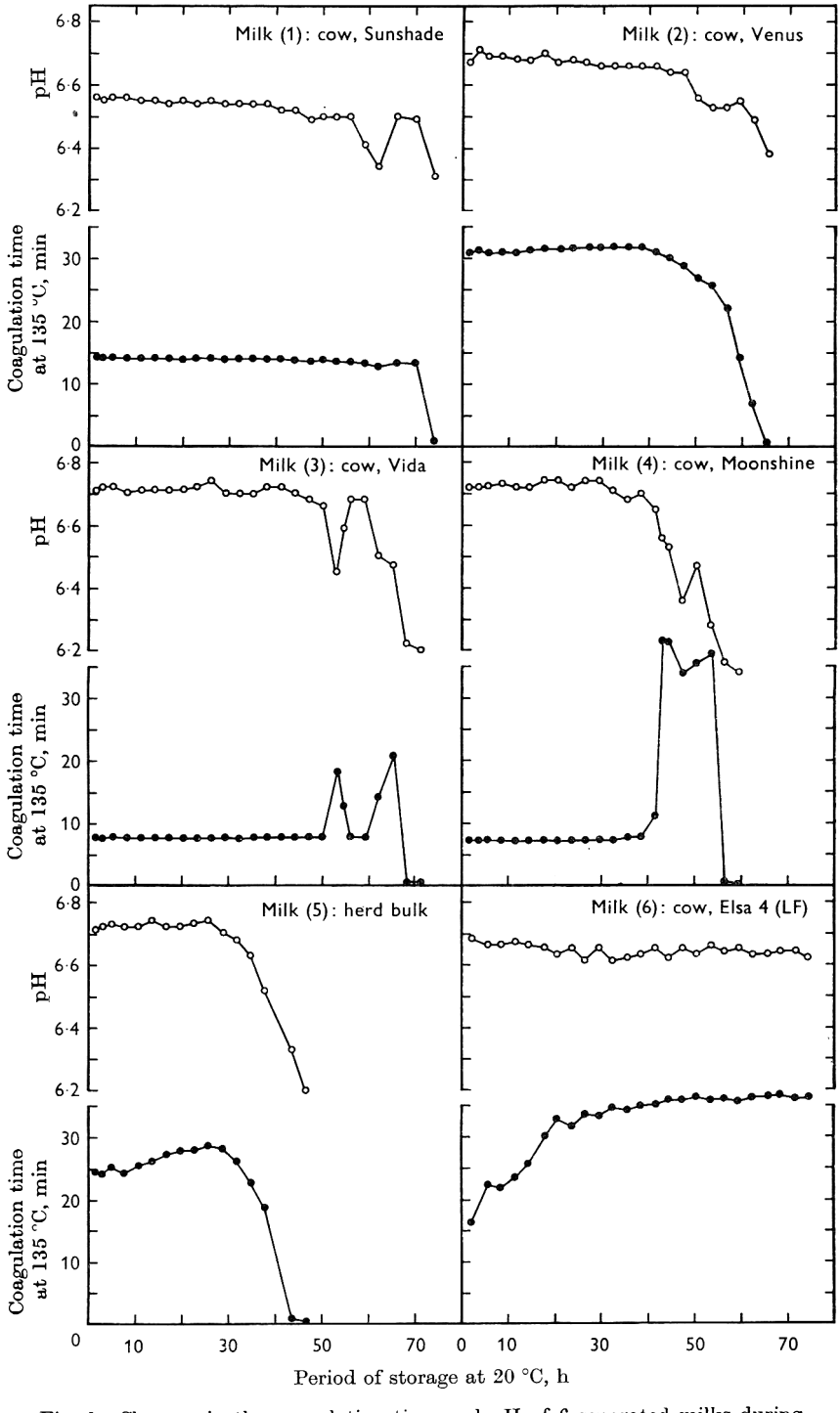


Fig. 1. Changes in the coagulation time and pH of 6 separated milks during storage in the dark at 20 °C.

change in pH, but it was observed that all were of the poorly coagulating type (see Davies & White, 1966). Milks showing this gradual increase in coagulation time during storage have been designated 'labile'.

The rapid decrease in heat stability that eventually occurs when both normal (non-labile) and labile milk are stored at 20 °C can probably be attributed to the concurrent rapid decrease in pH. It has long been known that when acid is added to milk the heat stability of the milk usually decreases, especially when the pH reaches 6·4–6·2 (Miller & Sommer, 1940). More recent studies of the heat stability-pH relation (Rose, 1961; Tessier & Rose, 1964) have revealed the occurrence of 2 types of milk, one with a minimum and a maximum heat stability in the pH range 7·1–6·4 and one with only a gradually decreasing heat stability over this pH range. It would appear from Fig. 1 that milks (3) and (4) were of the former type and that during souring the pH passed through the region in which a maximum heat stability occurred.

Table 1. *Changes in the coagulation time of separated milks stored at 20 °C in flasks and tubes, in darkness and light (flasks, 100-ml conical, containing 40 ml milk; tubes, as used in heat stability test, containing 2·5 ml milk)*

		16 h in flask		16 h in tube	
Milk:Cow	Fresh	Darkness	Darkness, then 4–5 h light*	Darkness	Light
	Coagulation time at 135 °C, min				
(1):Baby (RF)	9·4	8·6	13·2	—	—
(2):Baby (RF)	13·4	13·0	36·6	—	—
(3):Elsa 4 (LF)	7·9	—	—	8·4	11·6
(4):Isolde (LH)	9·3	—	—	10·0	15·5
(5):Vesta (RH)	12·8	11·8	17·4	—	35·1
(6):Nanda (RH)	33·9	39·4	40·4	40·6	40·5
(7):Herd bulk	15·3	19·0	—	—	33·1
(8):Baby (RF)	13·3	13·2	42·7	18·9	37·2

* After 2·5 ml milk transferred to tube.

To determine the frequency of occurrence of the seemingly unreported labile type of milk, 1 sample of milk was taken from each of 54 cows in the Institute herd and coagulation time determined when fresh and after storage for 24 and 48 h in the dark at 20 °C. In addition, the chemical composition, pH and colony count of the fresh milks, the pH of the stored samples, and the colony count of the '48-h' samples were determined. Only 7 cows (13%) were found to be giving milk whose coagulation time increased during storage; one cow gave milk whose coagulation time halved during storage for 24 h. These 8 milks were all of the poorly coagulating type and in no instance was the change in heat stability during storage associated with a change in pH or a consistent pattern of change in colony count. There was some evidence that the production of labile milk by these cows was associated with the presence of subclinical mastitis, but subsequent experience has shown that labile milk can be obtained from apparently healthy cows. Although labile milk has been found to be invariably of the poorly coagulating type the converse is not always true.

In the storage experiments so far described the milks were kept at 20 °C in darkness

and it is clearly of interest to know the effect on heat stability of storing milk at 20 °C but exposed to light. Such an investigation has not yet been systematically carried out. However, in the course of other work involving 35 milks the opportunity was taken of comparing the coagulation time of the milks when fresh and after the following conditions of storage at 20 °C: (1) in flasks for 16 h in darkness, (2) in flasks for 16 h in darkness then in tubes for 4–5 h in light, (3) in tubes for 16 h in darkness and (4) in tubes for 16 h in light. For exposure to light, the flasks and tubes were placed about 1 m from an 80-W white strip light. The coagulation time of 27 of the milks was unaffected by the 4 different storage conditions; the results obtained with the 8 other milks are shown in Table 1. Milks (1–5), on the evidence presented so far, would have been classed as 'non-labile' because their heat stability did not change to any significant extent during storage in darkness yet the coagulation time of each increased during exposure to light. Milk (6) would have been classed as 'labile' as would have milk (7), but milk (7) differed from milk (6) in that exposure to light enhanced the increase in coagulation time. The values obtained with milk (8) are anomalous in that coagulation time increased in darkness only when the milk was stored in a tube. All these 8 milks were of the poorly coagulating type, some came from cows with subclinical mastitis and with none could the change in coagulation time be ascribed to a change in pH.

These experiments show that the category 'labile' should embrace milks showing at least 4 different types of change in coagulation time during storage at 20 °C, namely, (*a*) milk whose coagulation time increases in darkness, the increase being enhanced by exposure to light, (*b*) milk whose coagulation time increases in darkness and light to the same extent, (*c*) milk whose coagulation time increases only on exposure to light, and rarely, (*d*) milk whose coagulation time decreases during storage in darkness. The 14 milks found in the first 2 experiments to increase in heat stability during storage in darkness would presumably be of type (*a*) or (*b*). A practical consequence of, and possible reasons for, the occurrence of labile milks are discussed on p. 90.

Effect of ageing milk at 4 *and* −20 °*C*

The experiments described above indicated that normal, non-labile, separated milk could be stored at 20 °C for at least 30 h, and usually for 40 h, with no significant change in coagulation time. To find whether it would be possible to extend the useful life of milks, labile as well as normal, to a period of 1 week, storage at 4 °C was examined even although previously published work on this means of preservation had yielded conflicting results. For these studies, 40-ml samples of milk were kept in glass-stoppered Pyrex conical flasks (100-ml capacity) in a cold room at 4 ± 1 °C and in an incubator at 20 ± 1 °C. A separate flask of milk was used for each determination of coagulation time. A preliminary investigation showed that after milk had been stored at 4 °C and the temperature of the milk then quickly adjusted to 20 °C, the coagulation time obtained immediately was very similar to that obtained after intervals of 0·5, 1 and 2 h, i.e. an equilibration period at 20 °C is unnecessary.

In the first storage experiment, involving one sample of milk from each of 8 different cows, coagulation time was determined on the fresh milk, after 1 day's storage at 20 °C and daily after storage at 4 °C for up to 7 days. It was found that 7 of these

milks, none of which appeared to be of the labile type, could be kept at 4 °C for 7 days with no significant change in coagulation time. However, the remaining milk, which differed from the others in having a poor coagulation and coming from a cow with subclinical mastitis, showed erratic increases in coagulation time after 2–7 days at 4 °C.

Table 2. *Coagulation time of quarter and composite samples of separated milk, from cows free from subclinical mastitis, when fresh and after storage at* 20 *and* 4 °*C* (*coagulation time following storage at* 4 °*C was determined* 0·5 *and* 6 *h after the temperature of the milk had been quickly adjusted to* 20 °*C*)

Milk	Fresh	1 day at 20 °C	4 days at 4 °C		7 days at 4 °C	
			0·5 h, 20 °C	6 h, 20 °C	0·5 h, 20 °C	6 h, 20 °C
	Coagulation time at 135 °C, min					
Cow, Elsanora						
LF	8·5	8·4	9·0	8·9	9·0	9·0
LH	8·5	8·5	8·9	8·8	9·0	8·9
RF	8·1	8·3	8·7	8·4	8·8	8·8
RH	7·9	7·9	8·3	8·4	8·4	8·2
Composite	8·1	8·3	8·8	8·5	9·1	8·6
Cow, Della						
LF	12·5	12·2	12·0	12·1	11·8	11·8
LH	11·8	11·5	11·6	11·4	11·2	11·3
RF	12·9	12·7	12·6	12·6	12·2	12·2
RH	12·6	12·3	12·2	12·2	11·9	11·9
Composite	12·5	12·3	12·1	12·0	11·8	12·0
Cow, Iran						
LF	19·2	19·8	20·1	20·6	21·2	21·0
LH	21·9	22·0	21·7	22·2	21·8	22·3
RF	20·7	20·0	21·0	19·1	22·6	21·5
RH	20·7	20·0	21·1	20·9	21·9	20·3
Composite	22·8	21·3	21·6	22·0	22·2	22·0

To confirm and extend the above findings, a second experiment was carried out involving the storage of 45 milks comprising quarter and composite samples from 9 different cows. Coagulation time was determined on the fresh milk, after storage at 20 °C for 1 day, and after storage at 4 °C for 4 and 7 days. The coagulation time of the milks stored at 4 °C was determined 0·5 and 6 h after the temperature of the milks had been quickly adjusted to 20 °C as in practice these would be the minimum and maximum periods at 20 °C to which milk previously stored at 4 °C would be subjected. The chemical composition and pH of each milk when fresh and after the 3 storage periods were also determined. This experiment was so designed that, in addition to achieving the primary object, a within-cow comparison could be made of the coagulation time of quarter and composite samples from cows free from subclinical mastitis and from cows with one or more quarters affected by subclinical mastitis. Results representative of those obtained for cows with all quarters apparently healthy and of those with one or more quarters with subclinical mastitis are shown in Tables 2 and 3, respectively.

The values in Table 2 show that with healthy cows the fresh milk from each quarter and the fresh composite milk have a very similar coagulation time, and, what is even

more important, that this coagulation time can be held virtually constant for 7 days by storage at 4 °C. Coagulation time was the same whether determined 0·5 or 6 h after adjusting the temperature of the milk from 4 to 20 °C. The milks from the 2 other healthy cows studied also remained constant in heat stability during storage and all the samples from this group of 5 cows had a coagulation classed as 'good'.

