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Destruction of *Bacillus subtilis* spores with solutions of sodium hydroxide

By R. L. WHITEHOUSE and L. F. L. CLEGG
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(*Received* 4 *March* 19€3)

Summary. The rate of destruction of *Bacillus subtilis* spores by sodium hydroxide solutions in the range of atmospheric temperatures 34–82 °F was studied: (*a*) to provide information which might be used in practice for immersion cleaning of dairy utensils in sodium hydroxide solutions at low atmospheric temperatures, and (*b*) to examine the nature of the disinfection curves by slowing down the rate of disinfection. The results expressed as a three-dimensional graph showed that an increase in temperature from 34 to 82 °F had a more marked effect on spore destruction than an increase in concentration of sodium hydroxide from 1·5 to 5·0 %. The overall effect could be expressed as

$$t = \frac{2{\cdot}6 \times 10^8}{C^{1{\cdot}66}\theta^{3{\cdot}79}}$$

where t = time in hours for the 99 % destruction of *B. subtilis* spores, C = concentration of NaOH (%, w/v) and θ = temperature, °F. Since the time for the 99 % destruction of *B. subtilis* spores by a 2 % NaOH solution at 34 °F is 5 times that of a 5 % solution, immersion cleaning in cold climates might be assisted by increasing the concentration of NaOH from the normal 2–3 to 5 %. Results in disinfection tests at low temperatures were more variable than had been observed in previous work at higher temperatures. These surprising results prevented a careful study of the nature of the disinfection curves and confirmed previous conclusions that such curves are basically sigmoid.

Chemical disinfection is influenced by many factors, the most important being concentration of disinfectant, temperature and time of contact. In a process of cleaning and disinfection of milking equipment known as immersion cleaning, it has been recommended that the milking equipment should remain in contact with a 3 % solution of sodium hydroxide at atmospheric temperature for the whole of the time between milkings. Immersion cleaning was developed by Thiel, Clough & Clegg (1955) for use in a temperate climate where the temperature of the disinfectant solution is unlikely to be often below 0 °C. In the present work, experiments were undertaken to assess the disinfectant properties of sodium hydroxide solutions at low atmospheric temperatures which are likely to be experienced in unheated milkhouses on farms in countries where winters are colder than in temperate climates. For this work, spores of *B. subtilis* were used to obtain a slow disinfection process and facilitate investigations.

METHODS

Test organisms. In preliminary experiments, spores of *B. subtilis* N.I.R.D. 736 were tested, but in the final experiments the more heat-resistant spores of *B. subtilis* SM 761 (Franklin & Clegg, 1956) were used.

Preparation of spore suspensions. The spores of *B. subtilis* SM 761 were produced on a solid medium containing 1 ppm of manganese as recommended by Charney, Fisher & Hegarty (1951). This medium was distributed in approximately 100 ml quantities in 150 Roux bottles and autoclaved at 115 °C for 20 min. The surface of the agar in each bottle was inoculated with a suspension of organisms from a 24-h culture on nutrient agar. The growth was harvested in sterile distilled water after 7 days' incubation at 33–36 °C. This crude suspension was first shaken for $2\frac{1}{2}$ h in bottles containing glass beads, and then heated at 85 °C for 5 min to destroy vegetative organisms. The suspension was centrifuged at approximately 1000 rev/min (with a diameter of 18·5 in from the bottom of one tube to the bottom of the opposite tube) for approximately 1 min to throw down any remaining clumps and large pieces of debris. The sediment was discarded, and the supernatant containing the spores was centrifuged at 3000 rev/min (with a diameter of 16 in) for 40 min to throw down the spores. The supernatant was decanted and, after microscopic examination indicated the absence of spores, was discarded. The sediment of spores was resuspended in a small volume of sterile distilled water by mechanical shaking. The suspension in each tube was diluted to approximately two-thirds the capacity of the tube by the addition of sterile distilled water, and the centrifugation at 3000 rev/min repeated. The spores were washed in this manner 5 times and the final suspension adjusted to 100 ml with sterile distilled water.

Microscopic examination showed the concentration of spores to be approximately 10^{10}/ml. The volume of the suspension was adjusted to 150 ml giving a microscopic count of 7×10^9 spores/ml. The suspension was kept refrigerated at 4–5 °C in stoppered bottles.

Medium. Before choosing a culture medium for this work, three media were compared for their ability to stimulate spore germination using both *B. subtilis* N.I.R.D. 736 and *B. subtilis* SM 761. The media examined were: (*a*) starch milk agar (SMA), i.e. nutrient agar containing 1 % separated milk plus 0·1% soluble starch (Grinsted & Clegg, 1955); (*b*) nutrient agar containing 0·1 % soluble starch (Williams, Clegg & Wolf, 1957); and (*c*) nutrient agar containing 0·5 % L-asparagine plus 1 % caramelized glucose (Hachisuka, Kato, Asano & Kuno, 1955). Little difference was found between the counts on the three media though media (*a*) and (*c*) gave counts generally slightly higher than agar. For convenience SMA was used for subsequent work.

