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The effect of feeding dried sugar-beet pulp on the intake and production of dairy cows

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(*Received* 15 *October* 1965)

Summary. Three rations were compared in a 15-week winter feeding experiment using 12 Ayrshire cows. All the cows received silage 3 times a day and concentrates; ration A contained no sugar-beet pulp whilst ration B contained 6 lb of pulp and ration C 12 lb.

The total weights of dry matter consumed were 29·7, 32·3 and 33·4 lb/day, respectively, on treatments A, B and C. For every 1 lb of extra sugar-beet pulp dry matter eaten the decline in the dry weight of the other feeds consumed was 0·50 lb on treatment B and 0·55 lb on treatment C. The main decrease was in the silage part of the ration. The weights of starch equivalent (S.E.) consumed daily were 18·0, 19·8 and 20·7 lb on rations A, B and C, respectively, with mean daily milk yields of 38·2, 39·3 and 39·7 lb, giving a response of 0·55 lb milk/lb extra S.E. between rations A and C. The corresponding fat percentages of the milks did not differ significantly from each other but the solids-not-fat (SNF) percentages increased significantly from 8·76% on ration A to 8·88% on ration C. The average response per lb extra S.E. was 0·044% SNF and the increase was in the protein fraction of the milk. The cows were significantly heavier on rations B and C than on ration A. The inclusion of beet pulp in rations B and C had no adverse effect on the taste of the milk.

It has been shown previously that, when fodder-beet was added to a diet of hay, grass silage and concentrates, the total daily intake of dry matter was increased (Castle, Drysdale & Waite, 1961; Castle, Drysdale, Waite & Watson, 1963). This increase in dry-matter consumption increased the milk yield and the SNF percentage of the milk, but, when 30 lb of fodder-beet was fed per day, the increased consumption of dry matter also increased the margin between the income from milk sales and feed costs. It might be more economical to use sugar-beet pulp instead of fodder-beet since Broster (1960) calculated that the cost of S.E. from dried sugar-beet pulp was about three-quarters of that of mangolds. The composition of the dry matter of dried sugar-beet pulp (Evans, 1960) differs from that of fodder-beet (Castle *et al.* 1961, 1963) because the beet pulp is a residue remaining after the extraction of sugar. Thus, although the S.E. of the dry matter of fodder-beet is similar to that of beet pulp, the beet pulp contains more than double the amount of crude fibre compared with fodder-beet and about 20% less nitrogen-free extract. Because of these differences the beet pulp might not have as marked an effect on intake as fodder-beet, but to use beet pulp for milk production could still be more profitable.

To investigate this a feeding experiment was made in which a basal ration of grass silage and concentrates was supplemented with 2 levels of dried sugar-beet pulp.

EXPERIMENTAL

Experimental design

Nine Ayrshire cows and 3 first-calf heifers with an average of 2·7 lactations/animal were used. They had calved from 3 to 12 weeks (average 8 weeks) before the experiment began. They were housed in a byre with facilities for individual feeding, but were allowed into a bare yard each day from 10.00 a.m. to 12.00 noon.

The 12 animals were divided into 4 groups with the animals in each group as similar as possible in stage of lactation, milk yield and live weight. The groups were then paired and animals in the 2 groups were allotted at random to the treatment sequences devised by Cochran, Autrey & Cannon (1941) for pairs of 3×3 Latin squares.

The experiment lasted from 16 November 1964 to 28 February 1965, and was divided into 3 equal periods of 5 weeks, but in calculating the results the data from weeks 4 and 5 in each period were used.

Feeding

All the cows were offered a weighed amount of grass silage at approximately 7.00 a.m., 12.00 noon and 5.00 p.m. each day, and it was given in sufficient quantity to ensure that a residue was always available for weighing 3 h after feeding. The silage had been made in late May from herbage at the long leafy stage and without a preservative. In addition to the silage the animals received one of the following 3 treatments daily: A, no dried sugar-beet pulp; B, 6 lb dried sugar-beet pulp/cow; C, 12 lb dried sugar-beet pulp/cow. Before it was given to the cows, the dried beet pulp was soaked in water (6 lb pulp to 10 lb water) for a minimum of 3 h and was given in 3 equal feeds $\frac{1}{2}$–1 h before the silage was offered. Any uneaten beet pulp was removed from the troughs and weighed before the silage was given.

Concentrates were given at a rate of $3\frac{1}{2}$ lb/10 lb of milk. The amount given in period 1 was calculated from the average weight of milk produced by each cow in the 2 weeks before the experiment began. In periods 2 and 3 the amount fed was based on the equalized feeding principle of Lucas (1943), using the milk yields from the last week of periods 1 and 2, respectively. The weight of concentrates given was kept constant throughout any one period and half the total daily weight was given at each milking time. The composition of the concentrate mixture was (parts by weight): crushed barley, 7; flaked maize, 1; groundnut cake, $1\frac{3}{4}$; mineral pre-mix, $\frac{1}{4}$, containing salt, dicalcium phosphate, magnesite and feeding limestone.

On 5 consecutive days each week the quantities of each feed refused were weighed. The feeds were sampled for analyses as previously described (Castle *et al.* 1961).

The analyses and calculated nutrient values of the feeds are given in Table 1. The dry matter of the sugar-beet pulp contained 14·6 % water-soluble carbohydrates, whereas that of the silage had only 1·6 %. On a dry-matter basis, the silage contained more than twice as much crude fibre and 3 times as much lignin as the beet pulp.

Table 1. *The composition and calculated feeding value of the concentrate mixture, the grass silage and the dried sugar-beet pulp*

	Dry matter, %	% of the dry matter								
		Crude protein	Ether extract	Nitrogen-free extract	Crude fibre	Ash	Lignin	Water-soluble carbo-hydrates	Approxi-mate S.E.	Approxi-mate D.C.P.
Concentrate	84·4	20·6	3·6	66·4	4·9	4·5	4·2	N.D.	83*	16·1*
Grass silage	21·8	14·4	4·6	40·9	30·9	9·2	8·7	1·6	47†	9·4†
Dried molassed beet pulp	88·9	10·9	0·7	66·6	15·0	6·8	2·6	14·6	65*	6·4*

N.D., not determined.
* Calculated from chemical analyses and tables of Evans (1960).
† Calculated from the regression equations of Watson & Nash (1960).

Table 2. *Intakes on the* 3 *feeding treatments*

Treatment	Dry matter, lb per cow per day				Daily dry matter consumption as % of live weight	Starch equivalent, lb/day	Digestible crude protein, lb/day	Crude fibre, lb/day	Lignin, lb/day
	Sugar-beet pulp	Silage	Concen-trates	Total					
A	—	18·5	11·2	29·7	2·9	18·0	3·5	6·2	2·1
B	5·2	16·7	10·4	32·3	3·1	19·8	3·6	6·4	2·0
C	8·3	15·2	9·9	33·4	3·2	20·7	3·6	6·4	2·0
Standard error of a difference between two means	±0·60	±0·48	±0·35	±1·08	±0·06	±0·46	±0·06	±0·13	±0·03

Table 3. *The yield and chemical composition of the milk and the mean liveweights of the cows*

Treatment	Milk yield, lb/cow/day	Milk composition, %					Casein no.	Live weight, lb
		Fat	SNF	Crude protein*	Casein	Lactose		
A	38·2	4·10	8·76	3·34	2·61	4·58	78·1	1020
B	39·3	4·22	8·83	3·41	2·68	4·58	78·6	1041
C	39·7	4·12	8·88	3·42	2·69	4·58	78·5	1040
Standard error of a difference between two means	±0·56	±0·06	±0·04	±0·02	±0·02	±0·02	±0·25	±6

* N × 6·38.

Milk yield, milk quality and liveweight

The cows were milked twice a day and their yields recorded at each milking to the nearest ½ lb. Milk samples from consecutive evening and morning milkings were taken twice a week from each cow in weeks 3, 4 and 5 in each period and analysed for total solids, fat, crude protein, casein and lactose by methods previously described (Waite, White & Robertson, 1956). Samples of milk from each cow were taken fortnightly for the determination of the total and differential cell count (Blackburn, Laing & Malcolm, 1955) and for the detection of any pathogenic bacteria.

The cows were weighed weekly at about 10 a.m.

RESULTS AND DISCUSSION

Feed consumption

When animals were on silage and concentrates only (treatment A) they ate on average 29·7 lb dry matter/day, and this was increased significantly to 32·3 lb/day on treatment B ($P < 0{\cdot}05$) and to 33·4 lb on treatment C ($P < 0{\cdot}01$) when sugar-beet pulp was offered in addition (Table 2). Thus, as anticipated from its composition the addition of dried sugar-beet pulp to a basal ration containing silage was found to have an effect similar to that when fodder-beet was added, and the total dry matter intake was increased. When the sugar-beet pulp was eaten, an equal weight of the other feeds in the ration was not refused; on average, for each 1 lb of sugar-beet pulp dry matter eaten, there was a reduction in the weight of basal feeds consumed of 0·50 lb and 0·55 lb on treatments B and C, respectively. These values are slightly higher than the values of 0·45 and 0·40 lb found in previous experiments using fodder-beet (Castle *et al.* 1961, 1963). The intake of concentrates fell slightly from treatment A to treatment C, but the major decrease occurred with the grass silage, which fell significantly from 18·5 lb on treatment A to 16·7 lb on treatment B, and to 15·2 lb on treatment C ($P < 0{\cdot}01$). This decrease in silage consumption of 18 % from treatment A to treatment C is identical with the decrease found when 5·9 lb of fodder-beet dry matter was added to the ration (Castle *et al.* 1961). Of the 3 feeds offered in the present experiment, the grass silage had the highest crude fibre content (Table 1) and this may possibly have accounted for the fact that refusals of silage were greater than refusals of concentrates. The intake of crude fibre was, in fact, slightly higher on treatments B and C than on treatment A but the differences were not significant. The intake of lignin was slightly higher on treatment A than on B and C but all the differences were small.

The intakes of S.E. on treatments B and C were both significantly higher than on treatment A ($P < 0{\cdot}01$) but there were no significant differences in the intakes of digestible crude protein (D.C.P.) The daily intakes of S.E. expressed as a percentage of the cows' requirements according to Woodman's standards (Evans, 1960) were 111, 119 and 124 % on treatments A, B and C, respectively. The D.C.P. intake calculated by the same method was 106 % of Woodman's standards on all the treatments.

Milk yield, milk composition, and liveweight

The average yield of milk throughout the experiment was 39·1 lb/day with only small differences between treatments (Table 3). The yield on treatment C was significantly higher than that on treatment A ($P < 0{\cdot}05$), while the yield on treatment B was intermediate and not significantly different from either. The response per 1 lb of extra S.E. fed was 0·61 lb milk between treatments A and B, and 0·44 lb milk between treatments B and C. These values are low compared with responses of 0·9–1·0 lb found in other experiments (Castle, MacLusky, Waite & Watson, 1958; Castle *et al.* 1961), although a response of only 0·7 lb of milk/lb S.E. was found in an earlier experiment when 2 levels of root feeding were compared (Castle *et al.* 1963). The low response in the present experiment may be attributable to the relatively high level of feeding on treatment A, 111% of Woodman's standards for S.E., and also to the possibility that the S.E. of the silage was higher than the value calculated, and hence the total S.E. intakes on all 3 treatments were higher than was intended.

There were no significant differences between the fat percentages of the milk on the different treatments (Table 3) but the SNF percentage increased from 8·76% on treatment A to 8·88% on treatment C ($P < 0{\cdot}01$). For each extra 1 lb of S.E. fed the SNF percentage increased by an average of 0·044%. This value agrees well with the value of 0·05% found in previous experiments with fodder-beet (Castle *et al.* 1961, 1963) and suggests that molassed sugar-beet pulp is also a useful supplement for rations containing a high proportion of silage if an increase in the SNF percentage is required. It was shown by Bath & Rook (1965) that, compared with roughages, sugar-beet pulp gave a lower proportion of acetic acid in the rumen contents with compensating increases in the proportions of propionic and butyric acids. The ratio of the last 2 acids varied from one animal to another, but increases in either or both of these acids can cause increases in the SNF percentage of milk, and this could explain the increases in the SNF values found here.

The lactose percentages of the milk were the same on the 3 treatments and the increases in the SNF percentages were attributable to increases in protein content, the milk from treatments B and C having significantly higher crude protein and casein contents than the milk from treatment A ($P < 0{\cdot}01$). The casein numbers of the milks of the 3 treatments were not significantly different, and with a mean value of 78·4 they indicated that the milk composition was not seriously affected by mastitis (Waite, 1961; Waite, Abbot & Blackburn, 1963).

The average liveweights of the cows are given in Table 3. On treatments B and C, which contained sugar-beet pulp, the values were significantly ($P < 0{\cdot}01$) higher than on treatment A.

Occasionally a fishy taste has been observed in milk when beet pulp has been given to cattle (e.g. Watson, 1949), and in this respect 9 lb of pulp/day given in 2 feeds has been regarded as the safe upper limit (Broster, 1960). This is similar to the amount eaten in treatment C of the present experiment. Samples of milk from each cow were therefore tasted regularly by an experienced tasting panel of 5 people, but there was virtually no difference in the taste of the milks attributable to the feeding treatments.

Finally, although the addition of sugar-beet pulp to a diet of grass silage *ad lib.*

and restricted concentrates increased the intake of dry matter, the milk yield, and the SNF content of the milk, the addition did not increase the margin between the income from milk sales and feed costs using current prices for the feeds and the milk. This is mainly attributed to the small increase in milk yield per 1 lb extra S.E. fed, which in turn was due to the relatively high level of feeding on treatment A (111 % of Woodman's standards). With a lower quality basal diet supplying less S.E. the effect of the beet pulp would no doubt have been more pronounced.

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REFERENCES

BATH, I. H. & ROOK, J. A. F. (1965). *J. agric. Sci., Camb.*, **64**, 67.
BLACKBURN, P. S., LAING, C. & MALCOLM, J. F. (1955). *J. Dairy Res.* **22**, 37.
BROSTER, W. H. (1960). *Br. Sug. Beet. Rev.* **29**, 21, 71.
CASTLE, M. E., DRYSDALE, A. D. & WAITE, R. (1961). *J. Dairy Res.* **28**, 67.
CASTLE, M. E., DRYSDALE, A. D., WAITE, R. & WATSON, J. N. (1963). *J. Dairy Res.* **30**, 199.
CASTLE, M. E., MACLUSKY, D. S., WAITE, R. & WATSON, J. N. (1958). *J. Dairy Res.* **25**, 365.
COCHRAN, W. G., AUTREY, K. M. & CANNON, C. Y. (1941). *J. Dairy Sci.* **24**, 937.
EVANS, R. E. (1960). *Bull. Minist. Agric. Fish. Fd, Lond.*, no. 48.
LUCAS, H. L. (1943). *J. Dairy Sci.* **26**, 1011.
WAITE, R. (1961). *J. Dairy Res.* **28**, 75.
WAITE, R., ABBOT, J. & BLACKBURN, P. S. (1963). *J. Dairy Res.* **30**, 209.
WAITE, R., WHITE, J. C. D. & ROBERTSON, A. (1956). *J. Dairy Res.* **23**, 65.
WATSON, S. J. (1949). *Feeding of Livestock*, p. 165. London and New York: Thomas Nelson and Sons Ltd.
WATSON, S. J. & NASH, M. J. (1960). *The Conservation of Grass and Forage Crops*, p. 691. Edinburgh and London: Oliver and Boyd.

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Serological differentiation of cow's, buffalo's, goat's and sheep's milks

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Summary. Milks of the closely related species ox, buffalo, goat and sheep were differentiated by the precipitin test using antisera to blood serum proteins. The antisera were made specific for the homologous species by absorption. The component of milk tested was the lactalbumin–lactoglobulin fraction present in whey. Fraudulent sale of diluted buffalo's milk, cow's milk–buffalo's milk–water mixtures and reconstituted whole and skim-milk powders as genuine cow's milk was detected. Of 270 milks serologically tested, 233 were cow, 26 buffalo, 1 goat, 3 cow–buffalo mixtures, 2 reconstituted whole-milk powders and 5 reconstituted skim-milk powders. Of the samples serologically identified as cow, 135 had analytical figures for fat and solid-not-fat (SNF) only just outside or within the buffalo range.

Milk from any one species was detected in a concentration of 5% (v/v) in milk of a related species. Species identification was made of human milk stains in 2 medico-legal cases. Fresh cow's milk, reconstituted whole-milk powder and reconstituted skim-milk powder were differentiated.

INTRODUCTION

In Ceylon and other countries in the East, the differentiation of milks of closely related species is an important problem, for cow's, buffalo's and goat's milks are essential items in the daily diet. From time to time, cases arise where the analytical figures and physical characteristics of the milk indicate that adulterated buffalo's milk, reconstituted whole- or skim-milk powder or a buffalo's milk–skim-milk powder–water mixture is being fraudently sold as fresh cow's milk. Differentiation of cow's, buffalo's and goat's milk by standard analysis is by no means conclusive as some overlapping occurs in the respective figures for fat and SNF. It is therefore imperative to have a simple, reliable method of differentiating the various types of milk.

The conditions obtaining in this country are such that most of the milk samples received are about 24-h old at the time of analysis. Despite the instructions given to add a few drops of formalin to a bottle of milk, approximately 10% of the samples are either in the early stages of souring or have completely soured at the time of receipt. Any method, therefore, has to be applicable to samples containing formalin or in a sour condition.

Cow's milk has been differentiated from buffalo's milk by estimation of carotene (Singh, Yadav & Pathak, 1963), by electrophoretic methods (Sen & Sinha, 1961;

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Aschaffenburg & Sen, 1963; Rao, 1964) and by immuno-electrophoretic methods (Ambrosino, Liberatori & Ubertalle, 1963*a*, *b*). A micro-immunodiffusion technique has also been used (Ambrosino, Liberatori & Ubertalle, 1965) for the differentiation of whey from cow's, buffalo's, ewe's and goat's milk.

It is known that the direct precipitin test fails to distinguish between closely related species and that lactosera and haematosera act similarly in both milk and blood (Nuttall, 1904; Bordet *et al.* 1909).

A modified serological technique was used by Soldberg & Hadland (1953) for the detection of cow's milk added to goat's milk. Dutheil (1959) also employed the same method for the detection of cow's or goat's milk added to sheep's milk and determined the percentage of the adulterant milk by nitrogen estimation of the precipitate. Nair & Iya (1962) and Nair, Malik & Singh (1965) used a similar technique to detect buffalo's milk as an adulterant in cow's milk. Johke, Hageman & Larson (1964) studied the immunological relationships of α-lactalbumin and β-lactoglobulin in milks of various species and found that rabbit antisera against cow's α-lactalbumin and β-lactoglobulin showed strong cross-reactions with the milks of goats, ewes and water-buffaloes and slight reactions with camel's and mare's milk. Soldberg & Hadland (1953) also showed that intravenous injections of different fractions of whey proteins from cow's milk produced antisera which were not specific to homologous antigens. Similar results were also obtained by Dutheil (1959) with antisera prepared by injections of casein, lactoglobulin and lactalbumin absorbed on aluminium hydroxide.

This paper describes a method which distinguishes serologically between cow's, buffalo's, goat's and sheep's milk by testing the whey obtained by natural souring or by the addition of acetic acid. The antisera used were prepared with blood serum proteins as antigens and made specific by absorption with the appropriate blood sera. The methods of preparation of the antigen and antiserum, the absorption technique and the precipitin test employed have been described previously (Pinto, 1961).

MATERIALS AND METHODS

Preparation of antigen

From blood serum. Twenty ml blood serum was diluted with 80 ml normal saline and 150 ml absolute alcohol added gradually with vigorous shaking. The mixture was kept overnight in the refrigerator, and sucked dry at the pump. The protein was then transferred to a Petri dish and dried and stored in a desiccator.

From whey. Fresh cow's milk was allowed to sour naturally. The protein was precipitated from the filtered whey with excess absolute alcohol, filtered and dried.

For injection. The protein (0·7 g) was ground to a very fine powder in a mortar and suspended in 70 ml saline. This was sufficient for injecting 2 rabbits.

Immunization

Against blood serum. Blood serum protein suspension was injected intraperitoneally into each rabbit (weight approximately 2 kg) as follows: 1st day, 5 ml; 2nd day, 10 ml; 3rd day, 15 ml.

Against whey. Whey protein suspension was injected intraperitoneally as follows: 1st day, 10 ml; 2nd day, 20 ml; 3rd day, 30 ml.

In both methods the rabbits were bled from the marginal ear vein when the potency was a maximum, which was generally about the 13th day. The antisera were stored without preservative in sealed ampoules in the refrigerator (4 °C).

Absorption of antiserum

Dilutions (1 in 200, v/v) in saline of ox's, buffalo's, goat's and sheep's blood sera were prepared. The antisera and the dilutions of the heterologous sera were measured out dropwise into a small tube using micropipettes. The required proportions for each batch of antisera were obtained by preliminary titrations. The mixtures were well shaken, allowed to stand for 15 min at room temperature (28 °C) and then centrifuged at 4000 rev/min for about 10 min.

Preparation of whey

The whey for the test was obtained by natural souring or by the addition of a few drops of glacial acetic acid. When souring was complete, the whey was filtered into a small beaker, warmed, and neutralized with anhydrous Na_2CO_3, added a very little at a time, with stirring; litmus paper was used as indicator. The neutral whey was then centrifuged at 4000 rev/min and the clear supernatant pipetted off for the test. Any whey that could not be clarified satisfactorily by centrifugation was filtered by allowing it to seep through a tightly wedged plug of moist filter paper inside a Pasteur pipette.

Dilution of whey. Using the capillary method, the clear whey was assayed as follows. A protein precipitant, e.g. Spiegler's solution (8 g mercuric chloride, 4 g tartaric acid, 20 g glycerine and 200 ml water), or 50 % (w/v) of aqueous salicyl sulphonic acid, was tested against progressive dilutions of whey until the intensity of the precipitate formed matched that in a control capillary tube. This control tube contained the absorbed ox's antiserum and a cow's milk whey diluted with saline to such a degree that the precipitate formed after 20 min was an optimum from the point of view of it being well defined and confined to the junction of the whey and antiserum layers. The tubes with the whey and Spiegler solution were examined immediately after being set up and not after 20 min.

Precipitin test—capillary method of Brüning & Kraft (1927)

The clear antigen solution was drawn up almost half-way into a capillary tube about 9 cm long followed by an approximately equal volume of the antiserum taken from a drop placed on a glazed porcelain tile. The top of the tube was then sealed in the Bunsen flame. Similar tubes were set up with the other antisera. The tubes were kept in a vertical position, by means of spring clips fixed to a wooden stand, for 20 min and then examined by transmitted light against a black background. A white precipitate at the junction of the 2 liquid columns indicated a positive reaction.

Test for species

The absorbed (specific) antisera, prepared just before the test, were tested against the unknown, diluted whey. Controls with the 1 in 200 dilutions of the blood sera as well as cow's, buffalo's and goat's milk whey against the antisera were set up at the same time. The capillary tubes were examined after 20 min.

Milk stains on fabrics—forensic application

For species identification, an approximately 0·75 cm square portion was taken from the garment. This was cut up into fragments, placed in a small tube and a few drops of saline added. The fragment were then prodded with a thin glass rod for a few minutes, the tube left overnight in the refrigerator (4 °C) and centrifuged the following day. The supernatant was pipetted off, filtered using the method already described, and the clear extract tested against the antisera. It was not found necessary to add acetic acid to the extract and neutralize before testing as in the case of liquid milk.

RESULTS

Antisera. The antiserum prepared with cow's milk whey proteins was approximately of the same potency, gave similar cross-reactions with buffalo's and goat's blood sera and milk whey and was made specific in the same way as the corresponding antiserum prepared with ox blood serum proteins. The blood antisera were therefore used for all subsequent tests.

Table 1. *Reactions of unabsorbed antisera against homologous and heterologous blood sera*

		Reaction with serum*			
Antiserum	Antigen	Ox	Buffalo	Goat	Sheep
Ox	Blood serum protein	+ + + + +	+ + + +	+ +	+ +
	Milk whey protein	+ + + + +	+ + + +	+ +	Not tested
Buffalo	Blood serum protein	+ + + +	+ + + + + +	+ + +	+ + +
Goat	Blood serum protein	+ +	+ +	+ + + +	+ + +
Sheep	Blood serum protein	+	+	+ +	+ + +

* The degree of precipitation was based on visual examination; +, the weakest reaction.

Table 2. *Reactions of absorbed antisera against homologous and heterologous blood sera*

		Reaction with serum†			
Antiserum	Serum used for absorption*	Ox	Buffalo	Goat	Sheep
Ox Blood	Buffalo and goat (4:2:1)	+ + + +	−	−	−
Ox Milk	Buffalo and goat (4:2:1)	+ + +	−	−	Not tested
Buffalo	Ox and goat (4:2:1)	−	+ + + + +	−	−
Goat	Ox and buffalo (4:2:1)	−	−	+ + +	+ +
Goat	Sheep (3:1)	Not tested	Not tested	+ + +	−
Sheep	Goat (2:1)	Not tested	Not tested	−	+

* 1:200 dilutions of the absorbing sera were used. The ratio of antiserum to absorbing serum are indicated.

† +, The weakest reaction; −, no reaction.

Specific antisera. Table 1 gives the results obtained when the unabsorbed antisera were tested against the 1 in 200 dilutions of homologous and heterologous blood sera, and Table 2 the results after absorption.

For the differentiation of sheep's and goat's milk the goat's and sheep's antisera

were absorbed only with sheep's and goat's blood sera, respectively. No absorption was made with ox's and buffalo's sera as otherwise the loss of potency was too great. Ox's and buffalo's antiserum, when absorbed as indicated in Table 2, were specific.

Differentiation of goat's and sheep's milk. One goat's antiserum prepared gave only weak cross-reactions with ox's and buffalo's blood sera and whey but reacted with sheep's sera. The antiserum was made specific by the addition of 1 in 200 dilution of sheep's blood serum in the ratio 3 parts goat's antiserum to 1 part of sheep's serum. Another goat's antiserum did not react with ox's and buffalo's sera and whey when unabsorbed. It was made specific by the addition of the sheep's serum dilution in the ratio of 4 parts goat's antiserum to 1 part of sheep's serum. This absorbed goat's antiserum reacted strongly with goat's milk whey but gave no precipitin reaction at all with sheep's milk whey.

The first sheep's antiserum prepared did not react with ox's and buffalo's sera but could not be made specific by absorption with goat's serum. The second gave a slightly weaker reaction with goat's than with sheep's serum, but the antiserum could only be absorbed with considerable loss of potency and was therefore of no value for species differentiation.

The third sheep's antiserum prepared gave weak cross-reactions with ox's and buffalo's blood sera and milk whey. This was successfully absorbed with the goat's serum in the proportion 2 parts of sheep's antiserum to 1 part of goat's serum. Using this absorbed sheep's antiserum it was possible to distinguish between sheep's and goat's milk. The potency of this absorbed sheep's antiserum, however, was not as great as the average absorbed ox's, buffalo's and goat's antisera.

Identification of milk species

Since the absorbed antisera were specific, the neutralized whey could be tested directly against them. However, it was found necessary, especially in the case of an unadulterated milk, to dilute the whey before testing, as otherwise the copious precipitate formed spread out quickly and formed a turbid lower layer. The use of the diluted whey resulted in a sharply defined zone of precipitation. It was also observed that a slight haze, sometimes present in the whey even after centrifuging and filtering, was eliminated on dilution and subsequent centrifugation. Dilution of the antigen tested is usually resorted to in serological tests, e.g. species identification of blood stains, to eliminate the so-called 'mammalian reaction'. But, in the test described in this paper, dilution is not made for this purpose.

Of a total of 270 milk samples serologically tested, 238 were identified as cow's, 26 as buffalo's, 1 as goat's, and 3 as cow–buffalo mixtures. The remaining 2 samples gave no precipitate with any of the antisera, absorbed or unabsorbed. The analytical figures for these 2 samples were those of an average fresh cow's milk, i.e. 3·5% (w/v) fat for both and 9 and 10% SNF, respectively. However, the wheys of these 2 samples differed from fresh milk whey but resembled that of milk reconstituted from whole milk powder in the lower content of soluble protein precipitable with the protein precipitant (Spiegler solution) and in the negative serological reaction.

Of the 238 samples identified as cow's milk, 5 gave weak but definite reactions with the ox's antiserum. These 5 wheys differed from fresh milk whey but resembled that of milk reconstituted from skim-milk powder in the low content of soluble protein

and the weak serological reaction with ox's antiserum. The fat content of all these 5 milks was less than 1 %.

In all the tests performed suitable controls were used. These controls were chosen from cow's and buffalo's milk, and ox's, buffalo's and goat's flesh. The 1 in 200 dilutions of ox's, buffalo's and goat's blood sera were used as controls in all the tests.

Forensic application. Stains of human milk on garments in 2 cases of infanticide were identified by this method. The human antiserum used was prepared as usual with the blood serum proteins.

Effect of heat treatment of milk

Crawford & Grogan (1960) studied the effect of heat treatment on the antigenicity of milk proteins using parenteral sensitization and challenge methods, and immunochemical analysis by serum agar precipitation. They showed that heat-denatured milk no longer reacted antigenically with lactalbumin antibody and reacted only feebly with lactoglobulin antibody, while the reaction with casein antibody was reduced. Raw and homogenized milk were found by them to react with all 3 antisera.

Using the method described in this paper, no difficulty was experienced in the serological identification of raw or pasteurized milk. On the other hand, boiled milk, sterilized milk, reconstituted whole milk powder (6 commercial preparations tested) and condensed milk (5 commercial preparations) failed to give a precipitin reaction with the ox's antiserum. The wheys in all these samples contained proteins in solution as shown by their reactions with Spiegler solution, but the concentrations were very low compared with that of raw or pasteurized milk whey.

Skim-milk powder. Reconstituted skim-milk powder (8 commercial preparations tested) gave a relatively weak but definite positive reaction with the ox's antiserum. This positive reaction was even weaker than the reaction with fresh milk diluted with 3 parts water. The quantity of precipitable protein in the skim-milk whey was approximately 3 or 4 times that in the reconstituted whole-milk powder whey when tested with Spiegler solution.

Raw milk samples were heated up to 80, 85, 90, 95 and 100 °C, respectively, and immediately allowed to cool to room temperature (28 °C) before testing. Positive identification was possible in the case of milk heated to 90 but not to 95 °C and over. There was a gradual decrease in the quantity of precipitate formed with both the antiserum and Spiegler solution with increase in temperature; the Spiegler reaction was positive, though weak, even at 100 °C, and approximated to that given by the whole-milk powder. When the reconstituted skim-milk powder was heated to boiling and then tested, a negative reaction was obtained with the ox's antiserum. It would appear that the difference in reactivity between whole- and skim-milk powder was due to some difference in the drying process during manufacture, probably connected with temperature.

Fresh milk and milk powders. From the results obtained above it could be inferred that the differentiation of fresh milk, reconstituted whole-milk and skim-milk powders would be possible by the scheme outlined in Table 3. Attempts were made to prepare antisera by injecting proteins precipitated from whey of reconstituted whole- and skim-milk powders with excess alcohol. Even a second course of injections failed to produce antibodies detectable by the precipitin test.

Sensitivity of method. The serological tests were done on fresh cow's milk adulterated with water, buffalo's milk and goat's milk, respectively. Detection of 5% adulteration was possible without difficulty in every case.

Table 3. *Serological differentiation of fresh milk, whole-milk powder and skim-milk powder*

Neutral whey (undiluted)	Reaction with protein precipitant (Spiegler's solution)*	Reaction with ox antiserum*
Fresh milk	20	5
Reconstituted whole-milk powder	2	—
Reconstituted skim-milk powder	6	1

* Unit of reaction is arbitrary; —, indicates no reaction.

DISCUSSION

Soldberg & Hadland (1953), Dutheil (1959), Nair & Iya (1962) and Nair *et al.* (1965) used the skimmed fresh milks as antigens in the preparation of antisera and rendered the latter specific by absorption with the appropriate milk (Table 4). They used a microscope-slide coagulation technique and were able to detect 2% adulteration. This is to be compared with a detection of 5% adulteration achieved by the method described in this paper. However, when one considers the charge of fraudulent adulteration of milk, detection of one species in a mixture in a proportion as low as 2% is only of theoretical interest. The method used by these workers differs from the method described here in that it detects the casein component, not the lact-albumin–lactoglobulin fraction. Their methods are therefore of little use in the differentiation of fresh milk and reconstituted milk powder.

Adulteration of fresh milk with milk powders cannot be detected by any of these methods. The electrophoretic method appears to be the solution to this problem (Zimmermann, 1960).

It is of interest to note that, of the 233 samples serologically identified as fresh cow's milk by our method, the analytical figures for 135 were abnormal for cow's milk and fell just outside or within the range for buffalo's milk. The standards in this country for cow's milk are 3·5% fat and 8·5% SNF and the corresponding values for buffalo's milk are 7·0 and 9·0, respectively. Although there are no statutory standards in this country for goat's milk, on the average the fat and SNF values are in the region 5 and 9, respectively. The specific gravity and the serum-refraction values for all these abnormal samples were average and so were the percentages of inorganic salts of a few of the samples which were 'ashed'.

The abnormal figures for these cow's-milk samples could be accounted for either by faulty sampling of average-composition milks or by their being single-cow and not bulk-milk samples. The analyses of carefully sampled, individual cow's milks made by the Research Officer of the Government Milk Board, Ceylon, have shown that fat and SNF contents in the average buffalo range or higher is not incompatible with the above 135 samples being genuine cow's milk.

Table 4. *Comparison of various serological methods for identifying cow's, buffalo's, sheep's or goat's milk*

Method	Antigen for antiserum	Absorbent	Sample for test	Sensitivity of detection
Soldberg & Hadland (1953)	Skimmed cow's milk	Goat's milk	Skim-milk	2 % cow's in goat's milk
Dutheil (1959)	Skimmed cow's milk Skimmed goat's milk	Sheep's milk	Skim-milk	2 % cow's (goat's) in sheep's milk
Nair *et al.* (1965)	Skimmed buffalo's milk	Cow's milk casein (Merck)	Skim-milk	1–5 % buffalo's in cow's milk
As described in this paper	Blood sera proteins of ox, buffalo, goat and sheep	Heterologous blood sera	Whey	5 % of any one milk in any other

This work on the differentiation of cow's, buffalo's, goat's and sheep's milks finds a parallel in the recent investigations of Ambrosino *et al.* (1965), who used a micro-immunodiffusion technique in their study of the whey proteins of these animals. Such tests, however, require 15–24 h, whereas the method described here is rapid and gives results which are clear-cut and specific. A batch of 10 milks could be serologically identified in under 2 h, if acetic acid were added the previous day and souring completed overnight. It is considered that the success of this rapid technique is due mainly to the method of immunization, which results in the production of potent specific antisera.

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REFERENCES

AMBROSINO, C., LIBERATORI, J. & UBERTALLE, A. (1963*a*). *Ricerca scient.* II-B, **3**, 49.
AMBROSINO, C., LIBERATORI, J. & UBERTALLE, A. (1963*b*). *Ricerca scient.* II-B, **3**, 54.
AMBROSINO, C., LIBERATORI, J. & UBERTALLE, A. (1965). *Ricerca scient.* (In the Press.)
ASCHAFFENBURG, R. & SEN, A. (1963). *Nature, Lond.*, **197**, 797.
BORDET, J. *et al.* (1909). *Studies in Immunity*, translated by F. P. Gay. New York: John Wiley and Sons.
BRÜNING, A. & KRAFT, B. (1927). *Z. Unters. Lebensmittel*, **54**, 347.
CRAWFORD, L. V. & GROGAN, F. T. (1960). *Am. J. Dis. Child.* **100**, 581.
DUTHEIL, H. (1959). *15th Int. Dairy Congr.* **3**, 1521.
JOHKE, T., HAGEMAN, E. C. & LARSON, B. L. (1964). *J. Dairy Sci.* **47**, 28.
NAIR, P. G. & IYA, K. K. (1962). *Milchwissenshaft*, **17**, 477.
NAIR, P. G., MALIK, S. S. & SINGH, M. (1965). *Indian Dairym.* (In the Press.)
NUTTALL, G. H. F. (1904). *Blood Immunity and Blood Relationships.* Cambridge University Press.
PINTO, F. C. (1961). *Br. vet. J.* **117**, 540.
RAO, M. B. (1964). *Nature, Lond.*, **201**, 1217.
SEN, A. & SINHA, N. K. (1961). *Nature, Lond.*, **190**, 343.
SINGH, B. S., YADAV, P. C. & PATHAK, R. C. (1963). *Indian J. Dairy Sci.* **16**, 121.
SOLDBERG, P. & HADLAND, C. (1953). *13th Int. Dairy Congr.* **3**, 1287.
ZIMMERMANN, G. (1960). *Mitt. Geb. Lebensmittelunters. u. Hyg.* **51**, 151.

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A preliminary investigation of the importance of clostridia in the production of rancid flavour in Cheddar cheese

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Summary. Bacteriological examination of 15 pairs of Cheddar cheeses, of which one of each pair was made aseptically, showed that there was a relationship between the development of rancid flavour and the numbers of *Clostridium tyrobutyricum* present. Other species of clostridia, *Cl. beijerinkii*, *Cl. sporogenes* and *Cl. perfringens*, were also present but occurred only as spores. Conditions for the growth of *Cl. tyrobutyricum* appeared to be more favourable in the aseptically made cheese; it is suggested that the adventitious bacterial flora of the non-aseptically made cheese, which gains access during cheesemaking, has some inhibitory effect on the growth of this organism.

Whilst sweet cheese, such as Swiss, Edam and Russian varieties, is liable to spoilage by gas formation caused by multiplication of the lactate-fermenting clostridia such as *Clostridium tyrobutyricum* (van Beynum & Pette, 1935), this defect does not often occur in Cheddar cheese. It is probable that the rapid development of acidity, lower pH and higher salt concentration control clostridial growth in Cheddar cheese (Hirsch, McClintock & Mocquot, 1952). However, the absence of blowing does not preclude multiplication of the organisms and production of butyric acid; lactate-fermenting clostridia can grow without visible gas formation and butyric acid is always the main product of lactate fermentation (Ritter, Sahli, Schilt & Heuscher, 1963).

The rancid flavour which occasionally occurs as a defect in Cheddar cheese may be due to butyric acid production by micro-organisms. Hood & White (1931) were able to reproduce rancid flavour in 83 % of cheeses made from milk inoculated either with butyric acid bacteria or with material such as rancid cheese or silage known to contain these organisms. Castell (1942) isolated clostridia from a number of Cheddar cheeses and found that they had developed more quickly in rancid than in non-rancid cheeses. However, some attempts to induce rancid flavour by inoculating cheese milk with strains of *Cl. butyricum* previously isolated from rancid cheese were unsuccessful (Castell & Irvine, 1942). It has since been suggested (Goudkov & Sharpe, 1965) that only *Cl. tyrobutyricum* and not *Cl. butyricum* is able to multiply at the low pH of Cheddar cheese.

In order to obtain further information about the causes of rancid flavour some experimental Cheddar cheeses which developed a strong rancid flavour were investigated.

Some of the cheeses were made in an 'aseptic' vat (Mabbitt, Chapman & Sharpe, 1959) and these were found to contain much higher numbers of clostridia than the control cheese made under normal conditions. As lactobacilli are the main group of bacteria excluded in the 'aseptic' process of cheese-making, their interaction with clostridia was studied *in vitro*.

METHODS

Cheese. Fifteen pairs of 40-lb Cheddar cheeses, made on different occasions from March to June 1963, were examined. Single-herd milk heat-treated at 161 °F for 17 sec, and a single strain of starter, *Streptococcus cremoris* 924, was used throughout. One cheese of each pair was made in the 'aseptic' vat and the other, the control, was made under normal conditions. The 'aseptic' cheeses contained only starter organisms, and any aerobic or anaerobic spores, from the cheese milk, which had survived the heat treatment. The control cheeses also contained non-starter lactic acid bacteria and, occasionally, in later stages of ripening, propionibacteria which had entered the cheese during the making. The pH of all contol cheeses at 18 weeks was 5·0–5·1; the salt concentration was lower than normal, being 1·05–1·44%. Cheeses were examined for clostridia at 8–11 months maturation.

Sampling. Two bores of cheese were taken on each occasion and homogenates and serial dilutions prepared as described by Naylor & Sharpe (1958). The pH of the cheese was measured electrometrically.

Table 1. *Growth of different bacteria on selective medium containing polymyxin and neomycin*

Species of bacteria	No. of strains tested	Growth* on medium	Species of bacteria	No. of strains tested	Growth* on medium
Clostridium sporogenes	27	+	*Str. lactis*	1	−
Cl. tyrobutyricum	14	−	*Str. faecalis*	1	∓
Cl. perfringens	4	+	*Lactobacillus casei*	1	−
Cl. botulinum	2	±	*L. plantarum*	1	−
Cl. bifermentans	2	+	*Pediococcus cereviseae*	2	−
Cl. bifermentans	2	−	*Bacillus subtilis*	2	−
Cl. tertium	3	±	*B. licheniformis*	1	−
Cl. butyricum	3	−	*B. pumilis*	1	−
Cl. beijerinckii	3	−	*B. cereus*	1	−
Streptococcus cremoris	1	−	*B. lentus*	1	−
Str. diacetilactis	1	−			

* +, Growth as good as on RCMA (Hirsch & Grinstead, 1954); ±, growth slightly less than on RCMA; ∓, very strong inhibition; −, growth completely inhibited.

Isolation and enumeration of clostridia from cheese

Media. A selective medium (SMA) was developed for the isolation of vegetative cells and spores of proteolytic clostridia, consisting of: BBL Trypticase, 1·5%; yeast extract, 1%; oxoid no. 3 agar, 2%; neomycin, 50 μg/ml; polymyxin, 50 units/ml; pH before sterilization, 7·0. Sterile solutions of the antibiotics were added to the liquefied basal medium at 50 °C just before inoculation.

The growth of different types of organisms in this selective medium is shown in Table 1. The medium proved to be satisfactory for isolating spores and vegetative cells

of proteolytic clostridia from cheese, and other sources. Enterococci occasionally appeared on plating milk samples, but they could be distinguished readily by their colony appearance. Unfortunately, the saccharolytic clostridia *Cl. tyrobutyricum*, *Cl. butyricum* and *Cl. beijerinckii* were completely suppressed on this medium, and the non-selective RCM broth (RCMB) and agar (RCMA) of Hirsch & Grinstead (1954) had to be used, which also allowed the growth of lactic acid bacteria and aerobic sporeformers.

In the 'aseptic' cheeses non-starter lactic acid bacteria were absent and, since at the time of sampling the starter streptococci had died out, it was possible to enumerate both spores and vegetative cells of clostridia. On the other hand, for the control cheeses, only the spores of clostridia could be enumerated because the presence of large numbers of non-starter lactic acid bacteria necessitated heat treatment of samples before plating on RCMA.