Table 3. *Coagulation time of quarter and composite samples of separated milk, from cows with one or more quarters affected by subclinical mastitis, when fresh and after storage at* 20 *and* 4 °*C* (*coagulation time following storage at* 4 °*C was determined* 0·5 *and* 6 *h after the temperature of the milk had been quickly adjusted to* 20 °*C*)

Milk	Severity of subclinical mastitis*	Fresh	1 day at 20 °C	4 days at 4 °C		7 days at 4 °C	
				0·5 h, 20 °C	6 h, 20 °C	0·5 h, 20 °C	6 h, 20 °C
		Coagulation time at 135 °C, min					
Cow, Sable							
LF	0	25·8	25·1	24·9	24·5	24·2	24·1
LH	0	25·2	24·5	24·3	24·1	23·4	23·4
RF	0	24·6	23·5	23·7	23·3	22·8	22·7
RH	1	32·5	30·0	30·8	30·3	30·2	29·6
Composite	—	27·0	25·3	26·1	25·3	24·9	24·8
Cow, Irma							
LF	1	36·7	38·7	38·8	39·2	39·6	39·7
LH	2	11·4	28·2	43·6	46·6	48·5	49·6
RF	4	18·6	5·4	77·2	49·0	78·5	13·6
RH	3	8·0	41·0	48·1	53·7	56·3	57·5
Composite	—	11·2	36·8	50·5	53·5	55·2	55·8
Cow, Relish							
LF	5	2·9	2·2	2·7	2·4	2·4	2·4
LH	3	26·6	28·4	25·4	30·1	29·9	30·5
RF	2	36·8	38·4	38·0	38·9	38·4	39·1
RH	2	10·3	29·7	25·8	25·6	38·8	35·4
Composite	—	12·4	34·9	38·3	34·3	42·9	43·7

* 0, healthy quarter; 1–5 subclinical mastitis in order of increasing severity.

The values in Table 3 show the wide range in coagulation time, and the patterns of change encountered during storage, when subclinical mastitis occurs in one or more quarters. The samples from the 3 healthy quarters of the cow Sable had, as expected, very similar coagulation times before and after storage, whereas the sample from the slightly affected quarter had a coagulation time about 30 % longer. However, the sample from the affected quarter showed little variation during storage and had a coagulation classed as 'moderate'; the milk from the LF quarter of the cow Irma was of the same type. When subclinical mastitis was severe, e.g. LF quarter of the cow Relish, the milk was very unstable to heat throughout storage and had a coagulation classed as 'good'. This type of milk reacts to heat in much the same way as colostral milk (White & Davies, 1958). When, however, the degree of subclinical mastitis was intermediate, i.e. classed as 2–4 (Table 3), all the milks had a poor coagulation and showed a variety of pattern of change in heat stability during storage. In no instance was there any clear association between the changes in heat stability of these labile milks and minor fluctuations in pH during storage.

The feasibility of using storage at −20 °C for the preservation of milk to be used in heat stability studies was also examined. Samples of milk (200 ml), in polythene bottles (500 ml), were stored at −20 °C for 1 month. Nine non-labile milks from cows known to be healthy were used. The frozen milks were liquefied by immersing the bottles in a water bath at 37 °C for 0·5, 1, 2 or 3 h and coagulation time determined after adjusting the temperature of the milks to 20 °C and maintaining them at this temperature for 0·5, 6 or 24 h. From a comparison of the coagulation times of the fresh and stored samples, the mean deviation after storage was found to be −0·5 min, with the range being −2·1 to +1·2 min; in treating the frozen milks no particular combination of periods at 37 and 20 °C seemed better than any other. After one of the milk samples had been liquefied, a small amount of protein-like material remained adhering to the polythene bottle but this had no untoward effect on coagulation time. Thus, in heat stability studies, storage of milk at −20 °C appears to offer the possibility of carrying out experiments with the same milk for a period of at least 1 month.

The discovery in the preceding experiments that the coagulation time of some milks changes whether the milks are stored at 20 °C (in darkness or light) or at 4 °C, has an important practical consequence in heat stability investigations, i.e. when milk samples are obtained, it must first be determined whether they are of the normal or labile type if erroneous conclusions are to be avoided, particularly when trying to relate coagulation time to some chemical or physical property of the protein or milk. A convenient way of doing this is to collect milk from cows at the afternoon milking and determine as soon as possible the coagulation time of the fresh separated milk, taking note of whether the coagulation is good or poor; if poor, there is a strong possibility that the milk will be of the labile type. The bulk of the sample should then be stored at 4 °C or at 20 °C if preferred and if the experimental work can be accomplished during the following day. At the same time a 2·5-ml sample should be placed in each of 2 tubes (as used in the heat stability test) and the stoppered tubes left overnight at 20 °C or room temperature, one exposed to artificial light and the other in darkness. Next morning these tubes should be used to determine coagulation time and the values compared with that obtained with the fresh milk. This procedure should detect any of the 4 previously mentioned categories of labile milk. However, an additional safeguard, which was adopted where appropriate throughout the present investigation, is to determine the coagulation time of a milk at the beginning and end of each day's work so ensuring that if a change in stability occurs it will be detected. In view of the likelihood of a quarter with subclinical mastitis producing labile milk, foreknowledge of whether a cow is affected is useful in avoiding labile milks. If 4 quarter samples are taken from one cow and the coagulation time of each is the same, this, apart from the usual diagnostic tests such as cell count, lactose content and casein number, is fairly conclusive evidence of the absence of subclinical mastitis. However, the absence of subclinical mastitis does not appear to be an absolute guarantee against milk being labile as labile milks are occasionally obtained from apparently healthy cows. Whether this is due to a failure to detect very mild subclinical mastitis by the usual tests or whether the production of labile milk is a natural characteristic, transient or permanent, of certain cows, is at the moment uncertain. Although only 13 % of the cows in the Institute herd were found

to be giving labile milk when their milk was tested once in a 6-week period, the difficulty at other times of obtaining non-labile samples, even of the herd bulk milk, shows that the incidence of labile milk may well have occasionally been more than 13%, or that the influence of labile milk when mixed with normal milk is disproportionately large.

As most of the labile milks encountered during the present investigation came from cows with subclinical mastitis, it was thought that the marked proteolysis of casein that occurs during the storage of milk from cows so affected might be the main factor responsible for the changes in heat stability. However, the inability to correlate degree of proteolysis and degree of 'lability', and the fact that the proteolysis can be extensive in milk from cows with subclinical mastitis whether the milk is labile or not, probably precludes proteloysis as a causal factor. Nevertheless, it is considered that the phenomenon of poorly coagulating, labile milk is associated with some quantitative or qualitative abnormality in the protein of the fresh milk, either occurring naturally or as a consequence of disease. Such an abnormality might be a concentration above a certain critical level of blood serum albumin. The fact that exposure to light in most instances enhances the increase in heat stability of labile milks suggests that reactions involving sulphydryl groups cause milks to be labile (see Burton, 1959).

The authors thank Dr P. S. Blackburn for determining the differential cell counts, Dr Margaret M. Taylor for determining the colony counts and Miss V. D. Young for technical assistance.

REFERENCES

Blackburn, P. S., Laing, Constance, M. & Malcolm, D. F. (1955). *J. Dairy Res.* **22**, 37.
Burton, H. (1959). *15th Int. Dairy Congr.* **3**, 1729.
Cole, W. C. & Tarassuk, N. P. (1946). *J. Dairy Sci.* **29**, 421.
Davies, D. T. & White, J. C. D. (1960). *J. Dairy Res.* **27**, 171.
Davies, D. T. & White, J. C. D. (1966). *J. Dairy Res.* **33**, 67.
Miller, P. G. & Sommer, H. H. (1940). *J. Dairy Sci.* **23**, 405.
Rose, D. (1961). *J. Dairy Sci.* **44**, 430.
Tessier, H. & Rose, D. (1964). *J. Dairy Sci.* **47**, 1047.
White, J. C. D. & Davies, D. T. (1958). *J. Dairy Res.* **25**, 281.
White, J. C. D. & Davies, D. T. (1966). *J. Dairy Res.* **33**, 93.
Whitney, R. McL., Paulson, Katherine & Murthy, G. K. (1952). *J. Dairy Sci.* **35**, 937.

Printed in Great Britain

J. Dairy Res. (1966), **33**, 93
With 1 *plate*

The stability of milk protein to heat

III. Objective measurement of heat stability of milk

By J. C. D. WHITE and D. T. DAVIES
The Hannah Dairy Research Institute, Ayr, Scotland

(*Received* 30 *September* 1965)

Summary. An objective test has been developed to help in assessing the value, as a method for determining the heat stability of milk, of the subjective test described by Davies & White (1966*a*). The objective test showed that coagulation time, as determined by the subjective test, is a reasonably accurate measure of the induction period that precedes the onset of initial rapid coagulation, but that coagulation proceeds in 2 different ways depending primarily on whether the coagulation of the milk is good (initial clots large) or poor (initial clots small). The latter finding confirms the view that in studying heat stability these 2 categories of milk should be examined separately.

The subjective heat stability test for milk has been shown to measure with satisfactory precision the time required for visible clots to form throughout a milk heated under the particular conditions of the test (Davies & White, 1966*a*). The assumption is usually made that the 'coagulation time' obtained by this type of test adequately characterizes the stability of milk protein to heat and hence that values obtained for a series of milks are comparable. The validity of this assumption is questionable.

There are differences in the manner in which milks coagulate when heated: with some, relatively large clots form very quickly and rapidly increase in size until virtually all the protein appears to be coagulated; with others, the clots that are first visible are very small, they increase in size very slowly and the coagulation appears incomplete. There is also much variation in whether or not coagulation proper, as defined by coagulation time, is preceded by the appearance of isolated flecks in the milk or by the formation of a deposit on the walls of the tube. Thus, there is clearly a wide variety in the manner, both qualitative and no doubt quantitative—with respect to the rate at which coagulation proceeds once started—in which the protein in different milks is coagulated by heat. Coagulation time takes no account of these differences and hence 2 milks, each with the same coagulation time yet quite different in their manner of coagulation, will be regarded as equal in heat stability. The desirability of distinguishing 2 main types of milk when studying heat stability namely, those with a good coagulation (initial clots large) and those with a poor coagulation (initial clots small), has already been indicated (Davies & White, 1966*a*, 1966*b*) and further evidence to support this view is given in this part of the investiga-

tion in which the heat stability of milk was examined by an objective test as well as by the standard subjective test already described.

Whitney, Paulson & Murthy (1952) recognized the limitations of merely determining coagulation time and they developed an objective method to obtain a better characterization of the heat stability of a milk. In their method, a plot of the decrease in filterable nitrogen against time of heating at 120 °C is obtained, the curve so constructed giving an indication of the progressive coagulation of the milk protein. However, Whitney *et al.* (1952) presented curves for only 3 milks and although they made clear the likely value of this information in heat stability studies, no-one appears to have subsequently utilized the method. In view of the more detailed characterization of heat stability provided by this procedure, an objective test similar in principle to that of Whitney *et al.* (1952) has been developed.

EXPERIMENTAL

Objective heat stability test

The same apparatus is used in the objective test as in the subjective test (Davies & White, 1966*a*) except that the carriage (Plate 1) accommodates 9 tubes and each tube, having its own clamping device for keeping the stopper in position, can be removed independently from the bath. The conditions under which the milk is heated are exactly the same as in the standard procedure for the subjective test. In the objective test, 18 samples of the milk are heated for different periods, in 2 consecutive sets of 9, some shorter and some longer than that required for visible coagulation. The periods are chosen having regard to a preliminary determination of coagulation time by the subjective test and are randomly distributed between the 2 sets. The extent of protein aggregation and degradation with heating period is obtained by nitrogen determinations on the supernatant liquids from the cooled, centrifuged samples.

Procedure

Pipette 2·5 ml of separated milk at 20 °C into each of 20 tubes and stopper the tubes so that the bottom of each stopper is 3·5 cm above the surface of the milk. Fit 9 of the tubes with the clamping device, place the tubes on the carriage and tilt the carriage so that all the inside surface of each tube is wetted with milk. With the liquid paraffin at a temperature of 135 °C, insert the carriage in the bath and simultaneously start a stop-watch. Immediately start the rocking mechanism, which has previously been adjusted so that the tubes will perform 8 rocking c/min. Use the front tube to confirm coagulation time and type of coagulation. At predetermined intervals remove each of the other tubes, in random order of position on the carriage, and immediately immerse in a water bath at 20 °C. Ten minutes after the last tube is placed in the water bath, take the tubes out and centrifuge for 15 min at 300 *g*; one unheated control tube is also centrifuged. Remove a portion of the supernatant liquid from each tube by inserting the drawn-out, turned-up tip of a 5-ml bulb pipette for a distance of 9·2 cm (from top of tube) and applying suction. Transfer the samples of the supernatant liquids to 5-ml Pyrex tubes.

Repeat the whole of the above procedure with the second set of 9 tubes.

Make the following determinations on the supernatant liquids:

Total-N(TN). Pipette 0·2 ml supernatant liquid into a micro-Kjeldahl flask and determine the nitrogen (White & Davies, 1958), expressing the value as mg nitrogen/100 ml supernatant liquid.

Proteose-peptone-N plus non-protein-N (PPN + NPN). Pipette 0·5 ml supernatant liquid into a 5-ml volumetric flask; at this stage, heat the supernatant from the control milk by immersing the flask in water at 95–100 °C for 15 min and then allow to cool. Add 3·5 ml distilled water at 40 °C, swirl the flask to mix its contents, add 0·1 ml acetic acid solution (5 %, w/v), mix, and allow the flask to stand for 10 min. Add 0·1 ml sodium acetate solution (0·5 N), mix, and allow to cool to room temperature. Dilute to 5 ml with distilled water, mix, allow the precipitate to settle and filter (Whatman no. 42, 9 cm). Pipette 3·0 ml of the filtrate into a micro-Kjeldahl flask and determine the nitrogen, expressing the value as mg nitrogen/100 ml supernatant liquid and using a correction factor of 0·998 to allow for the volume of precipitate in the 5-ml flask.