Incubation period. Many workers, e.g. Franklin & Clegg (1956), have used extended incubation periods for the accurate estimation of surviving spores in liquid media. Comparison of incubation for 2, 3 and 7 days at 37 °C on solid media was made with both strains SM 761 and 736. As there were no differences between these treatments, a 2-day incubation period was used subsequently.

Germination stimulation. Two methods were investigated: (1) the effect of heating the spore suspension, and (2) the effect of subjecting the spore suspension to a short

treatment with sodium hydroxide solution. Tests with strain 736 showed that heating at 93 °C for 15 min reduced the count of viable spores, while heating at 80 °C for 10 min had no stimulatory or other effect on the number of spores detected. With strain SM 761, heating the spore suspension at 100 °C for periods up to 90 min had no stimulatory effect on germination. This spore suspension was heated at 85 °C for 5 min in the early stages of preparation and this may well have accounted for the absence of heat stimulation with the final preparation.

In preliminary disinfection trials with spores of *B. subtilis* SM 761 and 0, 1·5, 2, 3 and 5% sodium hydroxide solutions, the counts of viable organisms at 0 h (i.e. after a momentary contact with the test solution) were considerably higher using the solutions of sodium hydroxide than with the control using distilled water. The count was higher with the lower concentrations of sodium hydroxide and for the 1·5% sodium hydroxide solution was occasionally as high as 100 times the count for the control.

Using a second suspension of spores of *B. subtilis* SM 761 in further preliminary disinfection trials, the counts at 0 h with the sodium hydroxide solutions were again higher than with the controls, though the differences were considerably less than those obtained with the first suspension of spores.

Disinfection tests. A modification of a method similar to that used by Franklin & Clegg (1956) and other workers was used. Ninety-ml quantities of the test solutions in sterile 300 ml conical flasks were placed in a water-bath at the temperature under investigation well before inoculation to ensure that the correct temperature was attained. Sterile glass beads were used in the flasks to facilitate mixing of the contents. Closures were of cotton wool, but the plugs and necks of the flasks were covered with aluminium foil to prevent evaporation since the duration of some treatments was up to 100 h. The diluted spore suspension (5 ml spore suspension plus 50 ml sterile distilled water) was also allowed to attain the temperature under investigation. In preliminary experiments, 100-ml quantities of the test solutions were each inoculated with 1 ml of the undiluted spore suspension. However, to ensure more even inoculation of the five test solutions in the final experiments, 90 ml of each solution was inoculated with 10 ml of the thoroughly mixed diluted spore suspension. The concentrations of the test solutions were such that when 90 ml was diluted to 100 ml with the spore suspension in distilled water, the final concentrations were 1·5, 2, 3 and 5% (w/v) sodium hydroxide.

Each solution was inoculated with 10 ml of the diluted spore suspension and, after thorough shaking by hand to mix the contents, a 10 ml sample was removed and added to a medical flat bottle containing 10 ml of hydrochloric acid of the same normality as the alkali and 10 ml of sterile phosphate buffer at approximately pH 7 containing bromthymol blue indicator. After thorough shaking by hand, this neutralized solution was diluted serially using 99- and 9-ml quantities of sterile distilled water. Preliminary experiments showed the desirability of greater replication in order to increase the accuracy of the tests and so duplicate sets of dilutions were used in addition to duplicate estimations from each dilution. An Astell roll-tube apparatus was used to make the estimations of the number of viable organisms, and SMA containing 2% agar instead of the 1·3–1·5% as in most solid media was used to prevent slipping of the agar film in the roll-tube. Three to 3·5 ml of medium

were used per roll-tube. Each roll-tube was inoculated with 0·5 ml of the diluted suspension. The surface area of the agar layer in a roll-tube is approximately half that of the agar surface in a standard Petri dish; accordingly, only dilutions giving