Isolation and enumeration. For the 'aseptic' cheeses, dilutions of samples were plated on RCMA and SMA. The cheese homogenate was then heated to 78 °C for 10 min, dilutions were plated on RCMA and triplicate 1-ml amounts transferred to RCMB for estimation of most probable numbers. Samples of the control cheeses were treated similarly except that the unheated samples were not plated on RCMA. All plates were incubated anaerobically for 3 days at 37 °C.

Better germination and growth of the saccharolytic clostridia were obtained in RCM than in Robertson's cooked meat, Mossel's (1959) or Rosenberger's (1956) media. *Cl. tyrobutyricum* grew only very poorly in Mossel's medium, unfortified milk and in Robertson's cooked meat medium. Plate counts on RCMA were more satisfactory than dilution counts in RCMB because, in the broth, the more rapidly multiplying clostridia such as *Cl. perfringens* were likely to outgrow *Cl. tyrobutyricum*, which might require at least 3 days to reach the log phase of growth. It was also observed that clostridial growth was absent sometimes from low dilutions of cheese in RCMB, and present in high dilutions, probably because of growth suppression by aerobic sporeformers. Facultative aerobic sporeformers also appeared on RCMA in anaerobic conditions, but their colonies could be distinguished from obligate anaerobes by the benzidine test (Deibel & Evans, 1960), which was carried out at least 30 min after the plates were removed from the anaerobic jar.

Colonies from suitable plates were picked into RCMB for identification. If gas was produced in RCMB, dilutions were plated on to RCMA and representative colonies were picked for identification.

Identification of strains of clostridia. Bergey's scheme of classification (Breed, Murray & Smith, 1957) and the methods of Beerens, Castel & Put (1962) were followed.

The *basal medium* used for many of the tests consisted of: BBL Trypticase, 1·5 %; yeast extract, 1 %; cysteine, 0·05 %; pH of medium, 7·0.

Carbohydrate fermentation. To the basal medium 0·5 % Seitz-filtered carbohydrate was added. The extent of fermentation was determined after 10-days incubation by measurement of the fall in pH and production of gas.

Lactate fermentation. The medium used was prepared by adding 0·5 g Ca lactate and 0·5 g Na acetate/100 ml to the basal medium and adjusting the pH to 6·8. Ca lactate was found preferable to Na lactate (Bryant & Burkey, 1956). Fermentation was judged to have occurred when the pH rose to 8·0 or higher after 10-days incubation.

H_2S production. The basal medium containing 0·1 % glucose and 0·05 % ferric citrate was used. A positive reaction was indicated by blackening of the medium.

Nitrate reduction to nitrite. The basal medium containing 0·2 % glucose and 0·1 % KNO_3 was used. Reduction was detected by methods described in the *Manual of Microbiological Methods* (1957).

Gelatin liquefaction. The medium used was nutrient gelatin containing 0·1 % glucose and 0·05 % cysteine.

Litmus milk. The litmus milk contained 0·08 % cysteine.

Morphology and motility. These were studied in RCMB or in Robertson's cooked meat medium.

The standard inoculum for all tests was 0·1 % of a 1:10 dilution of an 18-h culture in RCMB or Robertson's cooked meat medium. All cultural and test media were boiled for 20–30 min before inoculation. Tests were incubated at 37 °C, aerobically, except for the lactate fermentation which was incubated anaerobically in order to increase the fermentation rate.

Interaction between lactic acid bacteria and Clostridium tyrobutyricum

RCMA, buffered with 1·0 % phosphate at pH 7·0 with bromocresol purple indicator added, was inoculated with 1 % of an actively growing RCMB culture of *Cl. tyrobutyricum* and plates poured. One loopful of an 18-h broth culture of lactic acid bacteria was spotted on to the surface, and plates were incubated anaerobically for 3 days. The inhibitory effect was then measured by the zone of inhibition of clostridial growth. The presence of the indicator and buffer made it unlikely that any inhibition observed was due to lactic acid. Two strains of clostridia were used as test organisms, one isolated from an experimental cheese, and the other obtained from Dr Pette, Ede, Holland. The effect of catalase on inhibition was tested by adding a solution of catalase (Boehringer Ltd.) to the cooled agar to give a final concentration of 10 μg/ml.

RESULTS

The physiological characteristics of the strains of clostridia isolated from cheese, and their identification, are shown in Table 2.

Types and numbers of clostridia in experimental cheeses

Table 3 shows that there is a close association between the presence and numbers of *Cl. tyrobutyricum* in the cheeses, the pH and rancid flavour. This species was found in 7 'aseptic' cheeses (2A, 3A, 4A, 5A, 6A, 10A and 12A), the pHs of which were always higher than 5·3, and in which pronounced rancidity occurred. Cheeses which did not contain this organism had a pH of less than 5·3 and no rancid flavour was detected. In all the 'aseptic' cheeses where *Cl. tyrobutyricum* was found, vegetative cells of the organism were present and in greater numbers than were spores, and in 2 of the cheeses, 6A and 12A, which were resampled after further periods of 4 and 10 weeks, respectively, the numbers of vegetative cells had increased. This multiplication was correlated with increased pH and more pronounced rancid flavour. In another cheese, 7A, where species other than *Cl. tyrobutyricum*, namely *Cl. sporogenes* and *Cl. beijerinckii*, were isolated, no rancidity was detected. In this case the pH was less

Table 2. *Morphological and biochemical characteristics used to identify clostridia isolated from Cheddar cheese*

Species of clostridia	Morphological appearance	Growth in litmus milk + 0·08 % cysteine	Motility	Liquefaction of gelatin	Production of H_2S	Production of nitrites	Fermentation: Glucose	Sucrose	Lactose	Mannitol	Salicin	Glycerol	Soluble starch	Ca lactate
Cl. tyrobutyricum	Thick rods varying from very short to long. Subterminal, ovoid spores, swelling the cells	Only slight reduction of litmus	+	−	−	+	+	−	−	+	−	W*	−	+
C. beijerinckii	Long, not very thick rods, straight and slightly curved. Spores large, oval, eccentric to subterminal	Stormy fermentation in 20–30 h	+	−	−	−	+	+	+	+	+	W	−	+
Cl. sporogenes	Average rods. Spores ovoid, subterminal, swelling the cells. Sporulate readily in RCMB	Slow coagulation, peptonization with putrefactive odour	+	+	+	−	+	−	−	−	−	W	−	−
Cl. perfringens	Short, very thick rods. Never sporulate in RCMB	Stormy fermentation in 10–18 h	−	+	+	+	+	+	+	−	−	+	+	−

* W = weak.

Table 3. *Numbers and species of clostridia and lactobacilli isolated from experimental Cheddar cheeses with and without a rancid flavour*

Cheese no.	Age of cheese* at sampling, months	pH	No. of clostridia × 10^{-2}/g cheese: Spores	No. of clostridia × 10^{-2}/g cheese: Spores and vegetative cells	Species of clostridia isolated	Species of lactobacilli previously isolated	Degree of rancidity in cheese†
‡2A	9·5	5·6	1000	2100	*tyrobutyricum*	–	+ + +
‡2B§	9·5	5·4	40	.	*tyrobutyricum*	*plantarum, brevis*	+ + +
3A	9·5	5·6	400	2000	*tyrobutyricum*	–	+ + +
3B§	9·5	5·4	3	.	*tyrobutyricum*	*plantarum*	+ +
4A	9·5	5·7	650	1700	*tyributyricum*	–	+ + +
4B§	9·5	5·6	17	.	*tyrobutyricum*	*plantarum*	+ + +
5A	9·0	5·6	42	69	*tyrobutyricum* + *sporogenes*	–	+ + +
5B	9·0	5·4	220	220	*sporogenes*	*brevis*	–
6A	9·0	5·35	1	9	*tyrobutyricum* + *perfringens*	–	+
6A	10·0	5·55	20	50	*tyrobutyricum*	–	+ + +
6B	9·0	5·05	< 1	.	–	*brevis*	–
6B	10·0	5·12	< 1	.	—	*brevis*	–
7A	9·0	5·1	2	2	*sporogenes, beijerinckii*	–	–
7A	10·0	5·2	1	.	*sporogenes, beijerinckii*	–	–
7B	9·0	5·05	1	.	*sporogenes, beijerinckii*	*brevis*	–
7B	10·0	5·2	1	.	*sporogenes, beijerinckii*	*brevis*	–
8A	9·0	5·1	< 0·1	< 0·1	–	–	–
8B	9·0	5·05	< 0·1	.	–	.	–
8AA	9·0	5·16	< 0·1	–	–	–	–
8BB	9·0	5·12	< 0·1	.	–	*brevis*	–
9A	9·0	5·1	< 0·1	–	–	–	–
9B	9·0	5·12	< 0·1	.	–	*brevis, streptobacteria*	–

Table 3 (*cont.*)

Cheese no.	Age of cheese* at sampling, months	pH	No. of clostridia × 10^{-2}/g cheese: Spores	No. of clostridia × 10^{-2}/g cheese: Spores and vegetative cells	Species of clostridia isolated	Species of lactobacilli previously isolated	Degree of rancidity in cheese†
10A	9·0	5·35	16	210	*tyrobutyricum*	—	+
10B	9·0	5·1	< 0·1	.	—	*brevis*, *casei*	—
11A	9·0	5·0	< 0·1	—	—	—	—
11B	9·0	5·12	< 0·1	.	—	*brevis*	—
12A	8·5	5·12	0·1	0·1	*tyrobutyricum*, *sporogenes*	—	—
12A	11·0	5·3	0·1	12	*tyrobutyricum*	—	++
12B	8·5	5·1	0·1	.	*tyrobutyricum*, *sporogenes*	*plantarum*	—
12B	11·0	5·05	0·1	.	*sporogenes*	*plantarum*	—
13A	9·0	5·1	< 0·1	—	—	—	—
13B	9·0	5·1	< 0·1	.	—	*brevis*	—
14A	9·0	5·1	< 0·1	—	—	—	—
14B	9·0	5·1	< 0·1	.	—	*brevis*	—
15A	9·0	5·1	< 0·1	—	—	—	—
15B	9·0	5·1	< 0·1	.	—	*brevis*	—

* Cheeses 2 and 3 were made in March; 4 and 5 were made in April; 6–12 were made in May; 13–15 were made in June.

† +++, Strong rancidity; ++, rancid; +, mild rancidity; —, no rancidity.

‡ A, Aseptic cheeses; B, control cheeses; ., not determined; —, not present.

§ Propionibacteria isolated in numbers of 10^5/g cheese.

Cheese no.	Addition to cheese milk	Sample examined	*No. organisms/ml milk or /g cheese: *Cl. tyrobutyricum* (spores)	*L. plantarum*	Starter *Streptococci*	pH of cheese	Flavour
IA	*Cl. tyrobutyricum* and 0·75 % salt	Inoculated milk	80	< 1	.	.	.
		Curd at milling	170	< 10	.	.	.
		Cheese: 10 days	140	< 10	2×10^8	4·9	.
		4 weeks	600	< 10	2×10^7	4·95	
		12 weeks	75	< 10	$7{\cdot}5 \times 10^3$	5·05	Normal
		23 weeks	130	< 10	$7{\cdot}4 \times 10^2$	4·8	
		32 weeks	120	< 10	$5{\cdot}8 \times 10^2$	4·95	
IB	*Cl. tyrobutyricum* and 1·5 % salt	Inoculated milk	80	< 1	.	.	.
		Curd at milling	170	< 10	.	.	.
		Cheese: 10 days	240	< 10	3×10^8	4·9	.
		4 weeks	150	< 10	6×10^6	4·9	
		12 weeks	120	< 10	7×10^2	5·1	Normal
		23 weeks	110	< 10	< 10	4·8	
		32 weeks	140	< 10	< 10	4·9	
IIA	*Cl. tyrobutyricum* *L. plantarum* and 0·75 % salt	Inoculated milk	40	4×10	.	.	.
		Curd at milling	93	4×10^3	.	.	.
		Cheese: 10 days	110	$> 10^5$	$8{\cdot}3 \times 10^8$	5·1	.
		4 weeks	115	2×10^8	$3{\cdot}1 \times 10^8$	4·95	
		12 weeks	75	6×10^7	3×10^5	4·95	Normal
		23 weeks	130	1×10^7	3×10^5	4·7	
		32 weeks	80	$1{\cdot}4 \times 10^6$	$3{\cdot}3 \times 10^4$	4·95	
IIB	*Cl. tyrobutyricum*, *L. plantarum* and 1·5 % salt	Iinoculated milk	40	4×10	.	.	.
		Curd at milling	93	4×10^3	.	.	.
		Cheese: 10 days	110	$> 10^5$	$6{\cdot}2 \times 10^8$	5·0	.
		4 weeks	40	2×10^8	$3{\cdot}2 \times 10^8$	4·9	
		12 weeks	85	$6{\cdot}7 \times 10^7$	6×10^4	5·15	Normal
		23 weeks	130	$1{\cdot}5 \times 10^7$	$2{\cdot}6 \times 10^4$	4·85	
		32 weeks	105	$2{\cdot}9 \times 10^6$	$1{\cdot}3 \times 10^5$	5·0	
III	*Cl. tyrobutyricum*, *L. plantarum*, 0·75 % salt, and catalase (20 μg/ml milk)	Inoculated milk	40	1×10^3	.	.	.
		Curd at milling	93	9×10^4	.	.	.
		Cheese: 10 days	70	1×10^8	$7{\cdot}7 \times 10^8$	4·8	.
		4 weeks	1×10^4	2×10^8	1×10^7	5·15	Sweet, sl. rancid

than 5·3, and no vegetative cells of these species were found; 4 weeks later no increase in the numbers had occurred.

In the control cheeses, the vegetative cells of *Cl. tyrobutyricum* could not be enumerated because their presence was masked by the non-starter lactic acid bacteria in the unheated samples. However, *Cl. tyrobutyricum* was found in heated samples from 3 control cheeses which had a rancid flavour (2B, 3B and 4B) and in each case the pH was more than 5·3. Cheese 12B contained small numbers of *Cl. tyrobutyricum* + *Cl. sporogenes* (10/g cheese) but the pH was 5·1, and it did not develop any rancidity. After a further 10 weeks only *Cl. sporogenes* was detected, showing that in this case the very small numbers of *Cl. tyrobutyricum* had not multiplied. In cheese 5B large numbers of *Cl. sporogenes* (22 000/g) were found, but were not accompanied by a high pH or rancidity. Propionibacteria were detected in cheeses 2B, 3B and 4B in numbers of about 105/g.

Conditions for development of *Cl. tyrobutyricum* appeared to be more favourable in the 'aseptic' cheeses than in the control ones: cheeses 5B, 6B, 10B and 12B were normal whilst the corresponding 'aseptic' cheeses had a strong rancid flavour. In cheeses 2B, 3B and 4B the numbers of spores were lower than in the corresponding 'aseptic' cheeses, and the rancid flavour developed much later in maturation. Each pair of cheeses was made from the same batch of heat-treated milk so that the initial number of spores must have been the same within each pair of cheeses. As an important difference between them was the presence of non-starter lactic acid bacteria in the controls, the effect of these organisms on the growth of *Cl. tyrobutyricum* was studied.

Interaction between Cl. tyrobutyricum and lactic acid bacteria isolated from control cheeses

The lactic acid bacteria isolated from the control cheeses consisted of strains of lactobacilli and pediococci. Table 3 shows the species of lactobacilli which occurred in the different cheeses. All the 12 strains of *Lactobacillus plantarum* tested, and the one strain of *L. casei*, formed marked zones of inhibition on RCMA plates seeded with *Cl. tyrobutyricum*. Seven of the strains of *L. brevis* prevented growth to a lesser extent. None of the other lactobacilli nor any of the strains of pediococci were inhibitory. Catalase (10 μg/ml) reversed the inhibitory effect of the lactobacilli completely, indicating that the production of H_2O_2 was responsible for the inhibition. As the lower salt content in the experimental cheeses may also have encouraged the growth of the clostridia, further cheese trials were undertaken to observe the effect of these factors on the growth of *Cl. tyrobutyricum*.

Effect of salt concentration and the presence of lactobacilli and of catalase on the growth of Cl. tyrobutyricum in Cheddar cheese

Five cheeses were made in the 'aseptic' vat, the milk being inoculated with 40–80 spores of *Cl. tyrobutyricum*/ml milk. One pair of cheeses was salted at 1·5% (the normal concentration). Another cheese received only 0·75%, while the remaining pair, although salted at 1·5% and 0·75%, respectively, were made from milk which was inoculated with a strain of *L. plantarum* previously shown to inhibit the growth of *Cl. tyrobutyricum*, *in vitro*. Lactobacilli and 20 μg catalase/ml were added to the milk for the fifth cheese (0·75% salt). The numbers of spores of *Cl. tyrobutyricum* in

these cheeses are shown in Table 4. Contrary to our previous findings, the lower concentration of salt and the absence of lactobacilli did not result in an increase in numbers of clostridia. Only the cheese with catalase showed an approximately 100-fold increase in the numbers of *Cl. tyrobutyricum* at 4 weeks together with the typical rancid flavour and increased pH found in the other series of cheeses. Further increases of 100-fold in numbers and in intensity of rancidity occurred during the next 5 months.

DISCUSSION

The marked association between rancidity and the presence of large numbers of *Cl. tyrobutyricum* in a series of Cheddar cheeses fortuitously contaminated with this organism strongly suggests that this organism can be responsible for rancidity in Cheddar cheese. This conclusion was supported by the finding that the same rancid flavour accompanied the multiplication of *Cl. tyrobutyricum* in a cheese made from milk deliberately contaminated with it, whereas rancidity did not occur in others in which there was no multiplication of clostridia.

There was no evidence that the other species of clostridia present multiplied in the cheese. The numbers of *Cl. beijerinckii*, *Cl. sporogenes* and *Cl. perfringens* were the same before and after heat treatment of the samples, indicating that only spores were present, and there was no increase on resampling the cheeses at a later period. Cheddar cheese does not appear to be suitable for their development. *Cl. tyrobutyricum* was able to multiply in the Cheddar cheese probably because of its tolerance of a low pH (Galesloot, 1961; Kutzner, 1963).

In the first series of cheeses, the contamination by *Cl. tyrobutyricum* must have come from the milk. Usually, milk contains only small numbers of this species; only one of 14 samples of milk examined by us contained more than one spore of *Cl. tyrobutyricum*/10 ml. Davis (1955) states that rancid flavour is detected most frequently in Cheddar cheese made during the spring, whilst Peterssen (1961) observed that the largest number of clostridial spores were present in milk during February and March, and decreased from May onwards. Most of our rancid-flavoured cheese was made in March and April, whilst cheese made later in the year was free from this fault. Only small numbers of spores may be needed initially to cause the development of rancid flavour in the cheese under the right conditions. In cheese 12A, no off-flavour was observed at 9·5 months when only 10 spores/g were found in the cheese, but at 11 months, when 1200 vegetative cells/g were found, rancid flavour had developed.

The results of a cheese trial in which the milk was inoculated with clostridia were unexpected, in that neither lower salt concentration nor absence of lactobacilli led to increased multiplication of the organisms. The conditions differed from the previous cheese-making in that heated spores were added to the heat-treated milk. In the first series, the naturally occurring clostridia must have been heated in the milk and this may have resulted in conditions more likely to stimulate germination. However, the rapid multiplication in the cheese containing catalase suggests that inhibitory peroxides other than that produced by lactobacilli were present in the cheeses. It thus appears that salt concentration and the presence of lactobacilli are not the only factors inhibiting clostridia in this type of cheese.

The development of *Cl. tyrobutyricum* in 3 of the control cheeses 2B, 3B and 4B may have been due partially to the presence of propionibacteria, which were detected only in these cheeses. Propionibacteria stimulate the growth and gas production of *Cl. tyrobutyricum* (Swartling & Lindgren, 1960; Hunter & Frazier, 1961), and also produce catalase which might reverse the inhibitory effect of peroxide.

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REFERENCES

Beerens, H., Castel, M. M. & Put, H. M. C. (1962). *Annls. Inst. Pasteur, Paris*, **103**, 117.
Beynum, J. van & Pette, J. W. (1935). *Zentbl. Bkt. ParasitKde* (II. Abt.), **93**, 198.
Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957). *Bergey's Manual of Determinative Bacteriology. London*: Baillière, Tindall and Cox.
Bryant, M. P. & Burkey, L. A. (1956). *J. Bact.* **71**, 43.
Castell, C. H. (1942). *Scient. Agric.* **23**, 131.
Castell, C. H. & Irvine, O. R. (1942). *Scient. Agric.* **23**, 176.
Davis, J. G. (1955). *A Dictionary of Dairying*. London: Leonard Hill.
Deibel, R. H. & Evans, J. B. (1960). *J. Bact.* **79**, 356.
Galesloot, Th. E. (1961). *Neth. Milk Dairy J.* **15**, 263.
Goudkov, A. V. & Sharpe, M. E. (1965). *J. appl. Bact.* **28**, 63.
Hirsch, A. & Grinsted, E. (1954). *J. Dairy Res.* **21**, 101.
Hirsch, A., McClintock, M. & Mocquot, G. (1952). *J. Dairy Res.* **19**, 179.
Hood, E. G. & White, A. H. (1931). *Bull. Dep. Agric. Dom. Can.* no. 146.
Hunter, J. E. & Frazier, W. C. (1961). *J. Dairy Sci.* **44**, 2176.
Kutzner, H. J. (1963). *Zentbl. Bakt. ParasitKde* (I. Abt. Orig.), **191**, 441.
Mabbitt, L. A., Chapman, H. R. & Sharpe, M. E. (1959). *J. Dairy Res.* **26**, 105.
Manual of Microbiological Methods (1957). McGraw-Hill Book Co. Inc.
Mossel, D. A. A. (1959). *J. Sci. Fd Agric.* **10**, 662.
Naylor, J. & Sharpe, M. E. (1958). *J. Dairy Res.* **25**, 92.
Peterssen, I. (1961). *Svenska Mejeritidn.* **53**, 329.
Ritter, W., Sahli, K. W., Schilt, P. & Heuscher, E. (1963). *Schweiz. Milchztg*, **89**, Nr. 47, Wissenschaft. Beil. **91**, 723.
Rogosa, M., Mitchell, J. A. & Wiseman, R. F. (1951). *J. dent. Res.* **30**, 682.
Rosenberger, R. F. (1956). *J. appl. Bact.* **19**, 173.
Swartling, P. & Lindgren, B. (1960). *Rep. Milk Dairy Res. Alnarp*, 62.

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The para-caseinate–phosphate complex of washed rennet curd: its composition and its decomposition by water, sodium chloride solutions, and lactic acid solutions

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Summary. Washed rennet curd releases significant quantities of calcium phosphate and relatively small quantities of calcium para-caseinate when shaken with water. Treatment with small quantities of sodium chloride in aqueous solution similarly differentiates between these 2 components, but larger quantities attack both phosphate and para-caseinate with equal severity. A more extensive release of Ca and inorganic P occurs when the curd is treated with lactic acid solutions; the comparative ease with which Mg is removed under these conditions suggests that Mg is present as magnesium citrate.

Research into the nature of the colloidal complex of milk has frequently involved analysis of milk and of whey produced therefrom, since whey has been accepted as representing the soluble phase. Indeed, for this purpose whey compares very favourably with milk diffusates and ultrafiltrates (Davies & White, 1960; deMan, 1962). That rennet curd might serve as a convenient source of the colloidal complex has not found such general acceptance. No doubt much of this hesitance is due to the recognition that rennet brings about a cleavage of the complex. However, as the concentration of soluble Ca and inorganic P are unaffected when rennin acts on milk, the colloidal Ca and inorganic P are also unaffecred, in quantity if not in location. Some rearrangement of the complex may be expected in view of the selective attack by rennet upon the κ-casein component. Such rearrangement would be inevitable if the colloidal phosphate is attached to κ-casein (Pyne & McGann, 1962).

The object of the present work was to examine the behaviour of the Ca para-caseinate–Ca phosphate complex towards water, salt solutions, and dilute lactic acid, in the hope that some light might be thrown on the nature of the association between the phosphate and para-caseinate; information relevant to cheese-making might also result.

Methods

Preparation of rennet curd

To 1 l separated milk at 35 °C, 1 ml of rennet extract was added. When sufficiently firm, the coagulum was cut into small cubes and the whey decanted through butter muslin in a Büchner funnel. When no more whey separated, the curd was again cut and any further quantities of whey passed through the filter. This was repeated until no more whey could be obtained. The curd was then cut into pieces approximately $\frac{1}{3}$ cm diam., stirred with 1 l of distilled water, allowed to settle, and the supernatant

liquid passed through the filter. The washing was repeated with a further litre of water and the curd transferred completely to the filter. By gentle pressure and suction it was dried as much as possible, cut into small fragments, and stored at 0 °C in a wide-mouth stoppered bottle to which a few drops of chloroform had been added. The mean moisture content of the washed curd was 22·6 % and ranged from 20·5 to 24·8 %.

Preparation of water, brine and lactic acid extracts of the curd

Known weights of the chloroform-preserved curd of known moisture content were shaken with measured volumes of solvent. Mechanical end-over-end shaking was employed for periods of 3–4 h except where otherwise stated. After overnight storage in the cold room, the samples were centrifuged at 2500 rev/min for 30 min. The supernatant liquid was then removed for analysis.

Titratable acidity

To overcome the difficulty of titrating acid solutions containing calcium and phosphate ions, all acid extracts were treated with neutral saturated potassium oxalate solution before titration to the phenolphthalein end point.

Determination of Ca, Mg and inorganic P

Owing to the presence of protein in the curd extracts it was necessary to carry out these determinations on the filtrates after precipitation with trichloroacetic acid (final concentration 12 %). Ca was precipitated as oxalate and determined with 0·05 N-$KMnO_4$. Addition of ammonium phosphate and ammonia precipitated $MgNH_4PO_4.6H_2O$ from the calcium-free filtrate. After filtration the precipitate was dissolved in dilute HNO_3 and re-precipitated with ammonium phosphate and ammonia. The washed precipitate was finally dissolved in dilute HNO_3 and the P determined colorimetrically as vanadium phosphomolybdate. Inorganic P was determined on the 12 % trichloracetic acid filtrates by the same colorimetric method. On several occasions the yellow solution was turbid and it was then necessary to centrifuge and use the clear supernatant in the absorptiometer.

Determination of protein

The method of Rowland (1938) was employed except that ammonia was absorbed by boric acid solution and titrated with 0·02 N-HCl using methyl red–methylene blue indicator.

RESULTS

The composition of washed rennet curd

In the washing of the curd about 80 ml distilled water/g dry matter were employed —a volume which was considered adequate for the removal of entrained whey. Nevertheless, it was considered desirable to obtain experimental confirmation of this conclusion. The moisture content of the unwashed curd enabled an assessment of the quantity of entrained whey to be made. Analyses of the whey and of the 2 wash liquors showed that the first wash removed 85 % of entrained whey solids, and that the second removed 16 %. Two washings were therefore necessary for complete

removal of whey solids, but at the same time it was found that about 1·4 mg Ca/g and 1·35 mg inorganic P/g were dissolved from the curd. That such losses of Ca and inorganic P may occur during washing is evident from Table 1. While the values for Ca and P are in agreement with those of Monib (1962), both series are lower than those of the milk complex as determined by White & Davies (1958). These results led to a more detailed examination of the action of water on the washed curd.

Table 1. *The composition of washed rennet curd compared with that of the caseinate–phosphate of milk*

(Mg/g dry matter)

	Washed rennet curd				Caseinate–phosphate complex of milk (White & Davies, 1958), 12 samples	
	Present values, 9 samples		Monib (1962), 9 samples			
	Mean	Range	Mean	Range	Mean	Range
Ca	29·9	28·0–32·9	30·3	29·7–32·2	31·5	28·2–34·2
Inorg. P	10·2	9·3–11·6	10·3	9·3–11·0	11·4	10·2–12·2
Mg	1·4	1·3–1·5	—	—	1·7	1·5–1·8
Ratio Ca/P	2·93	—	2·94	—	2·77	—

The solubility of the para-caseinate–phosphate complex in water

Table 2 shows that the washed curd is further depleted of Ca, inorganic P, and protein by shaking with water. The quantities thus removed are clearly dependent upon the time of contact and the volume of water per g dry matter. It is noteworthy that the ratio Ca/inorganic P in the extract is less than that of the curd and less than that of the calcium phosphate of the caseinate–phosphate complex of milk (1·7), as determined by White & Davies (1958). The ratio mineral matter/protein in the extract is much greater than the curd value. That the quantities of Ca and inorganic P removed from the curd diminish with successive treatments suggests that the process of aqueous solution is concerned especially with the more easily accessible locations of calcium phosphate within the curd.

The mean concentration of Ca, inorganic P, and protein of all aqueous extracts was 4·7, 3·5, and 75 mg/100 ml, respectively. A saturated solution of laboratory tricalcium phosphate contained 1·78 mg Ca and 1·95 mg P/100 ml. The corresponding values for dicalcium phosphate were 2·37 and 2·26, respectively. The greater concentrations of the curd extracts were due, at least in part, to the presence of protein. A highly concentrated extract was prepared by treatment of the curd with the smallest practicable quantity of water (17 ml/g dry matter). This extract contained 12·2 mg Ca, 9·8 mg inorganic P and 210 mg protein/100 ml. Moreover, it possessed considerable sequestering powers for further additions of Ca and P. It was found possible to add 31·6 mg Ca and 18·6 mg P/100 ml without a precipitate appearing over a period of 5 days, the pH of the final solution being 6·8.

The solubility of the para-caseinate–phosphate complex in sodium chloride solutions

Table 3 gives the results of 7 trials in which the curd was shaken for 3–4 h with the salt solution. The influence of the volume of salt solution and the weight of NaCl

per g of dry matter on the amounts of Ca, inorganic P, and protein extracted from the curd is clear. Trial 7 indicates that the extraction of protein is particularly sensitive to variation in the volume of extracting solution. Monib (1962) observed that 0·85 N-NaCl had a strong peptizing effect on the protein of washed rennet curd, particularly where more than 50 ml/g dry matter was used. The ratio Ca/P in the extract of trial 1 suggests that calcium phosphate accounts for most of the dissolved mineral matter. In the other trials, where the weight of NaCl or the volume of solution were much greater, the higher Ca/P ratio indicates an additional solublization of Ca from calcium para-caseinate, presumably by a process of base exchange. In trial 1, successive treatments with salt solution extracted diminishing quantities of Ca and inorganic P. The more rapid decline of inorganic P suggests that, as with aqueous extraction, the first treatment is mainly concerned with the more easily accessible phosphate. A comparison of Tables 2 and 3 shows that, at comparable levels of the solvent/curd ratio, appreciably more Ca, inorganic P and protein are dissolved by salt solutions.

Table 2. *The aqueous extraction of Ca, inorganic P, and protein from washed rennet curd*

Curd sample	Treatment	Ml water/g dry matter	Amount extracted, mg/g dry matter			Ratio Ca/P
			Ca	Inorg. P	Protein	
1	Cold room, 48 h	42·5	2·1	1·5	43·0	1·40
		82·8	2·2	1·8	53·4	1·22
2	Shaken 6 h	50·0	2·5	2·1	29·5	1·19
	Shaken 24 h	50·0	3·3	2·3	35·0	1·43
3	Shaken 5 h	48·4	2·6	2·1	29·2	1·24
	Shaken 5 h, left 18 h in cold room	48·4	2·9	2·2	42·2	1·32
4	3 successive shakings for	62·7	2·1	1·6	48·0	1·31
	24 h using fresh water	62·7	1·9	1·3	45·0	1·46
	each time	62·7	1·4	0·9	33·6	1·55
14	5–48 h contact samples	17–120	2·4*	1·9*	41·4*	1·26*

* Mean values.

Table 3. *The extraction of Ca, inorganic P and protein from washed rennet curd by sodium chloride solutions*

Curd sample	Ml solution/g dry matter	Concentration of NaCl, moles/l	NaCl, g/g dry matter	Mg extracted/g dry matter			Ratio Ca/P extracted
				Ca	P	Protein	
1 1st treatment	21·3	0·15	0·18	5·8	3·2	29·4	1·79
1 2nd treatment	21·3	0·15	0·18	4·4	2·1	41·9	2·08
1 3rd treatment	21·3	0·15	0·18	4·0	1·6	44·1	2·48
2	37·8	3·21	7·10	10·7	3·1	—	3·44
3	40·2	6·00	14·10	10·1	3·2	—	3·16
4	66·7	3·24	12·60	15·7	5·7	—	2·73
5	389·0	0·10	2·30	15·9	5·3	—	3·02
6	389·0	0·20	4·60	18·1	6·1	—	2·95
7*a*	52·0	1·98	6·0	—	—	52·1	—
7*b*	114·0	0·92	6·0	—	—	160·0	—
7c	211·0	0·49	6·0	—	—	295·0	—

Decomposition of the para-caseinate–phosphate complex by dilute lactic acid solutions

The extraction of Ca and inorganic P from curd by brine involves no chemical reaction other than replacement of para-caseinate Ca by Na. Lactic acid, however, may react as follows: (*a*) conversion of the basic and neutral calcium phosphates to $Ca(H_2PO_4)_2$ and calcium lactate; (*b*) conversion of calcium para-caseinate to calcium lactate with an equivalent increase in the acid groups of the protein; (c) combination with iso-electric protein to give (Prot. NH_3^+ COOH)Lact'.

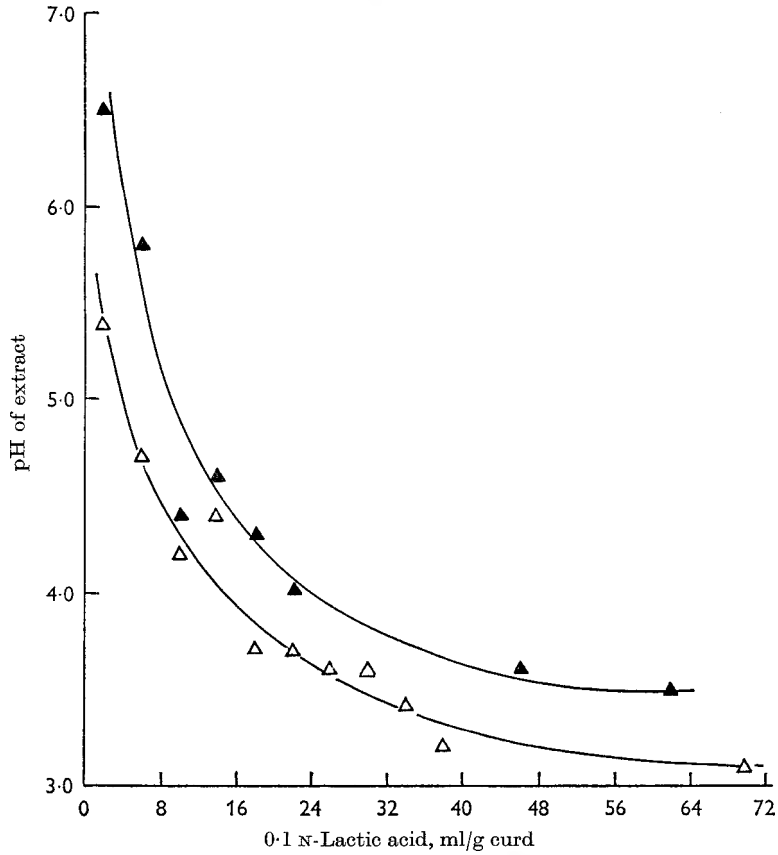

Fig. 1. The change in pH of the curd extract with the amount of lactic acid in the solution used for extraction. Total volume: △, 50 ± 22 ml/g curd; ▲, 400 ± 6 ml/g curd.

Figures 1–5 summarize the information obtained from this investigation. In view of the previously observed effects of the volume of solvent on the extraction of mineral matter from the curd (see also Monib, 1962), the values are presented for both moderate and higher volumes of added solvent.

Acidity of the lactic acid extracts

Figure 1 illustrates the strong buffer action of the complex in the region of pH 3–4. Some neutralization of added lactic acid may be expected so long as any accessible phosphate groups remain in the curd. If the protein remains undissolved, the acid

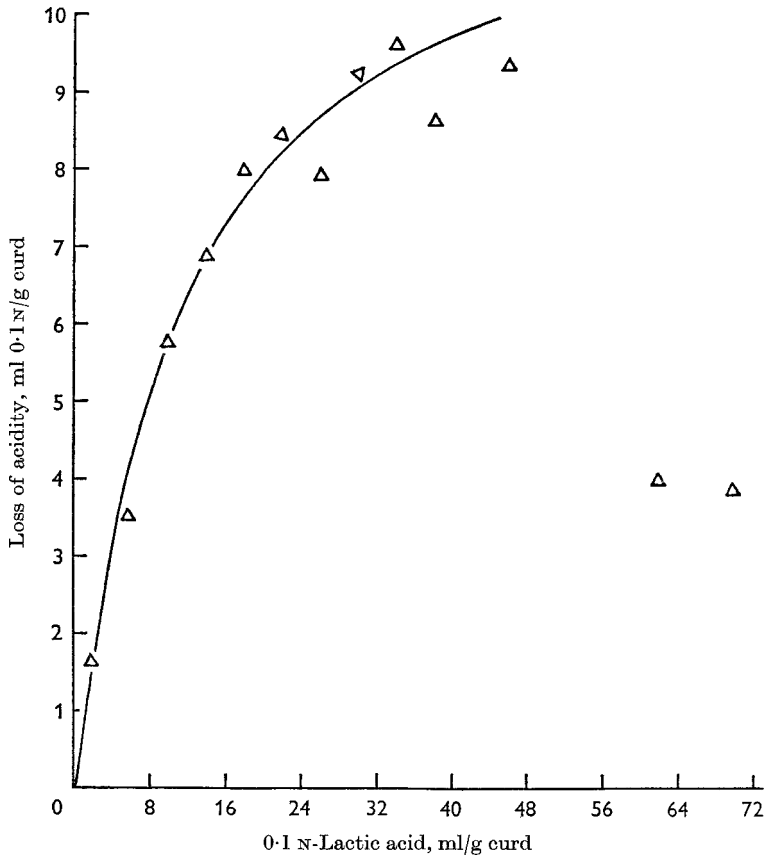

Fig. 2. Neutralizing capacity of washed rennet curd with different concentrations of lactic acid.

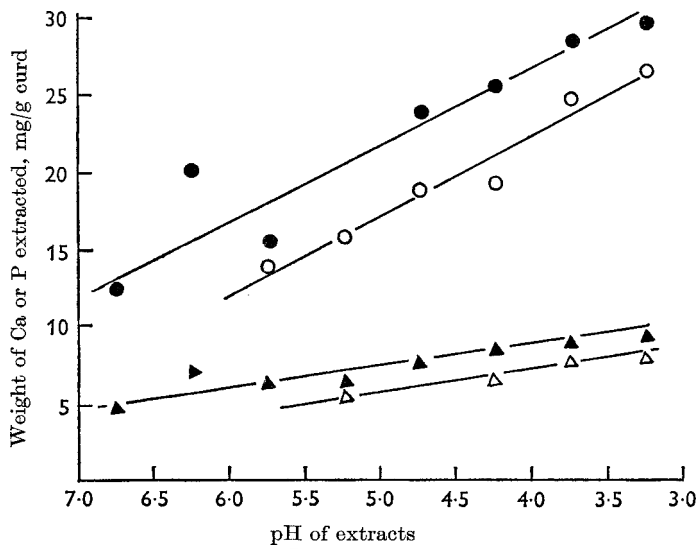

Fig. 3. Ca and P extracted from washed rennet curd at different final pHs of extract. ○, Ca; △, P; total volume 50 ml ± 22/g. ●, Ca; ▲, P; total volume 400 ml ± 69/g.

extracts will show further reductions of acidity due to reactions (*b*) and (*c*). These effects are illustrated in Figs. 1 and 2, in which the loss of acidity curve is almost linear up to the region of the isoelectric point. Beyond this region, the acid protein enters the solution in increasing quantities (Fig. 5), thereby eliminating or reducing effects (*b*) and (*c*). These considerations assume practical significance in cheese-making, where increases in whey acidity may be employed to assess the progress of

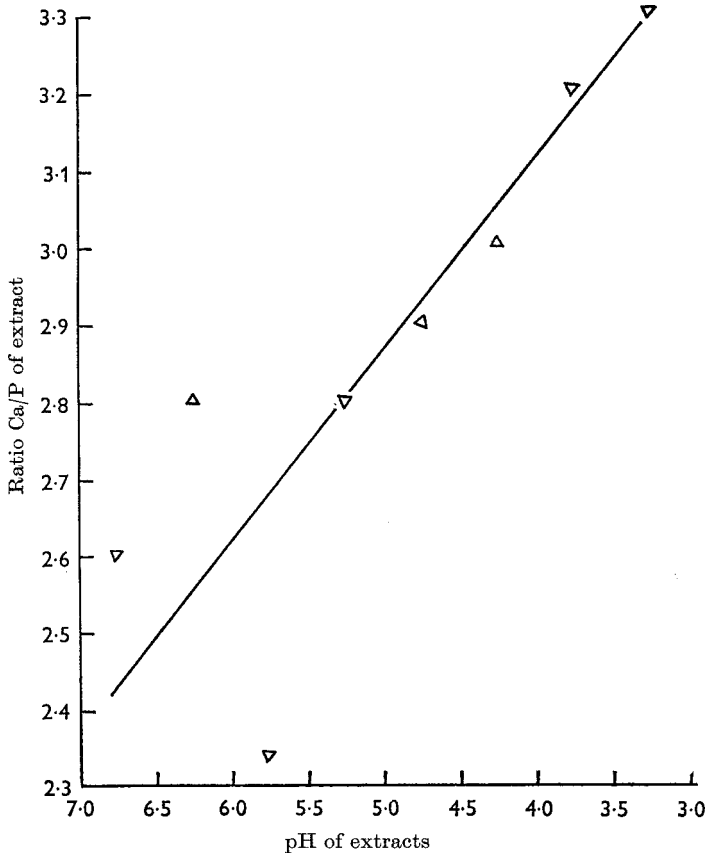

Fig. 4. pH and ratio Ca/P of curd extracts.

lactic acid development. Obviously, such increases represent only part of the total acid development. A complete assessment would require analyses of both curd and whey. Similar considerations would apply to studies of lactic acid development in milk and liquid milk products involving analyses of the soluble phase only.

Ca, Mg and inorganic P extraction by lactic acid

The sensitivity of the para-caseinate–phosphate complex towards added acid is manifest in Figs. 1, 3 and 5. Addition of as little as 2 ml 0·1 N-lactic acid brought into solution 15·2 mg Ca/g and 5·3 mg inorganic P/g for the lower dispersion and 13·9 mg Ca/g and 5·3 mg inorganic P/g for the higher. These values represent an extraction efficiency of approximately 50 %. At the same pH value the more dilute suspensions remove 15–25 % more Ca, and 18–33 % more inorganic P than the less dilute ones.