Notes on procedure

(1) If only one centrifuging treatment is used in the above kind of test, the most appropriate treatment is difficult to choose and has to be a compromise. If too mild, the centrifuging treatment may not reveal degrees of heat-induced aggregation of protein invisible to the eye, whereas if too severe, it will sediment protein in unheated milk and may not reveal differences in degree of aggregation in milk with a good coagulation and milk with a poor coagulation. In the present investigation, it was decided to use the fairly mild centrifuging treatment of 300 *g* for 15 min and reserve for later study the use of a range of centrifuging treatments. Although it was known that the centrifuging treatment adopted would be most unlikely to sediment protein from unheated milks, an unheated 'control' was given the same centrifuging and analytical treatment as the heated milks to obtain 'unheated' reference values for TN and PPN + NPN.

(2) In the present method the collection of the supernatant liquids by suction was preferred to the filtration used by Whitney *et al.* (1952) because of its greater convenience. The level in the tubes above which the supernatant liquid is removed (9·2 cm from the top, 3·0 cm from the bottom) was chosen so that the tip of the suction device would be about 1 cm above the maximum amount of sediment likely to be obtained.

(3) In fractionating the nitrogen, the assumptions are made that all the PPN + NPN, whether native or heat-produced, is retained in the supernatant liquids and that its determination could be satisfactorily carried out in the supernatants from heated milk. The procedure is a scaled-down version of Rowland's method (1938) which, although now known to give high values (Aschaffenburg & Drewry, 1959) is very convenient for comparative purposes. Tests showed that the pH of the 5-ml buffered solutions containing 0·5 ml of supernatant liquid, whether from unheated or heated milk, was within the range 4·6–4·8.

(4) Although the reproducibility of the objective test was not specifically examined, the fact that smooth curves were obtained when percentage of protein aggregated was plotted against heating period, despite the periods being randomized over 2 sets

of milk samples, and that identical curves could be obtained with fresh and stored samples of the same milk, was fairly conclusive evidence that the procedure has a satisfactory reproducibility.

(5) After the onset of visible coagulation, further prolonged heating often caused blockages in the tubes through the formation of very large aggregates of protein. These blockages led to restricted movement of the residual liquid, but in no instance did this have any apparent effect on the shape of the coagulation curve. It would appear, therefore, that after prolonged heating transference of protein from the dispersed to the coagulated state takes place whether the milk is moving or not.

Milk samples

The separated milks used in this part of the investigation were obtained from the same sources and were prepared, analysed, and stored, in the same way as described by Davies & White (1966*a*). The differential cell count of all the whole milks was determined (Blackburn, Laing & Malcolm, 1955).

RESULTS

The information that can be obtained by the objective heat stability test is shown in Table 1, which contains a selection of the results obtained with one milk. The term 'aggregated protein' means protein sufficiently aggregated, whether visibly or not, as a result of the heat treatment to be sedimented into the bottom 3 cm of the tube by the centrifuging conditions used.

The heat stability of 12 milks, chosen to provide a range of coagulation time and type of coagulation, was determined by the objective test and the curves obtained are shown in Figs. 1 and 2. The curves showing the percentage of the original protein (nitrogen) aggregated after various periods at 135 °C can be separated according to their shape into 2 types, those in Fig. 1 which for convenience are called the 'normal' type and those in Fig. 2 which are called 'abnormal'. The 6 normal curves have the following characteristics: (1) they show an induction period during which very little aggregation of protein appears to occur, (2) the induction period is followed by a sudden aggregation that precedes visible coagulation by only 1–2 min, and (3) coagulation proceeds without interruption, but at a rate varying from milk to milk, until finally, of the original protein present, about 80 % is aggregated, 15 % is converted to proteose-peptone and NPN, and only 5 % remains suspended. It can be seen that after the induction period milk (1) had the highest rate of coagulation and milk (6) the lowest. The objective measure of the rate of coagulation provided by the curves was consistent with the subjective, qualitative description of the manner in which these milks coagulated, namely 'very good' for milk (1), through 'good' and 'moderate' to the 'poor' recorded for milk (6). There was no relation between the slope of the curves and the concentration of total protein or casein in the milks.

The milks which furnished the results in Fig. 1 were normal in chemical composition and cell count and none of the milks was of the labile type (see Davies & White, 1966*b*). It is, therefore, appropriate that the coagulation curves in Fig. 1 should be regarded as the normal type, and characteristic of milk from healthy cows and of

Table 1. *A selection of results obtained by the objective heat stability test with a separated milk having a coagulation time at 135 °C of 18·3 min (milk 5, Fig. 1)*

Period at 135 °C, min	(*a*) TN	(*b*) PPN + NPN	(*a*–*b*) Suspended protein N (*x*)	(492·8–*a*) Aggregated* protein N (*y*)	(*b*–40·5) Heat-produced PPN + NPN (*z*)	$\frac{x \times 100}{452 \cdot 3}$ Suspended protein N	$\frac{y \times 100}{425 \cdot 3}$ Aggregated protein N	$\frac{z \times 100}{452 \cdot 3}$ Heat-produced PPN + NPN	$\frac{x \times 100}{x+y}$ Suspended protein N	$\frac{y \times 100}{x+y}$ Aggregated protein N
	mg/100 ml supernatant		mg/100 ml heated milk			% of original protein N			% of protein N present	
0	492·8	40·5	452·3	0	0	100·0	0	0	100·0	0
5·0	482·3	48·4	433·9	10·5	7·9	95·9	2·3	1·8	97·6	2·4
15·0	479·5	66·0	413·5	13·3	25·5	91·4	3·0	5·6	96·9	3·1
17·0	468·3	70·7	397·6	24·5	30·2	87·9	5·4	6·7	94·2	5·8
17·5	425·0	70·2	354·8	67·8	29·7	78·4	15·0	6·6	84·0	16·0
18·8	367·0	73·0	294·0	125·8	32·5	65·0	27·8	7·2	70·0	30·0
19·3	340·4	74·4	266·0	152·4	33·9	58·8	33·7	7·5	63·6	36·4
22·5	262·8	77·2	185·6	230·0	36·7	41·0	50·9	8·1	44·7	55·3
30·5	167·1	90·2	76·9	325·7	49·7	17·0	72·0	11·0	19·1	80·9
40·0	135·6	100·4	35·2	357·2	59·9	7·8	79·0	13·2	9·0	91·0
60·0	134·2	115·3	18·9	358·6	74·8	4·2	79·3	16·5	5·0	95·0

* Protein that was aggregated sufficiently by heat treatment to be sedimented into bottom 3 cm of tube by centrifuging for 15 min at 300 *g*.

herd bulk milk of average composition, whose mode of coagulation is usually classed in the range moderate–very good.

The most striking feature of the 6 abnormal coagulation curves (Fig. 2) is the occurrence in 5 of them, milks (7–11), of a marked reduction in the rate of coagulation

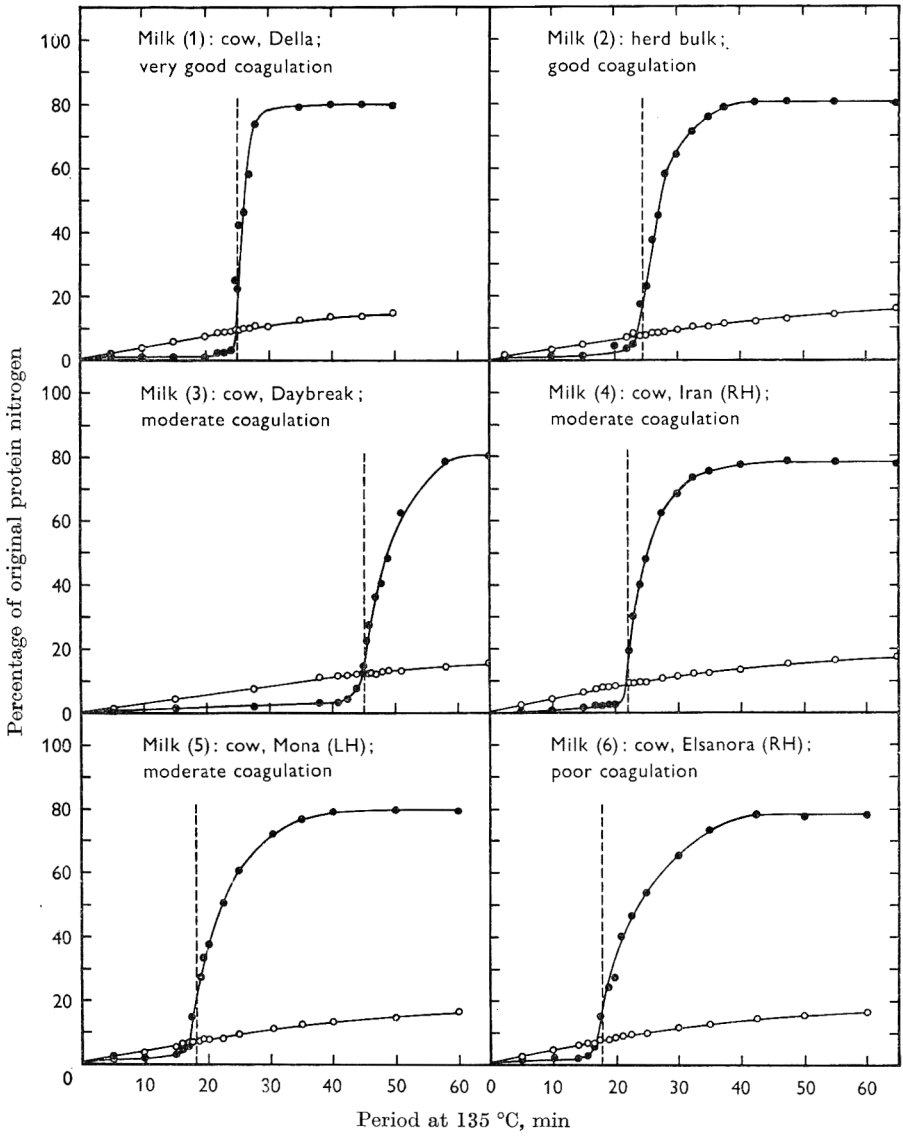


Fig. 1. Protein aggregated (●—●) and proteose-peptone plus non-protein nitrogen produced (○—○) in 6 'normal' separated milks after heating at 135 °C for various periods. The vertical broken lines indicate coagulation time, i.e. when clots were first visible.

after about 40 % of the protein originally present had coagulated. Up to this point of inflexion, coagulation proceeded quantitatively in much the same way as shown by the normal curves, except that in some instances there appeared to be slightly more aggregation before the visual detection of coagulation. Milk (12) exhibited a

different type of abnormality in that aggregation and then visible coagulation appeared to be a slow, continuous process with some aggregation during the induction period.

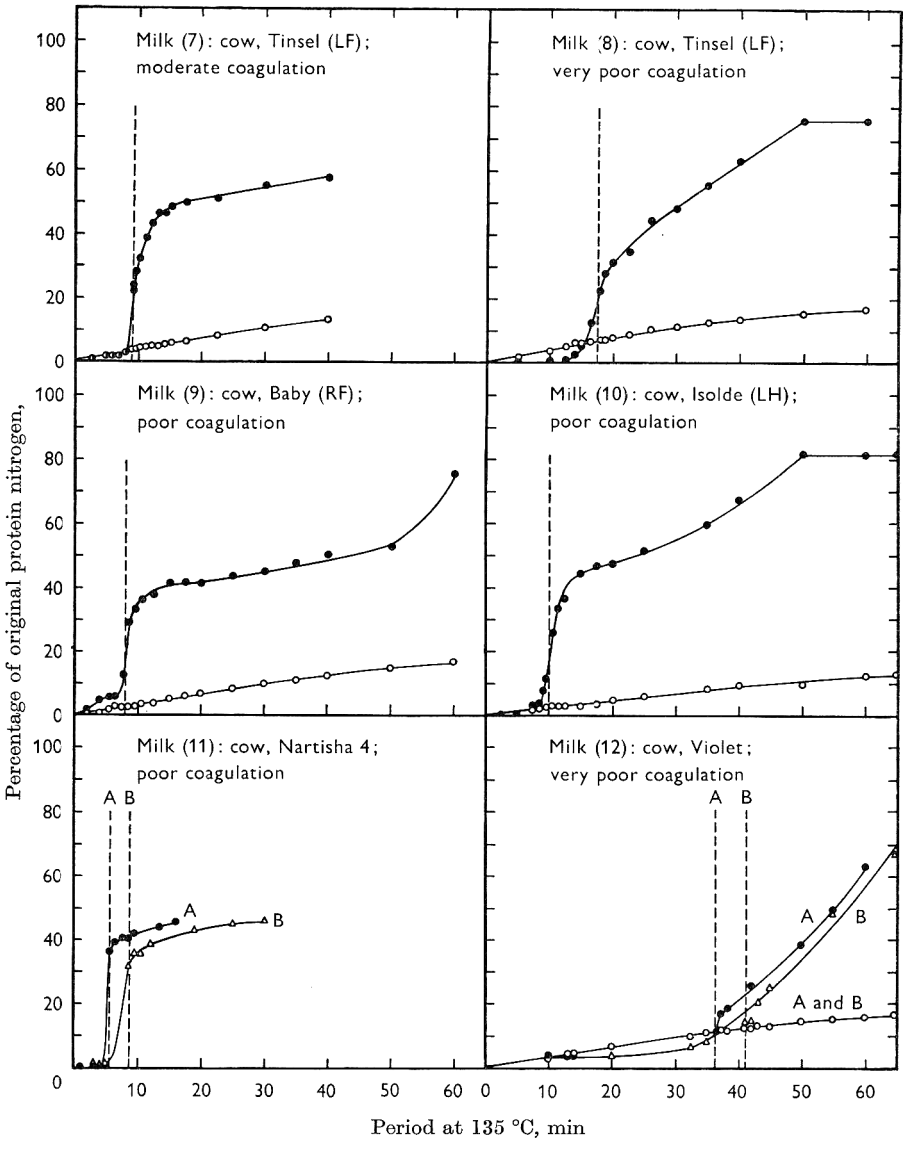


Fig. 2. Protein aggregated (●—●, △—△) and proteose-peptone plus non-protein nitrogen produced (○—○) in 6 'abnormal' separated milks after heating at 135 °C for various periods. The vertical broken lines indicate coagulation time, i.e. when clots were first visible. Samples 11 and 12 were of the type whose coagulation time increased on exposure to light and the results for protein aggregation for the first set of tubes (A) are presented separately from those of the second set of tubes (B).