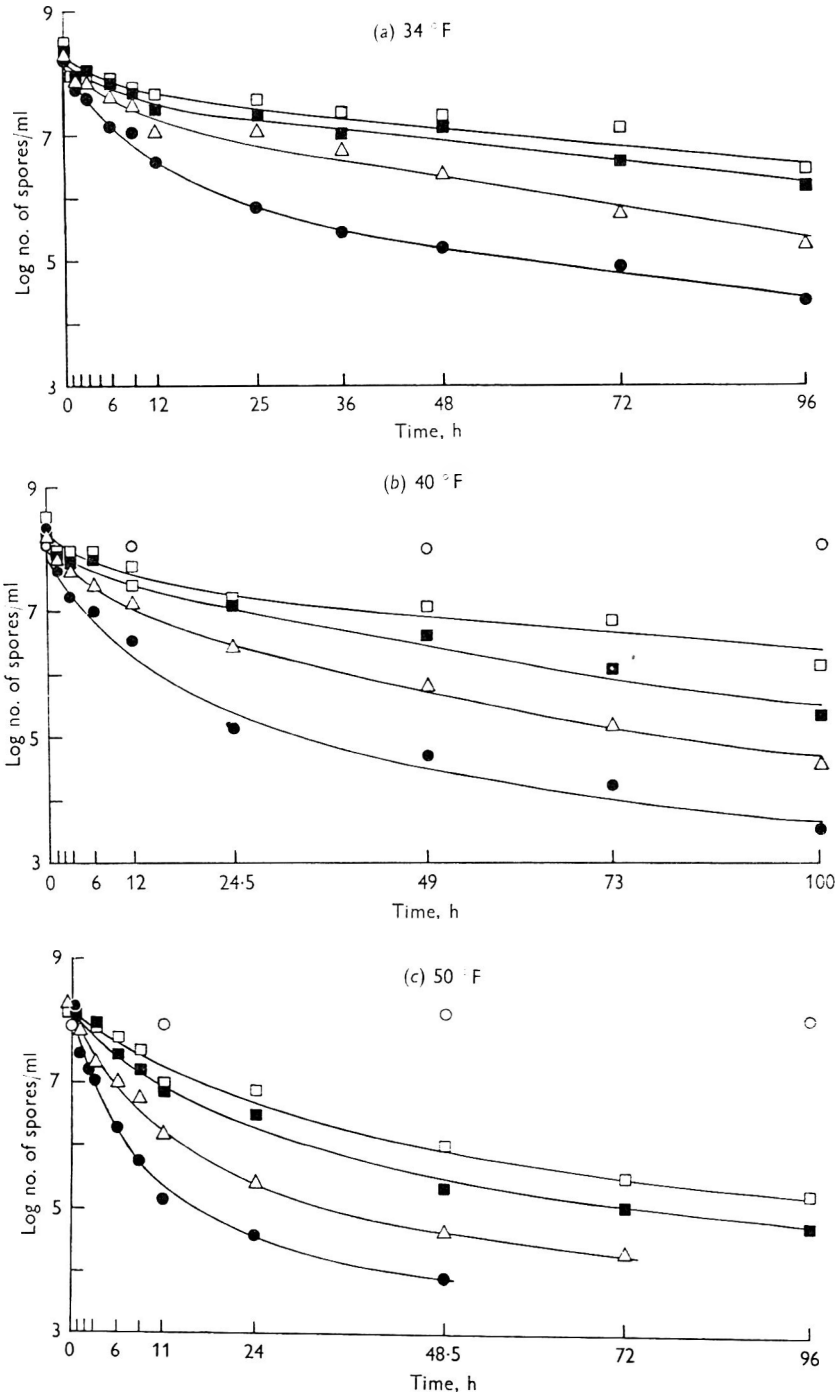

Fig. 1. For legend see opposite page.

counts of 15–250 colonies/roll-tube were used in estimating the number of viable organisms. Preliminary disinfection trials at 37, 50 and 82 °F were made to determine the dilutions to be cultured in the final trials. To be certain that the correct dilution was used, dilutions on either side of the predicted concentration were also cultured. For lower concentrations of viable organisms, 10-ml quantities of the undiluted neutralized solution were added to SMA contained in Roux bottles. If