Figure 4 shows that only in the region of the isoelectric point was the ratio of extracted Ca/inorganic P equal to that of the curd (2·93). At higher pH values corresponding to relatively small additions of lactic acid the ratio was less, again indicating that calcium phosphate is more easily attacked than calcium para-caseinate. The higher Ca/inorganic P ratio at lower pH values may be due to the cationic protein removing from solution negatively charged PO_4 ions or complex ions of the type

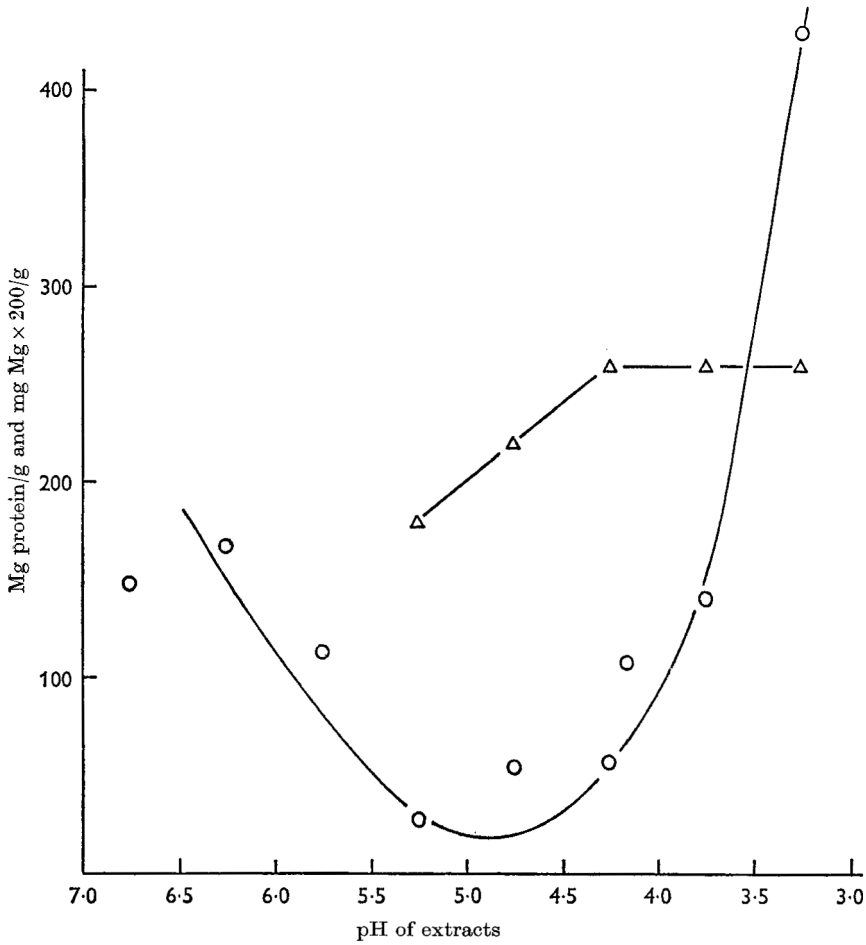

Fig. 5. pH of extracts of curd and amount of protein (○) or magnesium (△) extracted.

$Ca(HPO_4)_2''$. This would also explain why 100 % extraction of Ca or P was rarely achieved. Monib (1962), working with freeze-dried rennet curd, also reported values of extracted Ca and inorganic P the ratio of which increased from 2·4 to 3·5 with increasing additions of lactic acid. Belousov (1959) observed that for pH values of 5·5 upwards the solvent action of lactic acid was almost entirely confined to the phosphate. Mg was withdrawn from the curd with much greater ease than Ca or P (Fig. 5), being complete at pH 4·25, at which level only 70 % Ca and 68 % P were extracted.

In Table 4 a comparison is made at comparable pH values between the extraction of Ca and inorganic P from washed rennet curd and from the caseinate–phosphate

complex of milk. Except at the higher pH values these 2 constituents are withdrawn in greater quantities from the milk complex. At pH 5·87 the citrate, phosphate and calcium ions of the milk serum would impose restrictions on the entry of Ca and P from the colloidal phase. These impediments being absent in the curd suspensions, a higher extraction rate is possible. However, Belousov (1959), working with air-dried rennet curd, observed consistently higher values for milk within the range pH 4–6.

Table 4. *Extraction at comparable pH values of Ca and inorganic P from the para-caseinate–phosphate complex and from the caseinate–phosphate complex of milk (mg/g complex)*

	Ca				Inorg. P			
pH ...	5·87	5·31	4·89	4·58	5·87	5·31	4·89	4·58
Milk	7·1	16·9	23·9	28·9	2·7	6·1	9·0	9·9
Curd	13·0	15·7	17·8	19·1	4·5	5·5	6·0	6·6

DISCUSSION

The aqueous extracts of washed rennet curd contain Ca and P dissolved from the phosphate and only small amounts of dissolved calcium para-caseinate. Taking the mean protein extracted as 41·4 mg/g (Table 2), the equivalent of Ca as para-caseinate is 0·5 mg (White & Davies, 1958) leaving 1·9 mg/g Ca that must be derived from phosphate. The extracted phosphate therefore had a Ca/inorganic P ratio of 1·0. Saturated aqueous solutions of tricalcium phosphate were found to contain Ca and P in the ratio 0·91. The neutral dicalcium phosphate gave a saturated solution in which the ratio was 1·05. These considerations, together with the fact that the dissolved Ca and P showed no constant numerical relationship to dissolved protein, leads to the conclusion that the phosphate in the para-caseinate–phosphate complex is in a condition which enables it to dissolve independently of the caseinate. The data from sodium chloride treatment add further support to this view.

Successive treatments of the same curd with water or small amounts of sodium chloride in dilute solution provide evidence for the view that the initial action is mainly concerned with the more accessible phosphate locations within the curd. Small additions of acid also show some discrimination between the phosphate and caseinate. Mulder & Schipper (1959, 1962) consider that the colloidal calcium phosphate of milk is present as an amorphous phosphate adsorbed on calcium caseinate. The evidence of the present work suggests that colloidal calcium phosphate is similarly adsorbed on the calcium para-caseinate of rennet curd.

The comparative ease with which magnesium is extracted (Fig. 5) suggests that it may be present in the curd as magnesium citrate as suggested by Verma & Sommer (1957). The mean Mg content of washed rennet curd was 1·4 mg/g (Table 1). This is equivalent to 7·38 mg citric acid, a figure which agrees with the value 7·3 for the caseinate–phosphate complex of milk as determined by White & Davies (1958).

REFERENCES

BELOUSOV, A. P. (1959). 15*th Int. Dairy Congr.* **2**, 662.
DAVIES, D. T. & WHITE, J. C. D. (1960). *J. Dairy Res.* **27**, 171.
DEMAN, J. M. (1962). *J. Dairy Res.* **29**, 279.
MONIB, A. M. M. F. (1962). *Meded. LandbHoogesch. Wageningen*, **62** (10), 1.
MULDER, H. & SCHIPPER, C. J. (1959). *Neth. Milk Dairy J.* **13**, 137.
MULDER, H. & SCHIPPER, C. J. (1962). 16*th Int. Dairy Congr.* B, 529.
PYNE, G. T. & MCGANN, T. C. A. (1962). 16*th Int. Dairy Congr.* B, 611.
ROWLAND, S. J. (1938). *J. Dairy Res.* **9**, 42.
VERMA, I. S. & SOMMER, H. H. (1957). *J. Dairy Sci.* **40**, 331.
WHITE, J. C. D. & DAVIES, D. T. (1958). *J. Dairy Res.* **25**, 236.

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The effect of varying the interval between milkings on milk secretion

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Summary. The effect of 6-, 12-, 18-, 24-, 30- and 36-h milking intervals on milk secretion has been investigated. The effect of the previous milking intervals was eliminated by interposing a recovery period consisting of 12-h milking intervals between the experimental intervals. Bias due to the carry-over of residual milk was reduced by removing the residual milk at the end of each milking, after an injection of oxytocin.

The rate of secretion of milk and of the individual constituents decreased curvilinearly, with duration of the interval, but the degree of curvilinearity differed between constituents. The rates of decrease were in the following increasing order: sodium, chloride, fat, whey proteins, casein N, water, non-protein N, lactose and potassium. The effects of the treatments persisted for some time after the end of the experimental intervals, but the original rates of secretion were regained by the end of the recovery period. The differing effects on the secretion of individual constituents resulted in increases in the concentrations of sodium, chloride and whey proteins in the milk and decreases in those of lactose and potassium.

The earliest work on the rate of secretion of milk was carried out by Ragsdale, Turner & Brody (1924), who concluded that there was a progressive decline in the rate of milk secretion with increase in the length of milking interval, and that there was an even more marked effect on the secretion of fat. In the interpretation of the results the authors assumed that the milk obtained at a milking was that which had been secreted in the previous interval. Johansson (1940) demonstrated, however, that there is a considerable carry-over of milk and of fat from one milking interval to the next. He found that the carry-over of residual fat was in direct proportion to the amount of milk in the udder before milking. This was confirmed by Bailey, Clough & Dodd (1955), and Turner (1953, 1955) showed that it was true also for residual milk. In the type of experiment devised by Ragsdale *et al.* (1924) the carry-over would therefore bias the results so that the quantity of milk removed from the udder after short intervals would be greater than that secreted during the interval and, conversely, after a long milking interval the quantity removed would be less than the amount secreted. The effects would be even more marked for fat than for milk yield.

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Bailey *et al.* (1955) also showed that the rate of secretion is depressed not only during a long interval but also for a period after the end of the interval. The effect of this factor also was not recognized in the early experiments, and must be eliminated if the effect of milking interval on rate of secretion is to be determined accurately.

The biases caused by residual milk carry-over and previous interval effects have been fully discussed by Elliott, Dodd & Brumby (1960) and can be eliminated by a suitable choice of experimental design. In later experiments, where these biases have been either partially or wholly removed (Turner, 1955; Elliott *et al.* 1960; Elliott & Brumby, 1955; Schmidt, 1960), it has been shown that the relationship between the quantity of milk secreted and the duration of the milking interval is curvilinear, but that the depression in the rate of milk secretion becomes noticeable only after intervals of more than 12 h, varying between animals and with stage of lactation. The decrease in milk yield is accompanied by a decrease in the content of solids-not-fat (SNF) and an increase in the content of fat. Information on the effect of extended intervals on the rate of secretion of other constituents of milk is limited, but Hansson, Dassat & Claesson (1954) have observed a decrease in the lactose content and an increase in the chloride content at the end of intervals of 18 h or more. The effects of a milking interval of 60 h and of incomplete removal of milk from the udder on milk secretion have previously been investigated (Wheelock, Rook & Dodd, 1965*a*).

The authors of recent publications on the rate of milk secretion have not defined the term 'milk secretion' but they have used it to describe the processes that occur during the synthesis of milk within the secretory cells or during storage of the milk within the udder after transfer from the secretory cells. Folley (1947) has, however, defined milk secretion as the process by which milk is synthesized by the alveolar epithelial cells and passes from the cytoplasm of these cells into the alveolar lumen—a definition that does not recognize that there may be a diffusion of materials across the mammary epithelium, and a partial resorption of milk constituents before the milk is removed from the udder (Knutsson, 1964*a*). The products of the alveolar epithelial cells may also be modified by the addition of secretions from other cell types (Thaysen, 1960).

The results of several recent experiments suggest that modification of the secretion of the alveolar epithelial cells of the mammary gland does occur in the interval between the entry of the alveolar secretion into the alveolar lumen and the removal of the accumulated milk from the udder. Wheelock, Rook & Dodd (1965*b*) demonstrated that the milk present in the mammary gland is in continuous osmotic equilibrium with the blood and that water moves freely between the mammary gland and the blood to maintain this equilibrium. Knutsson (1964*a*) showed that following the introduction of sodium, potassium, chloride and phosphorus into the udder via the teat there was a net transfer of these constituents to blood. A continuous small excretion of lactose in urine from some cows which were milked twice daily has been observed by Wheelock & Rook (1966), and losses increased markedly in all cows when milking was suspended for periods up to 39 h, observations that can most readily be explained in terms of a resorption of milk lactose into the blood from the mammary gland.

Therefore, in describing the results of rate of secretion experiments in which bias due to carry-over of residual milk has been eliminated, it is necessary to recognize

that the measurements are of the final products of the mammary gland as distinct from the 'primary secretion' of the alveolar cells which may be altered in the alveolar lumen, ducts and cistern. A detailed examination of the effects of milking interval on the secretion of milk and individual milk constituents should contribute to the understanding of lactation.

EXPERIMENTAL

Experimental design

The experimental milking intervals were of 6-, 12-, 18-, 24-, 30- and 36-h duration. In expt. 1, with 6 cows (1, 2, 3, 5, 6 and 8), the sequence of intervals was introduced according to a 6×6 Latin square, balanced with respect to residual effects. To eliminate the effect of previous milking interval, recovery periods consisting of 12-h milking intervals were interposed between each of the experimental intervals, the number of intervals varying with the length of the preceding milking interval: after milking intervals of 6, 12, 18, 24, 30 and 36 h, the number of 12-h intervals was 2, 2, 3, 4, 5 and 6, respectively. Bias due to the carry-over of residual milk was largely eliminated by removing most of the residual milk at milking. This was done by giving an injection of 20 i.u. oxytocin (Syntocinon, Sandoz Products Ltd., London, W.1) during milking by means of an indwelling polythene cannula inserted into the jugular vein before the start of the experiment.

Effects specifically related to the use of oxytocin have been observed previously (Wheelock, Rook & Dodd, 1965*c*). In expt. 2, with 2 cows (4 and 7), the experimental intervals were introduced in the same order as with cows 6 and 8, respectively, in expt. 1, but injection of oxytocin and the removal of residual milk were omitted.

In both experiments there was a pre-experimental period of 2 weeks. During the last 5 days of the pre-experimental period all animals were milked at regular 12-h intervals. In the computation of the results the control value has been obtained by taking the average for the two 12-h intervals before the introduction of the treatments and the last 12-h interval of each recovery period.

Animals and management

Cows in the 1st (cow 2), 2nd (cows 3, 6, 7, 8) or 3rd (cows 1, 4, 5) lactation and in the 2nd or 3rd month of lactation were used in the experiments. Frequent bacteriological examinations of their milk throughout the current lactation had not detected any infection of the udder.

Animals were housed individually in loose boxes and given a diet of hay (12 lb/day) and a concentrate mixture balanced for milk production (2 lb + 4 lb/gal of milk produced daily in the pre-experimental period). During the pre-experimental period they were fed twice a day after milking and during the experimental period they were fed 4 times a day at 6-h intervals. Drinking water was continuously available. At the end of each interval all quarters were milked. Milking was carried out with a bucket designed for the separate collection of the milk of individual quarters.

Sampling and methods of analysis

At each milking the milk yield of individual quarters was recorded and a representative sample taken from the milk of the right-hind quarter. The samples were

analysed for total solids, fat, lactose, total N, non-casein N, non-protein N, sodium, potassium and chloride by methods previously described (Wheelock *et al.* 1965*b*). The effect of treatments on the rate of secretion of milk of individual quarters within animals was similar, so that the values for the right-hind quarter can be taken as typical for the whole animal.

During the course of the experiments aseptically taken fore-milk samples were tested by bacteriological plating and Whiteside tests (Crossman, Dodd, Lee & Neave, 1950).

RESULTS

In the calculation of the rate of secretion the assumption is made that the milk obtained at a given milking was secreted in the interval since the last milking. Because of residual milk effects the values for expt. 2 during the longer experimental intervals are an underestimate of the actual rate of secretion. The results for cow 6 have been excluded because she refused all her food for a period during the experiment and showed a marked decline in milk yield. The results for the remaining cows in expt. 1 may be treated individually since, within the limits of experimental error, the secretion rates were fully recovered by the end of the appropriate recovery period.

Milk yield (Figs. 1, 4, 5; Tables 1, 2)

For short periods during the 30- and 36-h intervals milk dripped from the teats of cow 1 and cow 4 and so their rates of secretion are slightly underestimated.

Individual cows varied in their response to the treatments (Table 2) and in some cows decreases in the rate of milk secretion were observed during the 18-h interval, but invariably there was an effect with intervals of 24 h or longer. In expt. 1 the most marked decrease was observed in a heifer (cow 2) which was giving a lower daily yield than any of the other cows in the experiment, whereas the response of cow 5, with the highest daily yield, was similar to that of other cows giving a much lower yield. The yields of milk obtained at the end of intervals of 24, 30 and 36 h were usually similar (Figs. 4, 5). During the recovery period in both experiments the secretion rate increased rapidly until it was about 90 % of the control rate and then increased much more slowly.

Milk fat (Figs. 1, 4, 5; Tables 1, 3)

There was a depression in the secretion rate of fat during the longer experimental intervals (Figs. 1, 4, 5; Table 3); that this is a decrease in the secretion of fat and not an artifact because of failure to remove all the fat at milking is demonstrated in Table 1. This shows that there was a decrease in the secretion rate of fat during the experimental and recovery intervals. Fig. 1 also shows that the concentration of fat was high in the milk obtained at the end of the 6-h interval. The concentration then decreased with increasing length of experimental interval to a minimum at the end of the 18-h interval and then increased again.

Constituents other than fat (Figs. 2–5; Tables 1, 4–7)

The secretion rates of SNF, lactose, potassium, casein N, non-casein N and non-protein N were similar to those for milk yield during the experimental and recovery

intervals in both experiments (Figs. 2–5). In expt. 1, sodium showed no decrease in secretion rate during the experimental interval (Figs. 2, 4) but there was a decrease during the 1st 12-h interval following the 24-, 30- and 36-h experimental intervals. Recovery was completed before the end of the recovery period and there was a marked increase above the control value in 2 of the 12-h intervals after the experimental interval. In expt. 2 (Fig. 5), there was a small decrease in the secretion rate of sodium during the 24-, 30- and 36-h intervals, but there was no effect on the rate when carry-over was eliminated by considering the experimental interval and recovery period together (Table 1). In both experiments the secretion of chloride was similar to that of sodium (Figs. 4, 5). In general, the differences between animals for the individual constituents were similar to those for milk yield. Values for individual cows for lactose and sodium are shown in Tables 4 and 5.

There was no difference between the experiments in the effects of treatments on the concentration of individual constituents. Characteristic changes in the contents of lactose, potassium, sodium and chloride when expressed on a water basis were observed. In the milk obtained at the end of the 24-, 30- and 36-h intervals there were decreases in the concentrations of lactose and potassium and increases in those of sodium and chloride. During the recovery period there was a progressive return to the original value. Changes in the content of non-casein N were similar to those for sodium and chloride but less marked. Previously (Wheelock *et al.* 1965*a*) it has been shown that increase in non-casein N when milk accumulated in the udder was mainly due to blood serum proteins. The content of non-protein N was unchanged except for a small decrease in the recovery period following the 36-h interval. Peskett (1934) showed that the concentration of urea in milk was similar to that in blood. Since we observed no marked change in the concentration of non-protein N, which largely consists of urea, although there were considerable changes in yield, it is likely that the secretion in milk of non-protein N is determined by the concentration in the blood. There was no consistent pattern of change observed for casein N content.

Although the secretion rates of all constituents except sodium were decreased with increase in milking interval, their rates of decline were different. This is indicated by the relative changes in the concentrations of constituents but is shown more clearly by relating the concentrations (Tables 6, 7). The effects on potassium and lactose were similar throughout the experimental period but there was a greater effect on chloride than on sodium during the 30- and 36-h experimental intervals and the 12-h intervals immediately after. There was a more marked effect on casein N than on non-casein N during the same intervals. During the 30- and 36-h intervals and the 1st 12-h intervals in the subsequent recovery period there was a more marked effect on lactose than on casein N. Comparison of Tables 6 and 7 emphasizes the similarity of the responses in the 2 experiments.

DISCUSSION

With the exception of sodium and chloride in expt. 1, the present results confirm that the rate of secretion of milk and of a wide range of milk constituents decreases curvilinearly with duration of milking interval. The extent of curvilinearity varied from cow to cow, although such variation was not obviously related to the level of milk yield; and also from constituent to constituent; the effects on milk yield,

lactose, potassium and casein N were invariably more marked than those on sodium, chloride, non-casein N and fat. These differences between the constituents are similar to those observed with incomplete milking (Wheelock *et al.* 1965*a*).

Apart from differences in the secretion rates of sodium and chloride during the longer intervals, responses to treatments in both experiments were similar (Figs. 4, 5). It appears, therefore, that the use of oxytocin does not have any major effect on the response to the treatment. It is likely that the greater effect of treatments in expt. 2 than in expt. 1 results from the larger volumes of milk present in the udder in expt. 2; in addition to that secreted during an interval, these volumes would include the residual milk carried over from the previous interval.

The rates of secretion in Figs. 1–3 are the average rates for the period since the last milking. As secretion continued at the maximum rate for about the first 18 h, there must have been marked decreases in the rates during the latter part of the longer experimental intervals as shown in Figs. 4 and 5. The results for the 12-h intervals following the experimental interval show that the effects of the long intervals persisted for some time after the end of the interval. These findings are in agreement with previous work (Bailey *et al.* 1955; Elliott *et al.* 1960).

The effects of the length of milking intervals on the concentration of fat in the milk obtained is similar to that found by Elliott *et al.* (1960) and Schmidt (1960). The fat concentration was highest in milk obtained after the shortest and longest milking intervals. This result is dissimilar to that of Bailey *et al.* (1955), who found that the concentration of fat progressively increased from the shortest to the longest interval. The difference between these results is likely to be largely due to different experimental designs. The method used by Elliott *et al.* (1960) and Schmidt (1960) to eliminate bias due to the carry-over of variable quantities of residual fat between consecutive milking intervals was to remove the residual milk after oxytocin injections. In the work of Bailey *et al.* (1955) the bias was eliminated by an experimental technique which equalized the carry-over of residual fat to consecutive intervals. It is recognized that the use of oxytocin does not remove all of the residual milk (Johannson, 1952; Knutsson, 1964*b*) and because of the high content of fat in residual milk the carry-over from one milking interval to the next may be sufficient to alter the fat content of the milk removed after the next interval, particularly if this were a short interval following a longer interval. We believe that the incomplete removal of residual milk at the previous interval accounts for the high fat content observed in our experiments in the milk obtained at the end of the 6-h interval. This bias would also occur after the longer intervals but would be so small in relation to the total quantity of fat that it would have little effect on the measured concentration of fat. For these reasons, the estimate of milking interval on fat secretion measured by Bailey *et al.* (1955) at least during the shorter intervals is more likely to be free of bias due to carry-over than those presented above or those of Elliott *et al.* (1960) or Schmidt (1960).

The observed decrease in the secretion rate of lactose during extended milking intervals will be partly the result of an increased resorption of lactose from the mammary gland towards the end of the interval (Wheelock & Rook, 1966) but the reduced secretion rate in a succeeding short interval indicates that there is also a decrease in the primary rate of secretion by the alveolar cells. In experiments of this

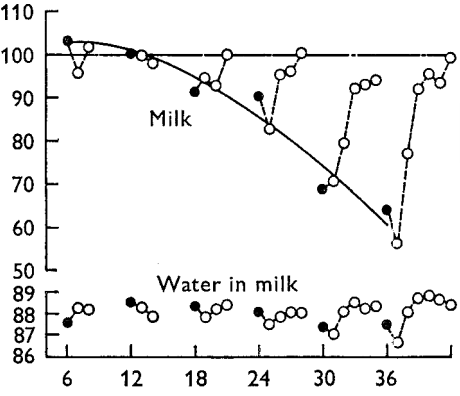
type it is not possible to partition the changes in secretion rates into changes due to resorption and to a decrease in primary secretion rate but Figs. 4 and 5 demonstrate that during the last 6 h of the 30- and 36-h intervals the rate of resorption of lactose was equal to, or greater than, the primary rate of secretion. Resorption of other synthesized constituents may also occur when milk accumulates within the mammary gland.

The effect on the secretion of those milk constituents which are normally present in blood may be more complex. These constituents may arise in part within the intracellular fluid of the alveolar cells and their rate of secretion could be expected to vary with the rate of the primary secretion of synthesized milk constituents. It has, however, previously been shown (Wheelock *et al.* 1965*a*), and the present results confirm this, that accumulation of milk within the udder causes a relative and in some instances an absolute increase in the rate of secretion of sodium, chloride and whey proteins. It is probable that the major source of the sodium and chloride of milk is a transudate originating in the extracellular fluids (cf. Barry & Rowland, 1953) and that engorgement of the udder with milk, which presumably affects the permeability of udder tissue, alters the rate of entry of the transudate into the udder.

Table 1. *The effect of milking interval on the rate of secretion (g/12 h) of milk and its constituents during the treatment and recovery periods*

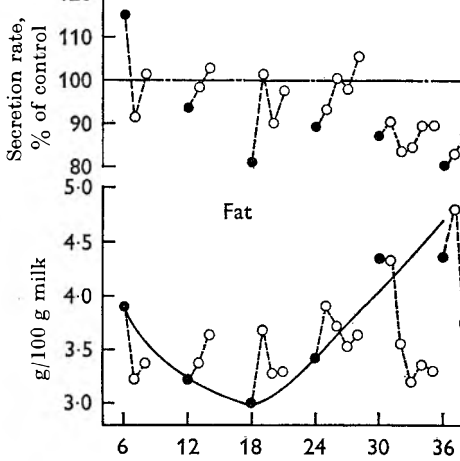
(In each case the length of the recovery period is equal to twice the length of the experimental interval. Values are the average for the right-hind quarter of 5 cows in expt. 1 and 2 cows in expt. 2.)

		Length of experimental interval, h					
Constituent	Expt.	6	12	18	24	30	36
Milk	1	2799	2830	2704	2627	2301	2265
	2	3095	3055	2679	2683	2443	2399
Fat	1	96·6	96·4	88·3	94·7	88·5	83·5
	2	108·9	99·0	85·2	93·2	94·1	95·7
SNF	1	239·5	238·2	229·0	223·1	193·3	186·2
	2	267·9	267·2	228·4	224·3	201·8	199·7
Non-casein N	1	3·21	3·19	3·09	3·01	2·80	2·71
	2	3·26	3·23	2·83	2·84	2·86	2·67
Non-protein N	1	0·80	0·78	0·78	0·74	0·63	0·58
	2	0·85	0·88	0·74	0·71	0·65	0·64
Casein N	1	10·34	10·31	9·80	9·30	8·48	8·09
	2	11·10	11·00	9·49	9·52	8·68	8·06
Lactose	1	124·4	124·6	120·0	117·4	99·7	95·3
	2	145·0	148·0	123·7	121·5	109·8	106·5
Potassium	1	4·98	5·09	4·91	4·63	4·12	3·99
	2	4·86	4·81	4·32	4·32	3·90	3·88
Sodium	1	1·08	1·07	1·00	1·07	1·04	1·06
	2	1·33	1·21	1·25	1·29	1·19	1·22
Chloride	1	2·72	2·91	2·74	2·66	2·54	2·77
	2	2·78	2·48	2·64	2·94	2·75	2·72



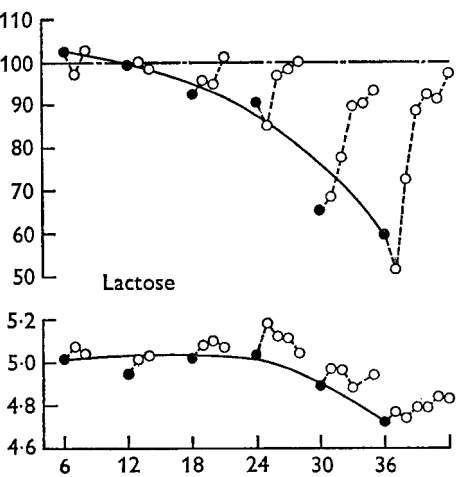
Milking interval, h

Fig. 1*a*



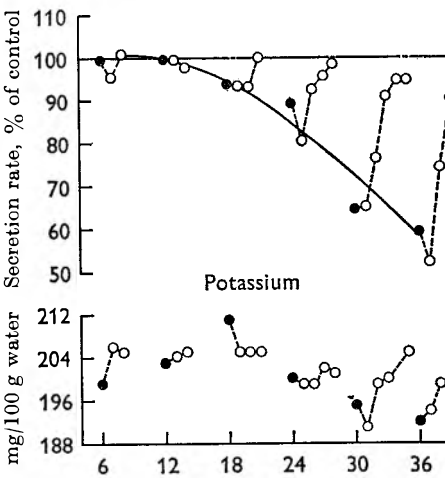
Milking interval, h

Fig. 1*b*



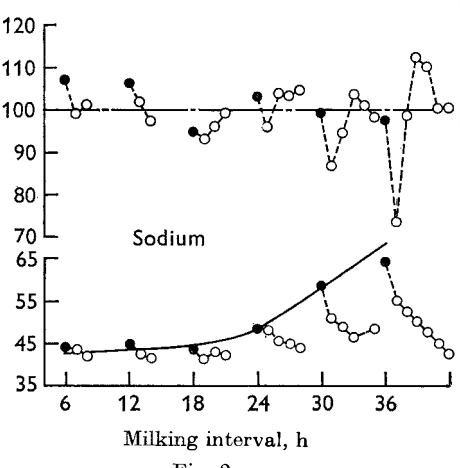
Milking interval, h

Fig. 2*a*



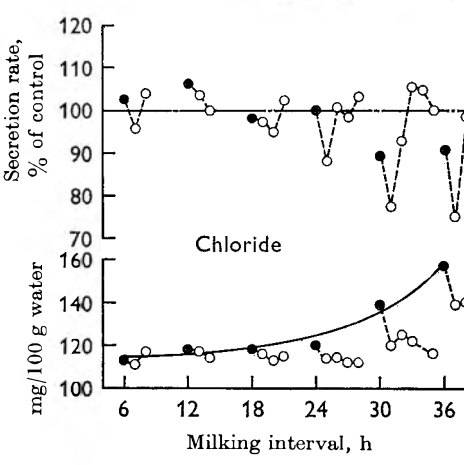
Milking interval, h

Fig. 2*b*



Milking interval, h

Fig. 2*c*



Milking interval, h

Fig. 2*d*

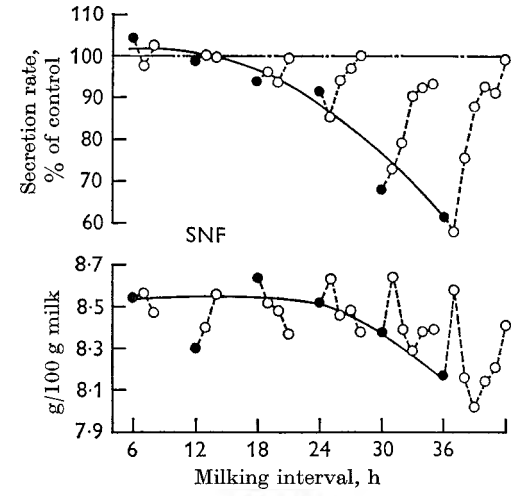


Fig. 3*a*

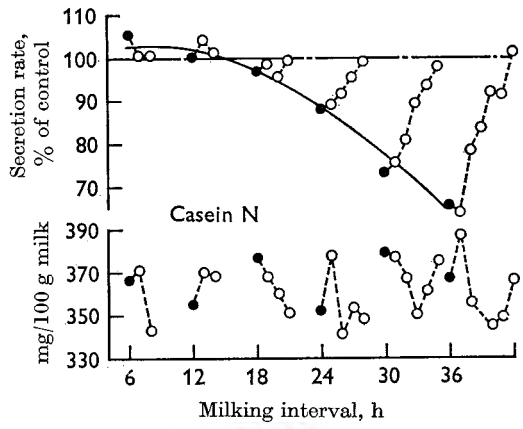


Fig. 3*b*

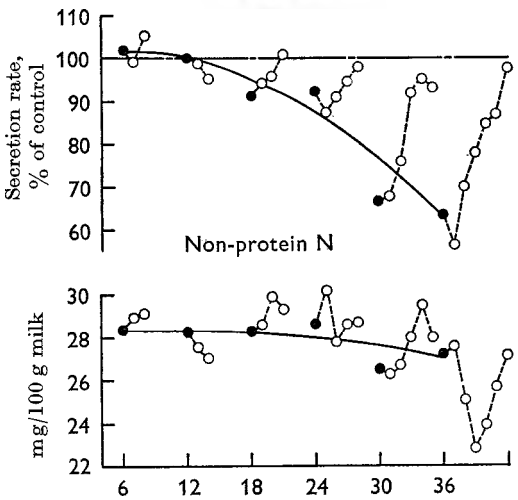


Fig. 3*c*

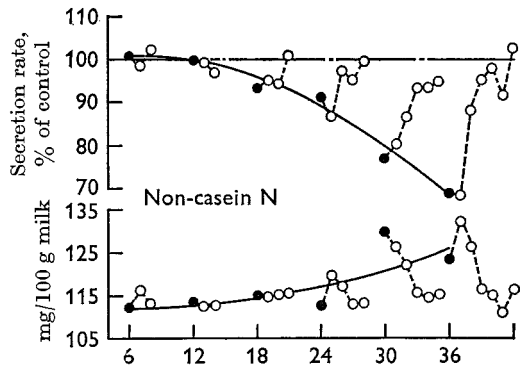


Fig. 3*d*

Fig. 3. The effect of milking interval on the rate of secretion and on the content of SNF, casein N, non-protein N and non-casein N in milk. Values are the mean for the experimental and for the recovery intervals of the 5 cows in expt. 1. ●, Experimental interval; ○, recovery interval.

Fig. 1. The effect of milking interval on the rate of secretion of milk and of fat and on the content of water and of fat in milk. Values are the mean for the experimental and for the recovery intervals of the 5 cows in expt. 1. ●, Experimental interval; ○, recovery interval.

Fig. 2. The effect of milking interval on the rate of secretion and on the content of lactose, potassium, sodium and chloride in milk. Values are the mean for the experimental and for the recovery intervals of the 5 cows in expt. 1. ●, Experimental interval; ○, recovery interval.

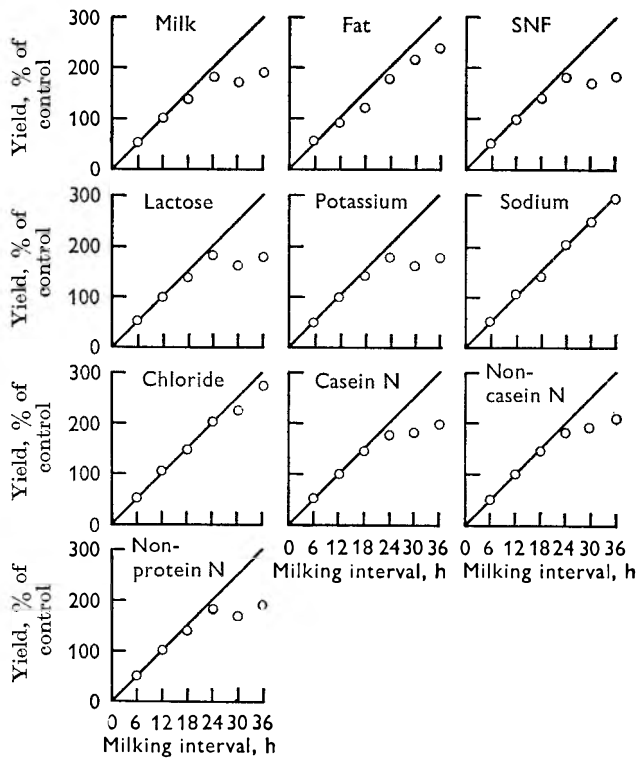


Fig. 4. The effect of milking interval on the yield of milk and of individual constituents. The yield, obtained at the end of an experimental interval, is expressed as a percentage of the control yield. Values are the mean for the 5 cows in expt. 1. The line represents the expected values if the control rate of secretion was maintained during the entire experimental interval.

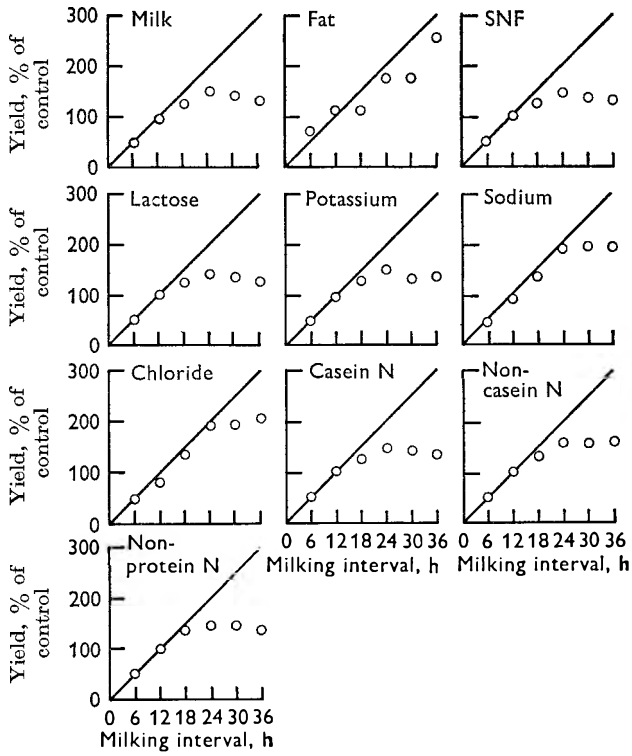


Fig. 5. The effect of milking interval on the yield of milk and of individual constituents. The yield, obtained at the end of an experimental interval, is expressed as a percentage of the control yield. Values are the mean for the 2 cows in expt. 2. The line represents the expected value if the control rate of secretion was maintained during the entire experimental interval.

Table 2. *The effect of milking interval on the rate of secretion of milk*

(The rate of secretion of milk in the right-hind quarter during each milking interval expressed as a percentage of the control rate of secretion. The values in bold type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

	Length of experimental interval, h																												Cont rate secret g/12
	6			12			18				24					30						36							
Cow																													
1	**104**	91	102	**93**	102	97	**97**	106	105	108	**83**	87	107	103	107	**74**	79	90	99	96	92	**64**	59	93	92	98	102	95	381
2	**107**	98	95	**105**	103	100	**101**	94	102	95	**92**	85	100	100	104	**56**	71	93	100	105	102	**49**	50	75	91	100	99	102	194
3	**114**	90	105	**95**	91	96	**82**	99	87	94	**99**	84	98	98	102	**65**	62	77	86	88	91	**71**	45	61	80	90	104	93	216
5	**95**	107	111	**107**	104	100	**86**	85	92	99	**91**	81	84	93	98	**68**	83	90	89	87	94	**66**	78	91	100	99	100	99	434
8	**95**	93	95	**101**	100	97	**91**	90	77	103	**86**	77	87	88	91	**80**	57	47	86	89	91	**69**	50	66	97	91	64	108	195
4	**106**	104	109	**99**	97	104	**89**	94	91	81	**81**	86	95	91	95	**68**	78	76	93	98	100	**52**	75	86	102	103	101	99	365
7	**97**	97	100	**89**	105	108	**79**	94	85	93	**69**	103	92	101	95	**45**	91	93	93	90	102	**43**	89	90	96	95	93	97	245

Table 3. *The effect of milking interval on the rate of secretion of fat*

(The rate of secretion of fat in the right-hind quarter during each milking interval expressed as a percentage of the control rate of secretion. The values in bold type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

	Length of experimental interval, h																												Cont rate secret g/12
	6			12			18				24					30						36							
Cow																													
1	**129**	88	107	**93**	101	111	**76**	104	90	92	**85**	93	123	106	115	**81**	105	97	103	102	94	**79**	77	95	83	87	90	89	138
2	**116**	107	103	**90**	105	104	**104**	102	108	95	**88**	92	103	104	105	**81**	81	102	101	95	93	**73**	68	85	85	87	90	92	76
3	**136**	79	108	**90**	88	101	**64**	80	96	95	**98**	98	100	99	104	**79**	82	81	45	83	84	**81**	78	73	84	77	102	82	88
5	**87**	97	94	**102**	106	108	**78**	94	88	99	**98**	101	95	104	112	**111**	106	91	92	86	95	**85**	115	100	102	102	104	97	104
8	**107**	86	94	**93**	92	90	**83**	96	68	106	**77**	88	82	78	91	**84**	79	48	80	83	82	**84**	78	84	96	73	35	105	80
4	**161**	118	125	**138**	98	124	**73**	135	113	65	**102**	128	125	94	102	**75**	182	103	109	113	134	**102**	123	100	107	100	103	113	100

(The rate of secretion of lactose in the right-hind quarter during each milking interval expressed as a percentage of the control rate of sceretion. The values in bold type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

Cow	Length of experimental interval, h																													Contr rate o secretio g/12 h
	6			12			18				24					30						36								
1	**103**	93	105	**96**	103	97	**100**	110	110	114	**86**	90	110	106	103	**73**	78	89	97	95	92	**61**	56	89	92	95	98	91	169·0
2	**109**	101	99	**104**	104	100	**102**	97	106	98	**92**	88	102	101	103	**46**	69	89	96	101	100	**44**	45	70	88	98	97	101	88·7
3	**114**	93	107	**95**	92	96	**81**	96	87	91	**99**	85	99	102	104	**63**	59	74	82	86	92	**70**	37	57	78	87	101	91	88·0
5	**97**	109	112	**103**	105	102	**85**	85	93	100	**93**	85	89	95	98	**70**	86	93	90	91	93	**64**	77	88	95	98	100	93	191·0
8	**90**	90	91	**99**	96	95	**93**	90	78	102	**84**	77	85	89	92	**76**	50	45	83	88	90	**61**	44	59	91	85	61	109	87·8
4	**107**	105	111	**104**	101	108	**90**	94	89	83	**80**	83	92	89	92	**65**	75	74	88	95	97	**45**	73	84	100	101	100	100	168·9
7	**98**	99	97	**96**	110	112	**76**	91	86	95	**64**	104	92	104	92	**42**	93	95	93	91	95	**40**	86	85	93	91	91	96	112·9

Table 5. *The effect of milking interval on the rate of secretion of sodium*

(The rate of secretion of sodium in the right-hind quarter during each milking interval expressed as a percentage of the control rate of secretion. The values in heavy type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

Cow	Length of experimental interval, h																													Contr rate o secretio g/12 h
	6			12			18				24					30						36								
1	**109**	92	104	**94**	102	94	**104**	103	101	106	**94**	102	120	110	113	**104**	102	112	111	103	99	**94**	79	117	110	111	109	96	1·28
2	**100**	89	83	**108**	100	100	**102**	86	100	86	**112**	114	117	111	109	**116**	94	116	119	116	105	**90**	64	97	111	116	114	113	0·64
3	**117**	89	105	**101**	87	92	**93**	102	100	98	**109**	89	100	99	99	**72**	70	90	95	93	93	**93**	65	78	99	107	120	100	1·06
5	**95**	112	110	**117**	103	96	**78**	77	94	99	**108**	84	82	98	101	**92**	94	95	90	91	92	**99**	92	109	113	110	104	100	1·53
8	**113**	112	104	**112**	117	105	**97**	97	85	106	**92**	92	100	97	100	**104**	74	60	103	103	103	**112**	68	91	128	108	54	94	0·78
4	**85**	109	105	**90**	87	98	**91**	101	97	90	**98**	96	101	96	96	**97**	90	87	101	97	102	**87**	84	96	110	100	96	103	1·56
7	**101**	103	106	**90**	97	97	**90**	106	92	111	**91**	111	101	108	101	**59**	95	95	97	94	99	**61**	106	111	109	105	101	106	1·05

Table 6. *The effect of milking interval on the ratios of various milk constituents*

(Mean values, with S.E., expressed as a percentage of the control value are given for the right-hind quarter of the 5 cows in expt. 1. The values in heavy type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

Length of experimental interval, h	Potassium/ lactose	Chloride/ sodium	Casein N/ non-casein N	Casein N/ lactose	Sodium/ lactose
6	**96·9 ± 1·44**	**91·7 ± 2·19**	**104·6 ± 5·25**	**102·5 ± 4·65**	**104·6 ± 5·48**
12-h recovery intervals	98·0 ± 0·87	94·1 ± 3·35	101·8 ± 1·77	103·8 ± 2·84	97·3 ± 9·39
	98·4 ± 0·84	98·1 ± 3·40	98·3 ± 1·92	98·1 ± 3·47	98·7 ± 4·63
12	**100·2 ± 1·04**	**94·1 ± 7·02**	**100·1 ± 1·06**	**101·2 ± 2·16**	**106·4 ± 3·09**
12-h recovery intervals	99·6 ± 1·16	98·2 ± 3·53	105·0 ± 1·05	104·0 ± 1·80	103·4 ± 4·94
	99·3 ± 0·66	98·8 ± 3·11	104·1 ± 0·66	102·5 ± 1·38	99·1 ± 2·84
18	**101·7 ± 1·54**	**100·5 ± 3·85**	**103·7 ± 2·15**	**104·9 ± 0·55**	**102·2 ± 3·60**
12-h recovery intervals	97·6 ± 2·44	100·9 ± 4·34	103·8 ± 2·88	104·8 ± 2·59	97·0 ± 3·73
	98·1 ± 1·06	95·5 ± 1·68	101·4 ± 2·53	101·0 ± 3·47	101·6 ± 4·24
	99·4 ± 1·80	99·3 ± 2·14	98·2 ± 2·45	98·2 ± 2·68	98·0 ± 3·56
24	**96·0 ± 7·2**	**93·8 ± 3·96**	**100·9 ± 6·22**	**101·6 ± 0·94**	**112·6 ± 2·53**
12-h recovery intervals	94·7 ± 1·56	88·4 ± 3·24	102·8 ± 4·09	104·7 ± 3·96	113·1 ± 5·61
	94·6 ± 1·74	93·5 ± 2·73	94·3 ± 3·26	94·5 ± 3·80	107·5 ± 2·26
	97·1 ± 1·29	91·8 ± 15·2	99·9 ± 2·24	96·6 ± 1·40	103·2 ± 2·26
	97·3 ± 1·75	94·7 ± 1·97	99·4 ± 2·95	97·5 ± 1·87	103·3 ± 2·33
30	**99·3 ± 2·46**	**89·7 ± 2·09**	**97·6 ± 5·59**	**111·3 ± 9·93**	**130·7 ± 5·01**
12-h recovery intervals	96·0 ± 3·31	87·0 ± 2·46	93·9 ± 4·00	111·5 ± 4·29	126·6 ± 6·60
	98·0 ± 1·45	94·4 ± 1·34	91·2 ± 6·79	104·9 ± 3·04	122·5 ± 5·03
	101·5 ± 2·08	98·1 ± 1·53	96·7 ± 4·63	99·8 ± 0·56	115·0 ± 4·20
	—	—	99·9 ± 1·82	—	—
	101·5 ± 0·94	98·4 ± 3·42	102·7 ± 3·15	104·6 ± 4·05	104·9 ± 2·30
36	**97·2 ± 7·42**	**89·7 ± 3·71**	**96·8 ± 2·93**	**111·4 ± 3·87**	**165·7 ± 12·28**
12-h recovery intervals	102·2 ± 3·40	91·4 ± 1·76	93·1 ± 3·31	129·2 ± 12·95	147·5 ± 9·28
	102·9 ± 1·80	96·4 ± 2·93	88·4 ± 5·10	107·2 ± 10·58	136·6 ± 4·47
	102·7 ± 1·96	98·8 ± 1·66	—	—	127·8 ± 3·67
	105·2 ± 1·62	103·2 ± 1·25	94·3 ± 2·66	99·6 ± 2·73	119·2 ± 2·24
	101·9 ± 3·15	100·8 ± 1·44	100·3 ± 2·30	98·6 ± 3·06	107·7 ± 5·66
	104·3 ± 0·93	104·5 ± 2·91	98·9 ± 2·37	104·1 ± 2·44	115·8 ± 4·87

Table 7. *The effect of milking interval on the ratios of various milk constituents*

(Mean values expressed as a percentage of the control value are given for the right-hand quarters of the 2 cows in expt. 2. The values in heavy type are for the experimental intervals and the following values are for the subsequent 12-h recovery intervals.)