All the milks giving the abnormal coagulation curves had the type of coagulation classed as 'poor' or 'very poor' except that for milk (7), which was classed as 'moderate'. Milks (7) and (8) were obtained from the same quarter of the same cow

when 15 and 64 %, respectively, through its first lactation. This cow appeared to be free of subclinical mastitis throughout the lactation period and the 2 milks were normal by the usual criteria. It would seem, therefore, that an abnormal coagulation curve can be obtained with milk from a healthy cow and may remain a characteristic of the milk throughout most of a lactation period. Milks (9–12) were of the labile type and milks (10–12), and possibly milk (9), were obtained from a quarter or quarters with subclinical mastitis. Milks (9) and (10) were known to be of the type that increased in heat stability only when exposed to light, and interference from this phenomenon was avoided by protecting these milks from light up to the moment heating started. This precaution was not taken with milks (11) and (12) and their coagulation time increased during the experimental procedure, the results obtained with the first and second set of tubes being indicated in Fig. 2 by A and B, respectively. The prognostics that a milk will give an abnormal heat coagulation curve would appear therefore to be, in order of decreasing generality, labile heat stability, poor coagulation and that it comes from a quarter or quarters with a mild to moderate degree of subclinical mastitis.

The coagulation curves in Figs. 1 and 2 were obtained after the milks had been stored in an incubator at 20 °C for about 16 h. To establish whether the same coagulation curve would be obtained after further storage, the curves for milks (4), (6) and (8) were re-determined after storage at 4° C for 1 week and for milks (6) and (8) after storage at −20 °C for 1 month. In every instance there was no significant change in coagulation time, type of coagulation or coagulation curve. Thus, for milks from healthy cows at least, the coagulation curve is a genuine characteristic of a milk.

It can be seen from Figs. 1 and 2 that heat-produced PPN + NPN (as a percentage of original protein nitrogen) increased at much the same rate in all the milks except milk (10); the corresponding results for milk (11) are not presented because proteolysis occurred in the control samples. The rate decreased only slightly as coagulation of the protein proceeded, whether this took the form indicated by the normal or by the abnormal coagulation curves. Thus, this degradation of milk protein, as well as the dephosphorylation of casein (Belec & Jenness, 1962), appears to be simply a concomitant of the processes involved in the heat coagulation of milk and is not a rate determinant.

DISCUSSION

In assessing the value of results obtained by the objective test it has to be remembered that the shape of the heat coagulation curves are governed to some extent by the particular, rather arbitrary, centrifuging treatment given the heated milks. Thus, as a means of determining how much of the original protein is coagulated after a certain period at 135 °C, the curves for different milks will have relative rather than absolute significance. Also, the test does not provide a complete differentiation of milks according to the size of the initial clots, although the mild centrifuging treatment used was found to be more efficacious in doing this than more severe centrifuging treatments. Despite these limitations, the objective test provided useful information.

The coagulation curves indicate that when separated milk is heated in a rocking

tube, coagulation is preceded by an induction period of variable duration during which the suspended protein particles (caseinate micelles with denatured serum protein) increase in size only to a very limited degree. This conclusion is supported by the results of Whitney *et al.* (1952) which, though few, were obtained by using more severe centrifuging conditions that would be expected to facilitate the detection of small increases in the size of the protein particles.

With bulk milk and milk from healthy individual cows, normal in composition and non-labile, the end of the induction period is marked by a rapid, 'explosive' coagulation of protein, in which the clots initially seen are usually relatively large and which continues, until virtually all the protein present is coagulated. The larger the initial clots the more rapidly does coagulation proceed to completion. With these normal milks, the objective test detects the onset of the rapid coagulation only 1–2 min before the visual subjective test and thus coagulation time, already shown to be capable of being determined with satisfactory precision (Davies & White, 1966*a*), is also seen to be a reasonably accurate measurement.

With milk whose coagulation time is labile or where the initially formed clots are relatively small, or where composition is slightly abnormal because of subclinical mastitis, there is generally a pronounced reduction in the rate of heat coagulation after about 40 % of the original protein has coagulated. It may be that in this 2-stage type of coagulation, the first is a premature coagulation of caseinate micelles with an abnormally large amount of adsorbed serum protein, and that the second is more akin to a normal coagulation occurring now in a 'milk' deficient in casein. Coagulation time may exceed the induction period of these abnormal milks by up to 4–5 min.

That 2 different types of coagulation curve can be obtained which are distinctive in the main of milks with a good coagulation and of milks with a poor coagulation, suggests that the mechanism of coagulation may differ in these 2 classes of milk. This supports the view expressed in Parts I and II of this investigation (Davies & White, 1966*a*, 1966*b*), that these 2 classes of milk should be examined separately when trying to relate coagulation time to some chemical or physical property of the milk. It would appear that an extension of the objective test to obtain coagulation curves corresponding to different centrifuging conditions, different temperatures and different protein concentrations should provide kinetic and thermodynamic data that would give indications of the mechanism of heat coagulation.

The authors thank Dr P. S. Blackburn for determining the differential cell counts and Miss V. D. Young for technical assistance.

REFERENCES

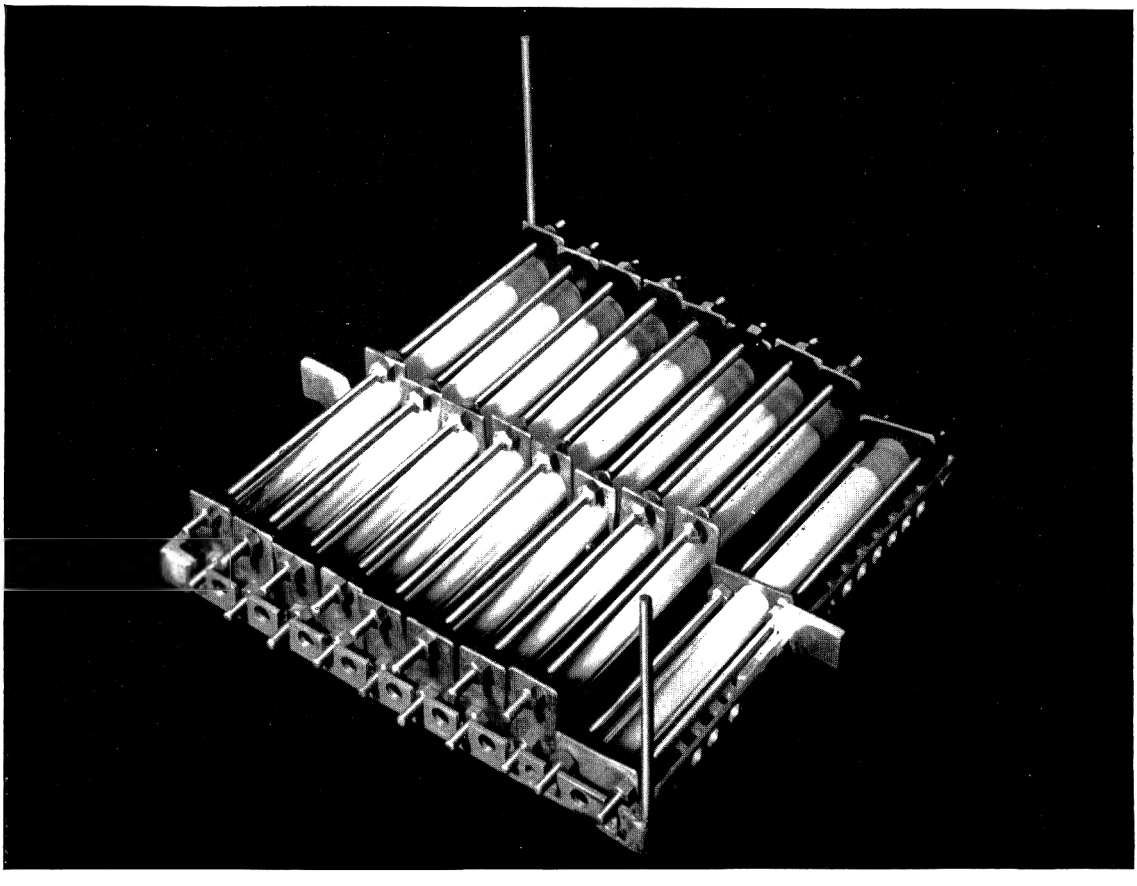
Aschaffenburg, R. & Drewry, J. (1959). *15th Int. Dairy Congr.* **3**, 1631.
Belec, J. & Jenness, R. (1962). *J. Dairy Sci.* **45**, 20.
Blackburn, P. S., Laing, C. M. & Malcolm, D. F. (1955). *J. Dairy Res.* **22**, 37.
Davies, D. T. & White, J. C. D. (1966*a*). *J. Dairy Res.* **33**, 67
Davies, D. T. & White, J. C. D. (1966*b*). *J. Dairy Res.* **33**, 83
Rowland, S. J. (1938). *J. Dairy Res.* **9**, 42.
White, J. C. D. & Davies, D. T. (1958). *J. Dairy Res.* **25**, 236.
Whitney, R. McL., Paulson, Katherine & Murthy, G. K. (1952). *J. Dairy Sci.* **35**, 937.

EXPLANATION OF PLATE

Plate 1

Carriage with sample tubes as used in objective test for determining the heat stability of separated milk.

Printed in Great Britain



Reviews of the progress of dairy science

Section F. Milk-borne disease

By J. H. McCOY

Public Health Laboratory Service, Kingston-upon-Hull, Yorks

(*Received* 1 *March* 1965)

CONTENTS

INTRODUCTION

Since the completion of the area eradication plan for bovine tuberculosis in 1960 (1) milk in Great Britain is no longer the vehicle of transmission to man of bovine tuberculosis, for many years the most serious and most widespread food-borne infection. Savage (2) in 1912 regarded the 'lamentable want of even ordinary care' with which milk was treated as responsible for 'an immense deal of sickness, ill health and death'. Wilson (3) in 1942 regarded milk as one of the most dangerous articles of diet, responsible for transmitting pathogenic bacteria derived not only from the udders of diseased cattle, but from the human personnel engaged in milk handling and from contaminated water used for washing utensils. The number of human deaths from non-pulmonary bovine tuberculosis in England and Wales and Scotland was estimated at 2612 in 1931 (4), 2007 in 1937 (3), and 1581 in 1944 (5), due almost wholly to the consumption of raw infected milk. There are now in Great Britain 'almost certainly more deaths from salmonella infections (excluding enteric fevers) than from bovine tuberculosis, and many hundred times as many infections' (6). Twenty-seven fatal cases of salmonella infection were recorded and 13104 persons were known to have been infected in 1963 (7).

The factors which, over the last 50 years, have brought about this change in the status of milk as a vehicle of human infection will first be discussed. Two general principles, cleanliness and safety, underlie any such discussion. The terms are not synonymous. A clean milk is not necessarily safe: a safe milk is not necessarily clean. Clean milk implies that the milk is produced with the minimum of added bacterial contamination, and is transported, processed and delivered under conditions which neither permit the multiplication of organisms already present, nor add to their number. The purpose of clean milk production is to ensure that the product when delivered to the consumer will remain fresh under domestic conditions of storage for at least 24 h. Safe milk implies that the milk is free from pathogenic organisms. Clean milk is achieved by the application of general hygienic principles to the production, processing and distribution of milk: safe milk is achieved in part by cleanliness, in part by heat treatment, in part by the eradication of bovine disease. This distinction is of the utmost importance. Clean production, processing and distribution may prevent contamination of milk with organisms of human or animal origin, but can have no effect on micro-organisms already present when the milk is drawn from a diseased cow. Heat treatment destroys organisms in milk at the time of processing but neglect of cleanliness may lead to post-treatment contamination with raw milk or polluted water. Only one milk-transmitted bovine disease of man, bovine tuberculosis, has been eradicated in Great Britain. Brucella, salmonella (7) and numerous other infections of dairy cattle are still transmitted to man through raw milk.