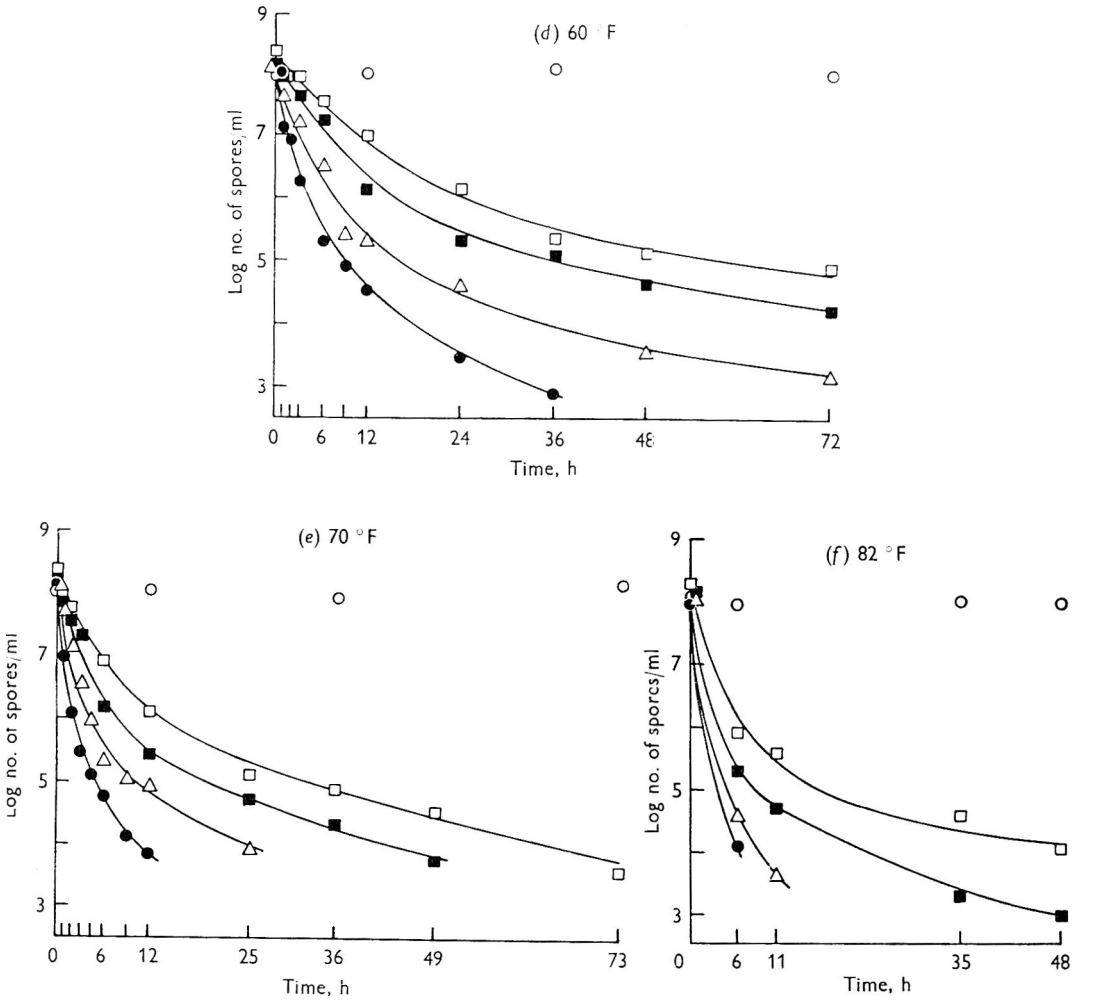

Fig. 1*a–f*. The effect of different concentrations of sodium hydroxide solutions on *B. subtilis* spores at various temperatures. ○, 0 % NaOH; □, 1·5 % NaOH; ■, 2 % NaOH; △, 3 % NaOH; ●, 5 % NaOH.

only one of the dilutions cultured was in the countable range, each estimate of survivors was based on 4 separate counts since duplicate dilutions and duplicate roll-tubes for each dilution were used. If 2 consecutive dilutions were in the countable range, the arithmetic mean of the two estimates was used and thus the final estimate was based on 8 individual determinations.

Dilutions in test tubes were shaken mechanically with the aid of a Vortex Jr. mixer, and bottles containing the neutralized treatment solutions were shaken vigorously by hand. The roll-tubes were incubated at 37 °C for 2 days.

RESULTS

The disinfectant properties of 1·5, 2, 3 and 5 % sodium hydroxide solutions at 34, 40, 50, 60, 70 and 82 °F were investigated. The results are given in Fig. 1 *a–f*, where the logarithms of the numbers of surviving spores/ml are plotted against time for each concentration of sodium hydroxide and at each temperature. The times required to produce 99 % destruction of the spores are given in Table 1 for each

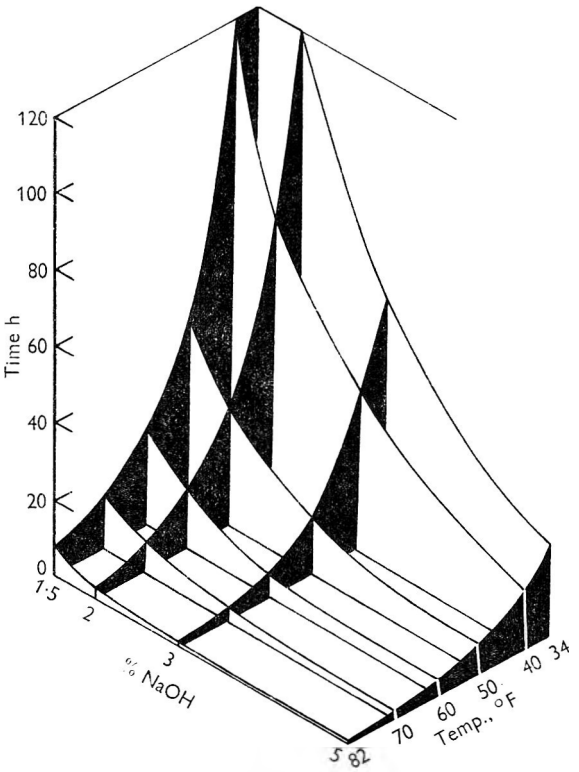

Fig. 2. The times required to produce 99 % destruction of *B. subtilis* spores at different concentrations of sodium hydroxide and at different temperatures.

Table 1. *The effect of concentration of sodium hydroxide solutions and temperature on the time required to produce* 99 % *destruction of* B. subtilis SM 761 *spores*

Concn NaOH, %	Time of treatment (h) required to produce 99 % destruction of *B. subtilis* SM 761 spores at Temperature, °F					
	34	40	50	60	70	82
1·5	150	120	49	26	13	7·5
2	120	76	32	16	8	3·2
3	62	44	15	5	3·2	1·6
5	24	16	7	4	2	1·5

temperature and for each concentration, and these results are plotted in the form of a three-dimensional graph in Fig. 2. The overall effect can be expressed as

$$t = \frac{2{\cdot}6 \times 10^8}{C^{1{\cdot}66}\theta^{3{\cdot}79}},$$

where t = time in h for the 99 % destruction of *B. subtilis* spores, C = concentration of NaOH (%, w/v) and θ = temperature, °F.