Length of experimental interval, h	Potassium/ lactose	Chloride/ sodium	Casein N/ non-casein N	Casein N/ lactose	Sodium/ lactose
6	**94·8**	**103·6**	**102·6**	**100·0**	**91·4**
12-h recovery intervals	97·7	90·9	96·6	96·7	103·8
	98·4	99·5	99·1	101·0	101·4
12	**95·0**	**88·8**	**101·2**	**100·2**	**89·5**
12-h recovery intervals	93·9	87·5	101·3	94·2	87·6
	92·8	95·4	105·3	99·8	88·5
18	**103·0**	**99·8**	**96·6**	**101·6**	**108·9**
12-h recovery intervals	100·0	90·7	99·1	99·9	111·5
	98·1	92·1	96·3	97·3	108·5
	—	—	99·1	—	—
24	**103·4**	**100·0**	**93·8**	**102·0**	**133·3**
12-h recovery intervals	96·4	100·4	93·3	97·2	111·1
	100·8	100·1	94·3	96·1	109·4
	100·4	103·7	97·2	96·3	105·0
	104·2	100·4	114·5	112·4	106·4
30	**98·9**	**98·2**	**91·2**	**104·3**	**143·9**
12-h recovery intervals	—	—	73·6	—	—
	100·5	102·3	88·7	95·6	108·6
	102·5	102·0	89·4	97·0	109·2
	104·9	106·5	87·8	94·4	103·4
	105·5	102·2	94·1	104·6	104·4
36	**106·6**	**92·0**	**84·3**	**104·6**	**172·7**
12-h recovery intervals	104·4	99·8	91·5	103·2	119·0
	107·1	100·3	85·7	97·1	122·7
	102·4	101·8	82·6	90·6	112·5
	103·8	101·0	93·5	97·4	107·0
	104·5	103·8	88·3	94·3	103·0
	98·4	97·7	93·2	96·7	106·3

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REFERENCES

BAILEY, G. L., CLOUGH, P. A. & DODD, F. H. (1955). *J. Dairy Res.* **22**, 22.
BARRY, J. M. & ROWLAND, S. J. (1953). *Biochem. J.* **54**, 575.
CROSSMAN, J. V., DODD, F. H., LEE, J. M. & NEAVE, F. K. (1950). *J. Dairy Res.* **17**, 128.
ELLIOTT, G. M. & BRUMBY, P. J. (1955). *Nature, Lond.*, **176**, 305.
ELLIOTT, G. M., DODD, F. H. & BRUMBY, P. J. (1960). *J. Dairy Res.* **27**, 293.
FOLLEY, S. J. (1947). *Br. med. Bull.* **5**, 142.
HANSSON, A., DASSAT, P. & CLAESSON, O. (1954). *Riv. Zootec.* **27**, 316.
JOHANSSON, I. (1940). Preprint *Int. Dairy Congr.* Congress not held.
JOHANSSON, I. (1952). *Acta Agric. scand.* **2**, 82.
KNUTSSON, P. G. (1964*a*). *LantbrHögsk. Annlr.* **30**, 477.
KNUTSSON, P. G. (1964*b*). Thesis, Uppsala, 6.
PESKETT, G. L. (1934). *Biochem. J.* **28**, 1657.
RAGSDALE, A. C., TURNER, C. W. & BRODY, S. (1924). *J. Dairy Sci.* **7**, 239.
SCHMIDT, G. H. (1960). *J. Dairy Sci.* **43**, 213.
THAYSEN, J. H. (1960). *Handb. exp. Pharmak.* **13**, 504.
TURNER, H. G. (1953). *Aust. J. agric. Res.* **4**, 118.
TURNER, H. G. (1955). *Aust. J. agric. Res.* **6**, 514.
WHEELOCK, J. V. & ROOK, J. A. F. (1966). *J. Dairy Res.* **33**, 37.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965*a*). *J. Dairy Res.* **32**, 237.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965*b*). *J. Dairy Res.* **32**, 79.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965*c*). *J. Dairy Res.* **32**, 255.

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Mechanics of machine milking

II. The flow-rate pattern within single pulsation cycles

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Summary. The milk flowing during a single pulsation cycle was collected in a circle of contiguous cups which rotated in a chamber at 1 rev/pulsation cycle just below the end of the teatcup liner. The mean flow rate during the time taken for each collecting cup to pass under the milk stream was calculated and the flow-rate curve for the milk-flow period of the pulsation cycle plotted. Flow rates were measured at 130, 97, 65, 32 and 16 c/min, and also after the pulsator had been stopped with the liner open for 0·5 min (0 pulsation).

It was concluded from the series of flow-rate curves at the different pulsation rates that flow rate from the teat increased in about 0·05 sec to a steady value which continued for 0·5 sec or so, and then declined over a period of about 1·5 sec to a new constant value approximately equal to that shown after milk had flowed continuously from the teat for 0·5 min.

These results suggest that once the pressure difference across the streak canal during milking forces the teat sphincter open a considerable time elapses before the muscle control system responds, and that a further much longer period elapses before the full closing force of the sphincter is exerted. Thus, it would appear that at pulsation rates of about 50 c/min and above, the streak canal is closed by pressure exerted on the teat by the closing liner, the sphincter muscle playing no active part because its response rate is slow compared with the pulsation rate. At lower pulsation rates the flow rate declines during each cycle because the sphincter muscle has time to exert a closing force to a greater or lesser extent depending on the duration of the milk-flow period.

Clough & Dodd (1956) and Clough (1963) showed that maximum flow during milking increased with increasing pulsation rate, and suggested as a likely explanation that flow rate within each pulsation cycle rises rapidly to a peak value and thereafter declines. Thus, with a constant pulsation ratio, the quantity of milk flowing in a period of time consisting of short pulsation cycles would be greater than the quantity flowing in the same time with fewer but correspondingly longer cycles. In the present work a simple mechanical device has been used to investigate further the pattern of flow rate from the teat within a single pulsation cycle at a variety of pulsation rates during maximum flow of milking, and a constant pulsation ratio of 50% (teatcup liner

more than half open for 50 % of the pulsation cycle). Similar information early in milking, and at the end of milking within a few pulsation cycles of cessation of milk flow, was also obtained. In addition, some measurements were made during maximum flow of milking with a pulsation ratio of 67 % to compare longer milk-flow periods within cycles at particular pulsation rates with those obtained with a pulsation ratio of 50 %.

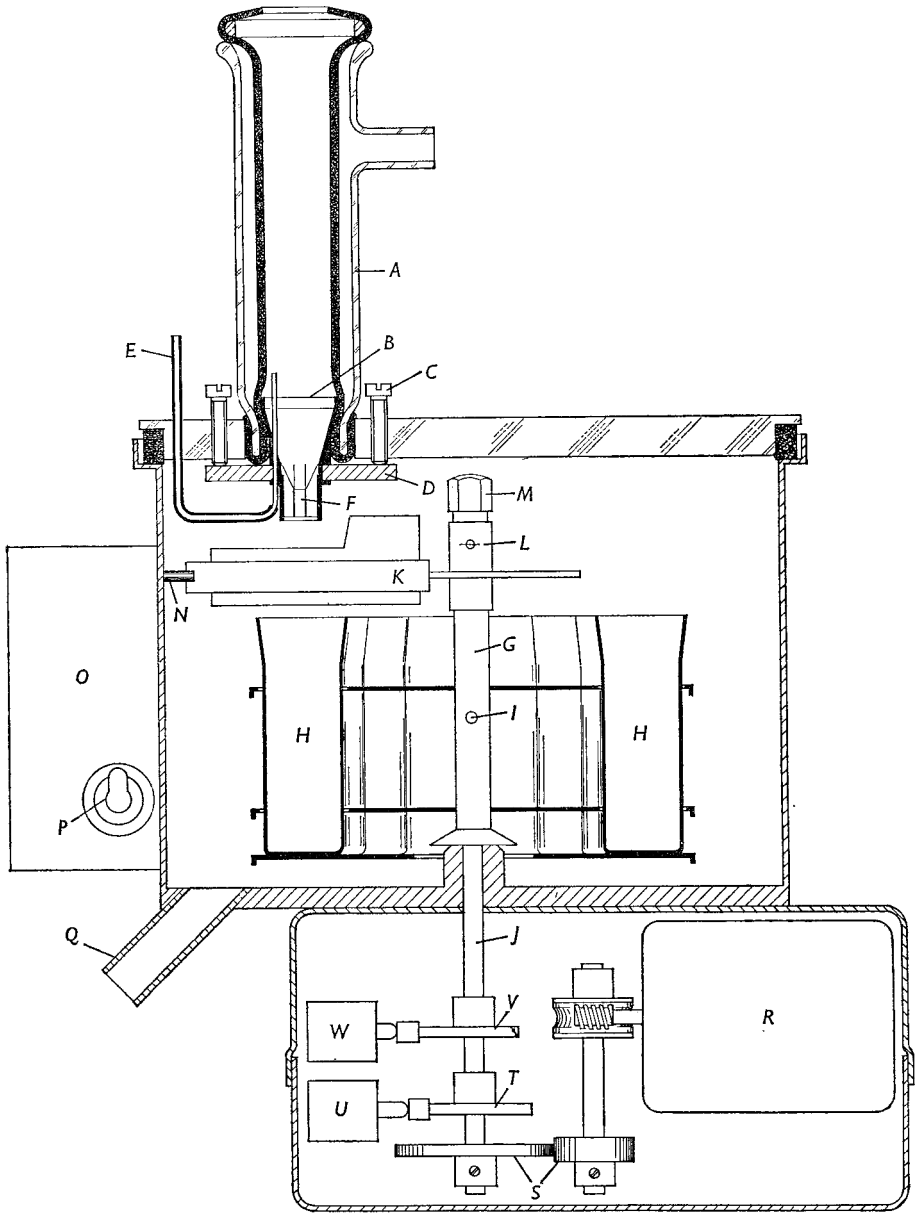


Fig. 1. Legend at foot of facing page.

APPARATUS AND METHODS

Apparatus for determining flow-rate curves during the milk-flow period of single pulsation cycles

A photograph of the apparatus in use is reproduced in Plate 1 and the relationships of the main components are shown in Fig. 1. The principle was to collect the milk obtained from a teat during the flow period of a single pulsation cycle in a circle of contiguous cups rotating at constant speed under the stream of milk flowing from the liner. In the apparatus finally evolved, the teatcup assembly was rigidly clamped to the lid of a chamber in which the collecting cups, arranged in a circular carrier rotating at 1 rev/pulsation cycle, passed below the open end of the liner. A deflector trough directly below the teatcup liner prevented milk from entering the collecting cups except during one selected cycle. The deflector was attached to the central shaft of the constantly rotating carrier by means of a friction clutch, and was normally restrained from rotating with the carrier by a catch which could be withdrawn by a solenoid. The electric circuit of the solenoid was so arranged that the deflector trough moved away from its position directly under the teatcup assembly just before the liner opened. Since the carrier with the circle of collecting cups rotated at 1 rev/c, the deflector also returned to its normal position while the liner was closed. Milk flowing from the teat during the part of the intervening period when the liner was open entered the collecting cups. From the weight of milk in each cup and the time taken by it to pass under the milk stream, a mean flow rate during that time was calculated, the results from all the cups enabling a flow-rate curve to be plotted. However, the beginning of milk flow into the first cup to contain milk and the end of flow into the last could occur at any time during the passage of these cups under the outlet of the liner, so that flow rates representing the beginning and end of the milk-flow period could not be calculated.

During milking the apparatus was held by both hands of the operator so that the

Fig. 1. Diagram of the rotating-cups apparatus. *Lid and teatcup assembly.* An Alfa–Laval 20003 B extruded liner with mouth ring was inserted under tension in a glass teatcup, *A*, with its end turned back over the necked end of the teatcup. Cone *B* compressed the liner on to the inner shoulder of the teatcup when drawn down by screws *C* and plate *D* fitting into milled recesses in the protruding part of the cone, thus sealing the teatcup assembly on to the lid and holding it firmly in place. Air admission through tube *E* at 8 l/min measured at 15 inHg pressure, a bore of $\frac{7}{16}$ in. in the cylindrical end of the cone, and a flow straightener *F*, all assisted in transferring milk smoothly to the collecting cups.

Main chamber. The central tube *G* of the carrier for the collecting cups *H* had a cross-pin, *I*, which engaged in a slot in the head of drive shaft *J* so that the carrier rotated continuously at 1 rev/pulsation cycle. Milk deflector *K* was attached to tube *G* by a friction clutch *L* and nut *M*. The deflector remained stationary under the liner when retained by catch *N* or it rotated with the collecting-cup carrier when the catch was withdrawn by a solenoid in case *O* under the control of switch *P*. Spigot *Q* on the main chamber was attached by a $\frac{1}{2}$-in. bore rubber tube to a milk interceptor vessel and thence to a source of controlled vacuum.

Lower chamber. Shaft *J* was driven by a geared squirrel-cage motor unit *R* through interchangeable pairs of gears *S* at 1 rev/pulsation cycle. An adjustable cam, *T*, operated a microswitch, *U*, to control current to a magnetic pulsator for any desired proportion of the pulsation cycle. Cam *V* was set in the correct phase relationship to cam *T* so that microswitch *W* briefly energized the solenoid, releasing milk deflector *K* while the liner was closed. To initiate the single revolution of the deflector, switch *P* in the same circuit was closed when the liner was seen to be open. The subsequent closing of microswitch *W* energized the solenoid and also a stepping relay to disconnect the electric supply after the single impulse.

teat and quarter appeared to be withstanding the same downward force as when milking with a normal cluster. After 1 min to establish maximum flow with certainty, the milk from a single pulsation cycle was allowed to flow into the collecting cups by operating the switch controlling the deflector. The assembly was then immediately removed from the teat, another carrier with empty collecting cups inserted and, after allowing 20 sec to establish normal flow, milk from a single cycle was collected in the second series of cups. Two similar measurements could then be made on another teat, all 4 within 4 min of the beginning of milking. The collecting cups were adjusted to the same weight ± 2 mg when dry. Weights of milk in the cups were recorded to the nearest 0·01 g.

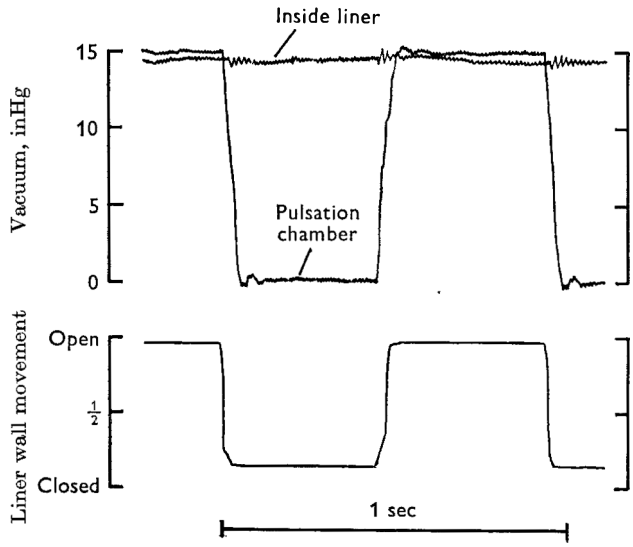


Fig. 2. Typical pressure changes in the pulsation chamber and inside the liner under the teat during milking, and liner wall movement.

Pressure conditions in the teatcup assembly and liner wall movement

The pulsation system was designed to give rapid changes of pressure in the pulsation chamber of the teatcup with consequent rapid movement of the liner wall. In this way, possible restriction of flow from the teat by the liner during opening and closing was confined to negligible periods of time. Typical pressure conditions in the pulsation chamber and inside the liner under the teat during milking, and consequent liner wall movement (Thiel, Clough & Akam, 1964) are shown in Fig. 2. The liner moved from about one-quarter open to three-quarters open, and also in the reverse direction, in not more than 0·02 sec. The milking vacuum inside the liner remained within the range 14·5–15·0 inHg throughout the work.

Distortion of the flow-rate pattern by the measuring equipment

It was evident from preliminary measurements with cows that, at pulsation rates of 65 c/min and above, the flow-rate curves within cycles showed that milk flowed from the liner for a longer period than it flowed from the teat, as judged by the time the liner was more than half open. The possibility that distortion of the flow-rate

pattern occurred during transit of milk from the teat to the rotating collecting cups was confirmed by cine photography at 100 frames/sec with the lid and teatcup assembly placed on an open-topped glass vessel, so that milk flow from the liner nozzle could be observed. At the various pulsation rates tried, milk flow from the nozzle started about 0·05 sec after the liner passed the half-open position, persisted

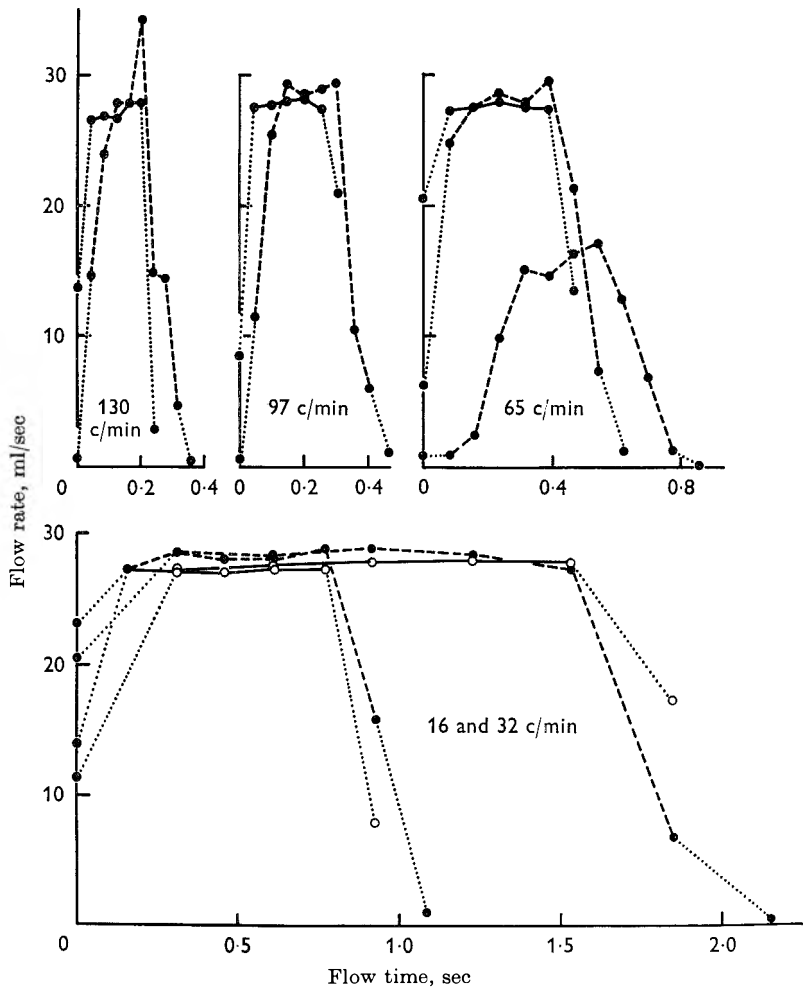


Fig. 3. Flow-rate curves of milk from a simulated teat at various pulsation rates and a pulsation ratio of 50 %. The direction of milk issuing from the teat was 20° to the vertical axis; diam. of teat orifice 0·080 in. Full lines, simulated teat mounted in the lid a little above the deflector in the measuring apparatus; dashes, simulated teat in the teatcup assembly. Lowest curve at 65 c/min was measured with a teat orifice diam. of 0·063 in. with a small plate below the orifice directing the milk horizontally on to the liner wall. Values plotted are the means of 5 replicates and are on the time-scale at the middle of the traverse time of each collecting cup. Flow rates assigned to the first and last cup containing milk which entered in an indeterminate period are joined to the usable part of the curves by dotted lines.

as a dense stream for about 0·02 sec after the liner was half closed and then as a diminishing stream and separate drops for a further 0·17 sec or so. Thus, at 130 c/min and a pulsation ratio of 50 %, milk flowed from the liner for most of each pulsation

cycle. Violent ejection of milk from the liner as the internal volume of the liner decreased on closing was also seen on projecting the film at 16 frames/sec. But the cineradiographic observations of Ardran, Kemp, Clough & Dodd (1958) showed clearly that milk flow from the teat starts before the liner is half open and ceases abruptly at the half-closed position. Therefore, distortion of the flow pattern during transit of milk through the liner certainly occurred.

Distortion of the flow-rate pattern during transit of milk from the teat to the collecting cups obviously imposes difficulties of interpretation of flow-rate curves obtained with the rotating-cups apparatus. A simulated teat was therefore built to measure directly the extent to which distortion could occur. A cylindrical metal teat (0·9 in. outside diam. and 0·5 in. bore) attached to an open vessel of about 2 l capacity was closed at its lower end by replaceable nozzles 0·4 in. long, through which holes could be drilled at various angles and of various diameters. Milk flow through the nozzle was controlled by an 0·2 in. diam. rod, tipped with a rubber disk of the same diameter, mounted inside the simulated teat and driven up to uncover and down to close the hole in the nozzle by separate solenoids. Adjustable delay circuits controlled by the cyclical electric supply to the pulsator enabled the opening and closing of the milk valve to be synchronized using an oscilloscope to the half-open and half-closed positions of the liner to less than 5 msec.

Some results using the simulated teat are given in Fig. 3, details of the experiments being given in the legend. The hole in the nozzle was at an angle of 20° to the vertical axis, which experience showed gave flow-rate curves at least as rounded as those obtained when milking cows. When this form of the simulated teat was mounted in the lid a little above the deflector in the rotating-cups apparatus, the shape of the flow-rate curves was a square wave if allowance was made for the uncertainties introduced by the indeterminate collecting periods of the first and last cups to contain milk. When, however, the same form of the simulated teat was placed in the teatcup assembly, distortion of the front of the flow-rate curve persisting for about 0·08 sec was clearly shown. (Milk would, on average, have begun entering the first cup to receive milk at the mid-point of its traverse and would, on average, have reached steady flow by about the mid-point of traverse of the last cup representing rising flow.) The period of distorted flow rate at the end of the curves was longer (0·15 sec or more), as expected. In another experiment the forward velocity of milk impinging on the liner was reduced as far as possible by a small plate fixed a little below the nozzle so that the milk was sprayed horizontally on to the wall of the liner. Flow from the liner with a pulsation rate of 65 c/min continued for almost the whole cycle (Fig. 3, lowest curve at 65 c/min). It was concluded from these and similar experiments that the rotating-cups apparatus would not enable a slow rise of flow rate at the teat to be distinguished with certainty from a square-wave flow pattern at the teat which was distorted as the milk moved through the liner to the collecting cups of the measuring apparatus.

A further limitation of the technique described here was the enhanced flow of milk from the liner induced by ejection of air and milk as the liner closed. As the position of any collecting cup with respect to the nozzle was not fixed in relation to liner wall movement, it was fortuitous whether this brief augmented milk flow reached a cup representing the end of the main milk-flow period or a cup measuring

the declining flow. If the former, a peak appeared at the end of the main flow period which tended to be higher the shorter the transit time of each collecting cup (Fig. 3, 130 and 65 c/min).

EXPERIMENTAL

Shape of flow-rate curves and mean flow rate during the milk-flow period of single pulsation cycles measured at maximum flow of milking

Measurements of flow characteristics during the milk-flow period of single pulsation cycles were made with the rotary cups apparatus using the cows described in Table 1. The nominal pulsation ratio was 50 % and the vacuum 15 inHg. Pulsation rates of 130, 97, 65, 32, 16 and 0 were used, the measurements at 0 pulsation being made in the following way. Having established maximum flow of milking with pulsation at 16 c/min the pulsator was stopped with the liner fully open so that milk flowed continuously from the teat. After 0·5 min the flow rate was measured with the rotating cups revolving at 16 rev/min.

Table 1. *Details of the cows used*

				Hind quarters			
				Yield at a milking		Maximum flow*	
Code no.	Breed	Lactation age, years	Weeks calved	left, lb	right, lb	left, lb	right, lb
1	Friesian	4	33	6·1	5·6	3·0	2·4
2	Ayrshire	3	9	9·8	10·4	2·1	2·2
3	Ayrshire	5	40	5·1	5·3	1·7	1·6
4	Friesian	3	30	7·4	8·2	2·0	1·6
5	Ayrshire	4	37	5·1	4·6	1·4	1·3
6	Friesian	3	43	7·7	7·2	1·5	1·4

* Highest yield in any 1 min when milking with a pulsation rate of 65 c/min and a pulsation ratio of 50 %; vacuum, 15 inHg.

A 6 × 6 Latin square design was adopted for the experiment. Measurements were made on 6 cows (Table 1), each cow being subjected to one of the 6 pulsation rates on each of 6 successive days at morning milking only. At each milking, duplicate measurements of flow-rate pattern within single pulsation cycles were made on both hind teats of each cow during maximum flow of milking. With the pulsation system used, a change in pulsation rate involved a change in pulsation ratio because the time delay from the beginning of air admission to the pulsation chamber to the half-closed position of the liner was appreciably shorter than the delay from the beginning of re-evacuation to the half-open position (Fig. 2). To maintain a constant pulsation ratio of 50 % each time the pulsation rate was changed between milking successive cows, it would have been necessary to adjust the cam controlling the magnetic pulsator. This was not possible in the time available. Therefore, the apparatus was set to give a 50 % ratio at 65 c/min; the change in ratio was from 54 % at 16 c/min to 46 % at 130 c/min.

Flow-rate curves during the milk-flow period of single pulsation cycles. To illustrate the nature of the results obtained and the method adopted of summarizing them, all the information referring to one cow is given in Fig. 4. It may be seen that for this set of data 5 graph points define the main part of each flow-rate curve at pulsation

rates of 32–130 c/min and 6 points at 16 c/min. This difference is, no doubt, associated with change in pulsation ratio with change in pulsation rate. At 0 pulsation, with milk flowing from the teat continuously, all collecting cups received similar amounts of milk, except those partly covered by the deflector or adjacent to it, and therefore likely to receive spray made by the sides of the deflector as they passed through the milk stream.

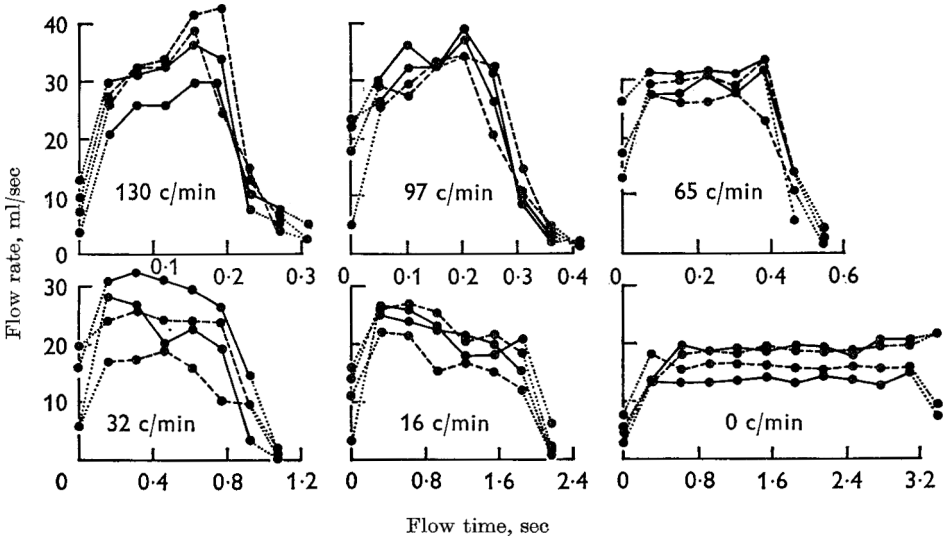


Fig. 4. Complete flow-rate data during the milk-flow period of single pulsation cycles for cow 2 at pulsation rates of 0–130 c/min; nominal pulsation ratio 50 %. Full lines, right hind teat; dashes, left hind teat. Flow rates assigned to the first and last cups containing milk which entered in an indeterminate period are joined to the curves by dotted lines.

In Fig. 4, the time at which the centre of the first cup containing milk passed under the centre of the liner has been taken as zero time on the horizontal axis. In fact for any one determination of flow-rate pattern the beginning of milk flow into the first cup may have occurred at any instant during its passage under the outlet of the liner. Nevertheless, for any series of measurements of flow-rate pattern the mid-point of traverse of the first cups to contain milk becomes the best estimate available of the beginning of milk flow for the whole series, and was so used even when plotting the results of single measurements of flow-rate pattern as in Fig. 4. The values of flow rate shown at zero time were calculated on the assumption that milk flowed into the first cups for their full time of traverse. These individual values do not define the beginning of the curve but serve to show the absence of a marked pattern.

By inspection it was judged that the 4 curves at each pulsation rate for each cow, similar to those shown in Fig. 4, were sufficiently similar in shape to justify combining them. Values for the first and last collecting cups containing milk were discarded. Also, the information already given for experiments with a simulated teat justified discarding values associated with the steeply falling parts of the curves. The mean curves thus obtained for all 6 cows are given in Fig. 5. Again the general shapes of the curves for the 6 cows at each pulsation rate were judged to be similar

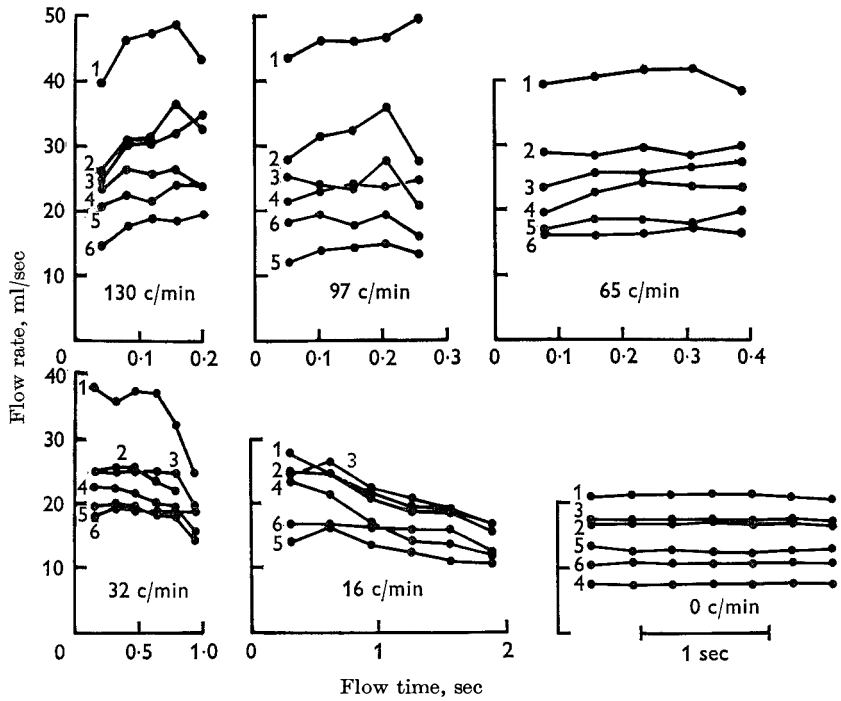


Fig. 5. Mean flow-rate curves during the milk-flow period of single pulsation cycles for each of 6 cows at 6 pulsation rates: nominal pulsation ratio, 50 %. Each curve is identified by the code number of the cow to which it refers.

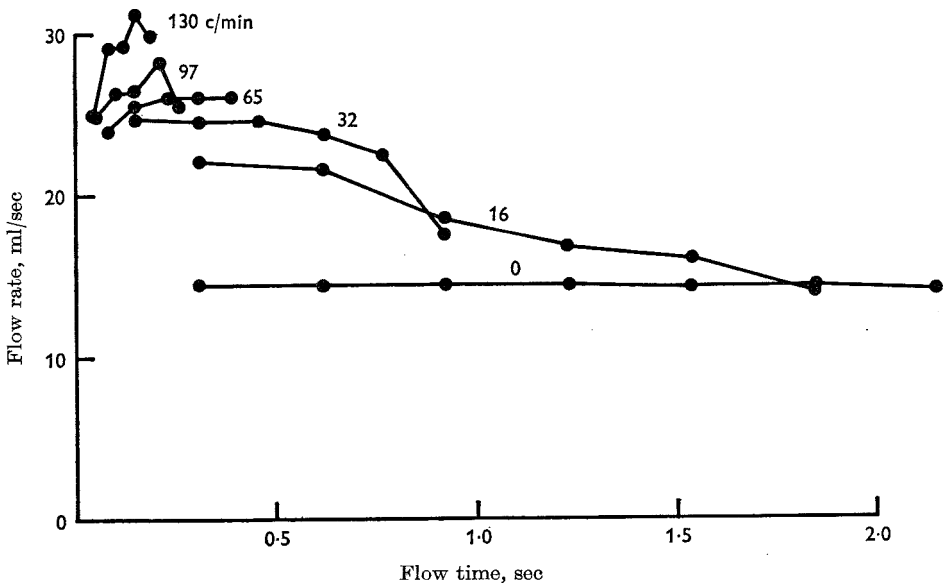


Fig. 6. Mean flow-rate curves during the milk-flow period of single pulsation cycles for all 6 cows at 6 pulsation rates: nominal pulsation ratio 50 %.

and were combined. These curves, all plotted to the same time-scale, are given in Fig. 6.

Mean flow rate during the milk-flow period of single pulsation cycles. The total milk flowing in single pulsation cycles of the Latin square experiment was estimated as the sum of the weights of milk in all collecting cups containing milk. The time during which this milk flowed from the teat was estimated from the nominal pulsation rate

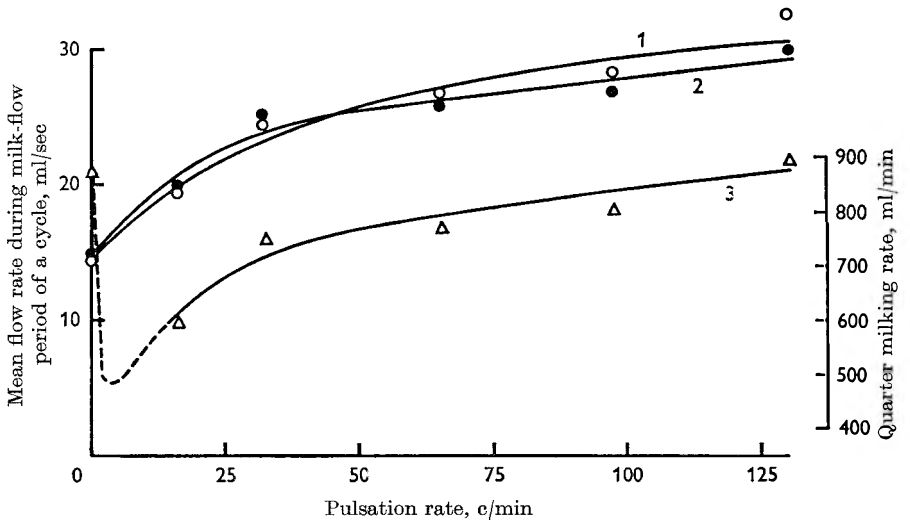


Fig. 7. The effect of pulsation rate on flow rate from a teat during the milk-flow period of single pulsation cycles: mean values for 6 cows at 6 pulsation rates and a pulsation ratio of 50 %. Curve 1, uncorrected values; curve 2, corrected values (see text). Curve 3, average quantity of milk flowing from a teat during 1 min calculated from the values used for curve 2 assuming a pulsation ratio of 50 %.

and the percentage of each cycle that the liner was more than half open. The pulsation ratios were measured by cine photography when milking the cows with the rotary cups apparatus on the day after completnig measurements of flow-rate patterns.

An analysis of variance of the mean flow rates during the milk-flow period of single cycles is given in Table 2 and the overall mean flow rates at the various pulsation rates are given in Table 3. The overall mean flow rates are plotted against pulsation rate in Fig. 7 (curve 1).

Various corrections were applied to the mean flow-rate data from which curve 1 in Fig. 7 was plotted. Correcting to a constant pulsation ratio of 50 % changed the values at the different pulsation rates by 0–3 %. Some deviations in pulsation rate from the nominal values were noted during the experimental work, varying from zero at 16 c/min to 1·4 % at 130 c/min. Finally, it is known from the cineradiographic work of Ardran *et al.* (1958) that, with increasing pulsation rate, milk flows from the teat for an increasingly greater proportion of the cycle than estimated when it is assumed that milk flows only when the liner is more than half open. However, the data quoted do not permit an accurate estimate of corrections to be applied to the present results. To show that this effect is not likely to alter the conclusions, corrections—which in our view are fairly substantial—of zero at 16 c/min rising linearly with

pulsation rate to 5% at 130 c/min have been applied. When all these corrections were made to the overall mean flow rates of curve 1 in Fig. 7, curve 2 was obtained.

Curve 3 in Fig. 7 shows the mean rate of milk flow over a period of 1 min from the

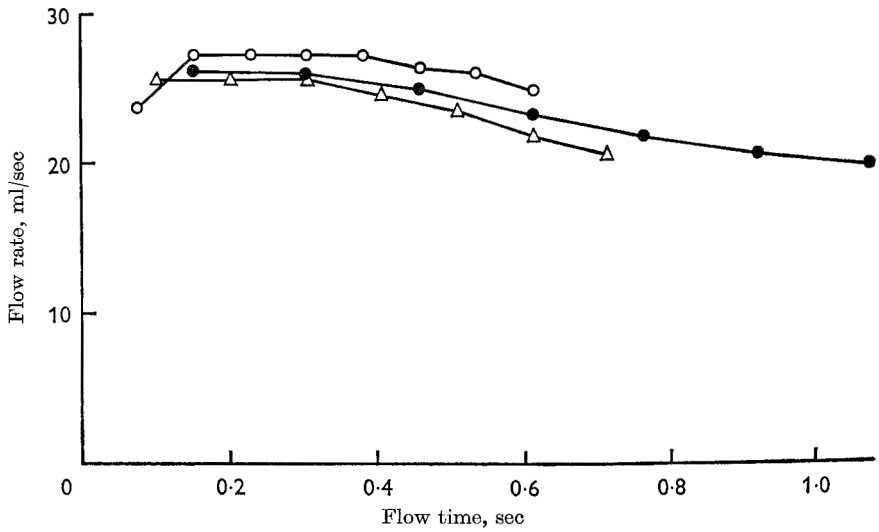


Fig. 8. Mean flow-rate curves during the milk-flow period of single pulsation cycles at a pulsation ratio of 67% and pulsation rates of 65 (○—○), 43 (△—△) and 32 (●—●) c/min.