Apart from specific measures taken to produce clean safe milk the influence of general improvements in environmental conditions in reducing human disease cannot be disregarded. The disappearance of summer diarrhoea, an infective disease of infants mainly under 2 years of age, epidemic in urban districts in the summer months and undoubtedly conveyed by food and milk, cannot be attributed to any specific factor, but rather to a multitude of factors. The last major epidemic of this disease occurred in 1921. Its decline was attributed (3) to: largely, improvements in the cleanliness of the milk supply; a partial replacement of loose by bottled milk; the substitution of heated milk for infant feeding; and the virtual abolition of flies in large towns by the supplanting of horse by motor traffic. Flies did not bring infection from where they bred but acted as carriers when they came into contact with infected matter (8), transmitting infection within and between households.

The relative emphasis on cleanliness and safety has altered with the years. In 1912 (2) efforts were being directed towards a 'pure' supply of milk; in 1942 (3) towards a safe supply.

In retrospect, the main measures which have contributed to the safety of the milk supply in Great Britain would appear to have been in roughly chronological order: the provision of unpolluted water supplies; changes in the production and distribution of milk; the pasteurization of milk; the development of bacteriological methods for the control of water supplies; the cleanliness of milk; and the adequacy of heat treatment.

WATER SUPPLY AND SEWAGE DISPOSAL

The nineteenth century in England and Wales was characterized by a vast expansion of population, which increased more than $3\frac{1}{2}$ times to approximately 37 millions. The distribution of population, which at the beginning of the century was predominantly rural (71 %), began to alter soon after the middle of the century owing to the great improvement in roads and railways, so that by the end of the century almost half the population lived in the larger towns and fewer than one quarter in rural districts (9). In many towns at the beginning of the century water supplies were derived almost entirely from wells, many of them polluted. It was common practice to draw water supplies from rivers without adequate purification. Inadequate water supplies rendered any satisfactory approach to the problem of drainage impossible. In urban areas most rivers were little better than elongated cesspools (10). By the end of the century, however, the evils of rapid urban growth, inadequate and polluted water supplies, inefficient or absent sewage disposal, and gross overcrowding, which together had rendered possible the epidemics of cholera, typhoid, and typhus fevers earlier in the century, had been largely mitigated, so that by the beginning of the twentieth century the town dweller in Britain had been given safe water and an efficient sewage disposal system, and therefore enjoyed relative freedom from water-borne disease.

A measure of the progress attained in the provision of unpolluted water supplies is afforded by the reduction in deaths resulting from cholera and typhoid fevers during the nineteenth century. Cholera is not an indigenous infection. The great epidemics in England and Wales followed its importation from abroad. The second and largest epidemic following its importation in 1849 caused more than 53 000 deaths in England and Wales (11): the fifth and last importation in 1893, 135 deaths (12). Typhoid fever, an indigenous disease, declined more slowly. In the 1870's, typhoid fever annually accounted for roughly 9000 deaths: by 1917 for less than 1000 (11).

The main methods of purifying sewage-polluted waters to potable standards are storage, filtration and sterilization. The first successful slow sand filter was constructed at Paisley in 1804 (13). Chlorine, in the form of chloride of lime, was first used for the sterilization of water mains in the Maidstone water-borne typhoid epidemic of 1897 (14). The first use of chlorine as sodium hypochlorite for the direct sterilization of water itself was in the Lincoln water-borne typhoid epidemic of 1904 (14). These, however, were isolated cases and chlorination was not generally adopted until the 1920's. The Croydon water-borne typhoid outbreak, which occurred in 1937, led to the routine sterilization of all major water supplies. As a result of these measures, indigenous cases of typhoid fever in England and Wales had, in the late 1950's, been reduced to rather less than 100 cases annually (15), a high proportion of which was in visitors and holiday makers returning from abroad.

FARM WATER SUPPLIES

The provision of unpolluted water supplies, however, was confined mainly to urban areas able to afford the capital outlay. Many supplies to villages and small communities remained untreated. Farm water supplies were often derived from unsatisfactory sources. Savage (2), in 1912, commented that frequently the only drinking water for

cows was obtained from ponds, ditches and streams 'not uncommonly of very doubtful purity', while water for washing milk vessels, although frequently of good quality, was 'far from rarely derived from wells of very doubtful purity and sometimes of certain impurity'. Forty years later little change was noted in farm water supplies. More than half the milk-producing farms in England and Wales were dependent on private water supplies, of which approximately 90 % were derived from shallow wells and springs, relatively few of which were satisfactory owing to deterioration, bad siting, and the absence of adequate protection (16). The Milk and Dairies Regulations (1949) (17) however, setting out conditions for production and handling of milk, required the provision of a suitable and sufficient water supply as a condition of registration. This ensured that at the time of licensing or registration the water supply was suitable for milk production. Suitability of a supply was assessed on a topographical survey of the sources of pollution of the supply, plus laboratory examination of a series of samples (18).

PRODUCTION AND DISTRIBUTION OF MILK

Before railways, the liquid-milk market could be supplied from within only a very small radius and stall-fed cattle were housed within the larger towns. More distant dairying areas made butter and cheese, but the distinction between liquid-milk producing and manufacturing districts disappeared with rail transport and the development of wholesaling. In 1861 London received supplies of almost 22 million gal from stall-fed cows, 16000 within the Metropolitan area, and a further 6000 in outlying parishes, whilst only one million gal arrived by rail; but by 1891 milk from town sheds in the Metropolis accounted for only 15 % of London's supplies, while 40 million gal 83 %, of the total supply, arrived by rail from districts up to 150 miles away.

Producer retailers, however, long survived near towns and in 1918 still supplied more than a quarter of retail milk. By 1936 the proportion of liquid milk consumed sold by producer retailers had fallen to one-sixth (19), and by the 1960's to rather less than one-twentieth (20). In 1964 there were still over 10000 producer retailers in England and Wales (20).

Technical progress in treatment and transport has now reached a stage in which milk can be carried without appreciable deterioration from almost any producing area to any market in the country.

The change from town milk to milk from distant areas had profound effects on the public health. With cowsheds near to the distribution areas, there was little delay in distribution, and consequently little multiplication of bacteria between collection and distribution. In addition, there was little mixing of milk from different sheds. Milk from distant areas, however, was not distributed for some considerable time after collection and was transported under bad conditions. (2) Town milk in general was exposed to contamination only during production and on delivery to the consumer. Distant milk was exposed to additional contamination during transit and during distribution to the retailer. Bulking of consignments from different consumers greatly increased the infectivity of milk. The impaired keeping quality of distant milk probably accounted for the early introduction of pasteurization for London's milk supplies.

PASTEURIZATION OF MILK

Pasteurization, devised by Pasteur in the 1860's to prevent the souring of wine and beer, was soon found to retard the souring of milk, and was gradually introduced into the larger towns of Europe and America for this purpose (3).

In London, pasteurization in a crude form began to be practised about 1903, and by 1911 about 20 % of the milk sold was heat-treated in some way or another. Pasteurization under licence did not begin until after the introduction of the Milk (Special Designations) Order (1922), but by then a considerable proportion of London's milk was already being heat-treated. By 1930 the proportion of milk heat-treated in London had risen to 90 %, and by 1939 to about 98 %. In the county boroughs the corresponding figures for 1930 and 1939 were approximately 35 and 60 % (21).

Up to 1941, the holder method was the only official method of pasteurization in England and Wales. Pasteurization consisted in retaining the milk at a temperature of not less than 62·8 °C and not more than 65·6 °C for at least 30 min, followed by its immediate cooling to at least 12·8 °C. The process was controlled by indicating and recording thermometers and temperature records were to be preserved for at least one month.

In 1941 the use of the High Temperature Short Time (HTST) method of pasteurization was introduced in order to encourage the heat-treatment of milk and so prevent a repetition of the great losses through souring that occurred during the hot summer of 1940. The Regulations (22) provided that the milk be held at a temperature of not less than 72·2 °C for at least 15 sec, that the apparatus be thermostatically controlled, and that it be provided with an automatic device to divert the flow of any milk not so treated. These provisions led to a further increase in the heat-treatment of milk, mainly in the larger centres of population, so that by 1955 the proportion of milk pasteurized in England and Wales had risen to 88 % (23).

Unfortunately, in the 1949 Regulations the temperature for the HTST process was lowered by 1 degF to 161°, i.e. 71·7 °C. For purposes of safety this temperature is on the low side for the destruction of *Rickettsia burnetii*; the organism that causes Q fever in man, and cannot be relied upon completely for destroying this organism when it is present in large numbers in the milk (24). Otherwise, pasteurized milk can be regarded as perfectly safe.

The effect of pasteurization in reducing the incidence of bovine tuberculosis is most effectively shown by the fall in death-rates from abdominal tuberculosis in children under the age of five (21) (23).

Table 1 shows that in London, where by 1944 about 98 % of milk was pasteurized, milk-borne tuberculosis had fallen to a twenty-third of what it was in 1921; in the rural districts, on the other hand, where the proportion of pasteurized milk consumed was far less, it had fallen to only a quarter.

Indirectly the table also demonstrates the effect of bulking in increasing the infectivity of milk supplies. Though the figures are not accurately known, in 1921 in London the amount of pasteurized milk sold probably represented about 50 % of the total, and in rural districts very much less, while comparatively little tuberculin tested (TT) milk was sold in either area (23). The higher proportion of heat-treated

milk sold in London in 1921 probably accounts for the death rate for London at that time being lower than in the rural districts in spite of the much greater degree of infectivity of the milk coming in to London resulting from bulking.

Table 1. *Deaths from abdominal tuberculosis per million children under 5 years of age living in each area*

	1921	1930	1938	1944	1953
London administrative county	136	24	12	6	4
Combined county boroughs	437	157	63	35	1
Combined urban districts	366	134	77	42	1
Combined rural districts	252	92	63	60	12

LABORATORY CONTROL OF WATER AND MILK SUPPLIES

Technical advances in the provision of safe water, and of clean and safe milk, hastened the demand for bacteriological and chemical techniques to assess the adequacy of the processes employed. Chemical techniques for the control of the purity of water supplies, whilst adequate for the detection of gross to moderate pollution, were found to be several hundred times less sensitive than bacteriological methods in detecting pollution.

It soon became apparent that for the control of water and milk supplies it was of greater importance to examine numerous samples by simple tests than occasional samples by more complicated tests. As pasteurization of milk became more common, the need for a test to demonstrate that in fact the process had been adequately carried out became urgent. In 1927, for example, in Montreal over 5000 cases of typhoid fever followed the consumption of milk thought to have been pasteurized. A considerable portion of the milk was found to have been distributed without pasteurization (25), and the supervisor of the plant was found to be a typhoid carrier; one of the employees developed typhoid fever whilst working on the plant (26). An outbreak of paratyphoid fever in 1932 in Ontario resulted from raw milk by-passing the heating unit of a pasteurizing plant through a common outlet for the raw and pasteurized milk tanks (27). Finally, an outbreak of scarlet fever in Glasgow in 1931 was traced to pasteurized milk produced in an unlicensed plant, in which the heating was inadequate owing to a broken valve (28).

Chemical methods developed on the Continent for the control of high-temperature pasteurization proved unsuitable for the control of the holder process used in Great Britain.

In the mid 1930's simple, reproducible, bacteriological tests became available for the control of the cleanliness of water and milk supplies. At the same time, a chemical test was developed for the control of pasteurization.

Laboratory control of water supplies

Bacteriological examination. Existing methods for the bacteriological examination of water supplies were codified in 1934 (29). Methods were described for estimating the numbers of presumptive coliform organisms and of *Escherichia coli* in water, by tube dilution tests in MacConkey's lactose–bile salt–peptone broth. Standards were suggested for non-chlorinated piped supplies and for chlorinated supplies. The use of

these unified techniques rendered comparable both the results obtained by workers in different parts of the country and the interpretations of these results. The methods described, with slight modifications, remain in use in Great Britain today.

Owing to the variability in 2 of the main constituents—bile salt and peptone—of MacConkey broth, its replacement by a chemically defined medium has long been considered desirable so that variation in results within and between laboratories might be minimized. Folpmers (30) used a glucose–glutamic acid medium incubated under anaerobic conditions for the routine examination of water samples in Amsterdam. The method as described had too many disadvantages to be practicable for the routine examination of large numbers of samples. Burman & Oliver (31) devised a modification which could be used aerobically in the same way as MacConkey broth. Comparison of the modified medium with MacConkey broth showed that, while the glutamic acid media produced slightly higher isolations of *Esch. coli*, results were slow to appear, and the total number of presumptive positive tubes was reduced. Gray (32) later introduced a simple formate–lactose–glutamate medium which improved the recovery of true coliform organisms with equal speed and with fewer false positive reactions than MacConkey broth, but which had the disadvantage that a proportion of minimally positive tubes could not be recognized easily. In addition, the medium proved unsuitable for the 44 °C test.

The medium of Burman & Oliver (31) has been modified (33) to ensure earlier detection of acid or gas. Gray (34) has increased the nutrient properties of his medium to ensure greater production of gas.

Membrane filtration in water examination. The value of membrane filtration in water examination is that results can be obtained in a shorter time than by standard methods, with marked reductions in the amounts of culture media and glassware required. Taylor & Burman (35) have described the techniques in current use at the Metropolitan Water Board laboratories, where some 50000 water samples are examined annually.

Frequency of sampling. The control of the hygienic quality of water supplies is based on the frequent and regular bacteriological examination of water entering the distribution system and in the distribution system. Recommendations (36) (37) have recently been made regarding the frequency of examination of raw and treated water supplies in relation to the size of the population served.

In routine control of the safety of water supplies it is, of course, more important to examine numerous samples by a simple test than occasional samples by more complicated tests or series of tests. The frequencies recommended are minimum frequencies for routine bacteriological examination.