The disinfection curves obtained are generally concave, i.e. disinfection commences at a rapid rate and then gradually and progressively slows down. There is a direct relationship between overall rate of disinfection and both (i) concentration of sodium hydroxide solution in the range of 1·5–5·0 %, and (ii) temperature in the range 34–82 °F.

The concentration of spores in the second suspension of *B. subtilis* SM 761 estimated by microscopic count was approximately 7×10^9/ml. The concentration of viable spores for the control treatment (i.e. distilled water) estimated on SMA was approximately 1×10^8/ml. The counts at 0 h for the treatment solutions were slightly higher than the counts for the control, suggesting a treatment stimulation and indicating that the medium used did not induce complete germination of spores. The highest number of viable organisms estimated was still less than 10 % of the direct microscopic count.

Over the range of conditions tested, the increase in temperature had a more marked effect on spore destruction than did the increase in concentration of the disinfectant.

DISCUSSION

General

This work had two main purposes. One was to provide data on the effectiveness of disinfection by sodium hydroxide at low temperatures, and the other was to study the nature of the disinfection curves. It was hoped that by studying the slow rate of disinfection of spores at low temperatures the evidence of sigmoid death curves, observed in other work (see for example, Fig. 3 of Franklin & Clegg, 1956), might be confirmed. In earlier work it was also demonstrated that various shapes of disinfection curves could be induced by an alteration of the conditions, particularly the rate of disinfection (Withell, 1942; Jordan & Jacobs, 1945).

In the present work greater variation in results was observed than in the work of Franklin & Clegg (1956), although the same organism was used and the conditions of test were similar. There were slight differences in the culture media, but the main difference was the much lower range of temperatures of disinfection used in the present work which slowed down the rate of disinfection. However, the possibility of differences due to the use of different preparations of the same organism must not be discounted. To elucidate this point would require more tests over a wider range of conditions than has been undertaken in the present project.

The relationship between the effect of temperature and the effect of concentration on rate of disinfection in the present work (Fig. 2) is similar to that previously reported (Fig. 8 of Clegg, 1956).

Suggested application of the findings

It is known that in immersion cleaning a 3% solution of sodium hydroxide at approximately 50–60 °F produces a satisfactory bactericidal effect. In the present disinfection studies a 3% solution of sodium hydroxide at 50 °F took approximately 15 h to produce 99% destruction of *B. subtilis* SM 761 spores. Reducing the concentration of sodium hydroxide to 2% doubled the time required to produce the same degree of disinfection. With 3% sodium hydroxide solution, reducing the temperature from 50 to 40 °F trebled the time taken to produce 99% destruction. With 2% sodium hydroxide solution at 40 °F the time required to bring about 99% destruction was approximately 5 times that with a 3% solution at 50 °F.

The solution used for immersion cleaning just before it is changed at the end of a month's use is about 2% sodium hydroxide. In farm trials Thiel *et al.* (1955) showed no difference between the effectiveness of 1 and 5% sodium hydroxide during the warmer period of the year (spring–autumn). However, farm trials are not normally as sensitive as laboratory tests. Since the disinfectant properties of sodium hydroxide solutions are markedly affected by both temperature and concentration, it would seem possible in the light of our results that *in cold weather* an improvement in immersion cleaning might occur if the concentration of sodium hydroxide were increased to 5%.

While it is generally held that milking utensils require (and receive) less care in cold weather, too much reliance should not be placed on this for immersion cleaning. If there should for any reason be a heavy contamination of spore-bearing organisms on the cow's udder (e.g. from loafing barns) then the immersion solution should be effective under the coldest operating conditions. An alternative would be to increase the temperature of the immersion solution either by direct heating or by heating the milk-house. While the latter is necessary in some cold areas to keep liquids from freezing, too much heat in a milk-house is not desirable. It would be more convenient and economical to increase the concentration of sodium hydroxide to 5% than to apply heat directly or indirectly to the immersion solution.

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Lactic casein whey powder in rations for pigs

I. The substitution of un-neutralized lactic casein whey powder for barley meal in an all-meal ration for growing pigs

By A. C. DUNKIN

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(*Received* 28 *September* 1962)

Summary. The results of 2 experiments indicated that casein whey powder can successfully constitute a relatively high proportion of an all-meal diet for growing pigs. Pigs given a diet consisting of a basal meal allowance per day of $\frac{1}{2}$ lb meat meal and $1\frac{1}{2}$ lb barley meal plus increasing amounts of casein whey powder took significantly less time and required significantly less food to grow from 50 lb to 120–140 lb liveweight, than either the control pigs or those given diets containing smaller basal meal allowances but correspondingly more whey powder. In the best diet whey powder comprised approximately 40 % of the total food consumed. In one experiment, diets containing more whey powder than this resulted in slower growth rates, relative to that of the controls, from 110 lb liveweight onwards.