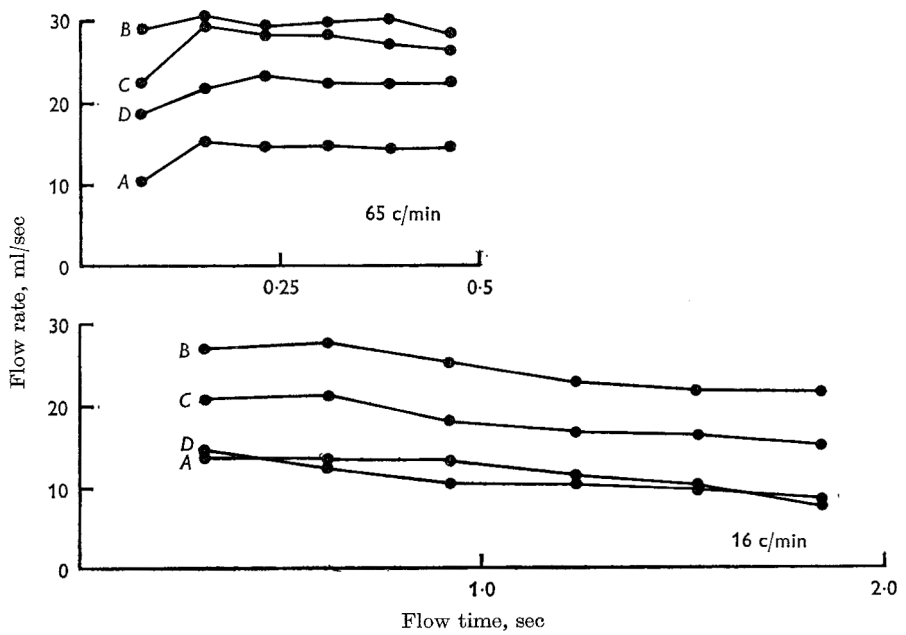


Fig. 9. Mean flow-rate curves for 6 cows during the milk-flow period of single pulsation cycles at pulsation rates of 65 and 16 c/min and a pulsation ratio of 50%. *A*, within 15 sec of placing the teatcup assembly on the teat; *B*, during maximum flow; *C*, near the end of milking as judged by the appearance of the quarter; *D*, repeat of *C* (in half the instances 5 or less pulsation cycles from complete cessation of milk flow).

12 experimental quarters with a pulsation ratio of 50 % at the various pulsation rates and also when continuously flowing with 0 pulsation. The values were calculated from those in curve 2.

Shape of flow-rate curves during the milk-flow period of single cycles with a pulsation ratio of 67 %

Duplicate measurements of flow-rate pattern during the milk-flow period of single cycles at maximum flow of milking were made with the rotating-cups apparatus on the hind teats of the 6 cows used in the previous experiment. Pulsation rates of 65, 43 and 16 c/min were used at a pulsation ratio of 67 %. The mean flow-rate curves shown in Fig. 8 were derived in the way already described.

Shape of flow-rate curves during the milk-flow period of single cycles at the beginning, during maximum flow, and at the end of milking of single quarters

Single measurements of flow rate during the milk-flow period of single cycles were made on the right hind teats of the same 6 cows at 4 stages during milking. The mean flow-rate curves obtained at pulsation rates of 65 and 16 c/min are shown in Fig. 9.

DISCUSSION

From the group of curves representing pulsation rates of 0–65 c/min (Fig. 6) it may be concluded with confidence that milk flow during each cycle continued for some time at a steady rate and then declined. Also, there is a general similarity between the flow rate at the end of a 2-sec flow period (pulsation rate, 16 c/min) and the flow rate at the end of continuous milk flow for 0·5 min (0 pulsation). These features lead to the hypothesis that milk flow from the teat at the beginning of each cycle soon rises to a value which remains constant for 0·5 sec or so, then declines steadily for about the next 1·5 sec and thereafter remains steady. Clearly, if the total duration of milk flow in a cycle is less than about 2 sec, the full sequence of events will be cut short by the closing liner.

At 65 c/min the rate of flow into the rotating cups attained a steady value after about 0·2 sec, but at 97 and 130 c/min the overall mean curves show considerable irregularity (Fig. 6). Alternative explanations of these patterns are that flow rate from the teat once the liner opened continued to rise for some time (0·2 sec or more), or that flow rate from the teat rose rapidly to a steady value but the system of measuring the pattern introduced distortion. It was shown in preliminary work using a cine camera that milk flowed from the nozzle at the base of the liner for a considerably longer period in each cycle than it flowed from the teat. Some distortion of the flow-rate pattern as milk moved from the teat to the outlet of the liner therefore occurred. Further experiments with a simulated teat showed that conditions of contact of milk with the liner wall, likely to occur when milking cows, caused distortion which could very readily account for the apparent slow rise of flow rate shown in Fig. 6 at the 3 highest pulsation rates. Also, a peak in flow rate as the liner closed (simultaneous ejection of milk and air) was often shown when working with the simulated teat, similar in height to those shown near the end of the curves for 97 and 130 c/min. It may therefore be concluded that the results obtained with the present apparatus

are not inconsistent with the hypothesis of a rapid rise in flow rate to a steady value, but the precise shape of the early part of the flow-rate curve remains uncertain as a consequence of measuring the flow-rate pattern external to the liner rather than at the end of the teat. Some indication of the limits of this uncertainty may be derived from the curves in Fig. 6. At 65 c/min the first flow rate is plotted 0·08 sec after flow into the collecting cups is estimated to have begun and is 6 % below the mean flow rate. Corresponding figures for the curves at 97 c/min and 130 c/min are 0·05 sec and 6 %, and 0·04 sec and 14 %, respectively.

The effect of extending the milk-flow period of each cycle by increasing the pulsation ratio was in agreement with the hypothesis that an initial constant flow rate begins to fall off after a time. Comparing Figs. 6 and 8 it may be seen that the mean flow-rate curves for the particular animals used showed no evidence of decline with a pulsation ratio of 50 % at 65 c/min but a definite decline with a ratio of 67 %.

Table 2. *Analysis of variance of mean flow rate during the milk-flow period of single pulsation cycles, ml/sec*

	D.F.	Mean square
Cows	5	1299·9***
Days	5	118·5NS
Pulsation rates	5	1003·5***
Residual	20	67·02
Between hind quarters of the same cow	6	40·14***
Rates × quarters of the same cow	30	8·50NS
Duplicates	72	6·19

*** Significant at 0·1 % level; NS, not significant at 5 % level.

Table 3. *Mean flow rate during the milk-flow period of single pulsation cycles at various pulsation rates*

Pulsation rate, c/min	0	16	32	65	97	130	S.E. of a mean (20 D.F.)
Mean flow rate, ml/sec	14·4	19·3	24·4	26·6	28·2	32·4	± 1·67

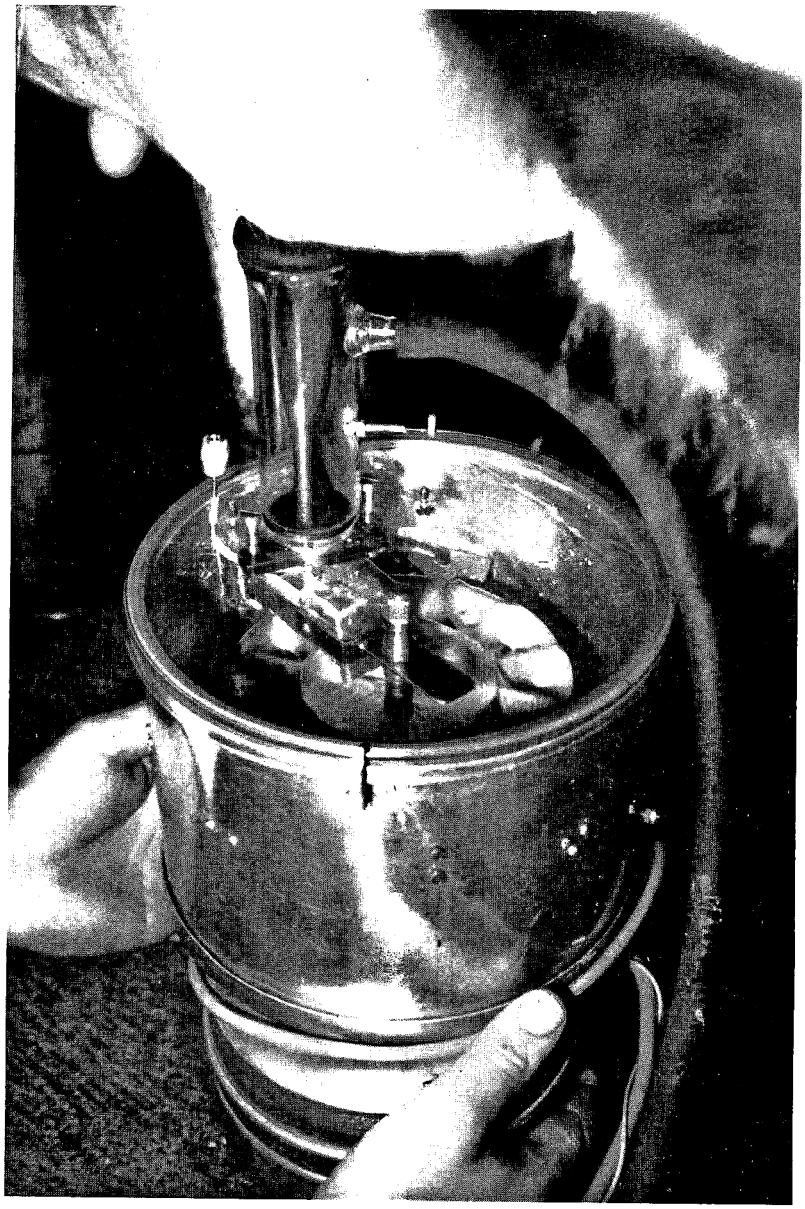
Means which are underscored by the same line do not differ significantly at the 5 % level.

As well as a change in general shape of the flow-rate curve within cycles with increasing pulsation rate, the mean flow rate during the milk-flow period of single cycles was also affected. Table 2 shows that, although there was significant difference in mean flow rate between hind quarters of the same cow, such difference was not significantly affected by pulsation rate. The table also shows marked differences in mean flow rate between cows and a marked overall effect of pulsation rate. The Latin square analysis does not give a direct measure of the interaction between pulsation rates and cows, but this may be estimated by combining the residual and the non-significant mean square for days. The resulting mean square (77·3 with 25 deg. of freedom) is significantly less than the mean square for pulsation rates and significantly greater than the interaction between rates and quarters of the same cow. This implies that, although differences may be detected between the effects of pulsation rate on different cows, there is sufficient agreement to sustain an overall mean pulsation rate effect. This last point justifies combining the data to give the curves of Fig. 7.

The relationship between mean flow rate during the flow period of single cycles and pulsation rate shown in Fig. 7 (curve 1), even when freed from bias as far as possible (curve 2), consists of a rapidly increasing mean flow rate up to 50 or 60 c/min and then a more gradual increase which was still evident at the maximum pulsation rate used of 130 c/min (Table 3). It is of interest that a calculated maximum flow of milking (curve 3) gave about equal values for 0 pulsation and 130 c/min, all other pulsation rates giving lower milking rates.

Any explanation of the results obtained should account for the characteristic change in flow rate beginning after about 0·5 sec and also the increased mean flow rate during the milk-flow period of single cycles which occurred over the entire range of increasing pulsation rate. As the pulsation rate was increased, the flow rate early in each cycle consistently increased (Fig. 6), which seems to imply that the general level of flow rate in a cycle is affected by the duration of preceding cycles. This suggests that response times of the sphincter muscle and its control system may be involved. Consider the flow-rate curves at 32 and 16 c/min. Once the pressure difference across the streak canal forced open the sphincter, the particular flow rates found would presumably result from the pressure difference, the intrinsic dimensions of the streak canal, the elasticity of its walls, and residual force exerted by the sphincter. The beginning of decline in flow rate after about 0·5 sec might indicate the response time of the muscle control system. The subsequent period of declining flow would then be a measure of the period required for the sphincter to acquire maximum closing force giving a new constant flow rate. (The flow rate after a flow period of 2 sec was about equal to the flow rate measured after continuous flow from the teat for 0·5 min.) The sphincter would presumably continue to contract after the streak canal had been occluded by the closing liner. Thus, the shorter the cycle the shorter the time available for contraction of the sphincter, and consequently the lower the residual force exerted by the muscle when next expanded. Hence, with increasing pulsation rate, milk flow begins at progressively higher rates.

Flow-rate patterns during the milk-flow periods of single cycles at the beginning, during maximum flow, and very close to the end of milking of individual quarters are puzzling (Fig. 9). Not unexpectedly, the mean flow rates during the milk-flow periods were low at the beginning and end of milking compared with peak flow. What is puzzling is that all the flow-rate curves at each pulsation rate were more or less parallel throughout their length, even though the flow rates were very different at the various stages of milking. This seems to imply that there was no depletion of milk in the teat cistern during each cycle (otherwise milk flow would have been expected to fail) which agrees with the findings of Witzel & McDonald (1964) that pressure in the teat cistern remains at atmospheric or above until cessation of milk flow. If, at both pulsation rates, reduced flow rate at the beginning and end of milking was caused by some additional resistance to flow, then at 16 c/min the postulated closing force exerted by the sphincter muscle would be expected to have a lower proportionate influence at the low flow rates compared with its effect at peak flow. However, the results show the reverse; at 16 c/min (Fig. 9), curve *B* shows a decline of 20 % and curve *D* of 40 %. As the data are limited there is of course no certainty that the parallel appearance of the curves is other than fortuitous.



We are grateful to Mr I. R. Cutts for supplying the control system on the simulated teat apparatus and to Mr F. A. Ravening for constructing the remaining apparatus.

REFERENCES

ARDRAN, G. M., KEMP, F. H., CLOUGH, P. A. & DODD, F. H. (1958). *J. Dairy Res.* **25**, 154.
CLOUGH, P. A. (1963). *Alfa-Laval Symposium no.* 1 *on Machine Milking*, p. 42. Tumba, Sweden: Alfa-Laval.
CLOUGH, P. A. & DODD, F. H. (1956). *Agriculture, Lond.*, **63**, 344.
THIEL, C. C., CLOUGH, P. A. & AKAM, D. N. (1964). *J. Dairy Res.* **31**, 303.
WITZEL, D. A. & MCDONALD, J. S. (1964). *J. Dairy Sci.* **47**, 1378.

EXPLANATION OF PLATE

The rotating-cups apparatus in use. The deflector, the rotating-cups assembly, and the top of the driving shaft can be seen under the Perspex lid.

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The variation in the cell count of cow's milk throughout lactation and from one lactation to the next

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SUMMARY. Total and differential cell counts were obtained for approximately 38000 fore-milk samples taken from cows of the Institute herd during a period of 12 years.

For most of the period of the experiment the incidence of subclinical mastitis associated with coagulase-positive staphylococci was about 5% of all the quarters in milk, but for a period of about 12 months in the 7th and 8th year of the experiment the herd was free from infection with coagulase-positive staphylococci.

The average cell count for cows in each lactation up to the 7th was calculated, and in addition each lactation was divided into 4 equal periods, and the average cell count for each period determined.

It was found that the average total cell count increased from one lactation to the next, and that this rise was due mainly to an increase in the number of polymorphs in the samples, the counts of cells other than polymorphs remaining relatively constant.

It was also found that the average total cell count rose during the course of any one lactation; but this was due to an increase in both the number of polymorphs and the number of cells other than polymorphs.

During the period when the herd was free from infection with coagulase-positive staphylococci the average lactation age of the cows was 4·3 lactations, and the average total cell count was 0·30 million cells/ml, a figure which was the same as that obtained throughout the experiment for animals in their 1st lactation.

Possible relationships between the rise in cell counts and the pathology of mastitis are discussed.

As part of a long-term study of mastitis as it occurs in the Hannah Institute herd, fore-milk samples have been taken for cell counts at fortnightly intervals from most of the cows in the herd over a period of 12 years (1953–65). From the results so obtained, the average cell counts for the milk of the cows in their 1st–7th lactations have been calculated. In addition, each lactation has been divided into 4 equal periods and the average cell count for each period calculated. The object was to find what differences occurred in the average total cell count during any one lactation and also from one lactation to another.

METHOD

The milk samples (a total of about 38000) were all quarter samples of fore-milk taken just before the evening milking. The numbers of cows used were as follows: 1st lactation, 57; 2nd lactation, 64; 3rd lactation, 68; 4th lactation, 67; 5th lactation, 57; 6th lactation, 50; 7th lactation, 47.

The total and differential cell counts were made by the method described by Blackburn (1956), which was based on earlier work by Blackburn & Macadam (1954) and Blackburn, Laing & Malcolm (1955).

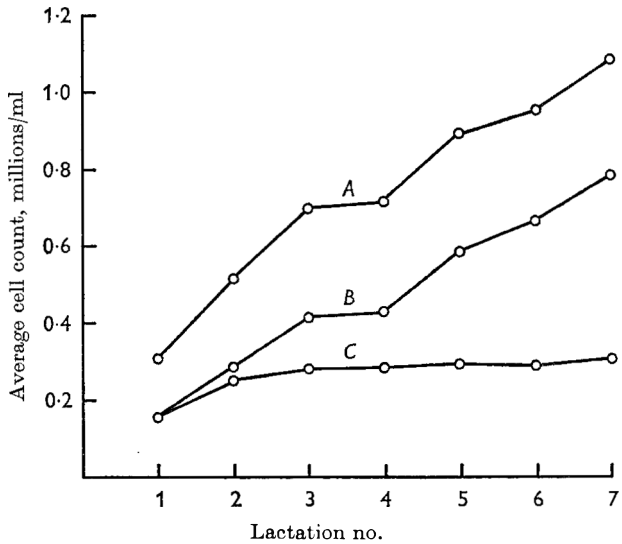

Fig. 1. Average cell count for each of 7 lactations. *A*, total cell count; *B*, polymorph count; *C*, count of cells other than polymorphs.

RESULTS

Average cell count for the whole lactation. The average total cell count (Fig. 1, line *A*) for all the cows in their 1st lactation was 0·30 million cells/ml and in succeeding lactations it increased to 0·51, 0·69, 0·71, 0·88 and 0·95, until in the 7th lactation it reached 1·08 million cells/ml. The graph of the average polymorph count, for each lactation (Fig. 1, *B*) after the 2nd, runs almost parallel to that for the average total count. This means that the increase in cell count from lactation to lactation was due mainly to an increase in the number of polymorphs in the samples. This is confirmed by Fig. 1, *C*, which shows that there was very little increase in the average count of cells other than polymorphs from the 2nd to the 7th lactation. The percentage of the total count that was due to polymorphs increased from the 1st to the 7th lactation, the figures being 50 for the 1st, 53 for the 2nd, 59 for the 3rd, 62 for the 4th, 67 for the 5th, 70 for the 6th and 73 for the 7th lactation.

Average cell count in 4 equal periods of a lactation. Each lactation was divided into 4 equal periods (periods 1–4). Fig. 2 shows the average total cell counts for each of the 4 periods for 7 lactations. With only 4 exceptions there was a rise in the average total cell count from one lactation to the next for all 4 periods. In the 1st lactation,

the average total cell count was slightly less in period 2 than it was in period 1, but otherwise in each lactation the count was higher in each successive period. The most marked rise in cell count in each lactation was from period 3 to period 4, and this rise was especially more marked in the 2nd–7th lactations.

Fig. 3 shows the average polymorph count for the 4 periods of each lactation. Each line follows closely the lines showing the average total count in Fig. 2, but at a lower level. This shows that the increase in cell count from lactation to lactation in each of the periods was mainly due to a rise in the number of polymorphs. This is con-

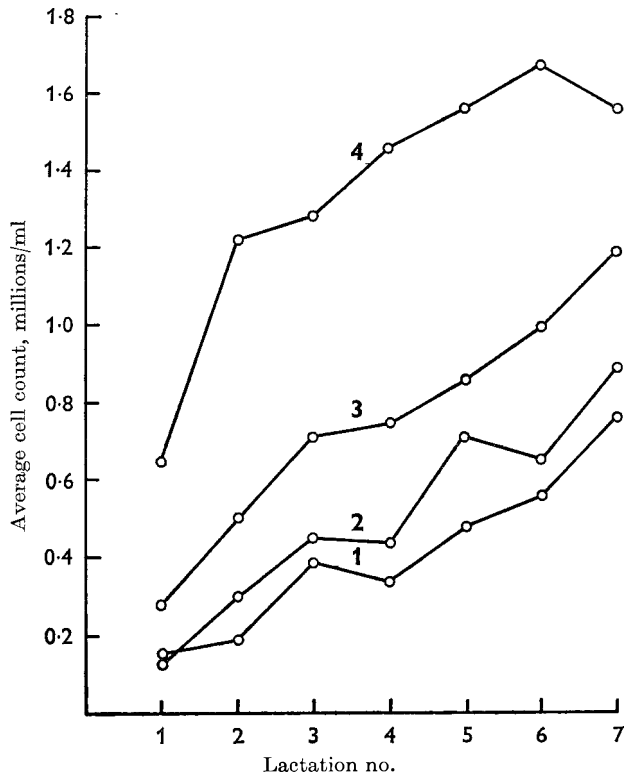

Fig. 2. Average total cell count in four equal periods (**1–4**) of each lactation.

firmed by Fig. 4, which shows the average count of cells other than polymorphs. No marked rise occurred from lactation to lactation except between lactations 1 and 2, where the rise in the number of cells other than polymorphs in each period was about the same as the rise in the number of polymorphs. The rise in cell count from one period to the next in any one lactation, however, was not due only to an increase in the number of polymorphs. Cells other than polymorphs also increased in number as lactation advanced. In each lactation the increase in total cell count from period 1 to period 2 and from period 2 to period 3 was due more to other cells than to polymorphs. The increase in total count from period 3 to period 4 in the 1st and 2nd lactation was also due more to other cells than to polymorphs. In subsequent lactations, however, the increase as lactation advanced was due more to polymorphs than to other cells (see Figs. 2, 3, 4).

Of the 38000 samples used in this work, 591 samples had counts of over 5 million cells/ml and came from quarters showing some symptom of clinical mastitis. In calculating the results shown in Fig. 5 these 591 samples have been excluded from the 38000. The average total cell count for all cows in the 1st lactation was 0·26 million

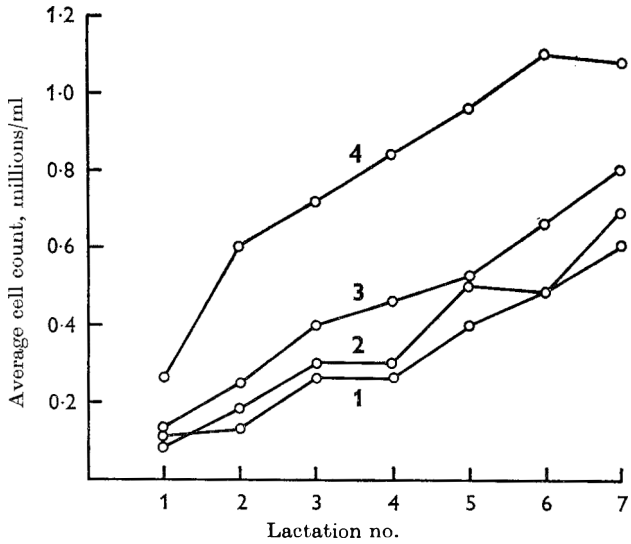

Fig. 3. Average polymorph count in four equal periods (1–4) of each lactation.

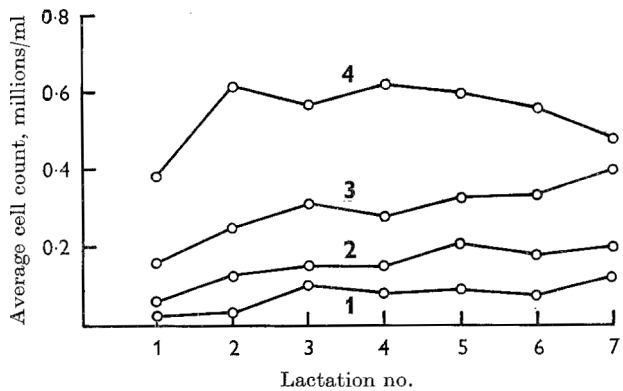

Fig. 4. Average count of cells other than polymorphs in four equal periods (1–4) of each lactation.

cells/ml, and in succeeding lactations it increased to 0·44, 0·55, 0·58, 0·61 and 0·71 until in the 7th lactation it reached 0·78 million cells/ml. The rise in the average total cell count from one lactation to the next is not so marked as when the 591 samples are included (Fig. 1), but the increase is still evident. Line *B* in Fig. 5 shows the average polymorph count in each lactation, and line *C* the count of cells other than polymorphs. From the 3rd lactation onwards the increase in the average total cell count was accounted for entirely by a rise in the number of polymorphs.

At the beginning of the experiment the incidence of subclinical mastitis associated with coagulase-positive staphylococci was high, about 25 % of the quarters in milk being affected, but extensive treatment quickly reduced the incidence to less than 5%.

Owing to treatment and disposal of chronically infected animals, the herd became free of infection with coagulase-positive staphylococci for a period of more than 12 months in the 7th and 8th year of the experiment. The cows in the herd during that period had an average lactation age of 4·3 lactations. The average total cell count of the milk of these animals during this period was 0·30 million cells/ml, which was the same as for cows in their 1st lactation. The percentage of the total cells that was polymorphs, however, was 80 % compared with 50 % for cows in their 1st lactation.

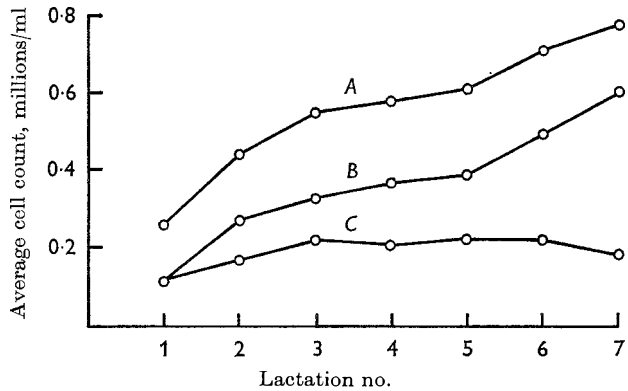

Fig. 5. Average cell count for the whole lactation excluding cell counts of over 5 million/ml. *A*, total cell count; *B*, polymorph count; *C*, count of cells other than polymorphs.

DISCUSSION

The results show clearly that in a herd affected with subclinical mastitis an increase in the average total cell count occurs from one lactation to the next. It is clear also that the increase is due to an increase in the number of polymorphs, the number of cells other than polymorphs remaining practically constant. Pattison (1951) describes the histological findings in experimental streptococcal mastitis in the goat. The minimal reaction to the inoculation of living organisms was a patchy migration of neutrophils into secreting acini and a rather more generalized increase of interacinar cellularity. He divided the changes in what he regarded as the marked reaction into those occurring in the lobules and those occurring in the ducts. Those occurring in the lobules arose from a migration of neutrophils into secreting acini, followed by a heaping up of acinar epithelium. These changes were progressive up to 4 days and then the affected lobules started to involute. Blackburn (1952) described similar changes in naturally occurring mastitis in the cow. He found that involution of affected lobules could be detected in life by the increased number of epithelial cells found in the milk. Waite & Blackburn (1963) differentiated the lesions found in the lobules into mild, moderate and severe acute inflammation, depending on the amount of cellular exudate found in the affected acini. It would appear, therefore, that the increase in cell count from one lactation to the next was due to an increase in the severity of the inflammatory lesions, and not to an increase in their extent. If there had been an increase in the extent of the lobular lesions there would have been an increase in the number of epithelial cells shed into the milk. On the other hand, the increase in the number of polymorphs could have been due to an increase in the extent of subacute lesions in the ducts. It was found by Blackburn (1952) that such

lesions added large numbers of polymorphs to the milk. The udders of most of the animals from which the samples were taken in this experiment were examined histologically when the animals were cast from the herd. An estimate was made of the extent of subacute inflammatory lesions in the ducts and also of the extent of periductal fibrosis. The presence of periductal fibrosis was taken as evidence that there had been subacute lesions in the ducts at some time in the life of the animal concerned. No periductal fibrosis was found in the udders of animals slaughtered after the 1st–4th lactations, but small amounts of subacute inflammation were found in some of the udders. On average, there was an increase in the amount of subacute inflammation from the 1st to the 4th lactation. In udders from animals slaughtered after subsequent lactations there was, on average, a decrease in the amount of subacute inflammation in the ducts, but a gradual increase in the amount of periductal fibrosis. It is, therefore, possible that the increase in the average number of polymorphs found in the milk samples from one lactation to the next was due to an increase in the extent of subacute inflammation of the ducts, as well as to an increase in the severity of the lobular lesions.

The increase in the average total cell count during any one lactation was found to be due to an increase in both the number of polymorphs and the number of other cells. This would seem to point to an increase in the severity and extent of the inflammation throughout a lactation. In the terminal part of a lactation, during the course of involution of the udder, epithelial cells are shed from the secreting acini into the milk (Blackburn, 1952). The rise in the number of cells other than polymorphs, the majority of which were epithelial cells, from period 3 to period 4 in any lactation, could have been due to some extent to the normal shedding of epithelial cells which occurs in late lactation.

The increase in the total average cell count from one lactation to the next was shown whether or not the samples with counts of over 5 million cells/ml were included, and in both cases the increase in the average total cell count was accounted for mainly by an increase in the number of polymorphs present.

The average total cell count of all the milk samples taken when the herd was free from infection with coagulase-positive staphylococci was 0·3 million cells/ml. The percentage of cells which were polymorphs was, however, 80 compared with 50 for animals in their 1st lactation, an observation which suggests that the lesions were of less extent (few epithelial cells) but of greater severity (more polymorphs).

The fact that the infection-free animals with an average lactation age of 4·3 lactations had an average total cell count of the same level as animals in their 1st lactation is evidence to refute the statement that has been made that the increased cell count in the milk of older cows is physiological.

REFERENCES

BLACKBURN, P. S. (1952). Thesis, University of Glasgow.
BLACKBURN, P. S. (1956). *J. Dairy Res.* **23**, 225.
BLACKBURN, P. S., LAING, C. M. & MALCOLM, J. F. (1955). *J. Dairy Res.* **22**, 37.
BLACKBURN, P. S. & MACADAM, I. (1954). *J. Dairy Res.* **21**, 31.
PATTISON, I. H. (1951). *J. comp. Path. Ther.* **61**, 71.
WAITE, R. & BLACKBURN, P. S. (1963). *J. Dairy Res.* **30**, 23.

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The effect of bacterial infections of the udder on the yield and composition of cow's milk

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SUMMARY. The effect of bacterial infections, produced by infusions of *Streptococcus dysgalactiae* and of *Staphylococcus pyogenes* into one quarter of the udder, on milk yield and composition in heifers has been investigated. After a quarter became infected there was invariably a decrease in the yield of milk and in the concentrations of lactose and potassium, while there was an increase in those of sodium, chloride and non-casein proteins. These effects persisted into the 2nd lactation if the infection was not eliminated. If the infection was eliminated during the 1st lactation or during the dry period before 2nd calving there was a complete recovery in the composition of the milk in the 2nd lactation, but the recovery in milk yield was not complete.

It is well known that bacterial infection of an udder quarter usually causes a depression in yield and a change in the composition of the milk; evidence of a decrease in the content of lactose and an increase in the contents of sodium, chloride and non-casein proteins is particularly well documented (see e.g. McDowall, 1945, and review by Munch-Petersen, 1938). There is, however, little information on the magnitude and persistence of the changes or on the extent to which these are related to the severity of an infection, or of other factors.

Seelemann (1932) demonstrated that the yield of milk was often reduced in infected cows even in the absence of clinical signs of mastitis and Crossman, Dodd, Lee & Neave (1950) observed that subclinical streptococcal and staphylococcal infections may persist for months and either cause a gradual reduction in the proportionate yield of a quarter or have no measurable effect, and that both these results can be observed simultaneously in different quarters of the same udder.

Rowland, Neave, Dodd & Oliver (1959) compared milk from 92 quarters infected with *Staph. pyogenes* with milk from an uninfected quarter of the same cows. They found that on average the infected quarters gave less milk of lower fat and solids-not-fat (SNF) contents than corresponding uninfected quarters and that when infections were eliminated there was an immediate partial recovery in milk composition though not in milk yield. In the following lactation the recovery in fat and SNF contents was complete but that of milk yield was only partial. The quantitative assessments of the effect of bacterial infection on milk composition that have been made are in the main

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unreliable, as the techniques adopted are open to serious criticism. Invariably, indirect tests have been used for detection of an infection—among others, Johns & Hastings (1938) have demonstrated the unreliability of such tests and have pointed out that, even when bacteriological methods are used, it is essential to test on a number of occasions, as bacteria are not necessarily shed at each milking even when an infection is present. Also, chemical analyses have usually been carried out either on the bulk milk of the 4 quarters of an udder or on the fore-milk of individual quarters, instead of on the bulk milk of the separate quarters.

The experiments reported in this paper were designed to obtain more complete and quantitative information on the effect of induced bacterial infections on the composition of the milk, to determine how the changes develop immediately after the initiation of an infection, the relationship between the changes in the various milk constituents and the recovery that follows the elimination of infection.

EXPERIMENTAL

Animals and management

Three Friesian (57, 33 and 72) and 2 Ayrshire cows (Y62 and Y63) in their 1st lactation and free from udder infection from first calving were selected from the Institute herd. Before the experiments commenced the composition of the milk from the separate quarters of each cow was similar. The animals were kept with other cows in the herd, in a covered yard during winter and out at grass during the summer. Milking was done in a cowshed where strict hygiene precautions were taken to limit the spread of infection. Cows were milked with a machine designed for the separate collection of the milk from each quarter. Otherwise the feeding and management of the experimental animals was similar to that of other animals in the herd.

Experimental procedure

In each of the 7 experiments (expts. 1–7) 2 udder quarters of each cow were selected randomly; one was used as a control; the other, after a short introductory period, was infused through the teat canal with 0·25 ml of a suspension of organisms in skim-milk. Both quarters were sampled simultaneously and the composition of the healthy quarter used as an index of the composition of the milk which the infected quarter would have produced in the absence of infection. In this way it was possible to assess quantitatively the changes in composition of an infected quarter over long periods, since stage of lactation effects could be ignored. A similar technique was used by Vanlandingham, Weakley, Moore & Henderson (1941), who analysed fore-milk samples only, and by Rowland *et al.* (1959), who analysed samples of the total milk produced by each quarter. Details of the type and the number of organisms infused and the number recovered in fore-milk samples as determined by the number of colony-forming units are given in Figs. 1–7. The 2 strains, *m* and *d*, of *Staph. pyogenes* used have been described by Sharpe, Neave & Reiter (1962). The one strain of *Str. dysgalactiae* used had been isolated in 1958 from a case of clinical mastitis and freeze-dried.

Sampling

The yield of milk from control and experimental quarters was recorded. Samples of the bulk milk from each quarter were taken for chemical analysis at one or two milkings before the infusion of the experimental quarter with bacteria, at each milking for a number of days after the infusion and at less frequent intervals throughout the remainder of the lactation and, in expts. 3–7, throughout part of the 2nd lactation also. A fore-milk sample, taken after careful disinfection of the teat orifice with alcohol, was used for bacteriological tests. A quarter was considered to be infected if pathogenic bacteria were recovered frequently from the fore-milk and if there was a marked increase in the cell count.

Analysis

The bacteriological methods used have been described by Crossman *et al.* (1950). Total cell counts were made using the strip method described by Dodd, Oliver & Neave (1957).

The mean cell count (1 strip) of the fore-milk of the individual control quarters ranged from 44 000/ml to 130 000/ml and that of the infected quarters from 880 000/ml to 20×10^6/ml. Small numbers of pathogenic bacteria were recovered on isolated occasions from the control quarters during the experiments.

Samples were analysed for fat, total solids, lactose, potassium, sodium, chloride and total N, non-casein N and non-protein N by methods described previously (Wheelock, Rook & Dodd, 1965*a*). The non-casein proteins were fractionated by the methods of Aschaffenburg & Drewry (1959).

Samples were first analysed for chloride, as an index of the extent of any change in composition. In the first days after the start of the infection, if the value fluctuated from milking to milking, each sample was analysed in detail. Otherwise, only selected samples or alternatively weighted composites prepared from samples obtained at 2 or 4 consecutive milkings were analysed in full.

RESULTS

Milk yield (Tables 1 and 2, Figs. 1–7)

Comparison of the yield of an infected quarter with the yield of the same quarter in the period before infection is acceptable in the days immediately following infection, but over longer periods is invalid because of the lactational changes in milk yield. The yield of an infected quarter has therefore been expressed as a percentage of that of the control quarter, but it should be recognized that a decrease in the yield of an infected quarter may result in a compensating increase in the yield of the control quarter.

Infection following infusion of bacteria invariably caused a decrease in milk yield but the magnitude of the decrease was not closely related either to the organism infused or to the severity of infection as judged by clinical signs. Apart from a partial recovery in certain experiments shortly after the infection developed (Figs. 3 and 7) the yield of milk remained depressed throughout the lactation, even in quarters in which the infection was eliminated spontaneously or by antibiotic therapy. In 2 of the

3 experiments in which the infection was eliminated before 2nd calving there was a recovery in milk yield in the 2nd lactation, but this was not complete (Figs. 3 and 4; cf. Crossman *et al.* 1950).

Concentration of milk constituents (Tables 1 and 3, Figs. 1–7)

The concentrations of lactose and potassium were invariably decreased and the concentrations of sodium, chloride, residual albumin N and proteose–peptone plus globulin N were invariably increased by an infection. The β-lactoglobulin concentration was unchanged and there was no consistent pattern for the concentrations of casein N or fat. SNF content, reflecting mainly the resultant effect of the decrease in lactose content and the increase in non-casein proteins, was usually decreased slightly, but not invariably so (Table 1). Changes in composition were often particularly marked in the first few days after the infusion of bacteria when clinical signs were common, and there was invariably a partial recovery in composition during the 1st lactation and a further recovery by the beginning of the 2nd lactation. Elimination of infection during the course of the 1st lactation or dry period enhanced recovery in composition and resulted in a complete recovery by the beginning of the 2nd lactation (Figs. 3, 4 and 6). The most severe effects on composition were observed in those experiments in which there was the greatest depression in milk yield (Figs. 3, 6 and 7). The magnitude of the changes in concentration of the various water-soluble milk constituents were closely related one to another, and during the development of an infection to the effect on milk yield. The correlations between sodium, chloride, potassium and lactose for 3 of the experiments in which severe infections were induced are shown in Table 3.

Yield of milk constituents (Tables 2 and 4)

Invariably the yields of the synthesized constituents—fat, casein, β-lactoglobulin and lactose—were depressed in the period following infection, but, whereas the extent of the depressions of fat, casein and β-lactoglobulin were roughly related to the decrease in the yield of milk, there was a more marked effect with lactose during the time of infection only. The yield of potassium was affected in the same way as that of lactose. With sodium, however, the increase in concentration tended to compensate for the decrease in milk yield. In expts. 1–3 and 7, shortly after infection the yield of sodium from the infected quarter was increased, and in expts. 1, 3, 5, 6 and 7 the yield of sodium from the infected quarter, in spite of a much reduced yield of milk, was equal to, or greater than, the sodium yield of the control quarter throughout the period of infection. Although less marked, there was a similar trend for chloride residual albumin and proteose–peptone plus globulin.

DISCUSSION

In the present experiments, the infection that followed infusion of bacteria into an udder quarter invariably resulted in a decrease in milk yield, and the apparent depression varied from 70 to 20%. With the small number of animals used these differences cannot be ascribed to differences between pathogens or between animals but, nevertheless, it was evident that 2 infections caused by the same pathogen can

have different effects in different animals (cf. expts. 1 and 2; 3 and 5) or even in the same animal (cf. expts. 4 and 5). The effect of an infection on the concentrations of sodium, chloride, potassium and lactose was closely related to the severity of clinical symptoms, and where clinical symptoms regressed spontaneously or in response to antibiotic therapy there was a recovery in composition, although a complete recovery was not achieved until the next lactation (cf. Rowland *et al.* 1959).

The major changes in milk composition following an infection of the udder are similar to those observed in advanced lactation (Barry & Rowland, 1953) and when milk is allowed to accumulate within the udder (Wheelock, Rook & Dodd, 1965*b*; Wheelock, Rook, Dodd & Griffin, 1966). Barry & Rowland (1953), who studied the concentrations of sodium, potassium and chloride in blood and in milk at different stages of lactation, and their variation between infected and uninfected quarters of the same cow, concluded that the variations in the composition of the milk could result from the mixing in varying proportions of a milk similar to that produced early in lactation by udders free of bacterial infection, with a fluid referred to as a transudate differing slightly in composition from blood serum.

The temporary decrease in milk yield observed shortly after the infection developed in certain of the experiments may result from a depression in the activity of all the secretory cells and may resemble the temporary depression in secretory activity observed immediately after an extended milking interval (Wheelock *et al.* 1966). However, the more permanent decreases are probably caused by the failure of certain areas within the gland to function rather than to a decrease in the activity of all the cells; Waite & Blackburn (1963) have shown that bacterial infection is associated with a much greater degree of involution than is normally observed at a given stage of lactation. If, as seems likely, it is assumed that infections of the udder would affect similarly the ability of the gland to synthesize lactose, casein and fat, our observations can be explained only in part in terms of a dilution of a primary secretory fluid by a blood serum transudate, as in all instances there was a more marked effect on the concentration of lactose than on the concentration of casein or fat, and occasionally the contents of casein and fat were unaltered. A possible explanation is that a part of the lactose synthesized by the alveolar cells is later resorbed, possibly as the result of an exchange process with sodium, chloride and other water-soluble constituents of the plasma across membranes rendered semipermeable by bacterial action. This resorption could take place from milk within the ducts and cisterns of the udder, or alternatively from the secretory cell before the expulsion of the synthesized constituents into the lumen of the alveolus. The decrease in lactose content, and the increases in sodium and chloride content, that occur when milk is allowed to accumulate within the udder (Wheelock *et al.* 1965*b*; Wheelock *et al.* 1966) have been shown to be associated with the resorption of milk lactose into the blood and its excretion in the urine (Wheelock & Rook, 1966). A temporary accumulation of milk within areas of an infected quarter could occur owing to occlusion of the ducts by clots, or as a result of inflammation, and this would enhance resorption.