Laboratory control of milk supplies

Bacteriological examination. The report of a critical inquiry into the validity of the methods available for the bacteriological grading of milk was published in 1935 (38). The report provided experimental data for conclusions as to the relative value and precision of procedures then current, the plate count and the coliform test, in the bacteriological grading of milk. The plate count was shown to be an inaccurate and unreliable method of ascertaining the number of organisms in milk. The final result, even with a standard technique, was correct only within wide limits, $\pm 90\%$, and

bore no constant relationship to the total number of bacteria, either alive or dead, in the sample. Also, the correlation between the plate count and the keeping quality of the milk was shown to be comparatively low, as was the correlation between the plate count and the sanitary conditions of production.

The coliform count, which in this country affords the best and most delicate index of excretal pollution in the analysis of water, was shown when applied to milk, in which excretal organisms multiply, to afford only a very imperfect and often entirely misleading index of the extent of the original contamination. The correlation between the coliform count and the keeping quality of the milk was shown to be comparatively poor. The best test for the bacteriological grading of milk was considered to be a modification of the methylene blue reduction test (39) most widely used at that time.

The modified methylene blue test described differed from the plate count in being simple and inexpensive, and having a very small experimental error; moreover it could be carried out by relatively unskilled workers on large numbers of samples. The test required a minimum of equipment, classified milk on the basis of cleanliness into the maximum number of grades desirable, and on the whole afforded a very good index of the keeping quality of the milk. The test did not appear to be seriously affected by the degree of clumping of the bacteria in the milk as was the plate count. The Report finally suggested that, by the use of the modified methylene blue reduction test, it should be possible to examine the milk of every farmer at weekly or fortnightly intervals throughout the year at a cost only a fraction of that of the plate count.

Control of pasteurization. The enzyme phosphatase is inactivated in milk by a temperature of 62·8 °C applied for 30 min. At this temperature tubercle bacilli are killed in 5 min (40). The modern control of pasteurization dates from 1935, when a test for the presence of phosphatase was published (41).

The test was based on the ability of phosphatase to split off inorganic phosphate from disodium phenyl-phosphate. The liberated phenol was measured colorimetrically and the quantity of phosphatase calculated in arbitrary units. Since the regulations for holder pasteurization demanded that milk be held at 62·8–65·6 °C for at least 30 min, a wide margin of safety was provided. Milk exposed to 61·9 °C for 30 min or to 62·8 °C for only 20 min still contains detectable quantities of phosphatase, so that underheating or underholding could be detected.

The test suffered from some disadvantages. It demanded exact standardization of all reagents, a high degree of technical skill in its performance, and the complete absence of phenol from the environment in which the test was carried out. Nevertheless, the efficiency of pasteurization could now be controlled, and underheating, underholding, and post-pasteurization contamination with raw milk of over 0·2% detected.

In the current Milk (Special Designation) Regulations, 1963, the original Kay–Graham phosphatase test (41) has been replaced by a simpler and more rapid test introduced by Ascheffenburg & Mullen (42).

Control of sterilized milk. The application of the phosphatase test to sterilized milk proved of no value except to detect contamination with raw milk, as the blue colour in both the unincubated controls and the tests proper tended to exceed those normally

encountered with pasteurized milk. A simple turbidity test for the adequacy of sterilization was developed in 1947 (43) based on the complete denaturation of soluble protein by the heat applied.

MILK PRODUCERS (20) AND MILK CONSUMPTION

The number of registered milk producers in England and Wales has continued to decline from the peak number of 162000 in 1950 to 110000 in 1963 and to 105000 in 1964. The number of producer retailers in England and Wales remained level in 1963 and 1964 at approximately 10500.

In 1963–64 liquid sales of milk off farms to consumers in England and Wales amounted to 1452 million gal; 67 million gal (4·6 %) from producer retailers, 1385 million gal from wholesale producers via depots and processing dairies. In June 1963 only 20 % of producer-retailers' milk deliveries were heat-treated, compared with 98·8 % of milk deliveries of other distributors.

Estimated liquid consumption per head in England and Wales remained almost static at 4·9 pints per head per week from 1961 to 1964, excluding the farm population who obtain their milk from supplies retained on farms.

From the figures already given the numbers of the population consuming raw milk can be calculated. The farm population supplied direct is estimated at 0·7 million, the population supplied by producer-retailers at 1·7 millions, the population supplied from other distributors at 0·5 million. The total number of persons consuming raw milk in England and Wales is estimated therefore at approximately 2·9 million, roughly 6·3 % of the population. This figure may be an underestimate, as no figures are available for the proportion of fresh cream consumed raw, or of the consumption of raw milk by holiday-makers on farms.

Irrigation

Water sources in the United Kingdom are limited and the need for more effective conservation and use becomes daily more pressing. (44)

The intensive practice of irrigating pasture with town sewage effluent followed by direct grazing with dairy or beef cattle is, however, open to the serious objections that it may result in contamination of milk by human pathogenic organism and infestation of cattle with the beef tapeworm.

Sewage effluent differs from crude sewage only quantitatively in its content of organisms, and contains all the human pathogenic organisms excreted via the intestinal tract. Of these the most important are the organisms of typhoid and paratyphoid fevers, purely human pathogens incapable of causing disease in animals. The risk of contamination of milk supplies arises indirectly, through the risk of contaminating the surfaces of cattle by spray drift, or by contact with recently sprayed pasture.

The survival of typhoid and paratyphoid bacilli on grass irrigated with crude sewage has been studied (45 *a*, *b*). This crude sewage used in Hamburg contained typhoid–paratyphoid bacilli in 90 % of 1-ml samples examined. After spray irrigation one-third of 204 samples of irrigated grass were positive for salmonella—*S. bareilly*, *S. oranienburg*, *S. paratyphi B* and *S. typhi* being isolated. The percentage

of samples containing salmonellae remained unchanged for 3 weeks and then declined, so that by 6 weeks only 5 % of samples were contaminated. The range of the spray was 25 m. Salmonellae were isolated from all samples collected within this area, and from 75 % of samples collected within 50 m of the spray.

Milk forms an excellent medium for the growth of human intestinal pathogens and contamination of milk from muddy cows during milking has caused outbreaks of paratyphoid and typhoid infection amongst consumers. Paratyphoid fever occurred at Wootton, Isle of Wight, in 1946 (46) in consumers of milk from the same small dairy. The chain of infection was traced from 2 carriers in a distant house by way of a cesspool and stream to the mud of a field at the dairy farm in which the milch cattle grazed. Salmonellae of the the same phage type (*S. paratyphi B*) were isolated from the carrier, the cesspool, the stream, and from the muddy pasture.

It is now considered that the infection of the milk in the Bournemouth, Poole and Christchurch typhoid epidemic of 1936 (47) was caused in the same way.

The usual sewage-treatment processes—sedimentation, biological filtration and sludge digestion—have been shown to be ineffective in destroying ova of the beef tapeworm *Taenia saginata* harboured by humans. Even sand filtration of the final effluent cannot be relied on to produce effluent free from viable ova. In addition, viable ova have been found after 6 months digestion in sewage sludge (48). On grass, especially in the summer, desiccation is the most important factor in limiting the viability of ova. In one experiment, the mean survival time of a fairly large number of eggs was 23 weeks. In no case was the maximum life of the ova on grass determined (49).

Irrigation for grass production requires the application of water at intervals only days apart. Under these conditions, irrigation with sewage effluent would presumably ensure permanent contamination of the pasture.

CLEAN MILK PRODUCTION

Though the means of achieving completely satisfactory results from chemical cleaning and sterilizing on dairy farms are readily available, the present situation is still considered unsatisfactory mainly owing to failure to appreciate the basic requirements for chemical cleaning and sterilizing and to misplaced emphasis on sterilizing as opposed to cleaning (50). The basic requirements are defined as the regular application of suitable chemicals to milking equipment so as to leave it free from residues and bacteria. To attain these requirements the sequence of operations generally employed in the United Kingdom after each milking is: pre-sterilization rinsing of plant; hot detergent sterilizer wash; final rinse to remove detergent/sterilizer solution. The main factor limiting the efficiency of cleaning is the lack of adequate supplies of water.

BOVINE DISEASE

Tuberculosis

Since the completion of the area eradication plan for bovine tuberculosis in 1960, routine herd tests have continued at an annual or biennial interval (51). The incidence of reactors amongst home-bred cattle remained at 0·16 % in 1961, but fell to 0·11 %

in 1962. These figures included 330 reactors in 1961, and 62 in 1962, from consignments from the Irish Republic of 'once tested' store cattle. Reactors and dangerous contacts were slaughtered compulsorily. Twenty cattle in 1961 and 10 cattle in 1962 were slaughtered under the Tuberculosis Order of 1938 which provided for the notification and slaughter of cattle found to be affected with certain forms of tuberculosis. Fourteen of these cattle were found to be affected with advanced tuberculosis in 1961 and 4 in 1962; 4 of the cattle were affected with non-advanced tuberculosis and 2 were not affected in each of the 2 years.

Veterinary examination of dairy herds under the Milk and Dairies Regulations revealed 12 cows affected with tuberculosis in 1961 and 4 in 1962. Six notifications that tubercle bacilli had been found in bulk milk samples by biological examination were received from local authorities in 1961, 5 in 1962. Veterinary examination of the herds revealed one affected cow in each of two herds in 1961, one affected cow in one herd in 1962. In the remaining herds no affected animal was found.

Legislation. As many of the provisions contained in current Orders are now obsolete owing to the progress made in clearing tuberculosis from herds throughout the country, consolidated Orders revoking and re-enacting, with certain amendments, the existing Orders dealing with the control of bovine tuberculosis came into force in August 1964 (52). Provisions in existing Orders relating to the tuberculin testing of cattle and to the disposal of reactors necessary to maintain the tuberculosis-free status of the national herd are continued in force.

Eradication of bovine tuberculosis in the Irish Republic. This programme was introduced in 1954. By the end of 1962 15 out of 26 counties were declared attested and it was agreed that exports of 'once tested' store cattle should cease as from 31 December. Fat or store cattle imported into Great Britain from the Irish Republic are now of attested status or 'uncertificated', i.e. fat cattle without any status relative to tuberculosis imported for immediate slaughter.

The biological testing of milk. Although bovine tuberculosis in England and Wales has been largely eliminated by eradication and although over 96 % of retail milk has been subjected to heat-treatment, the biological examination of milk continues. The tests are expensive and use time and laboratory facilities which could possibly be better employed elsewhere. In 1961–63, 11 samples out of 47 680 (0·023 %) examined by 49 Public Health Laboratory Service laboratories were found to be positive for tuberculosis (53). This percentage (0·023) compares favourably with the figure of 0·5 % reported in 1953 (54) and with figures of up to 10 % common before 1940 (3).

It is suggested, therefore, that biological testing for tuberculosis be confined to the herds of producer retailers who sell raw milk, the frequency of examination depending on the volume of raw milk sold. For the largest producers 4 samples annually are considered reasonable, for the smaller producers 1 or 2 samples.

Some comment on the failure to find affected animals in herds whose milk on biological examination is shown to contain tubercle bacilli seems appropriate. Well-known reasons are the disposal of animals after sampling and before the results of biological examination are available. The test takes 6–10 weeks for completion and in the interval the affected animal may have been disposed of either because it has been dried off or for other reasons. Another well-known cause is the receipt of accommodation milk which may not necessarily have been disclosed at the time of

sampling on the farm, or which could not have been ascertained on churn sampling at a dairy or depot. Many samples for biological examination are, for convenience, collected from churn milk from producers on arrival at a dairy or depot. Mistakes in the identity of such samples have also been known to occur. It would seem essential that all samples for biological examination should be collected on the farm at the time of milking and that a note of the number of cows in milk and the number dry should be made at the time of sampling.

Mastitis

In Great Britain, mastitis remains the major source of economic loss to the dairy farmer. The incidence of clinical mastitis in 1957–58 was estimated at 10 % of the cows/annum (55), of subclinical mastitis 25 % (56). In terms of national milk yield this means an annual loss of milk valued at £5·6 million (57). To this figure must be added the cost of treatment and the cost of replacing chronically infected cows.

Staph. aureus is now the major pathogen in mastitis, both clinical and subclinical. *Str. agalactiae*, which was isolated from 40 % of all clinical cases in a survey in 1942, is now found in only 4 % (58). It has been suggested that the reason for this change in incidence is the result of the increased use of antibiotics, but it has been shown that the main reason for the increase in staphylococcal infection is the change from hand to machine milking (59).

Staphylococci are able to colonize the intact skin of the udder and teats and multiply there (60), and to be readily transferred from cow to cow on contaminated teat clusters (59). Despite the preponderance of *Staph. aureus* in bovine mastitis, outbreaks of human food poisoning of the staphylococcal toxin type from the consumption of milk or milk products are rare. The factors inhibiting the growth of staphylococci in raw milk have been shown to be the comparatively low temperature at which milk is normally kept, the frequent presence of bacteria other than staphylococci, and the presence of a heat-labile clumping factor in raw milk (61). In addition to these factors, the dilution of milk from an infected cow by milk from normal cows in the herd may dilute the toxin to below the active level (62).

Cheese has rarely acted as a vehicle in food poisoning owing mainly to the suppression of organisms by the acidity developing during manufacture. Modern techniques of cheese-making depend on the use of pure cultures of lactic acid bacteria as 'starters' which allow a greater control over the development of acidity throughout the whole of the process. Normally the development of acidity may be relied on to prevent the growth of staphylococci which may be present in the milk. Failure of the starter culture, due either to bacteriophage or to the presence of antibiotics in the milk, results in cheese lacking the taste, body and texture of the normal product. Such cheese is classed as second-grade cheese and for many years has been imported for processing with normal cheese without incident. In the absence of acidity, staphylococci present in the milk are able to grow in the curd and persist in the cheese.