There was a little more scouring on the diets containing the greater amounts of whey powder; but even where whey powder formed approximately 83 % of the total food consumed, the incidence of scouring was not of great practical importance. Moreover, there was considerable variation among animals within treatment groups in this respect.

A big increase in the production of casein in recent years has led to interest in the use of the resulting by-product, lactic casein whey powder, as a food for farm livestock, especially pigs.

No recent reports have been found in the literature regarding the use of dried casein whey for pigs. Some reports are available on the value of dried cheese whey as a major ingredient in rations for growing pigs, but it is known that this product differs considerably from casein whey powder in the amount and composition of the ash fraction and in the lactic acid content (Spellacy, 1953). Schmidt, Kliesch & Schmalenbach (1939) reported that up to 1·7 lb/day of dried whey could be given satisfactorily to fattening pigs in place of 75 % of the cereal normally fed in a mixture that also contained potato flakes, dried sugar-beet and fishmeal. Bünger (1940) stated that in quantities up to 1·7 lb/pig daily, dried whey was a useful food comparable in nutritive value to barley meal. In contrast, Krider, Becker, Curtin & Van Poucke (1949) found that the inclusion of as little as 4 or 8 % of a dried cheese whey product in a maize-soyabean oil meal ration given *ad lib.* to weanling pigs produced some diarrhoea and there was also some indication that growth rate and

food intake were affected adversely. Results of another experiment reported in the same paper indicated that the lactose contained in the whey powder was probably responsible for the watery faeces. More recently, Becker, Terrill, Jensen & Hanson (1957) have presented results which indicate that the level at which cheese whey powder can be given without producing diarrhoea or reducing food intake and growth rate depends upon the age of the pig and on the other constituents of the ration. While 10 % dried cheese whey powder, as a partial replacement for maize in a maize-soyabean oil meal ration given *ad lib.* to weanling pigs, gave results comparable with those of the control diet, the inclusion of 20 and 30 % whey powder resulted in significantly poorer growth. However, this was not associated with any increase in diarrhoea. On the other hand, with heavier pigs (approximately 85 lb initial live-weight) individually hand-fed to appetite with a semi-purified diet, no adverse effects occurred until whey powder formed at least 40 % of the diet (replacing an equivalent amount of starch). At a level of 60 % of whey powder in the diet, food intake and growth rate were reduced substantially and marked diarrhoea occurred.

In view of the compositional differences between casein whey and cheese whey and the variable results that have been reported for the latter, there was clearly a need for information on the feeding value of casein whey powder for pigs at different stages of growth and when included in different types of rations. Two trials were therefore made in which casein whey powder was given in conjunction with different fixed daily meal allowances to growing pigs. This basis of feeding was adopted because it is commonly used in New Zealand in the feeding of liquid whey. The results of further trials in which all-meal rations containing different fixed proportions of whey powder were given to growing pigs and to early-weaned pigs are reported elsewhere (Dunkin, 1963; Palmer, 1961).

EXPERIMENTAL

Experimental design and animals used. In both trials a randomized block design involving 4 treatments and 7 replications was used. Seven blocks of 4 litter-mates of similar weight were selected from first-cross (Berkshire × Large White) litters at 8–10 weeks of age. One member of each block was allocated randomly to each of the 4 treatments, the only limitations being that, as far as possible, all treatment groups contained the same number of castrated male and female pigs.

Experimental treatments and feeding. For trial 1, the daily meal allowances per pig for the respective treatments were:

Treatment	Daily meal allowance
1	½ lb meat meal + increasing amounts of whey powder to make up to feeding scale
2	½ lb meat meal + ½ lb barley meal + increasing amounts of whey powder to make up to feeding scale
3	½ lb meat meal + 1½ lb barley meal + increasing amounts of whey powder to make up to feeding scale
4	½ lb meat meal + increasing amounts of barley meal to make up to feeding scale.

Trial 2 was a repetition of the first trial except that for treatment 4 the barley meal was replaced by a mixture of barley meal and whey powder in the ratio of 7 parts by weight of barley meal to 1 part of whey powder.

For all the treatments, total daily food allowances were rationed according to the scale of dry-matter (D.M.) intake shown in Table 1 and assuming that the D.M.

contents of the meatmeal, barley meal and whey powder were 92, 86 and 95% respectively. The scale is essentially the same as the 'B' scale of daily 'meal' allowances given by Woodman (1960), assuming that the 'meal' contained 88% D.M.

With the aim of avoiding a sudden drastic change in their diet, the pigs on treatment 1 were given the basal meal allowance of treatment 2 (1 lb daily) for the first week of the trial, after which the allowance was reduced to ½ lb meat meal daily.

Once weekly, all the pigs received 10 g of an emulsion containing 5000 i.u. vitamin A and 500 i.u. vitamin D_3 per g.

Table 1. *Feeding scale**

Liveweight, lb	Dry matter, lb/day	Liveweight, lb	Dry matter, lb/day
41–44	2·06	101–104	3·61
61–64	2·58	121–124	4·13
81–84	3·10	141–144	4·59

* Intermediate values obtained by interpolation.