Any hypothesis should account also for the decrease in potassium content which invariably is roughly proportional to that in lactose content. If resorption was to occur mainly from milk within the ducts and cisterns of the gland, resorption of potassium would have to occur at the same relative rate as lactose if proportionality

was to be maintained. Alternatively, if resorption was to occur mainly from the contents of the secretory cell without exchange with sodium and chloride, there would be no need to postulate resorption of potassium, as the secretions of lactose and potassium from the alveolar cell into the lumen are probably closely linked (Rook & Wood, 1959); this would result in a reduced volume of primary secretion without a reduction in the secretion of fat or protein. The extent to which this process was coupled with a change in the volume of transudate would determine the changes in concentration of the various milk constituents.

If, as suggested earlier, the initial phase of the infection is followed by involution, the infected cells would eventually cease to secrete, and the lobules would atrophy, the permeability of the damaged tissue would decrease and the flow of transudate would be depressed. The composition of the milk would therefore tend to recover while the milk yield remained depressed.

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REFERENCES

ASCHAFFENBURG, R. & DREWRY, J. (1959). *15th Int. Dairy Congr.* **3**, 1631.
BARRY, J. M. & ROWLAND, S. J. (1953). *Biochem. J.* **54**, 575.
CROSSMAN, J. V., DODD, F. H., LEE, J. M. & NEAVE, F. K. (1950). *J. Dairy Res.* **17**, 128.
DODD, F. H., OLIVER, J. & NEAVE, F. K. (1957). *J. Dairy Res.* **24**, 20.
JOHNS, C. K. & HASTINGS, E. G. (1938). *Can. J. Res.* **16**, 6.
MCDOWALL, F. H. (1945). *N.Z. Jl Sci. Technol.* A, **27**, 258.
MUNCH-PETERSEN, E. (1938). *Bovine Mastitis: Survey of Literature to the End of* 1935. Weybridge: Imperial Bureau of Animal Health.
ROOK, J. A. F. & WOOD, M. (1959). *Nature, Lond.*, **184**, 647.
ROWLAND, S. J., NEAVE, F. K., DODD, F. H. & OLIVER, J. (1959). *15th Int. Dairy Congr.* **1**, 121.
SHARPE, M. E., NEAVE, F. K. & REITER, B. (1962). *J. appl. Bact.* **25**, 403.
SEELEMANN, M. (1932). *Die Streptokokkeninfektionen des Euters insbesondere der gelbe Galt.* Hannover: Schaper.
VANLANDINGHAM, A. H., WEAKLEY, C. E. JNR., MOORE, E. N. & HENDERSON, H. O. (1941). *J. Dairy Sci.* **24**, 383.
WAITE, R. & BLACKBURN, P. S. (1963). *J. Dairy Res.* **30**, 23.
WHEELOCK, J. V. & ROOK, J. A. F. (1966). *J. Dairy Res.* **33**, 37.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965*a*). *J. Dairy Res.* **32**, 79.
WHEELOCK, J. V., ROOK, J. A. F. & DODD, F. H. (1965*b*). *J. Dairy Res.* **32**, 237.
WHEELOCK, J. V., ROOK, J. A. F., DODD, F. H. & GRIFFIN, T. K. (1966). *J. Dairy Res.* **33**, 161.

Expt.	Cow	Lactation no.	Length of period	Yield	% I	% C	g/100 g I	g/100 g C	% I	% C	g/100 g I	g/100 g C	g/100 g I	g/100 g C	% I	% C	g/100 g I	g/100 g C	g/100 g I	g/100 g C	g/100 g I	g/100 g C	g/100 g I	g/100 g C	I
			1 milking (1)	100	2·75	3·08	340	329	4·72	4·71	176	176	37·1	34·1	108	104	8·41	8·33	18·8	19·1	35·2	36·6	24·6	24·0	26
			Infection with *Staph. pyogenes* strain *m*																						
1	57	1	6 days (12)	92·0	3·04	3·22	342	341	4·60	4·77	165	170	48·4	37·4	113	100	8·28	8·40	20·6	20·9	34·8	33·3	28·4	26·2	28
			6 months (24)	85·9	3·57	3·64	349	347	4·36	4·55	159	165	59·1	46·7	123	114	8·14	8·26	25·8	26·0	36·8	37·8	32·3	28·3	28
			1 milking (1)	100	3·89	3·86	426	425	4·84	4·81	155	155	37·1	37·1	77	77	9·24	9·24	31·2	36·9	33·8	34·1	34·0	28·4	21
			Infection with *Staph. pyogenes* strain *m*																						
2	33	1	3 days (6)	89·0	3·79	3·78	431	435	4·55	4·77	143	152	59·2	36·6	121	82	9·05	9·17	34·1	32·8	34·2	34·8	37·9	29·4	37
			6 months (24)	86·0	3·95	4·09	434	445	4·63	4·85	141	144	52·7	39·7	99	84	9·20	9·31	30·4	29·7	45·3	47·1	35·2	29·2	34
			1 day (2)	100	2·84	2·90	333	344	4·69	4·73	165	164	29·6	29·3	71	70	8·71	8·72	32·6	31·6	29·9	31·7	26·4	28·6	16
			Infection with *Str. dysgalactiae*																						
		1	8 days (16)	70·9	3·40	3·31	342	333	3·94	4·91	144	164	84·7	31·3	131	66	8·47	8·61	25·2	22·1	41·2	33·4	71·3	26·3	60
3	Y62		Spontaneous recovery of infection																						
			3 months (16)	35·3	3·51	3·77	363	388	4·27	4·67	146	161	57·7	33·4	101	72	8·57	8·87	31·2	31·6	40·1	41·0	36·9	27·9	47
			Dry period																						
		2	2 months (12)	86·1	2·73	2·75	362	375	4·69	4·73	166	166	33·8	32·2	80	75	8·78	8·84	27·2	27·8	—	—	—	—	25
			1 day (2)	100	3·22	3·28	313	343	4·99	4·98	166	166	32·4	33·4	53	53	8·82	8·91	25·6	24·3	29·6	31·8	23·2	22·4	27
			Infection with *Str. dysgalactiae*																						
		1	10 days (20)	47·0	4·06	3·91	429	382	4·06	4·76	155	172	72·0	32·8	110	59	9·18	8·89	31·7	22·3	29·3	29·5	76·2	32·2	80
			Infection eliminated by antibiotic therapy but quarter infected with *Staph. pyogenes* strain *m* shortly after																						
4	Y63		2 days (4)	34·8	4·14	3·65	455	420	3·96	4·58	146	172	68·4	34·0	113	68	9·33	9·09	34·0	28·4	37·1	27·7	64·3	31·5	91
			3 months (16)	51·3	3·18	2·87	418	438	4·37	4·58	157	168	52·9	36·1	94	64	9·18	9·17	26·9	27·3	34·9	35·7	35·1	34·8	34
			Dry period—spontaneous recovery during the dry period																						
		2	2 months (8)	82·6	2·79	2·55	381	386	4·67	4·66	170	172	35·7	35·9	87	87	8·66	8·71	24·1	23·5	—	—	—	—	19
			Infection with *Str. dysgalactiae*																						
		1	7 days (14)	68·3	4·53	3·88	391	387	4·23	4·70	156	171	61·7	33·1	100	64	8·79	8·90	24·9	22·1	31·1	30·3	56·6	33·3	61
5	Y63		3 months (16)	73·7	3·26	2·87	394	438	4·42	4·58	160	168	48·5	36·1	89	64	9·06	9·17	27·5	27·3	36·1	35·7	37·4	34·8	40
			Dry period																						
		2	2 months (12)	74·4	2·78	2·55	381	386	4·53	4·66	168	172	43·5	35·9	85	87	8·59	8·71	23·9	23·5	—	—	—	—	22
			1 day (2)	100	4·90	4·90	434	442	4·35	4·37	146	145	57·2	53·1	101	94	8·83	8·73	32·2	32·6	32·6	31·5	32·9	35·6	33
			Infection with *Staph. pyogenes* strain *d*																						
		1	4 days (8)	54·3	4·19	4·65	375	416	3·34	4·52	117	153	123·6	50·3	163	94	7·81	8·91	25·0	24·5	34·6	34·1	59·3	33·8	62
6	72		Infection eliminated by antibiotic therapy																						
			3 days (6)	46·4	4·76	5·03	438	466	4·04	4·37	134	141	71·3	52·3	122	102	9·04	9·20	31·5	32·4	43·0	45·2	59·5	37·6	53
			Dry period																						
		2	3 months (16)	60·7	3·33	3·35	392	386	4·57	4·65	158	160	35·9	36·8	92	86	8·84	8·85	29·9	31·1	—	—	—	—	22
			1 day (2)	100	4·86	4·90	438	442	4·38	4·37	146	145	54·3	53·1	96	94	8·95	8·93	32·3	32·6	31·6	31·5	33·0	35·6	33
			Infection with *Staph. pyogenes* strain *d*																						
		1	4 days (8)	78·8	4·35	4·65	387	416	3·22	4·52	117	153	129·9	50·3	171	94	7·98	8·91	26·0	24·5	36·5	34·1	80·9	33·8	86
7	72																								

Figures in parentheses are the number of samples from which the values have been derived.)

Expt.	Cow	Lactation	Length of period	Yield I	Yield C	Fat I	Fat C	Casein N I	Casein N C	Lactose I	Lactose C	K I	K C	Na I	Na C	Cl I	Cl C	SNF I	SNF C	Protein N I	Protein N C	β-lactoglobulin N I	β-lactoglobulin N C	Residual albumin N I	Residual albumin N C	Proteose-peptone + globulin N I	Proteose-peptone + globulin N C
1	57	1	1 milking (1)[a]	1675	1705	46·1	52·5	5·70	5·61	79·1	80·3	2·95	3·00	0·62	0·58	1·81	1·77	141	142	6·95	6·87	0·59	0·62	0·41	0·41	0·44	0·3
			Infection with *Staph. pyogenes* strain *m*																								
			2 days (4)	2508	2800	76·0	88·0	8·45	9·55	115	131	4·17	4·18	1·24	1·00	2·88	2·84	207	234	10·6	11·6	0·84	0·89	0·72	0·75	0·80	0·5
			2 days (4)	2013	2603	69·1	94·0	6·87	9·08	85·3	125	3·11	4·37	1·39	0·95	2·65	2·58	162	214	9·35	11·0	0·80	0·96	0·92	0·72	1·17	0·6
			6 months (24)	1503	1782	53·7	65·0	5·26	6·35	65·5	81·0	2·46	2·84	0·92	0·81	1·85	2·03	123	148	6·68	7·74	0·55	0·67	0·49	0·51	0·59	0·5
2	33	1	1 day (2)	2525	3425	—	—	10·8	14·6	122	165	3·91	5·31	0·94	1·27	1·90	2·57	231	313	13·0	17·5	0·85	1·17	0·86	0·97	0·53	0·8
			Infection with *Staph. pyogenes* strain *m*																								
			3 days (6)	2133	3263	80·9	123	9·2	13·9	97·0	160	3·05	4·96	1·26	1·19	2·28	2·67	193	298	11·5	16·7	0·73	1·14	0·87	0·96	0·79	0·7
			6 months (24)	1554	2436	61·4	99·7	6·8	10·8	72	118	2·19	3·50	0·82	0·90	1·54	2·04	141	227	8·5	13·3	0·70	1·15	0·55	0·71	0·53	0·6
3	Y62	1	1 day (2)	4195	5125	119	149	14·0	17·6	197	242	6·93	8·41	1·24	1·50	2·98	3·57	366	447	17·0	21·9	1·26	1·62	1·11	1·46	0·66	0·7
			Infection with *Str. dysgalactiae*																								
			4 days (8)	3084	4796	102	159	9·70	15·4	129	237	4·58	7·94	1·64	1·50	3·21	3·00	255	410	14·1	19·1	1·11	1·38	1·57	1·37	1·56	0·8
			3 days (6)	2683	4993	95·3	164	—	—	99	239	3·65	8·06	2·66	1·56	3·65	3·30	233	437	—	—	—	—	—	—	—	—
			Spontaneous recovery of infection																								
			3 months (16)	1219	4215	42·8	159	4·43	16·4	52	197	1·70	6·36	0·72	1·32	1·24	3·05	105	374	6·29	20·5	0·49	1·84	0·90	1·34	0·58	1·2
		2	Dry period																								
			2 months (12)	5105	7216	139	199	—	—	240	341	8·48	12·0	1·73	2·32	4·09	5·39	448	638	23·9	34·4	—	—	—	—	—	—
4	Y63	1	1 day (2)	3175	3680	102	121	9·94	12·6	159	183	5·26	6·32	1·03	1·23	—	—	280	328	12·5	15·6	0·94	1·17	0·74	0·82	0·89	1·0
			Infection with *Str. dysgalactiae*																								
			10 days (20)	1444	3556	58·7	139	5·5	13·5	57	170	2·15	6·09	1·00	1·17	1·56	2·09	133	316	8·90	16·4	0·42	1·05	1·10	1·12	1·16	0·7
			Infection eliminated by antibiotic therapy but quarter infected with *Staph. pyogenes* strain *m* shortly after																								
			2 days (4)	1130	3898	46·8	142	5·2	16·4	45	178	1·66	6·70	0·77	1·33	1·28	2·66	105	354	7·30	19·7	0·42	1·08	0·73	1·22	1·03	0·9
			3 months (16)	2614	3490	83·3	117	10·9	15·3	115	160	4·12	5·86	1·37	1·22	2·44	2·61	240	318	13·9	18·7	0·90	1·10	0·92	1·22	0·89	0·9
			Dry period—spontaneous recovery during dry period																								
		2	2 months (8)	4975	6979	139	178	18·9	26·9	232	325	8·47	12·0	1·77	2·51	4·25	—	433	599	22·7	32·4	—	—	—	—	0·62	1·2
			Infection with *Str. dysgalactiae*																								
5	Y63	1	6 days (12)	2727	3573	111	142	10·6	13·7	115	168	4·26	6·08	1·68	1·19	—	2·14	242	318	14·6	16·7	0·83	1·07	1·54	1·18	2·02	0·7
			4 days (8)	2758	3936	—	—	11·1	16·4	119	182	4·39	6·74	1·60	1·33	2·71	2·78	—	—	15·2	19·8	1·08	1·16	1·55	1·27	1·42	0·9
			3 months (16)	2832	3490	92·5	117	11·2	16·3	125	160	4·54	5·86	1·37	1·22	2·53	2·61	257	318	15·2	18·6	1·02	1·25	1·06	1·22	1·14	0·9
		2	Dry period																								
			2 months (8)	5712	6979	159	178	21·7	26·9	259	325	9·62	12·0	2·48	2·51	5·00	—	4·91	599	26·5	32·4	—	—	—	—	1·25	1·2
			1 day (2)	1480	1595	72·5	78·1	6·42	6·99	64·3	70·0	2·17	2·27	0·85	0·92	1·50	1·50	131	143	7·89	8·44	0·48	0·50	0·49	0·57	0·49	0·3
			Infection with *Staph. pyogenes* strain *d*																								
		1	2 days (4)	878	1475	40·9	77·0	3·44	6·67	31·7	65·0	1·15	2·31	0·93	0·75	1·31	1·45	72·9	137	4·70	8·24	0·28	0·49	0·45	0·50	0·54	0·4
6	72		Infection eliminated by antibiotic therapy																								
			2 days (4)	588	1158	32·2	68·4	2·68	5·82	24·2	50·0	0·76	1·58	0·43	0·60	0·72	1·18	54·6	109	7·14	7·22	0·55	0·58	0·44	0·35	0·79	0·4
			Dry period																								
		2	3 months (16)	3442	5163	115	173	13·5	20·0	157	240	5·45	8·26	1·23	1·90	3·19	4·47	304	457	16·4	24·5	—	—	—	—	0·76	1·3

Table 3. *The relationships between sodium, potassium, lactose and chloride in milk from an infected quarter*

(The concentrations of sodium, potassium and chloride are expressed in mg/100 g milk water and the concentration of lactose in g/100 g milk water.)

Correlation between	Regression equations	S.E. of regression coefficients	Correlation coefficient
	Expt. 3		
Na and Cl	$Na = 0{\cdot}96Cl - 43$	$6{\cdot}07 \times 10^{-2}$	0·97
	$Cl = 0{\cdot}97Na + 51$	$6{\cdot}15 \times 10^{-2}$	
Na and K	$Na = -1{\cdot}92K + 405$	$3{\cdot}21 \times 10^{-1}$	−0·79
	$K = -0{\cdot}33Na + 225$	$5{\cdot}57 \times 10^{-2}$	
Na and L	$Na = -59{\cdot}1L + 357$	4·35	−0·93
	$L = -0{\cdot}015Na + 5{\cdot}9$	$1{\cdot}12 \times 10^{-3}$	
K and Cl	$K = -0{\cdot}32Cl + 110$	$5{\cdot}93 \times 10^{-2}$	−0·77
	$Cl = -1{\cdot}86K + 443$	$3{\cdot}46 \times 10^{-1}$	
K and L	$K = 17{\cdot}5L + 87$	4·30	0·67
	$L = 0{\cdot}026K - 0{\cdot}3$	$6{\cdot}38 \times 10^{-3}$	
Cl and L	$Cl = -59{\cdot}1L + 406$	4·65	−0·94
	$L = -0{\cdot}015Cl + 6{\cdot}6$	$1{\cdot}19 \times 10^{-3}$	
	Expt. 4		
Na and Cl	$Na = 0{\cdot}56Cl + 3$	$6{\cdot}47 \times 10^{-2}$	0·81
	$Cl = 1{\cdot}17Na + 41$	$1{\cdot}35 \times 10^{-1}$	
Na and K	$Na = -1{\cdot}28K + 301$	$1{\cdot}64 \times 10^{-1}$	−0·77
	$K = -0{\cdot}47Na + 212$	$6{\cdot}07 \times 10^{-2}$	
Na and L	$Na = -47{\cdot}3L + 299$	2·68	−0·95
	$L = -0{\cdot}018Na + 6{\cdot}1$	$1{\cdot}06 \times 10^{-3}$	
K and Cl	$K = -0{\cdot}26Cl + 210$	$5{\cdot}24 \times 10^{-2}$	−0·62
	$Cl = -1{\cdot}47K + 389$	$2{\cdot}94 \times 10^{-1}$	
K and L	$K = 23{\cdot}5L + 65$	3·11	0·77
	$L = 0{\cdot}025K + 0{\cdot}3$	$3{\cdot}32 \times 10^{-3}$	
Cl and L	$Cl = -60{\cdot}5L + 415$	6·31	−0·87
	$L = -0{\cdot}012 + 6{\cdot}2$	$1{\cdot}20 \times 10^{-3}$	
	Expt. 7		
Na and Cl	$Na = 1{\cdot}14Cl - 72$	$6{\cdot}05 \times 10^{-2}$	0·98
	$Cl = 0{\cdot}84Na + 68$	$4{\cdot}44 \times 10^{-2}$	
Na and K	$Na = -1{\cdot}74K + 348$	$2{\cdot}43 \times 10^{-1}$	−0·86
	$K = -0{\cdot}43Na + 146$	$6{\cdot}05 \times 10^{-2}$	
Na and L	$Na = -53{\cdot}7L + 366$	3·11	−0·97
	$L = -0{\cdot}018Na + 6{\cdot}2$	$1{\cdot}02 \times 10^{-3}$	
K and Cl	$K = -0{\cdot}5Cl + 229$	$7{\cdot}26 \times 10^{-2}$	−0·86
	$Cl = -1{\cdot}47K + 380$	$2{\cdot}14 \times 10^{-1}$	
K and L	$K = 22{\cdot}4L + 54$	3·89	0·81
	$L = 0{\cdot}03K - 0{\cdot}3$	$5{\cdot}11 \times 10^{-3}$	
Cl and L	$Cl = -43{\cdot}8L + 345$	4·32	−0·93
	$L = -0{\cdot}02Cl + 7{\cdot}4$	$1{\cdot}93 \times 10^{-3}$	

L, lactose; Na, sodium; K, potassium; Cl, chloride.

Table 4. *Expt. 3. Effect of bacterial infection of the udder on the ratios in milk of casein to fat, lactose to fat and lactose to casein*

(Values are for consecutive periods throughout the experiment.)

Lactation	Condition of experimental quarter	No. of samples from which ratio has been derived	Casein/fat		Lactose/fat		Lactose/casein	
			Experimental quarter	Control quarter	Experimental quarter	Control quarter	Experimental quarter	Control quarter
1	Uninfected	2	1·18	1·18	1·66	1·62	1·41	1·38
	Infected with *Str. dysgalactiae*	8	0·95	0·97	1·26	1·49	1·33	1·54
		4	1·02	0·98	0·93	1·35	0·91	1·37
	Uninfected	4	1·04	1·03	1·21	1·24	1·17	1·20
2	Uninfected	8	1·32	1·35	1·72	1·70	1·30	1·26

Key to Figs. 1–7

Figs. 1–7. Changes in number of bacteria, clinical symptoms, cell counts, milk yield and composition due to infection of the udder. Clinical symptoms were estimated by straining all the milk from a quarter and expressing the number of clots on an arbitrary scale, 1–4. Concentrations of milk constituents are shown as the differences between the value for the infected quarter and the value for the control quarter. ○, Quarter not infected; ●, quarter infected. Points relating to values for consecutive milkings or days are joined by a full line and those values obtained after longer intervals are joined by a dotted line.

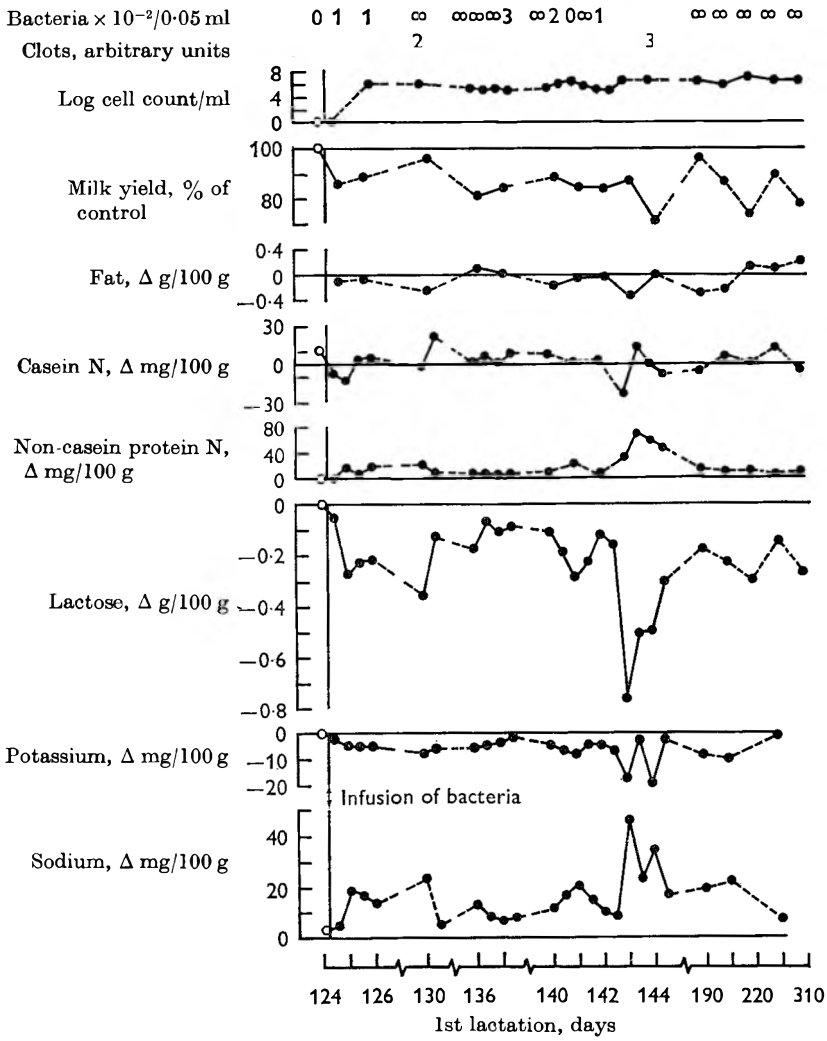

Fig. 1. Expt. 1, cow 57. The left-fore quarter was infused with 150000 viable cells *Staph. pyogenes* strain *m*. During the first 20 days clinical symptoms were observed on 2 occasions in association with marked changes in the contents of lactose, potassium and sodium in the milk. The infection persisted throughout the lactation.

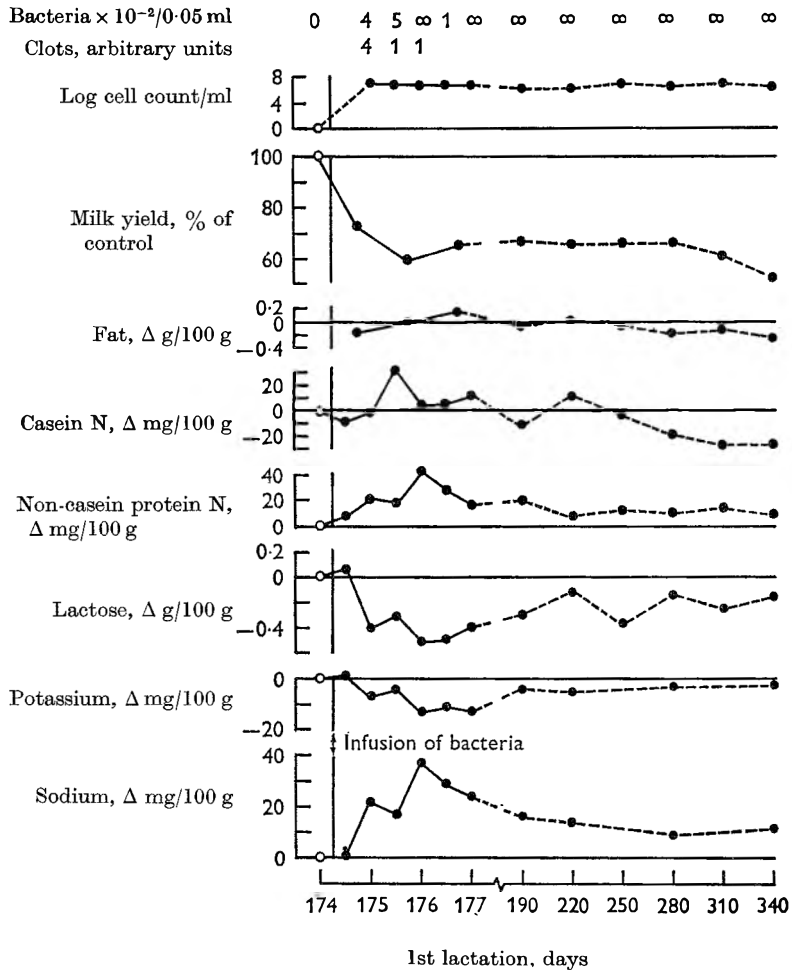

Fig. 2. Expt. 2, cow 33. The right-fore quarter was infused with 500000 viable cells *Staph. pyogenes* strain *m*. Two days later clinical symptoms were observed in association with a marked decrease in yield and in the contents of lactose and potassium and an increase in the contents of sodium, chloride and non-casein proteins. Subsequently, there was some recovery in the composition but none in the yield of milk.

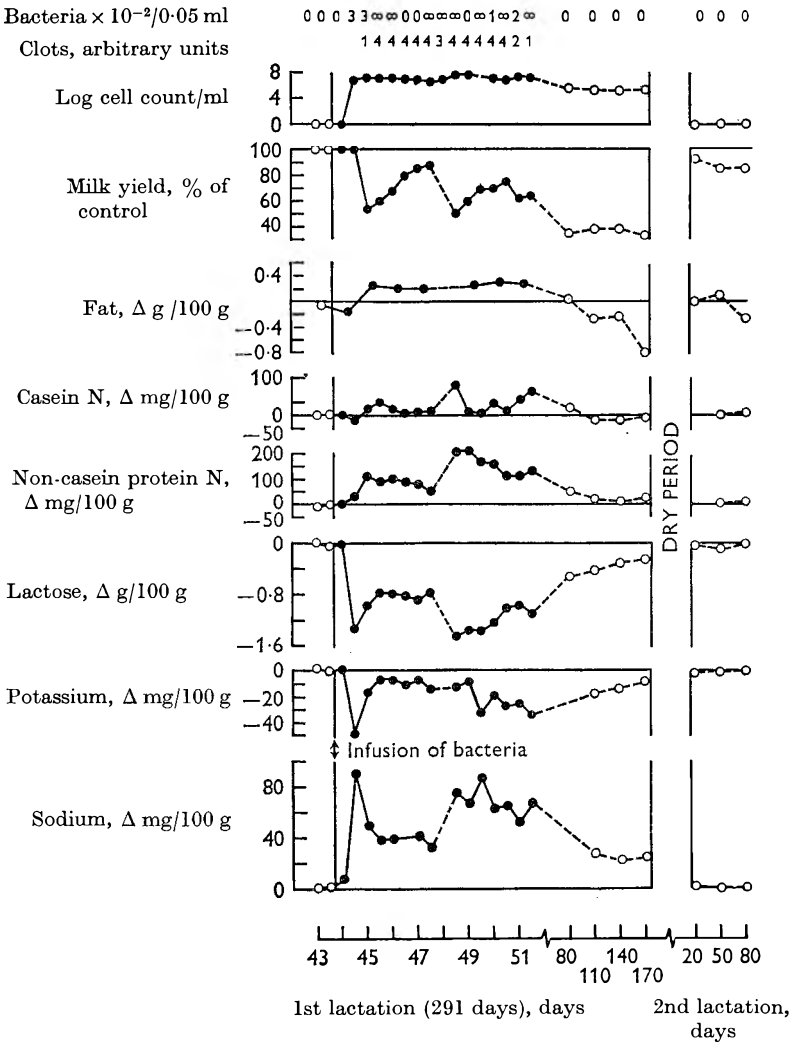

Fig. 3. Expt. 3, cow Y 62. The left-fore quarter was infused with 5 000 viable cells *Str. dysgalactiae*. The next day there was a rise in the rectal temperature of the cow, the infused quarter was swollen and the cow refused food. Two days after the infusion the quarter showed clinical symptoms of infection and a 50 % drop in yield, and then the yield fluctuated widely during the following 10 days. The magnitude of changes in the contents of lactose, potassium, sodium and non-casein proteins varied with the effect on milk yield. Nine days after the infusion there was a spontaneous recovery from the infection and this was followed by a gradual recovery in milk composition but not in yield. In the 2nd lactation the composition was fully recovered and the yield, which at the end of the 1st lactation had decreased to 40 % of that of the control quarter, recovered to about 90 %.

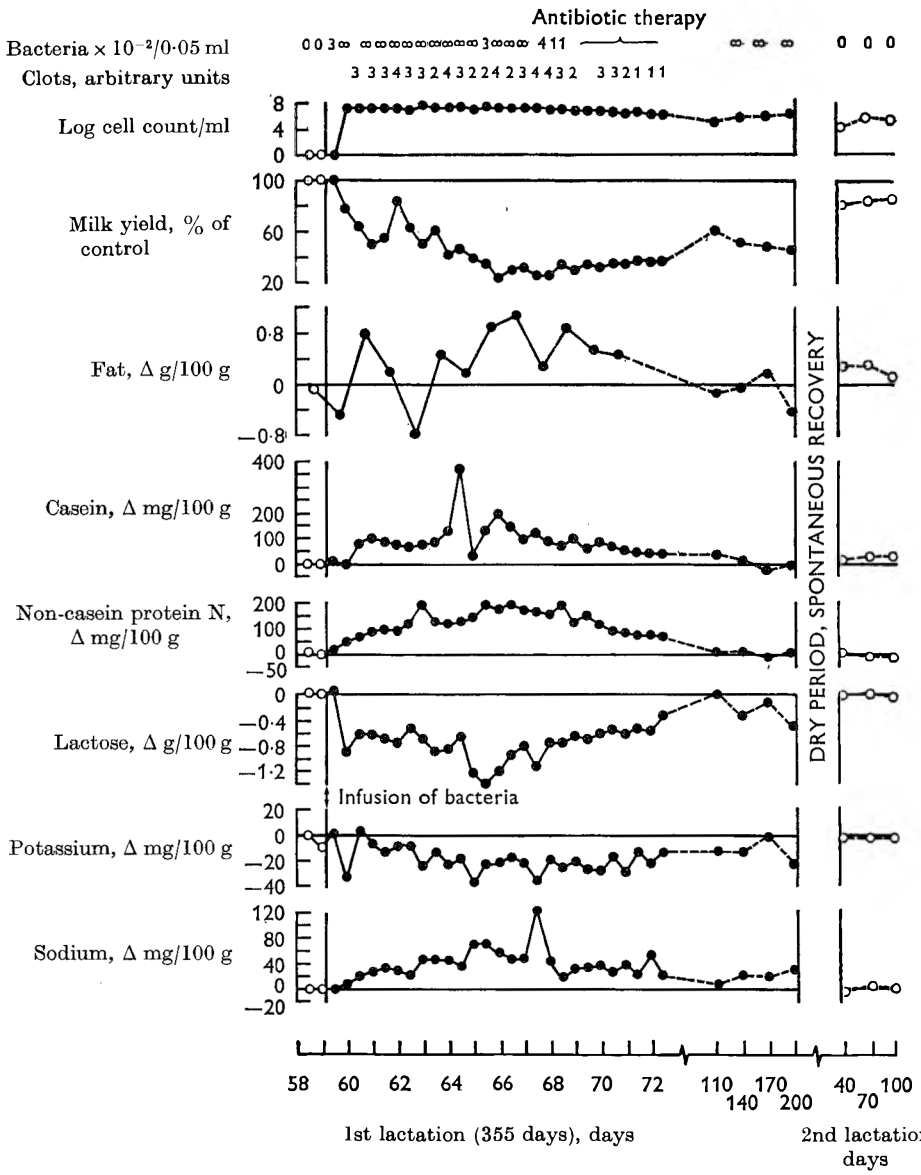

Fig. 4. Expt. 4, cow Y 63. The left-fore quarter was infused with 500 viable cells *Str. dysgalactiae*. Clinical symptoms were observed shortly afterwards and there was a rapid decrease in the yield of milk with decreases in the contents of lactose and potassium and increases in the contents of sodium and chloride. Ten days after infusion the infection was eliminated by antibiotic intramammary therapy and there followed a slight recovery in yield and composition. Six weeks after the antibiotic therapy the quarter developed an infection of *Staph. pyogenes* strain *m*, but without clinical symptoms. During the dry period this infection recovered spontaneously and in the 2nd lactation there was a complete recovery in composition but the milk yield recovered only partially.

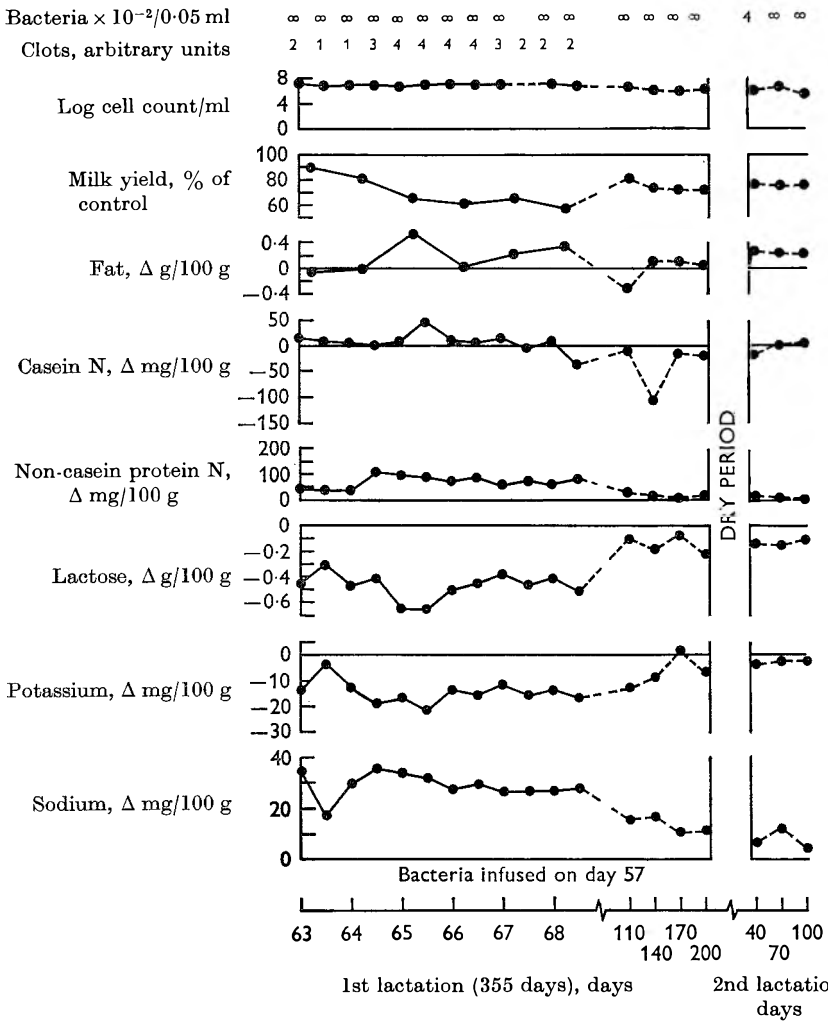

Fig. 5. Expt. 5, cow Y 63. Clinical symptoms developed 6 days after the infusion of the left-hind quarter with 500 viable cells *Staph. pyogenes* strain *d*. The milk yield decreased and there were the typical changes in composition. The quarter remained infected throughout the 1st lactation, the dry period and the start of the 2nd lactation, but clinical symptoms regressed and the quarter appeared normal again about 7 weeks after the infusion. There was a slight recovery in milk yield and composition in the last 3 months of the lactation. In the 2nd lactation, the milk yield of the quarter was depressed by about 25 % but there was an almost complete recovery in milk composition.

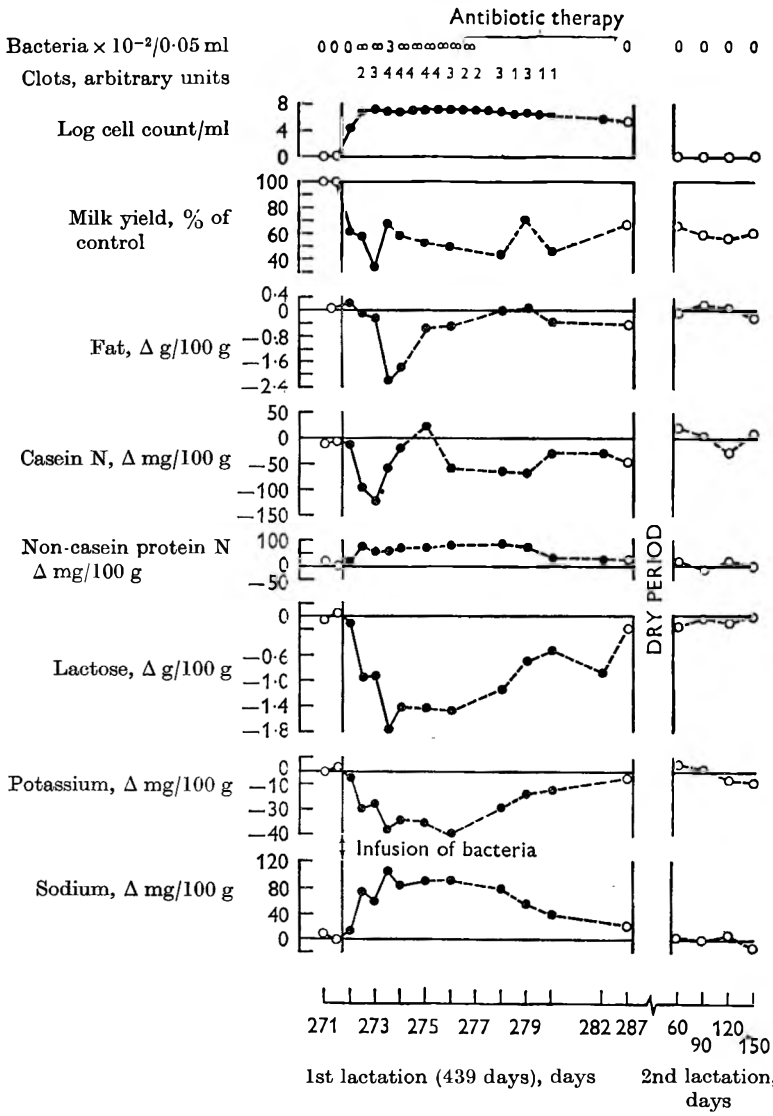

Fig. 6. Expt. 6, cow 72. The left-fore quarter was infected after an infusion of 2000 viable cells *Staph. pyogenes* strain *d*. There was an increase in the rectal temperature of the cow the next day. Clinical symptoms were observed within 2 days of the infusion and there was a decrease in yield and changes in composition. After antibiotic intra-mammary therapy the clinical symptoms quickly disappeared and there was a marked recovery in milk composition but little effect on milk yield. In the 2nd lactation the recovery in composition was complete but the yield was still depressed and to a similar extent.