Outbreaks of food poisoning in 2 hospitals in the London area in 1961 were attributed to the consumption of New Zealand second grade cheese intended for manufacturing purposes only (63). Samples of the suspected cheese showed an average count/g of approximately 200 million coagulase-positive staphylococci of phage

type 42D. 130 tons of the consignment were held up for examination. Later brands with *Staph. aureus* counts of less than 50000/g were released for processing unconditionally.

Later, after feeding experiments in which 1–2 oz quantities of cheese containing staphylococci ranging from 2·5 to 60 millions/g were eaten without incident by 40 persons, cheese containing up to 200 million staphylococci/g was released for processing after dilution to 5% with normal cheese. Cheese containing staphylococci in excess of 200 million/g was considered unfit for human consumption.

The strains of *Staph. aureus* isolated from the cheese were resistant to penicillin. Statements from some of the exporters indicated that the milk used for cheese making had not been pasteurized. The presence of penicillin-resistant staphylococci in such large numbers in cheese 9–12 months after production suggested that penicillin in the original milk, from the widespread use of this antibiotic in New Zealand to control mastitis, was the reason for starter failure, which had resulted in the classification of the cheese as grade 2.

After this outbreak, compulsory pasteurization of milk for cheese-making (161 °F for 15 sec) was introduced in New Zealand. Since then bacteriological examination of second-grade cheese has shown the absence of coagulase-positive staphylococci or their presence only in very low numbers.

Antibiotics in agriculture. In addition to their use in human medicine, antibiotics are used in veterinary medicine and in agriculture for animal-feed supplements, crop protection, and for food preservation (64, 65). In veterinary medicine, as in human medicine, the widespread use of antibiotics has been followed by the emergence of resistant strains of pathogenic bacteria which now cause a considerable proportion of the disease previously caused by sensitive strains. There is, however, no evidence that resistant strains are more or less virulent than their sensitive counterparts. The presence, however, of antibiotics in human foods, even in small quantities, is of greater potential hazard through the risk of inducing antibiotic sensitivity, or of producing allergic or even anaphylactic reactions in persons already sensitized. On commercial grounds the presence of antibiotics in milk for manufacture into cheese or yogurt is undesirable, because destruction of the starter cultures used may ruin production, or at best result in an inferior product.

Ten per cent of farm milks in England and Wales in 1961 were shown to contain penicillin in quantities greater than 0·01 i.u./ml (66). Tramer (67) points out that the findings of the survey mean, in effect, that were all the milk produced in this country pooled, the average concentration of penicillin would be 0·02 i.u./ml, a concentration quite unsuitable for making yogurt. In bulk tanker supplies received in London concentrations of up to 0·04 i.u./ml are not uncommon.

Estimation of antibiotics in milk. The tests used depend on the inhibition of an antibiotic-sensitive test organism. The indicator organism may be added to the milk (68): filter-paper disks dipped in milk may be placed on a nutrient agar plate containing the organism (69); or on a non-nutrient agar plate containing spores (70).

The identity of penicillin in milk can be confirmed by removal of the inhibitory effect by the addition of penicillinase. In the National Survey of 1961, 482 samples, (1·1%), were shown to contain inhibitors other than penicillin. Of the strains of staphylococci isolated from bovine mastitis 70% are now resistant to penicillin (51),

and the use of antibiotics other than penicillin for the treatment of this disease is increasing. Concern has been expressed that substances not used for treatment which might contaminate milk might produce inhibition of test plates. The Ministry of Agriculture (71) has indicated that gross contamination of milk with hypochlorites, quaternary ammonium compounds and iodophors would be necessary for a positive test result to be obtained. It is considered that rinse-water residues containing these substances in the concentrations normally used are unlikely to push into the 'fail' category milk which is not there already from other causes.

Brucellosis

Human infection. Ninety-seven cases were diagnosed by Public Health Laboratory Service laboratories in 1962, 136 in 1963 (72). A case of human infection with the British strain of *Brucella melitensis* (*Brucella abortus* type 5) was recorded (73) in a schoolboy of 12, who had consumed raw milk. The illness, of 5-months duration, was typical of undulant fever. The organism was isolated by clot culture and blood culture. The household normally received pasteurized milk from a wholesale dairy, but the roundsman, a producer-retailer with a small herd, occasionally delivered his own raw milk instead of pasteurized milk. *Brucella abortus* type 5 had been isolated by direct culture from a bulk sample from this herd one month before the onset of the patient's illness. Twelve out of 17 cows in the herd reacted to the milk ring test (M.R.T.) and 6 of them were found to be excreting the organism. Three of the infected animals were still excreting the organism a year later. This case was of particular interest, since it showed for the first time that the British melitensis strain was pathogenic for human beings.

The difficulties experienced in controlling infected milk by pasteurization where brucella infection has been shown to exist in a herd have been discussed (74). It is considered that control of infected milk by a pasteurization order, applied in strict accordance with Ministry of Health criteria (75), is inadequate to prevent the sale of contaminated milk.

Brucellosis in dairy cattle. The first official survey of the incidence of brucellosis in the dairy cattle of Britain was carried out in 1960/61 (76), on a random sample of herds (2262 herds containing 76453 cows). The purpose of the survey was to estimate the national and regional incidence of herd infection, the relation of uterine to milk infection in the same animal, the effect of the use of strain 19 vaccine, and the economic loss resulting from infection.

The relatively high proportion of British cattle that had been vaccinated as adults (7 %) precluded the use of serum agglutination tests for diagnosis. Instead, each can of milk produced on survey farms was examined by M.R.T. on 3 occasions at specified intervals during the survey year. When the M.R.T. on any can sample was positive, all the can samples from the farm or herd were tested biologically. If any biological test was positive, or if evidence of infection was obtained from a placenta, the milk from each cow in the herd was examined for brucella by culture.

These methods were adopted after preliminary studies on 29 herds containing 845 cows in the south of England had shown that biological examination of milk samples from cans detected a significantly higher proportion of infected herds than cultural examination with 4 plates/sample. In the examination of samples from

individual cows, the 4-plate cultural test was shown to detect 90 % of the samples that were positive by the biological test. This degree of accuracy was considered adequate for the survey.

Herd infection was estimated at 25–30 % of dairy herds. Infection in the national population of 3184000 dairy cows was estimated at 2·07 % (66000 cows). Udder infection was estimated to be in 0·91 % of cows, uterine infection in 0·98 %, infection of both sites in 0·18 %. Of herds found excreting brucellae, four-fifths were identified by isolation of brucellae from can-milk samples. The remaining one-fifth of herds was identified by examination of placentae. Brucellae were not found in can-milk samples from these herds.

Wales showed the lowest percentage of infected herds (12 %), followed by southern Scotland (17·5 %). The west Midlands showed the greatest percentage of infected herds (29·6 %) followed by south-west England (29·5 %) and Devon and Cornwall (26·0 %). The 4 remaining regions showed percentages of infected herds ranging from 19·4 % in eastern England to 25·7 % in northern England.

Brucellae were isolated from the milk of an average of 2·7 cows/infected herd. In 62 % of infected herds only 1 or 2 cows were found to be excreting the organism. Isolation of brucellae from the milk of individual cows increased from 0·6 % of cows in first lactation to 1·6 % in fourth and fifth lactations and thereafter declined slightly. Cows of the Channel Island breeds had more udder and more placental infection than average, cows of the Ayrshire breed less. Bought cows had more udder infection (1·5 %) than homebred cows (0·9 %), but did not differ appreciably in placental infection.

About 6 % of cows in the survey calved prematurely. Brucella infection was associated with 7 % of these abortions, or 0·4 % of all premature births. Strain 19 vaccine, when given to immature animals, appeared to halve the frequency of infection. There was no evidence of any useful results of adult vaccination. Adult vaccination appeared to be used on homebred cows mainly in response to an outbreak of abortion or on purchased cows as a prophylactic.

Economic losses. The major economic losses associated with brucellosis were estimated to be 11000 calves $3\frac{1}{2}$ million gal of milk, the cost of vaccination with S19 vaccine, and the losses consequent on 6000 retained placentae.

The eradication of brucellosis. The British Veterinary Association views with increasing disquiet the lack of vigorous measures to eradicate brucellosis in Great Britain, not only on account of the continuing risk to human and animal health but also because further delay may lead to difficulties in the export of livestock to countries where the disease has already been eradicated, or is in the process of being eradicated. The agreed policy of the Association (77) is the establishment of disease-free herds by the compulsory vaccination of all heifer calves during a preliminary period of 3 years, followed by a test and slaughter policy with compensation applied to all herds. The Association draws attention to the inadequacy of present legislation for the control and eventual eradication of brucellosis from animals and man in Great Britain.

Legislation for the control of brucellosis in England and Wales would appear to be limited to section 91 of the Food & Drugs Act 1955, which confers power to sample but does not necessarily mean that legal proceedings would subsequently be taken

under section 31, and to Pasteurization Orders to control the sale of infected milk. The scheme for the eradication of Brucellosis in Northern Ireland indicates the stages in the necessary legislation (78).

Salmonellosis in cattle

Gibson (79) has reviewed salmonellosis in dairy cattle, and has considered the public health aspects of infection.

Salmonellae are transmitted from cattle to man by meat, milk, and by direct contact. *S. dublin* and *S. typhi-murium* are the serotypes most frequently responsible for clinical disease in cattle; *S. dublin*, however, rarely causes human infections.

The importance of cattle as a reservoir of human salmonella infection was demonstrated by an investigation into salmonellae in abattoirs, butchers' shops, and home-produced meat (80). Cattle were considered to introduce salmonellae into abattoirs more frequently than any other animal species. *S. typhi-murium* was the serotype most frequently isolated from all sources and from human infections in the areas of the abattoirs.

Five outbreaks of salmonella infection following the consumption of raw milk were recorded in 1963 (81). One outbreak, due to *S. heidelberg* (82), followed the sale of milk in vending machines from a farm on which an outbreak of enteritis had occurred among cattle. Five persons on the farm were also infected. *S. heidelberg* and *S. montevideo* were isolated from cows and *S. heidelberg* from feeding stuffs on the farm. Feeding stuffs may have been infected through cross-contamination.

The other 4 outbreaks were due to *S. typhi-murium*. In one village outbreak 15 cases occurrred through consumption of raw milk from a farm where cows and feeding stuffs were infected with *S. typhi-murium* of the same phage type as that isolated from patients. In a second, *S. typhi-murium* of the same phage type was isolated from a dairy worker and from 30 cases and excreters who drank raw farm-bottled milk. In a farm outbreak the same phage type of *S. typhi-murium* was isolated from calves and from members of the farm family. Milk from the farm was not sold to the public. Farm-bottled milk was associated with one outbreak but the source was not discovered.

The 1964 Consolidated Orders for the control of bovine tuberculosis (52) introduced a new provision about the marking of cattle. All calves are now required to be marked by the age of 14 days, or earlier if moved off the farm to premises other than a slaughterhouse. This provision will be welcomed as facilitating the tracing of the source of infection in calf salmonellosis.

Pseudotuberculosis

In 1959, during an investigation into the possible viral origin of human mesenteric adenitis (83), *Pasteurella pseudotuberculosis* was isolated by culture from glands removed from 2 out of 17 cases of acute mesenteric adenitis. In a third case, histological examination of an excised gland showed the early features of *Pasteurella pseudo-tuberculosis* infection. All 3 cases showed serological evidence of infection.

A family outbreak of infection was described in 1961 (84). Histological appearances typical of infection were noted in a lymph gland removed during the removal of a

normal appendix from a child with symptoms of appendicitis. Serological evidence of infection was found in 3 children in the family and in the family dog.

Despite the widespread distribution of *Pasteurella pseudotuberculosis* in wild and domestic animals, there does not appear to be any authentic record of the isolation of the organism from cattle until 1962(85), when the organism was isolated from 2 bovine foetuses at Leeds and Cambridge. Serological evidence of infection was obtained from the 2 affected heifers and from 2 heifers in contact with one of them.

Human infectious disease conveyed by milk

Diphtheria. Since the immunization of the susceptible population in the 1940's and with the continuation of the immunization programme, diphtheria has become a rare disease in the United Kingdom. The infection still persists in other parts of the world.

In South Africa, during the routine investigation of a human case of diphtheria closely associated with the dairy on a farm 130 miles from Johannesburg, *C. diphtheriae* was isolated from 3 children considered to be carriers and from the teats of 2 out of 50 hand-milked cows on the farm(86). Milk samples from the cows proved negative. The milk supply from the farm was suspended for 12 days, deliveries being resumed when the infected cows and carriers had been isolated. No cases of diphtheria occurred which could be ascribed to contaminated milk.

Typhoid fever. Over 400 cases of typhoid fever occurred in Aberdeen in 1964. The outbreak was ascribed to the sale of a tin of corned beef, contaminated after sterilization by cooling in raw river water(87) polluted with crude sewage. Because a number of the earliest victims of the disease all received their milk from the same source, the general possibility of milk contamination was considered. As the number of cases increased, milk figured less and less in the case-histories, but there remained the possibility that anyone contracting the disease who played any part in the city's milk supply would constitutate a hazard. At the outbreak of the epidemic milk and other foods were regarded as a possible source of infection and investigated with negative results. To prevent subsequent spread of the disease by possible contamination of the milk by an infected person, all milk offered for sale in the Aberdeen area was pasteurized.