Feeds. The whey powder used was an un-neutralized spray-dried product derived from lactic casein whey. It was very hygroscopic, quickly becoming sticky and then setting hard on exposure to air. Samples of powder from the consignments used in trials 1 and 2 respectively had the following composition (%, w/w) moisture 13·2 and 7·1, crude protein 15·0 and 12·8, lactose (by anthrone method) 59·0 and 60·3, ash 9·6 and 10·9 and lactic acid by titration 6·4 and 6·9.

The consignments of meat meal used in the two trials were obtained from the same factory. Henneberg analysis of a sample of the meat meal used in the second trial gave the following results (%, w/w): moisture 9·9, crude protein 57·3, ether extract 14·4, ash 16·4 and nitrogen-free extract 2·1. Samples of two lots of barley meal used in the second trial contained respectively (%, w/w): moisture 14·5 and 15·5, crude protein 10·3 and 10·3, ether extract 1·3 and 1·1, ash 1·6 and 2·5, crude fibre 3·8 and 3·7 and nitrogen-free extract 68·6 and 66·9.

Management. All the pigs used had been weaned when 3 weeks old. At 8–10 weeks the selected animals were placed in individual pens in a totally enclosed, well-insulated Danish-type fattening house equipped with mechanical ventilation and with provision for thermostatic control of air temperature. Attempts were made to maintain a uniform temperature of approximately 67–69 °F within the house. Continuous thermograph recordings indicated that at a height of 4 ft 6 in from the floor the air temperature very rarely exceeded the range 60–70 °F.

From the time the pigs were placed in individual pens, until changed to their appropriate experimental rations at 48–51 lb liveweight, they each received ½ lb meat meal and ½ lb whey powder daily plus sufficient barley meal to provide a total ration in accordance with the feeding scale used during the trial.

The pigs were weighed twice weekly before the morning feed and their rations adjusted accordingly. The day's food allowance for each pig was weighed into a plastic bucket and water was added at the rate of 3 parts of water to 1 part of meal. For the first trial, where some of the whey powder was in hard lumps as a result of being stored in multi-layer paper bags, the mixture of meal and water was allowed

to stand overnight. This enabled the softened lumps of powder to be stirred in satisfactorily the following morning before the pigs were fed. For the second trial this procedure was unnecessary since the whey powder had been packed in plastic bags.

No additional drinking water and no bedding were provided. Each morning before the pens were cleaned, observations were made on the consistency of the dung voided by all the pigs during the previous 24 h period. The pigs remained in the experiment until they reached a minimum liveweight of 140 lb in trial 1 and of 120 lb in trial 2.

RESULTS

Health. In trial 1 all the pigs remained in good health. In trial 2, one pig (treatment 2) died 2 weeks after the trial began. Post mortem examination revealed evidence of excessive gas production in the small intestine and it appeared that death was due to an acute flatulent colic.

Table 2. *Observations of the consistency of the dung of pigs on different feeds*

(7 pigs per treatment except where indicated)

Treatment	No. of daily observations				χ^2 (for 6 d.f., $P = 0{\cdot}1$, $\chi^2 = 16{\cdot}8$)	No. of pigs with at least three consecutive daily 'scours'
	'Normal'	'Soft'	'Scour'	Total		
			Trial 1 (to 140 lb liveweight)			
1	397	81	54	532		3
2	438	50	33	521		2
3	421	47	31	499		1
4 (control)	470	45	13	528		1
Total	1726	223	131	2080	45·8**	
			Trial 2 (to 120 lb liveweight)			
1†	338	32	35	405		3
2†	336	26	32	394		3
3	372	33	13	418		1
4 (control)	453	14	1	468		0
Total	1499	105	81	1685	61·3**	

** $P < 0{\cdot}01$. † Six pigs only.

Several members of 2 of the experimental blocks in the second trial grew appreciably more slowly than those in the remaining blocks. In particular, the 2 pigs on treatment 1 grew very slowly. A course of sulphamezathine injections given to these 2 animals at 80–90 lb liveweight had no noticeably beneficial effect and one of them stopped growing entirely at 108 lb liveweight. It was removed from the trial and the appropriate results were discarded.

Observation of excreta. The results of daily observations on the consistency of the dung are presented in Table 2.

In both trials the incidence of scouring among the control pigs was negligible, but with the experimental animals it increased more or less progressively with increasing treatment levels of whey powder, reaching a maximum of 9–10 % of all observations for the pigs on treatment 1.