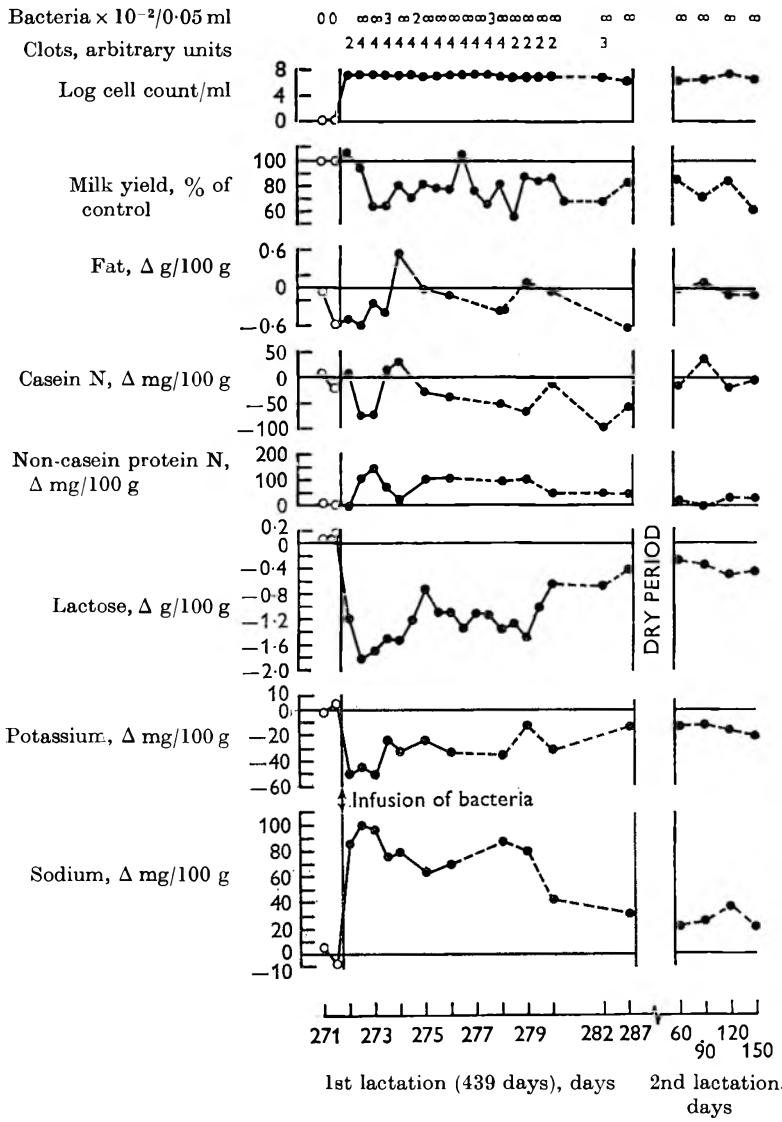

Fig. 7. Expt. 7, cow 72. The right-hind quarter was infused at the same time as the left-fore quarter (expt. 6) with 2×10^6 viable cells *Staph. pyogenes* strain *d*. Antibiotic therapy was not applied in this experiment and the infection persisted through the dry period into the 2nd lactation. Soon after infection the milk yield declined by about 30 % but did not decline further. There was a recovery in milk composition towards the end of lactation and the difference in milk composition between the infected and control quarters was small at the start of the 2nd lactation, but the milk yield was still depressed.

Effects of dilution, freezing and thawing and drying on the dispersibility of isolated fat globule membrane

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Summary. The effects of dilution, freezing and thawing, and drying on the dispersibility of fat-globule membrane (FGM) preparations were studied. Dilution of FGM favoured aggregation of the lipoprotein particles. Freezing and thawing of FGM had a similar effect. Drying FGM by roller-drying markedly decreased its dispersibility. Drying by low-temperature vacuum-drying only slightly increased the dispersibility of FGM, while freeze-drying and spray-drying gave larger increases in the dispersibility.

The effects of various methods of drying on the dispersibility of dried whole milk, skim-milk and cream have been reviewed by King (1965). In addition to gas content, particle size, specific surface area, lactose crystallization and other factors, the change in proteins and lipids during drying has a pronounced effect on the reconstitution of these products (Hostettler & Imhoff, 1953*a*, *b*; King, 1955*a*; Calapaj, 1962; Greenbank & Pallansch, 1962; Berlin, Howard & Pallansch, 1964; King, 1965). Among these factors, the release of free fat appears to be quite detrimental to dispersibility. The presence of free fat is indicative of damage to the fat-globule membrane (FGM). The amount of free fat released varies with the method of drying. Expressed as a percentage of the total fat the following values have been obtained: roller-dried whole-milk powder, 91·6–95·8 % (Lampitt & Bushill, 1931); low-temperature vacuum-dried whole milk, 5–7·5 % (Bullock, 1958); freeze-dried powder, 43–75 % (Nickerson, Coulter & Jenness, 1952); foam-dried powder, less than 10 % (Tamsma, Edmondson & Vettel, 1959); spray-dried powder, 3·3–14·2 % (Lampitt & Bushill, 1931), 12–19 % (Nickerson *et al.* 1952), 1–20 % (Litman & Ashworth, 1957), and 1·0–6·9 % (Reinke, Brunner & Trout, 1960).

Freezing and thawing of washed cream is also known to damage the structure of the FGM (King, 1955*b*; Cole, Kloepfel & Lusena, 1959).

This paper describes a study of the effects of various forms of drying, dilution, and freezing and thawing on the dispersion of the isolated membrane in an attempt to define the effects of these treatments on the lipoprotein system of the membrane.

MATERIALS AND METHODS

Milk. Raw whole milk was obtained at the University of Wisconsin. Each collection was 40 gal of morning milk (fat content, 5·0 %) obtained from healthy Jersey and Guernsey cows. Each batch of milk was collected from the same animals through-

out the experimental period. The milk was transferred immediately after milking to the processing laboratory without cooling.

Preparation of fat-globule membrane. The FGM was isolated essentially as described by Brunner, Duncan & Trout (1953), except that the cream was washed 3 times with distilled water at 37 °C. After washing, the cream was adjusted to 40 % fat and churned. This method yielded the combined FGM from the buttermilk and butterfat fractions. In addition, FGM was not precipitated from suspension after churning, but was immediately dried or frozen after pH adjustment to 6·85 when necessary. The total solids content of the FGM suspension was determined by the Mojonnier method and found to average about 2·1 % (w/v). This yield is slightly higher than expected from calculations based on literature values for membrane protein, phospholipid, and triglyceride (Jenness & Patton, 1959). Each of the following experiments was repeated twice, and analyses were run in duplicate. Control samples were run with each experiment.

Separate batches of FGM were frozen or dried according to the following conditions, and compared with control FGM for dispersibility.

Freezing. About 1 l of FGM preparation containing 2·1 % (w/v) total solids was frozen at −20 °C for 48 h and then thawed slowly at 4–8 °C.

Spray-drying. FGM was spray-dried in a Niro laboratory spray-dryer, and enough material was collected for dispersibility studies. Approximately 2·5 l of a preparation containing 2·1 % (w/v) solids were fed into the dryer at an air inlet temperature of 170 °C and an air outlet temperature of 70 °C.

Freeze-drying. An FGM preparation of 2·1 % (w/v) total solids was freeze-dried in a laboratory lyophilization apparatus. About 3 l was shell-frozen, and dried at a vacuum of 0·3 mmHg.

Roller-drying. About 2·5 l of FGM preparation of 2·1 % (w/v) total solids were roller-dried at atmospheric pressure in a small Buflovak dual roller-drier using steam under pressure to give a temperature of 133 °C at the surface of the roller. The temperature of the film as scraped from the roller was approximately 121 °C.

Vacuum-drying. About 800 ml of FGM preparation of 2·1 % (w/v) total solids were dried in the low-temperature vacuum apparatus described by Kielsmeier (1956). The vacuum on the FGM was maintained at 4·8 mmHg. The temperature of the product, monitored by thermocouples, dropped to 0 °C, rose from 0 to 10 °C in the first 10 min, from 10 to 40 °C in the following 20 min, and from 40 to 60 °C in the last 4 min.

Dispersibility test. For the dried FGM preparations a sufficient amount of dried FGM was added to distilled water to give 0·6–0·8 % (w/v) total solids. The control sample was of undried FGM and contained 0·65 % (w/v) total solids. One hundred ml of the control and reconstituted dried preparations were each treated with a single speed Waring Blendor (model 700 B) for exactly 1 min. Frozen and thawed samples were examined at a concentration of 2·1 % (w/v) total solids, and compared with controls at 2·1 % (w/v) total solids; comparisons were made with and without blending.

Duplicate samples were then subjected to analysis by ultracentrifugation. They were spun in tared cellulose tubes in a number 30 rotor in the Spinco model L ultracentrifuge at room temperature. The cellulose tubes were previously dried *in vacuo*

at 60 °C for 24 h before they were weighed. The 1st pellet was obtained by centrifuging at 5000 *g* for 20 min. The supernatant material was transferred to tared tubes and spun at 10000 *g* for 30 min. The 3rd, 4th and 5th pellets were obtained in the same manner at 20000, 40000, and 80000 *g* for 30 min, respectively. The supernatant liquid after the 80000 *g* spin, and the pellets, were dried *in vacuo* at 60 °C to constant weight. The yield of each pellet was expressed as a percentage of the total solids, and the cumulative percentage values were plotted against centrifugal force to give an ultracentrifuge profile.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of dilution on the degree of aggregation of the FGM preparation before and after blending. Sample D_1 is the 0·65 % (w/v) solids control compared with C_1, the 2·1 % (w/v) solids control without blendor treatment. Apparently, dilution has a destabilizing effect on the dispersed lipoproteins causing aggregation

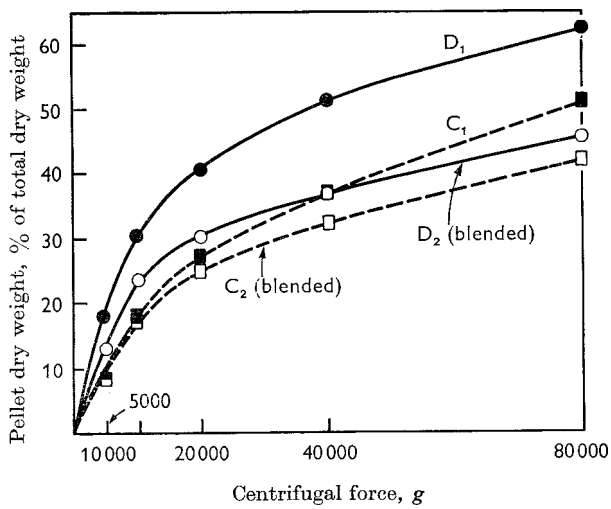

Fig. 1. The effect of dilution on the state of aggregation of the lipoproteins of the fat-globule membrane. ■, 2·1 % (w/v) total solids, untreated; □, 2·1 % (w/v) total solids, after blending; ●, 0·65 % (w/v) total solids, untreated; ○, 0·65 % (w/v) total solids, after blending.

of the particles, thus yielding greater quantities of lipoprotein in the pellet. Blendor treatment of the 2 control samples increased dispersion; but the difference between the samples still persisted after blending. Because of this concentration effect, control samples were compared with experimental samples at comparable concentrations.

The observed effect of dilution is consistent with the generally accepted phenomenon that proteins are less stable in dilute solutions. Perhaps the gegen ion atmosphere of the lipoproteins is altered by dilution to yield less stable systems. Blaauw (1960) observed that the free fat content of spray-dried milk decreased with increasing solids content of the preconcentrate. This might be due, in part, to the dilution effect on the stability of FGM lipoproteins.

As shown in Fig. 2, freezing and thawing the FGM preparation affected the ultracentrifuge profile. Comparing the untreated sample (denoted by 'Frozen 1' in Fig. 2)

and the C_1 control, it is apparent that freezing and thawing caused an aggregation of the lipoprotein particles. This could explain the very damaging effects of freezing and thawing on cream. Blendor treatment of the samples increased the dispersion; however, there was still a difference between the control and frozen samples.

Although control samples in Figs. 1 and 2 are labelled C_1 and C_2, these represent

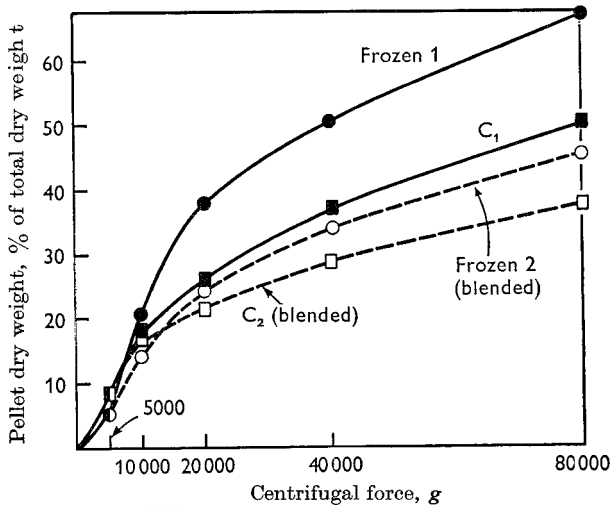

Fig. 2. The effect of freezing and thawing on the state of aggregation of the fat-globule membrane lipoproteins. ■, Untreated FGM preparation, 2·1 % (w/v) total solids; □, blended FGM preparation, 2·1 % (w/v) total solids; ●, FGM preparation after freezing and thawing; ○, FGM preparation after freezing, thawing and blending.

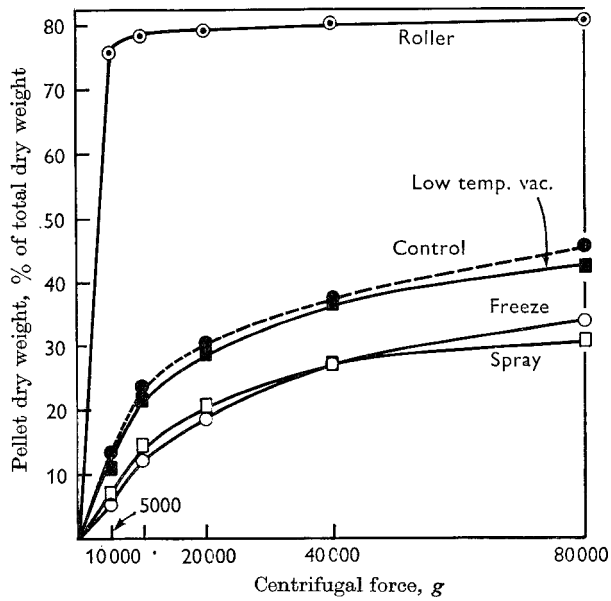

Fig. 3. The effect of drying on the dispersibility of the lipoproteins of the fat-globule membrane. ●, Undried FGM preparation at 0·65 % (w/v) total solids; ○, freeze-dried sample of FGM preparation; ■, low-temperature vacuum-dried sample of FGM preparation; □, spray-dried sample of FGM preparation.

different control samples, thus indicating the fairly good agreement between experiments.

Fig. 3 shows the effects of various types of drying on the dispersibility of FGM. It is evident that roller-drying is very deleterious to FGM, decreasing the dispersibility very markedly. There is, however, approximately 20% of the solids in the roller-dried FGM which remain dispersible. The high temperature of roller-drying is undoubtedly responsible for the low dispersibility of the FGM. Low-temperature vacuum-drying gave a curve similar to that of the control, but indicated a slight increase in dispersibility of the FGM. Freeze-dried and spray-dried samples gave ultracentrifuge profiles that were similar and indicated that the dispersibility of the FGM was increased compared with the control. Apparently, spray-drying is as gentle as freeze-drying for this complex lipoprotein system.

There appear to be two types of damage to the FGM material. One is the aggregating effect resulting from high heat treatment, from dilution and from freezing and thawing. The other is the increased dispersion resulting from freeze-drying and spray-drying. Since freeze-drying of whole milk yields high values for free fat compared with spray-drying and low-temperature vacuum-drying, it is difficult to extrapolate the results on FGM to whole-milk systems. The aggregating effects due to freezing and thawing seem to be obviated in the drying during freeze-drying. Perhaps the damage to the lipoprotein system results from the thawing over a prolonged period of time rather than from the freezing. However, a factor which must be considered in the above experiments is the slow rate of freezing in the bulk system, as compared to the fast rate of freezing in the freeze-drying process.

The effects on the lipoprotein system in freeze-drying and spray-drying are similar; however, the free fat in freeze-dried whole-milk powder is higher than in spray-dried whole milk. This higher free fat may be due to greater physical damage to the fat globules in the recovery and handling of the dried sample rather than to damage during the freeze-drying process.

FGM dried by low-temperature vacuum-drying gave nearly the same dispersibility as the control. Perhaps this method of drying yields a more nearly normal fat-globule membrane. Certainly the low free fat in whole-milk powder prepared by this process (Bullock, 1958) would indicate minimal damage to the fat-globule membrane during drying.

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REFERENCES

Blaauw, J. (1960). *Misset's Zuivel*, **66**, 1123; *Off. Org. K. ned. Zuivelb.* **52**, 1084 (*Dairy Sci. Abstr.* **24**, [52]).
Berlin, E., Howard, N. M. & Pallansch, M. J. (1964). *J. Dairy Sci.* **47**, 132.
Brunner, J. R., Duncan, C. W. & Trout, G. M. (1953). *Fd Res.* **18**, 454.
Bullock, D. H. (1958). Ph.D. Thesis, University of Wisconsin.
Calapaj, G. G. (1962). *Boll. Ist. sieroter. milan.* **41**, 512 (*Dairy Sci. Abstr.* **26**, [249]).
Cole, L. J. N., Kloepfel, D. & Lusena, C. V. (1959). *Can. J. Biochem. Physiol.* **37**, 821.

GREENBANK, G. R. & PALLANSCH, M. J. (1962). 16*th Int. Dairy Congr.* B, 1002.
HOSTETTLER, H. & IMHOFF, K. (1953*a*). 13*th Int. Dairy Congr.* **2**, 382.
HOSTETTLER, H. & IMHOFF, K. (1953*b*). 13*th Int. Dairy Congr.* **2**, 423.
JENNESS, R. & PATTON, S. (1959). *Principles of Dairy Chemistry*, pp. 270–272. New York: John Wiley.
KIELSMEIER, E. W. (1956). Ph.D. Thesis, University of Wisconsin.
KING, N. (1955*a*). *J. Dairy Res.* **22**, 205.
KING, N. (1955*b*). *The Milk Fat Globule Membrane and Some Associated Phenomena.* Farnham Royal: Commonwealth Agricultural Bureaux.
KING, N. (1965). *Dairy Sci. Abstr.* **27**, 91.
LAMPITT, L. D. & BUSHILL, J. H. (1931). *J. Soc. chem. Ind., Lond.*, **50**, 45T.
LITMAN, I. I. & ASHWORTH, U. S. (1957). *J. Dairy Sci.* **40**, 403.
NICKERSON, T. A., COULTER, S. T. & JENNESS, R. (1952). *J. Dairy Sci.* **35**, 77.
REINKE, E., BRUNNER, J. R. & TROUT, G. M. (1960). *Milk Prod. J.* **51** (9), 6.
TAMSMA, A., EDMONDSON, L. F. & VETTEL, H. E. (1959). *J. Dairy Sci.* **42**, 240.

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A reduction in milk yield associated with certain half-udder milking techniques

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During a series of lactation studies on ewes a half-udder technique was used for comparing twice- with thrice-a-day milking. The treatments were applied to the half-udders of 8 ewes in a Latin square design, in which each period lasted 7 days. Essentially the treatments were as follows: (A) a half-udder milked *twice* daily whilst the opposite half-udder was milked *thrice*; (B) a half-udder milked *thrice* daily whilst the opposite half-udder was also milked *thrice*; (C) a half-udder milked *twice* daily whilst the opposite half-udder was also milked *twice*.

Milking times were: for twice milking, 12 h and 24 h; for thrice milking, 8, 16 and 24 h. Thus, in the case of treatment A, the milking of both halves coincided only at the 24-h milking, whereas with treatments B and C both halves were always milked at the same time (coincidental milking). The mean yields of udder halves at a given milking and those yields expressed as a percentage of the yield at the coincidental milking at midnight are shown in Table 1. The mean reduction in yield at a non-coincidental milking ranged from 19 to 25% (significant at the 0·01% level).

Table 1. *Yield of milk, g obtained from half-udders of ewes when milked coincidentally with the opposite halves and when milked alone*

(At 08.00, 12.00 and 16.00 h, yields are also given as a percentage of the yield at 24.00 h when all half-udders were milked coincidentally.)

Treatment	Frequency of milking of half-udder	Milking times 08·00 h	12·00 h	16·00 h	24·00 h	Total yield over 24 h
A	One half thrice	225·3* 75·3%	—	235·9* 78·9%	299·1	760·3
	Other half twice	—	328·1* 75·6%	—	433·9	762·0
B	Both halves thrice	264·6 97·4%	—	254·3 93·6%	271·6	790·5
C	Both halves twice	—	396·0 99·8%	—	396·6	792·7

* Non-coincidental milking.

Half-udder techniques have been used for many milk-secretion studies (see Bailey, Clough, Dodd, Foot & Rowland, 1953; Elliott, 1959). The original data of one such report (Elliott, 1961) have now been re-examined in the light of the findings reported above. In this experiment the left fore and left hind quarters of 6 cows were milked thrice daily at 14.00, 22.00 and 06.00 h, and the right quarters twice daily at 18.00

and 06.00 h over a period of 39 days. This is similar to treatment A in the ewe experiment reported above, except that the coincidental milking was at 06.00 h and not at midnight. The results given in Table 2 confirm those of the sheep experiment. The milk yields of half-udders were lower at the non-coincidental milkings, the reduction varying from 10 to 15 % (significant at the 0·01 % level).

Table 2. *Yield of milk, (g) obtained by Elliott* (1961) *from half-udders of cows when milked coincidentally with the opposite halves and when milked alone*

(At 14.00, 18.00 and 22.00 h, yields are also given as a percentage of the yield at 06.00 h, when all half-udders were milked coincidentally.)

Treatment	Frequency of milking of half-udder	Milking times				Total yield over 24 h
		14.00 h	18.00 h	22.00 h	06.00 h	
As in A in Table I	One half thrice	2264*	—	2361*	2647	7292
		86·3 %	—	89·2 %	—	—
	Other half twice	—	2996*	—	3528	6524
			84·9 %			

* Non-coincidental milking.

These experiments indicate that milk yields of individual quarters are lower when they are milked at a time when not all the quarters of the gland are milked. This is possibly due to less effective milk ejection, though the reasons for this are, at present, obscure.

I am grateful to Dr G. M. Elliott for permission to use the data from her experiment.

REFERENCES

BAILEY, G. L., CLOUGH, P. A., DODD, F. H., FOOT, A. S. & ROWLAND, S. J. (1953). 13*th Int. Dairy Congr.* **2**, 76.
ELLIOTT, G. M. (1959). *Dairy Sci. Abstr.* **21**, 481.
ELLIOTT, G. M. (1961). *J. Dairy Res.* **28**, 209.

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Reviews of the progress of dairy science

Section D. Dairy chemistry. The formation and metabolism of methyl ketones and related compounds

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CONTENTS

The importance of methyl ketones as constituents of biological materials has, in some respects, undergone considerable reappraisal in recent years. It was early recognized that methyl ketones of intermediate chain length were metabolic products of certain fungi and made an important contribution to the flavour characteristics of mould-ripened cheese but the origin and importance of methyl ketones in other milk products was not clear. It is now established that methyl ketones may be derived from naturally occurring constituents of milk fat—namely the triglyceride-bound β-keto acids. However, the contribution of the methyl ketones from this source to the general flavour characteristics of milk products is still uncertain.

Investigations of the metabolism of methyl ketones by Penicillia and related fungi has been considerably hampered by difficulties in the preparation of cell-free extracts with enzyme activity and, as a consequence, the mechanism of formation of methyl ketones by fungi is not clearly understood at present. Nevertheless, there has been a steady accumulation of information in this field of research.

Although this review is especially concerned with the chemistry and metabolism of carbonyl compounds in dairy products—the methyl ketones in particular—it is appropriate to include a discussion of the analytical techniques which have been applied to the volatile carbonyls in biological materials in general.

Techniques of isolation and estimation of methyl ketones

Spectacular advances have been made in the application of physico-chemical analytical techniques to flavour research, but, despite the high sensitivity of techniques such as gas–liquid chromatography and mass spectrometry, there are some disconcerting aspects of flavour research: (*a*) the difficulties in obtaining representative samples of flavourful substances for analysis; (*b*) the need to calibrate organoleptic flavour evaluation against physico-chemical analytical flavour techniques; (c) the interaction of odours and the synergistic effect of compounds at subthreshold concentrations; (*d*) the acceptance of a particular flavour as desirable in one product and undesirable in another. Of the numerous publications which discuss these and other aspects of flavour research, the recent review by Lea (1963) is of special interest.

Direct sampling techniques. In the analysis of food volatiles, there are obvious advantages in being able to take a sample of the free-space vapours above a foodstuff without recourse to concentration techniques. It is certain that this method retains the original ratios of the flavour components better than other techniques. This approach has been used in several recent studies (Jennings, Viljhelmsson & Dunkley, 1962; Bassette, Ozeris and Whitnah, 1963; Morgan & Day, 1965) which combine head-space sampling with gas–liquid chromatographic analysis. In a further elaboration Kroger & Patton (1964) have used a hypodermic syringe as the sampling chamber in the measurement of the head-space gas of Cheddar cheese. The finely divided cheese was placed in the syringe half-filled with carrier gas, the syringe warmed to an appropriate temperature and the mixture of gases injected into the column.

Concentration techniques. Unfortunately, the concentration of carbonyls in samples to be analysed is frequently too low to permit direct sampling methods such as those referred to above. Enrichment techniques commonly employed are (*a*) circulation of air (or pure gas) through or over the product and condensation of the vapours in liquid oxygen (Day, Forss & Patton, 1957*a*), (*b*) steam distillation, frequently at reduced pressure and at temperatures of 40 °C or less to prevent heat-induced changes (Patton & Tharp, 1959), and (c) vacuum distillation and condensation using liquid nitrogen or oxygen (McGugan & Howsam, 1962; Scarpellino & Kosikowski, 1961) as coolant.

Of particular interest in this connexion is the study made by Nawar & Fagerson (1962) on the effects of the dissolving medium on the composition of the enriched volatiles. Using the C_3–C_7 odd-numbered methyl ketones in water or corn oil, each at a concentration of 200 ppm., they found that the relative proportions of the 5 methyl ketones obtained by circulating air for 1 h at 25 °C through the 2 solutions differed widely and also differed from the proportions present in the head space (Table 1). The recoveries of the methyl ketones are obviously better with the lipid than with the aqueous system.

Quantitative recoveries of volatile carbonyls (carbon number of C_3–C_{14}) from oils and dairy products have been obtained using short-path vacuum distillation (De Bruyn & Schogt, 1961; Lea & Swoboda, 1962; Libbey, Bills & Day, 1963; Kurtz, 1965) and condensation with liquid nitrogen or oxygen for later analysis, e.g. by gas–liquid chromatography.

Gas–liquid chromatography. Of the available analytical techniques gas–liquid

chromatography is probably the best single method for providing information about the composition of volatiles. Whereas derivatives of carbonyls are usually employed in other chromatographic methods of separation because of difficulties associated with the volatility of the carbonyls themselves, this property enables carbonyls to be separated at comparatively low temperatures by gas–liquid chromatography. However, chromatograms are not readily interpreted as an objective measure of flavour, and some of the problems associated with enrichment techniques used to prepare samples for gas–liquid chromatography have been discussed already. In addition, compounds at below the threshold concentration of organoleptic detection may exhibit a synergistic effect and thus contribute to flavour. For example, Nawar & Fagerson (1962) demonstrated that a mixture of 5 methyl ketones was detected organoleptically despite the concentration of each methyl ketone being well below the minimum threshold.

Table 1. *Effect of enrichment of volatiles from aqueous and lipid systems on their gas chromatographic analyses*

(Nawar & Fagerson, 1962)

Compound*	Relative area† direct head-space	Relative area† after enrichment	Ratio, enriched: direct
In aqueous system			
Acetone	10	268·0	26·8
2-Butanone	14	298·0	21·3
2-Pentanone	22	321·2	14·6
2-Hexanone	29	118·9	4·1
2-Heptanone	24	26·4	1·1
In lipid (corn oil) system			
Acetone	47	2547·4	54·2
2-Butanone	11	707·3	64·3
2-Pentanone	5·7	338·01	59·3
2-Hexanone	2·7	78·3	29 0
2-Heptanone	0·7	10·5	15·0

* Concentration of each compound, 200 ppm.
† Corrected for effective carbon number according to Sternberg *et al.* (1961).

Bearing in mind that most of the compounds of interest in flavour studies occur at extremely low concentrations it follows that the very sensitive hydrogen flame ionization or β-ionization detectors offer an advantage over thermal conductivity and other less sensitive detectors. Ionization detectors are able to measure simple organic compounds in the range from 0·08 to 0·002 ppm. (Condon, Scholley & Averill, 1960; Desty, Geach & Goldup, 1960), while the threshold level of detection by humans for different carbonyls varies considerably. For example, the flavour threshold for octan-2-one and heptan-2-one is, respectively, 1·6 ppm. (Lea & Swoboda, 1958) and 0·6 ppm. (Wong, Patton & Forss, 1958), while that for *n*-deca-2,4-dienal, which is a major carbonyl product of heat-decomposed methyl linoleate, is about 0·5 ppm. (Patton, Barnes & Evans, 1959). If, however, gas–liquid chromatographs equipped with destructive detectors, e.g. hydrogen flame, are employed for preparative purposes, by-pass systems must be used, which results in loss of effective

sensitivity. Numerous column procedures have been described for the separation of the volatile constituents of milk and milk products (Table 2).

The high resolving property of capillary columns is well illustrated by the separation of about 130 neutral components in the volatile fraction of Cheddar cheese (Day & Libbey, 1964) and by earlier experiments on the separation of aldehydes, methyl ketones, and alcohols by Nawar & Fagerson (1962). However, the gas–liquid chromatographic pattern of the total neutral volatiles from biological materials is most often very complex, and the reliable identification of components by retention times is not practical. Consequently, for this purpose, most workers use column, paper and thin-layer chromatographic procedures followed by colorimetry for the isolation and identification of carbonyls as their 2,4-dinitrophenylhydrazones (DNP hydrazones)—frequently in conjunction with direct gas–liquid chromatography of the carbonyls and other neutral volatiles. If total 2,4-DNP hydrazones are subjected to paper chromatography, some common components (e.g. butanone and *n*-butyraldehyde) are difficult to separate but the free carbonyls are cleanly resolved by gas–liquid chromatography.

Table 2. *Gas–liquid chromatographic methods for the separation of volatile carbonyls*

Column	Liquid phase	Temperature	Compounds separated	Reference
Packed	Silicone oil	110°	C_{3-11} methyl ketones	Forss *et al.* 1960
	Carbowax 400	70°	C_{3-11} methyl ketones	
Packed, 4·5 ft × $\frac{1}{8}$ in.	Pluronic F68	110–150°	C_2–C_7 aldehydes	Hawke *et al.* 1957
Capillary, 300 ft × 0·01 in.	Polypropylene glycol	73–174°	C_3–C_{11} methyl ketones	Day & Libbey, 1964
Packed, 11 ft × $\frac{1}{8}$ in.	20 % diethylene glycol succinate (DEGS)	70°	Alcohols, aldehydes and esters	Day & Libbey, 1964
	20 % Apiezon M	100°	Alcohols, aldehydes and esters	Day & Libbey, 1964
Capillary, 150 ft	Polypropylene glycol	—	C_3–C_9 methyl ketones	Nawar & Fagerson, 1962
Packed, 8 ft	10 % Apiezon L	150°	C_7–C_{13} methyl ketones	Muck *et al.* 1963
	20 % polyethylene glycol succinate	150°	C_7–C_{13} methyl ketones	Muck *et al.* 1963
Packed, 10 ft × $\frac{1}{8}$ in.	20 % carbowax 20M	100°	C_3–C_6 methyl ketones	Bassette *et al.* 1963
Packed, 10 ft × $\frac{1}{16}$ in.	20 % Apiezon M	70°	C_3–C_4 methyl ketones C_2–C_7 aldehydes	Libbey *et al.* 1963
Capillary, 150 ft × 0·02 in.	Diethylene glycol succinate (DEGS)	100°	C_3–C_8 methyl ketones C_2–C_7 aldehydes C_2–C_4 alcohols	McGugan & Howsam, 1962

The quantitative regeneration of carbonyls isolated from the 2,4-DNP hydrazone mixtures offers the advantage of obtaining flavour assessment of individual components and being able to use gas–liquid chromatography as a confirmatory method of identification. Several exchange reagents have been employed for the regeneration of ketones from their DNP hydrazones, and one of these—namely laevulinic acid and 10 % mineral acid (Keeney, 1957)—was used by Lawrence & Hawke (1963) to avoid the severe quenching of radioactivity which results when radioactive ketones are

converted to their DNP hydrazones. Ralls (1960) described a method with a precision of 10–20% in which DNP hydrazones are exchanged with excess α-ketoglutaric acid by heating at 250 °C for 10 sec and the products volatilized directly into a gas–liquid chromatograph. Unfortunately, however, α-ketoglutaric acid alone gives carbonyl products when pyrolysed. Jones & Monroe (1965) evaluated a number of different types of exchange reagents and found that a mixture of oxalic acid dihydrate and *p*-dimethylaminobenzaldehyde efficiently regenerated carbonyls from their DNP's, and only a small fast-moving peak chromatographed in the reagent blank. Using butyraldehyde DNP as internal standard, an accuracy of ± 10% for 95% of the determinations was achieved with mixtures of C_2–C_5 aldehydes and ketones.

Liquid–liquid column and paper chromatography. In recent years a number of excellent chromatographic procedures have been described for the preparation and separation of the 2,4-DNP hydrazones of carbonyl compounds occurring in dairy and other food products. Because these compounds frequently occur at very low levels, it is important that steps are taken to ensure that the extraction and chromatographic solvents are carbonyl-free. Celite columns impregnated with 2,4-DNP hydrazine, phosphoric acid and water (Schwartz & Parks, 1961) may be used to remove contaminant carbonyls from most solvents.

Although many of the carbonyls are sufficiently volatile to enable steam distillation to be used as a step in the isolation procedure, it is more satisfactory in many instances to use direct methods of isolation. Schwartz, Haller & Keeney (1963) described a procedure for the quantitative formation of very low levels of 2,4-DNP hydrazones (e.g. $2{\cdot}4 \times 10^{-3}$ μmole) in the presence of large amounts of fats and oils, and the subsequent isolation of the DNP hydrazones. The DNP hydrazones are prepared by passing the hexane solution of the fat–carbonyl mixture through the 2,4-DNP hydrazine/Celite column referred to above. The DNP hydrazones are separated from the neutral fat by adsorption on to activated magnesia–Celite columns in hexane solution and are then isolated by elution of the columns with a nitromethane–chloroform mixture. The individual DNP hydrazones are separated into classes on magnesia 2665/Celite columns (Schwartz, Parks & Keeney, 1962), the order of elution of each class of compounds using chloroform–hexane mixtures being methyl ketones, saturated aldehydes, 2-enals and 2,3-dienals. The short-chain members of each class, e.g. acetone, move more slowly than their homologues and are imperfectly separated from the class of compounds which follow in order of elution. However, such contaminants are usually recognized when partition or reverse-phase column chromatography (Parks, Keeney, Katz & Schwartz, 1964) is used to separate the individual members of the classes (Monty, 1958; Corbin, Schwartz & Keeney, 1960; Day, Bassette & Keeney, 1960).

Several reliable paper chromatographic methods have been developed for the identification of 2,4-DNP hydrazones of methyl ketones, either after fractionation on columns or in total steam distillates (Huelin, 1952; Klein & de Jong, 1956; Lynn, Steele & Staple, 1956).

Thin-layer chromatography. Considerable progress has been made in the application of thin-layer chromatography (TLC) to the separation of 2,4-DNP hydrazone derivatives of carbonyl compounds of interest in flavour studies. It is found that silica gel,

aluminium oxide or other adsorbents, in general, separate carbonyls or their derivatives into chemical classes but there is only limited subfractionation within classes. This was demonstrated by Urbach (1963), who showed that aluminium oxide G eluted with 4% diethyl ether in light petroleum readily separated the 2,4-DNP hydrazones of aldehydes, saturated methyl ketones and vinyl ketones but only gave slight separation of 2,4-DNP hydrazones of aldehydes of different degrees of unsaturation. Good resolution of these classes of compounds is achieved by using thin layers of basic zinc carbonate providing that water vapour is rigidly excluded from the chromatoplate jar (Badings, 1964). Mixtures of positional isomers of C_{10}–C_{27} alkones when chromatographed as the free carbonyls are resolved on silica gel G (Marcuse, 1962) but high volatility precludes the application of this method to the lower molecular weight compounds.

Silver nitrate or mercuric acetate-impregnated adsorbents, e.g. silica gel, provide some separation within chemical classes differing in the degree of unsaturation and also separate *cis–trans* isomers (Morris, 1962). Such separations appear to depend upon the formation of co-ordination complexes of olefin linkages with the metal ion (Barrett, Dallas & Padley, 1963). Since the stability of the complexes increases with increasing numbers of double bonds (Nicols, 1952; Winstein & Lucas, 1938), and is greater for unconjugated and *cis* bonds than for conjugated and *trans* bonds, useful separations using this technique are possible between a number of 2,4-DNP hydrazones of interest in flavour work. For example, nona-*trans*,2-*trans*,6-dienal has a higher R_F value than nona-*trans*,2-*cis*,6-dienal (Urbach, 1963). Urbach (1963), using aluminium oxide G containing 20% $AgNO_3$ (w/w) as adsorbent and diethyl-ether–light-petroleum solvent mixtures, also separated the 2,4-DNP hydrazine derivatives of alkanals, alkenals, alkadienals, methyl vinyl ketone and methyl ethyl ketone.

Reverse-phase systems separate members of homologous series according to carbon number. Urbach (1963) adapted the 2-phenoxyethanol–light-petroleum system used by Lynn *et al.* (1956) with paper chromatography to TLC on kieselguhr G plates. All the members of the normal homologous series of C_1–C_{14} alkanals, C_3–C_{13} alkan-2-ones, C_4–C_{10} alk-1-en-3-ones, $C_{3-11,\ 16}$ alk-2-enals, $C_{5-12,\ 14,\ 16,\ 18}$ alk-2-4-dienals and $C_{6,\ 7,\ 10}$ alk-3-en-2-ones were separated as the DNP hydrazone derivatives by a multiple ascent technique. Badings (1964) also separated different classes of 2,4-DNP's according to chain length on Carbowax-400-impregnated plates of basic zinc carbonate.

Libbey & Day (1964) have also used silica gel G impregnated with mineral oil (Shell Ondina 27) and a technique of continuous development to separate the C_{1-14} n-alkanal DNP hydrazones and C_3–$C_{9,\ 13}$ n-alkan-2-one DNP hydrazones.

Spectral methods. Concentrations of 2,4-DNP hydrazones in total extracts or in column eluates may be determined by measuring the absorbance of hexane or ethanol solutions at 340–362 mμm. However, the reactions and extractions should be carried out in closed vessels to prevent losses due to volatilization (Lawrence, 1965*a*).

Infra-red absorption spectra have proved of value for the identification of carbonyl groups in unknown mixtures (Dutra, Jennings & Tarassuk, 1959; Winter, Stoll, Warnhoff, Greuter & Büchi, 1963) and in purified fractions (Wong *et al.* 1958) as well as to investigate the precursors of methyl ketones in milk fat (Parks *et al.* 1964). In conjunction with other techniques it was used by Stark & Forss (1964) to distinguish

between oct-1-en-3-ol and the corresponding ketone in the volatiles of oxidized butter (Stark & Forss, 1962).

In addition, mass spectrometry (requiring a few microgrammes of sample) prove to be particularly useful in the identification of oxidation products (Stark & Forss, 1962, 1964; Day, Forss & Patton, 1957*b*). This method is likely to continue to be an extremely valuable technique in flavour studies, since only small amounts of the flavour constituents are usually available for analysis. Rapid-scan mass spectrometry in conjunction with gas–liquid chromatography (McFadden & Teranishi, 1963; Watson & Biemann, 1965) is very effective in the identification of mixtures of ketones, aldehydes and esters in steam volatiles of Cheddar cheese (Day & Libbey, 1964).

Forss (1964) has also demonstrated the value of far-ultraviolet spectroscopy in the identification of volatile ketones and aldehydes. The Australian workers prefer to determine the spectra of vapours rather than solutions because vapours give more complex spectra, thus providing a better fingerprint; in addition, vapours are easily collected from gas–liquid chromatographs.

Methyl ketones in milk and milk products

Precursors of methyl ketones. It is now well recognized that, while the even-numbered straight-chain fatty acids constitute the major components of natural fats, trace quantities of other fatty acids are also present. These include methyl branched-chain saturated acids with both odd and even numbers of carbon atoms and normal odd-numbered fatty acids (Shorland & Hansen, 1957). Investigations on butterfat have shown that these fatty acids comprise approximately 4% of the total fatty acids present.

More recently β-keto acids have been recognized as an additional class of trace constituent of the triglycerides of milk fat.

As long ago as 1933 Täufel, Thaler & Martinez (1933) found that alk-2-ones were generated from fat through the action of heat and water. A homologous series of methyl ketones with odd numbers of carbon atoms were found by Patton & Tharp (1959) to be produced from milk fat during steam distillation; they also demonstrated, from spectral changes after adding 2,4-dinitrophenylhydrazine, that keto groups must be present in the fat. These observations can now be explained by the discovery that the C_{4-16} β-ketoacids occur in milk fat as mixed esters of glycerol (Boldingh & Taylor, 1962) to the extent that 0·045% of the triglycerides of butterfat contain one keto acid and two fatty-acid moieties (Parks *et al.* 1964).

Van der Ven, Begemann & Schogt (1963) have shown that the presence of water is essential for the production of ketones from the β-keto acid esters of the fat. Vacuum degassing of dry butterfat yielded only traces of ketones in the distillate, whereas heating in the presence of water yielded considerable amounts of ketones. A model β-keto acid glyceride (1,3-didodecanoyl-2-β-ketododecanoylglycerol) behaved in an analogous manner. They also provided direct evidence for the occurrence of β-keto acid triglycerides in butter by reacting Girard-T reagent with butterfat and identifying the homologous series of pyrazolones which were formed in the following reaction sequence (R = odd numbered C_3–C_{13} series; R′ = constituent fatty acids of butterfat). Methyl ketones themselves do not yield pyrazolones when treated with hydrazine reagents.

More recently Parks *et al.* (1964) isolated the β-keto acid triglycerides by adsorption chromatography on magnesia–Celite columns followed by preparative thin-layer chromatography. They showed by infra-red spectra and chemical analysis that the methyl ketone precursors yield diglycerides on heating in a sealed tube for 40 h at 100 °C in the presence of moisture. Consequently, the data are consistent with the precursors of the C_3–C_{15} odd-numbered methyl ketones being triglycerides containing one β-keto acid and 2 fatty-acid moieties.

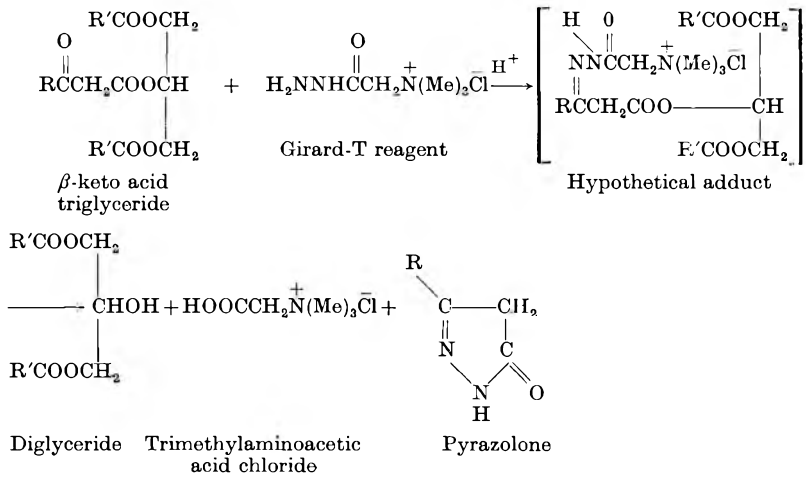


Fig. 1. From van der Ven, Begemann & Schogt, 1963.