The Aberdeen outbreak led to a re-examination of the evidence which had, in 1948, resulted in the conclusion on circumstantial evidence that milk had been the vehicle of infection in the Oswestry outbreak of typhoid fever, in which there were over 150 cases and seven deaths(88,89). Re-examination of the evidence showed that on the day when infection was most likely to have occurred the lunch supplied to nursing and some domestic staff, and the main meal for the night staff, consisted among other things of corned beef. Re-examination of the strain of the typhoid bacillus isolated from this outbreak established that it was phage type 34, the same as was responsible for the Aberdeen outbreak.

REFERENCES

(1) *Report on the Animal Health Services in Great Britain* (1960). London: H.M.S.O.
(2) Savage, W. G. (1912). *Milk and the Public Health.* London: MacMillan and Co. Ltd.
(3) Wilson, G. S. (1942). *The Pasteurization of Milk.* London: Edward Arnold and Co.
(4) Report (1934). *Economic Advisory Coun. Comm. Cattle Diseases.* London: H.M.S.O.

(5) Wilson, G. S., Blacklock, J. W. S. & Reilly, L. V. (1952). *Non-pulmonary Tuberculosis of Bovine Origin in Great Britain.* London: Nat. Ass. Prev. Tuberc.
(6) Editorial (1964). *Lancet*, ii, 999.
(7) Report (1964). *Mon. Bull. Minist. Hlth*, **23**, 189.
(8) Peters, O. H. (1910). *J. Hyg., Camb.*, **10**, 602.
(9) Benjamin, B. (1964). Rep. no. 4. *Centre for Urban Studies*, p. 14. London: University College.
(10) Ferguson, T. (1964). Rep. no. 4. *Centre for Urban Studies*, p. 5. London: University College.
(11) Thomson, D. (1955). *Mon. Bull. Minist. Hlth*, **14**, 106.
(12) Underwood, E. A. (1947). *Proc. R. Soc. Med.* **41**, 165.
(13) Institution of British Water Engineers (1950). *Manual of British Water Supply Practice.* Cambridge: W. Heffer and Sons.
(14) Scott, H. H. (1934). *Some Notable Epidemics.* London: Edward Arnold and Co.
(15) McCoy, J. H. (1963). *R. Soc. Promot. Hlth J.* **83**, 154.
(16) Cuthbert, W. A. (1952). *J. appl. Bact.* **17**, 76.
(17) Regulation (1949). *The Milk and Dairies Regulation*, no. 1588. London: H.M.S.O.
(18) Morgan, G. T. (1954). *J. appl. Bact.* **17**, 70.
(19) Barnes, F. A. (1958). *Publs Inst. Br. Geogr.* no. 25, p. 167.
(20) Dairy Facts and Figures (1964). *Fedn U.K. Milk Mktg Bds.* 81.
(21) Lethem, W. A. (1946). *Mon. Bull. Minist. Hlth*, **5**, 80.
(22) *Milk (Special Designation) Regulations* (1941). London: H.M.S.O.
(23) Lethem, W. A. (1955). *Mon. Bull. Minist. Hlth*, **14**, 144.
(24) Marmion, B. P., MacCallum, F. O., Rowlands, A. & Thiel, C. C. with McDonald, J. R. & Phipps, P. H. (1951). *Mon Bull. Minist. Hlth*, **10**, 119.
(25) Report (1927). *Publ. Hlth Rep., Wash.*, **42**, 1893.
(26) Pease, H. D. (1931). *Congr. Am. publ. Hlth Ass. Montreal*, 1931.
(27) McKay, A. L., Currey. D. V., McNabb, A. L. & Berry, A. F. (1932). *Con. publ. Hlth J.* **23**, 303.
(28) Seiler, H. E. (1932). *Med. Offr.* **48**, 45.
(29) Report (1934). *Rep. publ. Hlth med. Subj., Lond., no.* 71. London: H.M.S.O.
(30) Folpmers, T. (1948). *Antonie van Leeuwenhoek*, **14**, 58.
(31) Burman, N. P. & Oliver, C. W. (1952). *Proc. Soc. appl. Bact.* **15**. 1.
(32) Gray, R. D. (1959). *J. Hyg., Camb.*, **57**, 249.
(33) Report (1962). *39th Rep. Metrop. Wat. Bd, Lond.*
(34) Gray, R. D. (1964). *J. Hyg., Camb.*, **62**, 495.
(35) Taylor, E. W. & Burman, N. P. (1964). *J. appl. Bact.* **27**, 294.
(36) Report (1961). *European Standards for Drinking Water.* Geneva: Wld Hlth. Org.
(37) Report (1963). *International Standards for Drinking Water*, 2nd edn., Geneva: Wld Hlth Org.
(38) Report (1935). *Spec. Rep. Ser. med. Res. coun.* no. 206. London: H.M.S.O.
(39) Report (1934). *Standard Methods of Milk Analysis*, 6th edn. New York: Amer. Publ. Hlth Ass.
(40) North, C. E., Park, W. H., Moore, V. A., Rosenau, M. J., Armstrong, C., Wadsworth, A. B. & Phelps, E. B. (1925). *Publ. Hlth Bull.* no. 147. Washington D.C.: U.S. publ. Hlth Serv.
(41) Kay, H. D. & Graham, W. R. (1935). *J. Dairy Res.* **6**, 191.
(42) Aschaffenburg, R. & Mullen, J. E. C. (1949). *J. Dairy Res.* **16**, 58.
(43) Aschaffenburg, R. (1947). *Mon. Bull. Minist. Hlth*, **6**, 159.
(44) Report (1962). *Irrigation in Great Britain.* Nat. Resour. (Tech.) Comm. London: H.M.S.O.
(45*a*) Muller, G. (1955). *Kommunalwistschaft* no. 8, 409.
(45*b*) Muller, G. (1955). *LitBer. Wass. Abwass. Luft Boden*, **4**, 330.
(46) Wallace, W. S. & Mackenzie, R. D. (1947). *Mon. Bull. Minist. Hlth*, **6**. 32.
(47) Shaw, W. V. (1937). *Rep. publ. Hlth med. Subj., Lond.*, no. 81. London: H.M.S.O.
(48) Newton, L. W., Bennett, H. S. & Figgatt, W. B. (1948). *Am. J. Hyg.* **49**, 166.
(49) Jepson, A. & Roth, H. (1952). *14th Int. vet. Congr. London*, 1949.
(50) McCulloch. W. (1965). *J. Soc. Dairy Technol.* **18**, 36.
(51) Report (1964). *On the Animal Health Services in Great Britain*, 1961 *and* 1962. Ministr. Agric., Fish., Fd & Dept. Agric. Scotl. London: H.M.S.O.
(52) The diseases of animals: *The Tuberculosis Order* 1964. London: H.M.S.O.
(53) Report (1965). The biological testing of milk. *Mon. Bull. Minist. Hlth*, **24**, 34.
(54) Davis, J. G. (1953). *Dairy Inds*, **18**, 210.
(55) Leech, F. B., Davis, M. E., Macrae, W. D. & Withers, F. W. (1960). *Disease, Wastage and Husbandry in British Dairy herd*, 1957/58. London: H.M.S.O.
(56) Howell, D., Wilson, C. D. & Vessey, M. P. (1964). *Vet. Rec.* **76**, 1107.
(57) Wilson, C. D. (1961). *Vet. Rec.* **73**, 1019.
(58) Wilson, C. D. (1964). *J. Soc. Dairy Technol.* **17**, 142.
(59) Wilson, C. D. (1963). *Vet. Rec.* **75**, 1311.
(60) Davidson, I. (1961). *Vet. Rec.* **73**, 1015.
(61) Williams Smith, H. (1957). *Mon. Bull. Minist. Hlth*, **16**, 39.

(62) Jones, A. C., King, G. J. G., Fennell, H. & Stone, D. (1957). *Mon. Bull. Minist. Hlth*, **16**, 109.
(63) Epsom, J. E. (1964). *Med. Offr*, **112**, 105.
(64) Goldberg, H. S. (ed.) (1959). *Antibiotics—their chemistry and non-medical uses.* New York: Van Nostrand Co.
(65) Woodbine, M. (ed.) (1962). *Antibodies in Agriculture.* London: Butterworths.
(66) Report (1963). *Antibiotics in Milk in Great Britain.* London: H.M.S.O.
(67) Tramer, J. (1964). *J. Soc. Dairy Technol.* **17**, 95.
(68) Wright, R. C. & Tramer, J. (1961). *J. Soc. Dairy Technol.* **14**, 85.
(69) Galesloot, Th.E & Hassing, F. (1962). *Neth. Milk Dairy J.* **16**, 89.
(70) Kosikowski, F. V. & Ledford, R. A. (1960). *J. Am. vet. med. Ass.* **136**, 297.
(71) Comment (1964). *Vet. Rec.* **76**, 1502.
(72) Vernon, E. (1964). *Mon. Bull. Minist. Hlth*, **23**, 54.
(73) Barrow, G. I. & Peel, M. (1965). *Mon. Bull. Minist. Hlth*, **24**, 21.
(74) Parry, W. H. (1963). *Med. Offr*, **109**, 397.
(75) Bothwell, P. W., McDiarmid, A., Bartram, H. G., MacKenzie-Wintle, H. A. & Williamson, A. R. H. (1962). *Vet. Rec.* **74**, 1091.
(76) Report (1964). Minist. Agric., Fish, Fd rep. no. 4. *Animal Disease Surveys. Brucellosis in the British Dairy Herd.* London: H.M.S.O.
(77) British Veterinary Association. (1965). *Vet. Rec.* **77**, 135.
(78) Kerr, W. R. & Rankin, J. F. (1963). *J. Soc. Dairy Technol.* **16**, 214.
(79) Gibson, E. A. (1965). *J. Dairy Res.* **32**, 67.
(80) Report (1964). *J. Hyg., Camb.*, **62**, 283.
(81) Report (1964). *Mon. Bull. Minist. Hlth*, **23**, 189.
(82) Hutchinson, R. I. (1964). *Brit. med. J.* i, 479.
(83) Mair, N. S., Mair, H. J., Stirk, E. M. & Carson, J. G. (1960). *J. Clin. Path.* **13**, 432.
(84) Randall, K. J. & Mair, N. S. (1962). *Lancet*, i, 1942.
(85) Mair, N. S. & Harbourne, J. F. (1963). *Vet. Rec.* **75**, 559.
(86) Greathead, M. M. & Bischop, P. J. N. (1963). *S. Afr. med. J.* **37**, 1261.
(87) Report (1964). *The Aberdeen Typhoid Outbreak*, 1964. London: H.M.S.O.
(88) Bradley, W. H., Evans, L. W. & Taylor, I. (1951). *J. Hyg., Camb.*, **49**, 324.
(89) Jones, A. C. (1951). *J. Hyg., Camb.*, **49**, 335.

THE JOURNAL OF THE

SOCIETY OF DAIRY TECHNOLOGY

17 DEVONSHIRE STREET, LONDON, W.1. *Tel. Langham* 5059

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

The Subscription rate to non-members of the Society, payable in advance, is £2. 12*s*. net per volume (post free): single numbers, 15*s*. net. Subscriptions and all business enquiries should be sent to the Secretary, Society of Dairy Technology, 17 Devonshire Street, London, W. 1.

Each member of the Society receives one free copy of each number. One further copy of each number may (if available) be bought by members for 10*s*.

CONTENTS OF THE JANUARY ISSUE

CONTENTS OF THE JULY ISSUE

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

Advertising Offices: 9 GOUGH SQUARE · LONDON · E.C. 4

(CONTENTS OF VOL. XVII, No. 2, JUNE 1964

The Journal of General Microbiology

Editors
B. C. J. G. KNIGHT
and A. F. B. STANDFAST

Vol. 40, No. 3. September 1965

CONTENTS

The subscription rate is 80s. net per volume of three parts. Single parts 35s. each, plus postage.

Four volumes will be issued each year. Orders should be sent to

Cambridge University Press,
Bentley House, 200 Euston Road,
London, N.W. 1

DIRECTIONS TO CONTRIBUTORS

GENERAL

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

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

FORM OF PAPERS

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

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

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

TABLES

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

ILLUSTRATIONS

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

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

As a rule the photographs and diagrams should be about twice the size of the finished block and not larger over-all than the sheets on which the paper itself is typed. For general guidance in preparing diagrams, it is suggested that for a figure measuring 9 in. × 6 in. all lines, axes and curves, should have a thickness of 0·4 mm, thus ———. Graph symbols in order of preference should be ○ ●, △ ▲, □ ■, × +, and for a 9 in. × 6 in. graph the open circles should be $\frac{1}{8}$ in in diameter. The open triangles should be large enough to contain circles $\frac{3}{32}$ in. diameter and the open squares circles of $\frac{1}{8}$ in. diameter. The crosses should have lines $\frac{1}{4}$ in. long. The block symbols should be slightly smaller than the corresponding open symbols. Scale marks on the axes should be on the inner side of each axis and should be $\frac{1}{8}$ in. long.

REFERENCES

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

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

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

SYMBOLS AND ABBREVIATIONS

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

DESCRIPTIONS OF SOLUTIONS

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

REPRINTS

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

Journal of Dairy Research
Volume 33, Number 1, February 1966

CONTENTS

ORIGINAL ARTICLES

Printed in Great Britain at the University Printing House, Cambridge