Langler & Day (1964) calculated that the complete conversion of the β-keto esters to methyl ketones would require only about 0·0031 % water in the milk fat and found that treatment of fat with calcium hydride was effective in removing these last traces of water. In view of the difficulty in removing water to below this level, recent suggestions that methyl ketones may be formed from milk precursors by heating in the absence of water (Nawar, Cancel & Fagerson, 1962) will need re-appraisal. Schwartz, Spiegler & Parks (1965) have found that butteroil dried with CaH_2 may be heated for 24 h at 101 °C without methyl ketones being detected. On the other hand, maximum levels were obtained with as little as 0·008 % water after similar heat treatment for 6 h.

This need to decompose β-keto esters before methyl ketones can be formed may explain why Bassett & Harper (1958), who employed a cold extraction technique, did not obtain C_3–C_{15} methyl ketones from Cheddar cheese.

Methyl ketones formed from milk and milk fat. Acetone, pentan-2-one and heptan-2-one were identified as the principal volatile carbonyl compounds in distillates obtained at low temperature and reduced pressure from commercial evaporated milk (Wong *et al.* 1958; Parks & Patton, 1961; Dutra *et al.* 1959; Muck, Tobias & Whitney, 1963). Comparisons with cream and skim-milk (Wong *et al.* 1958) left little doubt that the methyl ketones arise from the lipid phase of the milk and they speculated that β-keto acids might occur in milk either as degradation products of fatty acids by β-oxidation or as intermediates in the synthesis of fatty acids from acetate in the mammary gland.

Langler & Day (1964) showed that large increases in ketone production from fat occurred at temperatures above 100 °C and that heating for 3 h at 140 °C was sufficient for maximum ketone formation from milk fat. Lawrence (1963), using steam distillation at 100 °C, found that it was necessary to collect 10 l of distillate to ensure maximum yields of methyl ketones from 10 g butterfat. Van Duin (1965) did not obtain methyl ketones when butterfat was heated for 2 h at 50 °C, although heating for 2 h at 75 °C produced measurable quantities. Nearly maximum yields were obtained at 125 °C. Individual methyl ketones varied markedly in their volatility in steam, the C_{5-11} methyl ketones being most readily recovered, whilst the complete recovery of C_3, C_{13} and C_{15} methyl ketones was more difficult. The average yields of methyl ketones obtained by exhaustive steam distillation of different milk fat samples were C_{15} (46 ppm.), C_7 (25 ppm.), C_{13} (24 ppm.), C_9 (16 ppm.), C_{11} (14 ppm.) and C_5 (13 ppm.). These results were similar to those of Langler & Day (1964) and of Boldingh & Taylor (1962), who steam distilled at 180 °C. Parks *et al.* (1964) decomposed the bound β-keto acids in sealed ampoules and extracted the methyl ketones directly into hexane; 17 ppm. of heptan-2-one and approximately half of this amount of pentan-2-one and of undecan-2-one were obtained. Allowing for some variation in the composition of milk fats from different sources, it appears that each of these techniques gives quantitative yields of methyl ketones from the β-keto acid esters in milk fat.

Methyl ketones in Cheddar cheese. Although it was once thought that the microflora of Cheddar cheese might be implicated in the formation of methyl ketones from fatty acids via β-oxidation and decarboxylation during ripening (Walker & Harvey, 1959) the presence of precursors of methyl ketones in milk now gives a ready explanation for the occurrence of methyl ketones in such cheese. This was confirmed by Lawrence (1963), who showed that there is a close similarity in the maximum yield of methyl ketone from both milk fat and fat obtained from Cheddar cheese by distillation. With both these substrates the distillation pattern is characterized by a slow formation of methyl ketone, whereas steam distillates of mould-ripened cheeses (Patton, 1950) contain high concentrations of methyl ketones in the early stages of distillation. The absence of significant amounts of methyl ketones with even numbers of carbon atoms in the steam distillates of Cheddar cheese in which synthetic triglycerides containing heptanoic, nonanoic and undecanoic acids have been incorporated during manufacture is further evidence against the formation of significant amounts of methyl ketones via β-oxidation–decarboxylation reactions catalysed by bacterial or other enzymes (Lawrence, 1964).

It is possible (Lawrence, 1964) that the limiting concentration of flavour-forming substrate referred to by Robertson & Perry (1961) might be the β-keto acid precursor of methyl ketones in milk. Harvey & Walker (1960) showed that increasing amounts of methyl ketones were isolated as the cheese matures, particularly after the typical Cheddar flavour becomes apparent. Steam distillation was not prolonged in these experiments and, furthermore, the C_5, C_7 and C_9 methyl ketones were obtained by distillations under reduced pressure at 45 °C. Consequently these results may have been a measure of the free methyl ketones present in the cheese at different stages of maturity. The possibility remains, therefore, that the breakdown of the β-keto esters may occur slowly under conditions of cheese ripening to produce the

same or a similar range of methyl ketones to that obtained under more rigorous conditions (Lawrence, 1963; Day & Libbey, 1964). Clarification of this point awaits further experiments along the lines of those conducted by Bassette & Harper (1958) and Schwartz & Parks (1963), who used solvent extraction rather than heat treatment to isolate methyl ketones in Cheddar cheese.

The occurrence of alcohols in the neutral volatile fraction of Cheddar cheese is not as well documented as the occurrence of methyl ketones. Butan-2-ol, ethanol and isopropyl alcohol have been identified by measurement of retention times on gas–liquid chromatograms obtained from total neutral volatiles (Scarpellino & Kosikowski, 1961; McGugan & Howsam, 1962).

It does not seem possible at present to decide whether cheese flavours can be attributed to (*a*) the influence of one very potent substance of characteristic flavour, or (*b*) the combined effects of contributions from a large number of substances, some or all at a level below their threshold concentration for organoleptic tests. It seems likely that methyl ketones contribute to the flavour of Cheddar cheese and their contribution may be enhanced by the presence of other compounds such as hydrogen sulphide (Walker, 1961), SH compounds (Kristoffersen, Gould & Purvis, 1964), volatile esters and δ-lactones (Day & Libbey, 1964; van der Ven, 1964). On the other hand, Patton (1963) found that the removal of volatile carbonyl compounds from steam distillates of Cheddar cheese did not decrease the Cheddar aroma, which was, however, decreased by the removal of the volatile (C_2–C_8) fatty acids. Consequently, Patton (1963) concluded that these acids are the main contributors to the basic Cheddar flavour.

Detailed discussions of the microbiological and chemical aspects of the ripening of Cheddar cheese have appeared in recent reviews (Mabbitt, 1961; Marth, 1963).

Biosynthesis of methyl ketone precursors. Before 1962 it was generally considered that the methyl ketones with odd numbers of carbon atoms which were obtained in the steam distillates of cheese fat were derived from β-oxidation of the C_4–C_{14} fatty acids of the fat (Walker & Harvey, 1959; Mabbitt, 1961). Although a considerable amount of work has been carried out on the oxidation of milk fat (Forss, Dunstone & Stark, 1960; Day & Lillard, 1960), it has been difficult to distinguish between oxidation and decomposition of fatty constituents (Patton & Tharp, 1959). However, it appears that aldehydes predominate as products of oxidative mechanisms whereas ketones are the major components formed by thermal degradation (Nawar *et al.* 1962). The latter workers concluded that the C_3–C_{11} methyl ketones are formed by heating milk fat in the absence of oxygen and further observed the presence of relatively small amounts of the even-numbered C_4–C_8 methyl ketones as well as the C_1–C_9 n-alkanals.

Wong *et al.* (1958) were the first to propose that the methyl ketones obtained from milk fat might be derived from β-keto acids which are natural constituents of milk fat. As discussed above, later work has confirmed this proposal.

Lawrence & Hawke (1963) investigated the origin of methyl ketones in steam distillates of milk fat obtained from a cow which had been injected with [1-^{14}C]-acetate. When the specific activities of the isolated C_7–C_{13} methyl ketones were measured, it was found that there was a marked similarity between the ^{14}C-labelling pattern of the methyl ketones and the fatty acids isolated by Popják, French, Hunter & Martin (1951) from a goat which had been injected with [1-^{14}C]acetate.

The C_{11} methyl ketone and the C_{10} fatty acid possessed the maximum radioactivity in the respective experiments and radioactivities fell away on either side of these maxima in a similar way.

In further experiments Lawrence & Hawke (1966) made direct comparisons of the labelling pattern of the methyl ketones and fatty acids in 3 separate samples of milk fat obtained 3, 9 and 22 h after injection of [1-^{14}C]acetate. Except for acetone in all milkings and C_9 methyl ketone in the 2nd milking, the methyl ketones had greater specific activities than the corresponding fatty acids. In the 9- and 22-h milkings comparisons of labelling in fatty acids and methyl ketones showed that the principal features were the close similarities in the specific activities of the 2 series of compounds in the range from C_5–C_{10} and the much higher specific activities of the methyl ketones in the C_{11}–C_{16} range.

Although there were several points of difference in the labelling patterns of the fatty acids and the corresponding methyl ketones with one less carbon atom, the results in general are consistent with the view that the β-keto acids, from which the methyl ketones are formed during steam distillation, form part of the same metabolic pool as the fatty acids undergoing incorporation into triglycerides. Consequently, the higher methyl ketones were very weakly labelled in the first 3 h, yet contained the major part of the activity at the end of 22 h. The change in labelling pattern of the fatty acids with time was in accord with similar experiments with the goat by Popják *et al.* (1951).

The high specific activities of the methyl ketones relative to the corresponding fatty acids may be a reflexion of the formation of β-keto acids as intermediates in fatty-acid synthesis prior to a rate-limiting step, or to a greater rate of incorporation of the β-keto acids than the fatty acids into the triglycerides. The close similarity between the labelling patterns and the specific activities of the low molecular weight fatty acids and the corresponding methyl ketones with one less carbon atom suggest that the β-keto acids and the corresponding fatty acids have a common precursor and are together incorporated into the triglycerides. The higher specific activities of the methyl ketones relative to the fatty acids was evidence that the β-keto acids are not synthesized from fatty acids by β-oxidation. The presence of small amounts of β-keto acid in triglycerides with similar specific activity to fatty acids of the same carbon number implies that there is some dissociation of the β-ketoacyl-enzyme (Lynen, 1961) or β-ketoacyl-acyl carrier protein (ACP) (Alberts, Majerus, Talamo & Vagelos, 1964) in the mammary gland prior to the dissociation of acyl-enzyme or acyl-ACP during triglyceride synthesis. In an extension of their study Lawrence & Hawke (1966) steam-distilled coconut oil, which like milk fat is characterized by a high proportion of C_8–C_{16} saturated acids, but no methyl ketones except acetone were obtained. This suggests that if C_6–C_{16} β-keto acids are incorporated into the triglycerides of coconut oil they are reduced *in situ*. The occurrence of β-keto acids in the triglycerides is possibly unique to milk fat and may result from the rapid removal of triglycerides from the site of synthesis during milk secretion.

The presence of unlabelled acetoacetate, but labelled higher β-keto acids, in milk triglycerides following injection of labelled acetate could be explained by the pathway outlined below and based upon the mechanism for fatty-acid synthesis proposed by Bressler & Wakil (1962). The quantitative importance of acetone in the mixed

methyl ketones from milk fat suggests that more acetoacetate than any of the higher β-keto acids is present in the metabolic pool.

Since both the C_5–C_{15} methyl ketone precursors and the short-chain fatty acids in milk fat are synthesized from acetate, the amounts of methyl ketone obtained on distillation might be expected to exhibit a seasonal variation similar to that observed with the quantitatively important short-chain fatty acids (Hansen & Shorland, 1952; Keeney, 1956). The greater amounts of C_5–C_{15} methyl ketones reported earlier (Lawrence, 1963) in samples of New Zealand butterfat (about 0·88 μmoles/g compared with 0·63 μmoles/g by Lawrence & Hawke (1966)) might be due to a seasonal effect.

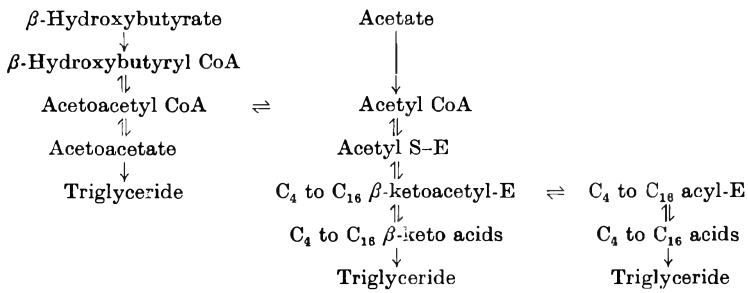


Fig. 2. A possible mechanism for the synthesis and incorporation of β-keto acids into milk fat (Lawrence & Hawke, 1966).

No correlation appears to exist between the relative amounts of ketone obtained after heat treatment and the relative amounts of the corresponding saturated fatty acid present in the triglycerides. For example, octanoic acid occurs in relatively low concentration in milk triglycerides, yet heptan-2-one, which would arise from the 8-carbon β-keto acid, generally occurs in relatively high concentration.

Discussion of the biosynthesis of precursors of the carbonyl compounds which are possibly concerned with flavour would not be complete without reference to normal and branched-chain aldehydes which are found in milk and milk products (Bassette, 1959). The aldehydes of intermediate molecular weight are products of heat treatment and storage but the lower molecular weight aldehydes are present in fresh milk (Parks & Patton, 1961). In a study of the volatile carbonyl constituents of fresh butter, Winter *et al.* (1963) suggested that formaldehyde, acetaldehyde, iso-butyraldehyde, iso-valeraldehyde and phenylacetaldehyde which occurred in butterfat at the 1–100 mg/100 kg level might be produced from the amino acids glycine, alanine, valine, leucine and phenylalanine, respectively, by an enzymic oxidative decarboxylation. This or a similar mechanism is supported by the work of Dutra *et al.* (1959), who showed by means of radioactive tracers that part of the acetaldehyde present in evaporated milk did indeed originate from alanine.

Methyl ketones in mould-ripened cheese

Whereas there is doubt about the importance of the contribution of methyl ketones to the overall flavour characteristics of Cheddar cheese, there is general agreement that the homologous series of these compounds makes an important contribution to the flavour and aroma of mould-ripened cheeses in which *Penicillium*

roqueforti or other moulds are the ripening agents. The importance of methyl ketones in this respect and the involvement of fungi in the formation of methyl ketones was first indicated by Stärkle (1924), who found that *P. roqueforti* and 2 species of aspergilli produced methyl ketones when grown for several weeks on fatty acids of intermediate chain length or on neutral fats, e.g. cacao oil. Patton (1950) isolated pentan-2-one, heptan-2-one and nonan-2-one from mould-ripened cheese and agreed with the conclusion of Hammer & Bryant (1937) that these methyl ketones, particularly heptan-2-one (Patton, 1951), have a flavour and aroma typical of mould-ripened cheese. Further work by Morgan & Anderson (1956) showed that all the C_3–C_{11} odd-numbered methyl ketones were present in the steam distillates of mould-ripened cheese. When a solvent extract of mould-ripened cheese was treated with 2,4-DNP hydrazine Schwartz & Parks (1963) also obtained small amounts of C_{13} and C_{15} methyl ketone in addition to the lower homologues reported by Morgan & Anderson (1956).

The relatively high levels of methyl ketones of intermediate chain length found in these cheeses exclude the possibility that the bound β-keto acids of milk are the main source of this group of carbonyls, although it is likely that the small amounts of the C_{13} and C_{15} methyl ketones which are found are formed from these precursors by natural breakdown rather than by microbial action (Schwartz & Parks, 1963). However, these workers found that no methyl ketones were liberated from the fat of mould-ripened cheese by heat treatment in the presence of water. This may indicate that the methyl-ketone precursors in milk are metabolized by the micro-organisms and thus make a small contribution to the final methyl ketone levels (Schwartz & Parks, 1963).

Heptan-2-ol and nonan-2-ol are also major components of the neutral volatile fraction of mould-ripened cheese and smaller amounts of pentan-2-ol have also been identified as their 3,5-dinitrobenzoates, and by infra-red spectroscopy and GLC retention times (Jackson & Hussong, 1958; Coffman, Smith & Andrews, 1960).

The formation of methyl ketones by fungi. The formation and metabolism of methyl ketones by fungi during the ripening process in mould-ripened cheese appears to involve 4 main enzymic mechanisms: (*a*) the liberation of free fatty acids from the triglycerides of milk fat by lipases; (*b*) oxidation of the free fatty acids to β-keto acids; (*c*) decarboxylation of β-keto acids to methyl ketones; (*d*) reduction of methyl ketones to secondary alcohols.

Although it is generally agreed that the synthesis of methyl ketones from fatty acids is among the metabolic processes of many fungal spores, there are conflicting reports on the ability of the vegetative cells of fungi to produce methyl ketones. In particular, Gehrig & Knight (1958, 1963) have claimed that the methyl-ketone-synthesizing activity is confined to the spores and is not a function of hyphal cells. Whether or not this uncertainty has arisen from the inhibitory effect of fatty acids at moderate and high concentrations and from problems relating to the permeability of cell membranes to substrates is not clear. As pointed out by Cochrane (1958), until more success has been achieved with cell-free preparations, too much emphasis should not be given to the variability in the metabolism of fungal material of different physiological ages.

Lipolysis of neutral lipids. Early interest in lipolysis by fungi was concerned with

the microbial spoilage of coconut oil due in particular to the formation of methyl ketones to give a 'ketone rancidity' (Stokoe, 1928). These ketones were predominantly heptan-2-one and to a lesser extent pentan-2-one, and the large amount of heptan-2-one was related to a considerable drop in the amount of octanoic acid ester remaining in the coconut oil. Very little free fatty acid accumulated during the incubation, which indicated that free fatty acids liberated by lipase activity were rapidly oxidized. However, Wilcox, Nelson & Wood (1955), using butterfat as substrate with lipase preparations from *P. roqueforti* and *Aspergillus lipolyticum*, obtained C_4, C_6 and C_8 fatty acids.

Several studies of the lipase action of various fungi reveal different specificities for substrate (Thibodeau & Macy, 1942; Fodor & Chari, 1949; Shipe, 1951; Wilcox *et al.* 1955). The activity of the lipases of *P. roqueforti* on butterfat fell within the range of activities found with tricaproin, tricaprylin and trilaurin—tributyrin being hydrolysed at a faster rate. The pH optima varied from 6·0 to 7·8. Morris & Jezeski (1953) obtained slightly different specificities for intra- and extracellular mycelial lipases, suggesting that there may be more than one enzyme involved.

Other workers, e.g. Thaler & Eisenlohr (1941), obtained methyl ketones using mycelia of *P. glaucum* and a variety of triglyceride substrates. High concentrations of triglycerides were readily oxidized to methyl ketones by spores of *P. roqueforti* when the equivalent concentrations of free acid were either only slowly oxidized or inhibitory (Lawrence & Hawke, 1964). Since no free fatty acids were observed to accumulate during incubation it would seem that if lipolysis occurred its rate did not exceed the rate of oxidation and therefore inhibition of respiration was avoided.

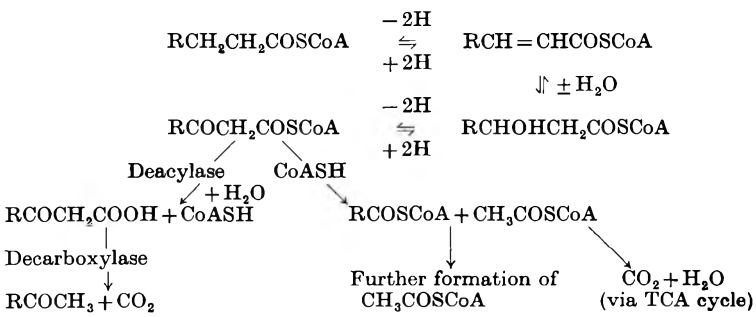
The interpretation of experiments concerned with the specificity of lipases of other than cell-free preparations is complicated by the influence of pH, concentration, etc. on the rates of oxidation of the individual fatty acids, which are superimposed on lipase activity. The relationship between the triglyceride structure of butterfat and the positional specificity of fungal lipases is uncertain.

Oxidation of free fatty acids. In contrast to mammalian, plant and bacterial systems, a reconstructed fatty-acid oxidizing system has not been prepared from fungi. This lack of success with fungi is in large part due to difficulties in disrupting cells of the mycelium and in obtaining a solution of the enzymes. Consequently, fungal systems so far have been unattractive for enzyme studies. These difficulties have led to numerous studies of the metabolism of intact cells which for many purposes are less satisfactory because of the variable toxicity of fatty-acid substrates, problems of permeability, and pH effects. However, the evidence available suggests very strongly that the same mechanisms for the oxidation of fatty acid to carbon dioxide and water are present in fungi as exist in other biological systems. Support for this comes from experiments with mycelia of *P. glaucum* (Thaler & Stählin, 1949). Saturated fatty acid, $\alpha\beta$-unsaturated fatty acid, and β-hydroxy fatty acid each gave rise to methyl ketones, thus providing evidence for the sequence saturated fatty acid $\rightarrow$ $\alpha\beta$-unsaturated fatty acid $\rightarrow$ β-hydroxy fatty acid $\rightarrow$ β-keto fatty acid $\rightarrow$ methyl ketone. In earlier work with mycelia of the same fungus, Thaler & Geist (1939) found that β-hydroxy C_4, C_8, C_{10}, C_{12} and C_{14} all gave methyl ketones—the C_8 and C_{12} β-hydroxy acids in 12–20 % yield. The maximum yield of methyl ketone from β-hydroxy and saturated fatty acids was at pH 6·0 and pH 3·0, respectively. Additional evidence

for a β-oxidation mechanism was provided by Thaler, Schottmayer, Stählin & Beck (1949), who compared α- and β-methyl fatty acids as substrates for growth of *P. glaucum* and for methyl ketone formation; methyl ketones were formed from α-methyl derivatives of butyric, caproic and lauric acids but not from β-methyl derivatives.

Spores of almost half the filamentous fungi tested by Gehrig & Knight (1961) converted caprylic acid to heptanone—including the spores of 9 out of 11 *Aspergillus* species and 9 out of 12 *Penicillium* species examined. The synthesis of heptan-2-one was found to be O_2-dependent and was proportional to the amount of O_2 consumed. The formation of heptan-2-one is of the greatest interest because it is usually one of the most abundant methyl ketones in mould-ripened cheese and has consequently been used as a criterion in many comparative experiments with fatty-acid substrates.

The original investigations (Stärkle, 1924; Stokoe, 1928; Hammer & Bryant, 1937) on the formation of methyl ketones showed that when single fatty acids are used as substrates certain fungal mycelia produce only the methyl ketones of one fewer carbon atoms. This was later confirmed with *P. roqueforti* spores using [1-^{14}C]-octanoate as substrate; $^{14}CO_2$ was produced and the heptan-2-one synthesized was unlabelled (Gehrig & Knight, 1963). When [2-^{14}C]octanoate was used as substrate (Lawrence, 1964) the specific activity of the heptan-2-one was very similar to that of the original fatty-acid substrate. With mycelium the rate of appearance of $^{14}CO_2$ was $1\frac{1}{2}$–3 times greater for [1-^{14}C]octanoate than when [2-^{14}C]octanoate was used as substrate. This confirms that ketone formation and β-oxidation proceed simultaneously and that the 2-C moiety (presumably acetate) formed by β-oxidation is further oxidized via the tricarboxylic cycle (e.g. Katz & Chaikoff, 1955). Dehydrogenase activity in fungi has been shown to be dependent on coenzyme A (Franke & Heinen, 1958*b*), and consequently the pathway of fatty-acid oxidation and methyl ketone formation may be represented by:



(Compare also Stokoe, 1928, and Jackson & Hussong, 1958.)

Such a mechanism of methyl ketone formation by direct deacylation is similar to a proposed pathway for the synthesis of ketone bodies in mammalian tissues (Drummond & Stern, 1960). The control mechanisms involved in the formation of methyl ketones are unknown and any hypothesis must allow for (*a*) the synthesis of only methyl ketones of one less carbon atom than the fatty acids used as substrate, and (*b*) β-oxidation to proceed simultaneously, Thus, if hexanoate and octanoate are

used as substrates separately, only pentan-2-one and heptan-2-one, respectively, are formed.

The influence of low concentrations of fatty acids on the growth of micro-organisms, in particular bacteria, has been reviewed by Nieman (1954), who suggested that growth interference is best interpreted as the result of changes in cell permeability by adsorption of the fatty acids at the cell membranes. Depending on conditions, such as concentration, a fatty acid may be either inhibitory or stimulatory to bacterial growth. Inhibitions of a similar nature have been observed with fungi and the inhibition of respiration by fatty acids in general reaches a maximum with increasing chain length up to a certain carbon number and then decreases (Girolami & Knight, 1955). pH has a considerable effect on the extent of inhibition (Gehrig & Knight, 1963) and on the point of the maximum effect in a homologous series (Lawrence & Hawke, unpublished). At pH 2·5 decanoate exhibits maximum inhibition of respiration, whereas at pH 5·2–6·0 dodecanoate has the maximum effect. Moreover, the C_4–C_{18} fatty acids were not inhibitory at pH values close to neutrality (Girolami & Knight, 1955). The same workers found that the extent of inhibition or stimulation of respiration and the formation of methyl ketone is also dependent upon the concentration of the fatty acids.

Methyl ketone formation is also pH dependent (e.g. Franke & Heinen, 1958*a*). Gehrig & Knight (1963) demonstrated that at 25–30 °C maximum formation of heptan-2-one from octanoate by spores occurred at pH values close to neutrality. The relationship between O_2 uptake and formation of heptan-2-one is also affected by pH—maximum uptake of O_2 occurring at pH 5·5–6·5 when heptan-2-one formation was low.

The likelihood of success in the preparation of cell-free extracts from fungi which oxidize fatty acids appears to vary with the species. Thus Franke & Heinen (1958*b*) prepared cell-free extracts from several moulds which possessed fatty-acid dehydrogenase activity, notably *A. niger* and *Penicillium* species. Dehydrogenase activity was found in mitochondrial-free extracts of mycelium, whereas fatty-acid dehydrogenase activity is confined to the mitochondria of other biological systems. Dehydrogenase activity was highest for palmitate, stearate and oleate and weak for the lower fatty acids and involved the coenzyme A thioesters of the fatty acids. Mukherjee (1951) found that fatty-acid dehydrogenase activity increased from C_4 to C_{10} and was absent with palmitate and stearate as substrate.

Gehrig & Knight (1963) were unsuccessful in obtaining cell-free preparations, which would produce methyl ketones from fatty acids, from spores of *P. roqueforti* although they used a variety of methods of disruption and supplemented the extracts with the usual co-factors for the oxidation of fatty acids. Lawrence (1964) found that cell-free extracts prepared by disrupting mycelium of *P. roqueforti* in the Hughes press oxidized low concentrations of octanoate after a lag period but methyl ketones were not detected.

Decarboxylation of β-keto acids to methyl ketones. Karrer & Haab (1948) found that mycelial acetone powders prepared from various penicillia were capable of decarboxylating β-keto acids to the corresponding methyl ketone but attempts to isolate the decarboxylase in cell-free extracts were unsuccessful. They concluded that the β-keto decarboxylases were bound to membranes or cell walls and this conclusion is sup-

ported by recent failure to prepare from *P. roqueforti* a soluble system capable of forming methyl ketones from fat (Lawrence, 1964). However, Lawrence (1965*b*) found that in spores there was little effect of pH, between the limits 4·5–7·0, on the formation of heptan-2-one from octanoic acid.

However, Franke, Platzeck & Eichhorn (1961), working with mycelia of *A. niger* and other mould species, obtained a decarboxylase in solution which was active on β-ketomonocarboxylic acids. The enzyme preparation decarboxylated β-keto acids with from 4 to 12 carbon atoms, but maximum activity was obtained with β-ketolaurate; the pH optimum increased from 5·5 to 7·0 with the increasing molecular weight of the substrate.

The metabolism of methyl ketones. Stokoe (1928) was the first to give a detailed consideration to the relationship between the formation of secondary alcohols and methyl ketones by fungi. Although he thought that the secondary alcohols were probably the primary product of decarboxylation of β-hydroxy fatty acids, he did not dismiss the possibility that they were formed by reduction of ketones. On the basis of the volatiles present at different stages in the ripening of mould-ripened cheese, Jackson & Hussong (1958) suggested that the methyl ketones are first formed and are subsequently reduced to the corresponding secondary alcohols. Further indirect evidence to favour this mechanism of secondary alcohol formation is the presence of C_8 and C_{10} β-keto acids in mould-ripened cheese (Bassett & Harper, 1958).

Direct evidence that methyl ketones are in fact precursors of secondary alcohols was provided by Franke, Platzeck & Eichhorn (1962) in experiments with mycelium suspensions and cell-free extracts of *A. niger*. Under anaerobic conditions methyl nonyl ketone, which had been synthesized from laurate, was slowly reduced by mycelium suspensions to the corresponding carbinol, a reaction which was reversed on return to aerobic conditions. Mycelial extracts contained an ammonium sulphate precipitable DPN-specific alcohol dehydrogenase which catalysed the reaction:

$$RCOCH_3 \underset{DPN^+}{\overset{DPNH+H^+}{\rightleftharpoons}} RCHOHCH_3$$

It seems that the mechanism of formation of secondary alcohol is similar to alcohol fermentation in yeast and to lactic acid formation in muscle. This is supported by the observed stimulation of carbinol formation by glucose (Franke *et al.* 1962). The amount of DPN^+ in cells is limited and the reduction of methyl ketone to secondary alcohol is a possible mechanism for the reoxidation of the reduced pyridine nucleotide formed during anaerobic glycolysis.

REFERENCES

Alberts, A. W., Majerus, P. W., Talamo, B. & Vagelos, P. R. (1964). *Biochemistry, N.Y.*, **3**, 1563.
Badings, H. T. (1964). *J. Chromat.* **14**, 265.
Barrett, C. B., Dallas, M. S. J. & Padley, F. B. (1963). *J. Am. Oil Chem. Soc.* **40**, 580.
Bassett, E. W. & Harper, W. J. (1958). *J. Dairy Sci.* **41**, 1206.
Bassette, R. (1959). *Diss. Abstr.* **20**, 267.
Bassette, R., Ozeris, S. & Whitnah, C. H. (1963). *J. Fd Sci.* **28**, 84.
Boldingh, J. & Taylor, R. J. (1962). *Nature, Lond.*, **194**, 909.
Bressler, R. & Wakil, S. J. (1962). *J. biol. Chem.* **237**, 1441.
Cochrane, V. W. (1958). *Physiology of Fungi.* New York: John Wiley and Sons.
Coffman, J. R., Smith, D. E. & Andrews, J. S. (1960). *Fd Res.* **25**, 663.

CONDON, R. D., SCHOLLEY, P. R. & AVERILL, W. (1960). *Proc. 3rd Symp. Gas Chromat.* London: Butterworths Scientific Publs.
CORBIN, E. A., SCHWARTZ, D. P. & KEENEY, M. (1960). *J. Chromat.* **3**, 322.
DAY, E. A., BASSETTE, R. & KEENEY, M. (1960). *J. Dairy Sci.* **43**, 463.
DAY, E. A., FORSS, D. A. & PATTON, S. (1957*a*). *J. Dairy Sci.* **40**, 922.
DAY, E. A., FORSS, D. A. & PATTON, S. (1957*b*). *J. Dairy Sci.* **40**, 932.
DAY, E. A. & LIBBEY, L. M. (1964). *J. Fd Sci.* **29**, 583.
DAY, E. A. & LILLARD, D. A. (1960). *J. Dairy Sci.* **43**, 585.
DE BRUYN, J. & SCHOGT, J. C. M. (1961). *J. Am. Oil Chem. Soc.* **38**, 40.
DESTY, D. H., GEACH, C. J. & GOLDUP, A. (1960). *Proc. 3rd Symp. Gas Chromat.* London: Butterworths Scientific Publs.
DRUMMOND, G. I. & STERN, J. R. (1960). *J. biol. Chem.* **235**, 313.
DUTRA, R. C., JENNINGS, W. G. & TARASSUK, N. P. (1959). *Fd Res.* **24**, 688.
DUIN, H. VAN (1965). *Off. Org. K. ned. Zuivelb.* **57**, 115 (*Dairy Sci. Abstr.* (1965), **27**, 256).
FODOR, P. J. & CHARI, A. (1949). *Enzymologia*, **13**, 258.
FORSS, D. A. (1964). *J. Dairy Sci.* **47**, 245.
FORSS, D. A., DUNSTONE, E. A. & STARK, W. (1960). *J. Dairy Res.* **27**, 211.
FRANKE, W. & HEINEN, W. (1958*a*). *Arch. Mikrobiol.* **31**, 50.
FRANKE, W. & HEINEN, W. (1958*b*). *Arch. Mikrobiol.* **31**, 359.
FRANKE, W., PLATZECK, A. & EICHHORN, G. (1961). *Arch. Mikrobiol.* **40**, 73.
FRANKE, W., PLATZECK, A. & EICHHORN, G. (1962). *Arch. Mikrobiol.* **41**, 154.
GEHRIG, R. F. & KNIGHT, S. G. (1958). *Nature, Lond.*, **182**, 1237.
GEHRIG, R. F. & KNIGHT, S. G. (1961). *Nature, Lond.*, **192**, 1185.
GEHRIG, R. F. & KNIGHT, S. G. (1963). *Appl. Microbiol.* **11**, 166.
GIROLAMI, R. L. & KNIGHT, S. G. (1955). *Appl. Microbiol.* **3**, 264.
HAMMER, B. W. & BRYANT, E. W. (1937). *Iowa State College J. Sci.* **11**, 281.
HANSEN, R. P. & SHORLAND, F. B. (1952). *Biochem. J.* **52**, 207.
HARVEY, R. J. & WALKER, J. R. L. (1960). *J. Dairy Res.* **27**, 335.
HAWKE, J. C., DUNKLEY, W. L. & HOOKER, C. N. (1957). *N.Z. Jl Sci. Technol.* B, **38**, 925.
HUELIN, F. E. (1952). *Aust. J. scient. Res.* B, **5**, 328.
JACKSON, H. W. & HUSSONG, R. V. (1958). *J. Dairy Sci.* **41**, 920.
JENNINGS, W. G., VILJHELMSSON, S. & DUNKLEY, W. L. (1962). *J. Fd Sci.* **27**, 306.
JONES, L. A. & MONROE, R. J. (1965). *Analyt. Chem.* **37**, 935.
KARRER, P. & HAAB, F. (1948). *Helv. chim. Acta*, **31**, 795.
KATZ, J. & CHAIKOFF, I. L. (1955). *Biochim. biophys. Acta*, **18**, 87.
KEENEY, M. (1956). *J. Ass. off. agric. Chem.* **39**, 212.
KEENEY, M. (1957). *Analyt. Chem.* **29**, 1489.
KLEIN, F. & JONG, K. DE (1956). *Recl Trav. chim. Pays-Bas Belg.* **75**, 1285.
KRISTOFFERSEN, T., GOULD, I. A. & PURVIS, G. A. (1964). *J. Dairy Sci.* **47**, 599.
KROGER, M. & PATTON, S. (1964). *J. Dairy Sci.* **47**, 296.
KURTZ, F. E. (1965). *J. Dairy Sci.* **48**, 269.
LANGLER, J. E. & DAY, E. A. (1964). *J. Dairy Sci.* **47**, 1291.
LAWRENCE, R. C. (1963). *J. Dairy Res.* **30**, 161.
LAWRENCE, R. C. (1964). Thesis, Massey University of Manawatu.
LAWRENCE, R. C. (1965*a*). *Nature, Lond.*, **205**, 1313.
LAWRENCE, R. C. (1965*b*). *Nature, Lond.*, **208**, 501.
LAWRENCE, R. C. & HAWKE, J. C. (1963). *Nature, Lond.*, **197**, 1276.
LAWRENCE, R. C. & HAWKE, J. C. (1966). *Biochem. J.* **98**, 25.
LEA, C. H. (1963). *Chemy Ind.* p. 1406.
LEA, C. H. & SWOBODA, P. A. T. (1958). *Chemy Ind.* p. 1289.
LEA, C. H. & SWOBODA, P. A. T. (1962). *J. Sci. Fd Agric.* **13**, 148.
LIBBEY, L. M., BILLS, D. D. & DAY, E. A. (1963). *J. Fd Sci.* **28**, 329.
LIBBEY, L. M. & DAY, E. A. (1964). *J. Chromat.* **14**, 273.
LYNEN, F. (1961). *Fedn Proc. Fedn Am. Socs exp. Biol.* **20**, 941.
LYNN, W. S., STEELE, L. A. & STAPLE, E. (1956). *Analyt. Chem.* **28**, 132.
MABBITT, L. A. (1961). *J. Dairy Res.* **28**, 303.
MARCUSE, R. (1962). *J. Chromat.* **7**, 407.
MARTH, E. H. (1963). *J. Dairy Sci.* **46**, 869.
MCFADDEN, W. H. & TERANISHI, R. (1963). *Nature, Lond.*, **200**, 329.
MCGUGAN, W. A. & HOWSAM, S. G. (1962). *J. Dairy Sci.* **45**, 495.
MONTY, K. J. (1958). *Analyt. Chem.* **30**, 1350.
MORGAN, M. E. & ANDERSON, E. O. (1956). *J. Dairy Sci.* **39**, 253.
MORGAN, M. E. & DAY, E. A. (1965). *J. Dairy Sci.* **48**, 1382.
MORRIS, H. A. & JEZESKI, J. J. (1953). *J. Dairy Sci.* **36**, 1285.

MORRIS, L. J. (1962). *Chemy Ind.* p. 1238.
MUCK, G. A., TOBIAS, J. & WHITNEY, R. McL. (1963). *J. Dairy Sci.* **46**, 774.
MUKHERJEE, S. (1951). *Archs Biochem. Biophys.* **33**, 364.
NAWAR, W. W., CANCEL, L. E. & FAGERSON, I. S. (1962). *J. Dairy Sci.* **45**, 1172.
NAWAR, W. W. & FAGERSON, I. S. (1962). *Fd Technol.* **16** (11), 107.
NIEMAN, C. (1954). *Bact. Rev.* **18**, 147.
NICOLS, P. L. (1952). *J. Am. chem. Soc.* **74**, 1091.
PARKS, O. W., KEENEY, M., KATZ, I. & SCHWARTZ, D. P. (1964), *J. Lipid Res.* **5**, 232.
PARKS, O. W. & PATTON, S. (1961). *J. Dairy Sci.* **44**, 1.
PATTON, S. (1950). *J. Dairy Sci.* **33**, 680.
PATTON, S. (1951). *Fd Engng*, Dec., p. 93.
PATTON, S. (1963). *J. Dairy Sci.* **46**, 856.
PATTON, S., BARNES, I. J. & EVANS, L. E. (1959). *J. Am. Oil Chem. Soc.* **36**, 280.
PATTON, S. & THARP, B. W. (1959). *J. Dairy Sci.* **42**, 49.
POPJÁK, G., FRENCH, H. T., HUNTER, G. D. & MARTIN, A. J. P. (1951). *Biochem. J.* **48**, 612.
RALLS, J. W. (1960). *Analyt. Chem.* **32**, 332.
ROBERTSON, P. S. & PERRY, K. D. (1961). *J. Dairy Res.* **28**, 245.
SCARPELLINO, R. & KOSIKOWSKI, F. V. (1961). *J. Dairy Sci.* **44**, 10.
SCHWARTZ, D. P., HALLER, H. S. & KEENEY, M. (1963). *Analyt. Chem.* **35**, 2191.
SCHWARTZ, D. P. & PARKS, O. W. (1961). *Analyt. Chem.* **33**, 1396.
SCHWARTZ, D. P. & PARKS, O. W. (1963). *J. Dairy Sci.* **46**, 989.
SCHWARTZ, D. P., PARKS, O. W. & KEENEY, M. (1962). *Analyt. Chem.* **34**, 669.
SCHWARTZ, D. P., SPIEGLER, P. S. & PARKS, O. W. (1965). *J. Dairy Sci.* **48**, 1387.
SHIPE, W. F. (1951). *Archs Biochem.* **30**, 165.
SHORLAND, F. B. & HANSEN, R. P. (1957). *Dairy Sci. Abstr.* **19**, 168.
STARK, W. & FORSS, D. A. (1962). *J. Dairy Res.* **29**, 173.
STARK, W. & FORSS, D. A. (1964). *J. Dairy Res.* **31**, 253.
STÄRKLE, M. (1924). *Biochem. Z.* **151**, 371.
STERNBERG, J. C., GALLAWAY, W. S. & JONES, D. T. L. (1962). *Proc. 3rd Int. Gas Chromat. Symp.* New York: Academic Press.
STOKOE, W. N. (1928). *Biochem. J.* **22**, 80.
TÄUFEL, K., THALER, H. & MARTINEZ, M. (1933). *Margarine Ind.* **26**, 37 (*Chem. Abstr.* (1934), **28**, 7045).
THALER, H. & EISENLOHR, W. (1941). *Fette Seifen AnstrMittel*, **48**, 316.
THALER, H. & GEIST, G. (1939). *Biochem. Z.* **302**, 369.
THALER, H., SCHOTTMAYER, A., STÄHLIN, I. & BECK, H. (1949). *Biochem. Z.* **320**, 87.
THALER, H. & STÄHLIN, I. (1949). *Biochem. Z.* **320**, 84.
THIBODEAU, R. & MACY, H. (1942). *Tech. Bull. Minn. agric. Exp. Stn*, no. 152.
URBACH, G. (1963). *J. Chromat.* **12**, 196.
VEN, B. VAN DER (1964). *Recl. Trav. chim. Pay-Bas Belg.* **83**, 976 (*Dairy Sci. Abstr.* (1965), **27**, 84).
VEN, B. VAN DER, BEGEMANN, P. H. & SCHOGT, J. C. M. (1963). *J. Lipid Res.* **4**, 91.
WALKER, J. R. L. (1961). *J. Dairy Res.* **28**, 1.
WALKER, J. R. L. & HARVEY, R. J. (1959). *J. Dairy Res.* **26**, 265.
WATSON, J. T. & BIEMANN, K. (1965). *Analyt. Chem.* **37**, 844.
WILCOX, J. C., NELSON, W. O. & WOOD, W. A. (1955). *J. Dairy Sci.* **38**, 775.
WINTER, M., STOLL, M., WARNHOFF, E. W., GREUTER, F. & BÜCHI, G. (1963). *J. Fd Sci.* **28**, 554.
WINSTEIN, S. & LUCAS, H. J. (1938). *J. Am. chem. Soc.* **60**, 836.
WONG, N. P., PATTON, S. & FORSS, D. A. (1958). *J. Dairy Sci.* **41**, 1699.

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