Although a highly significant treatment difference was found in respect of the number of observations in each dung category, there was much individual variation

Table 3. *Growth and food consumption in each of the two trials*

	Treatments					
	1 ½ lb meal + whey powder	2 1 lb meal + whey powder	3 2 lb meal + whey powder	4 Control (all-meal)	Standard error of means	Significance of difference
No. of pigs	7	7	7	7		
			Trial 1			
(*a*) 48–110 lb liveweight†						
No. of days	59·2	59·6	58·6	68·8	1·042	1, 2, 3, < 4**
Average daily gain, lb	1·05	1·03	1·06	0·90	—	—
Total food consumed, lb	183·2	185·2	186·3	227·4	3·4	1, 2, 3 < 4**
% whey powder‡	81	67	37	0	—	—
lb food consumed/lb gain	2·96	3·00	2·99	3·66	—	—
(*b*) 110–140 lb liveweight						
No. of days	26·1	26·3	21·9	22·6	0·49	3, 4, < 1, 2**
Average daily gain, lb	1·15	1·14	1·37	1·33	—	—
Total food consumed, lb	113·4	114·8	98·2	105·7	2·2	3 < 1, 2**; 3 < 4*; 4 < 1, 2*
% whey powder‡	89	77	56	0	—	—
lb food consumed/lb gain	3·78	3·83	3·27	3·56	—	—
(c) 48–140 lb liveweight						
No. of days	85·4	85·8	80·4	90·6	1·30	3 < 4**; 3 < 1, 2*; 1, 2, < 4**
Average daily gain, lb	1·08	1·07	1·14	1·02	—	—
Total food consumed, lb	297·0	299·6	284·0	335·0	4·6	1, 2, 3 < 4**; 3 < 2*
% whey powder‡	84	71	43	0	—	—
lb food consumed/lb gain	3·23	3·27	3·08	3·64	—	—
			Trial 2			
49–120 lb liveweight						
No. of days	68·1§	64·9§	59·9	66·6	1·76	3 < 1**; 3 < 2, 4*
Average daily gain, lb	1·04	1·10	1·18	1·07	—	—
Total food consumed, lb	214·3	208·2	194·9	224·0	6·1	3 < 4**; 3 < 1*
% whey powder‡	82	70	39	10	—	—
lb food consumed/lb gain	3·02	2·93	2·76	3·15	—	—

* $P < 0{\cdot}05$. ** $P < 0{\cdot}1$.

† Data for this period comprise means adjusted to the same mean pre-experimental period.

† Fresh weight basis.

§ Missing plot values calculated for one pig.

within treatments and some also occurred between blocks. The individual pig variation was indicated by the comparatively few pigs in each treatment group for which a period of scouring extending over 3 or more days was recorded, as distinct from an occasional 'scour' observation (Table 2). Nevertheless, this second criterion also revealed a treatment effect similar to that based on the total number of daily 'scour' observations.

Very little scouring occurred on any treatment after liveweights of approximately 100 lb had been attained. On the contrary, in the second trial from 80–90 lb liveweight, dung that was drier and harder than normal was sometimes voided by 4 pigs on treatment 1 (including the unthrifty pig) and 2 pigs on treatment 2. Although for the purposes of Table 2 these observations were included as 'normal', they constituted 12 and 3% of the total dung observations for treatments 1 and 2, respectively.

Also in the second trial from the same stage of growth, 3 pigs from each of the groups on treatments 1 and 2 sometimes passed urine from which a white amorphous material was deposited. The material was analysed qualitatively and found to contain phosphates, sodium and calcium.

Growth rate, food intake and food conversion efficiency. In trial 1 the whey powder appeared to be highly palatable and no food refusals were recorded. In the second trial several pigs refused small quantities of food in the early stages and 4 pigs refused food more persistently. However, no obvious treatment differences were noted in this respect, and with the exception of the unthrifty pig that was withdrawn from the trial the total estimated meal refusal for any pig did not exceed 11 lb.

The growth rate and food consumption results of trials 1 and 2 were examined over several stages of growth.

A summary of the results together with the standard errors of means and the significances of treatment differences are presented in Table 3. In trial 2 results were also calculated for the period 49–110 lb liveweight, but these are omitted from Table 3 since they were essentially similar to those for the slightly longer period.

For trial 1, significant regressions ($P < 0{\cdot}01$) were found of the time taken and the food required for the pigs to reach 110 lb liveweight on the length of the pre-experimental period in the trial pens. Accordingly, adjusted means were used in testing for individual treatment differences for this period of growth. Since the corresponding regressions for the overall trial period were not significant ($P < 0{\cdot}15$) the unadjusted means were used for the period and the period 110–140 lb liveweight.

From 49 to 110 lb liveweight the pigs in the 3 groups receiving whey powder had very similar performances and all 3 groups took significantly ($P < 0{\cdot}01$) less time and food than the control group. From 110 to 140 lb, however, the control pigs and those given 2 lb meal daily (treatment 3) significantly improved their position relative to those given the two highest whey powder diets. Overall, the pigs on treatment 3 took significantly less time and food to reach 140 lb liveweight than those given greater amounts of whey powder (treatments 1 and 2); while those on treatments 1 and 2, in turn, did significantly better than the control group.

In trial 2, the pigs on treatment 3 took significantly fewer days to reach 110 lb liveweight than the 3 remaining groups of animals which all grew at a similar rate.

As in the first trial, the control pigs required more food than the remainder, while those fed the highest whey diet (treatment 1) also needed significantly more food than the pigs on treatment 3.

DISCUSSION

The satisfactory results which were obtained in the present trials in which relatively high levels of casein whey powder were given to pigs during growth from 50 to 120–140 liveweight were in contrast to the poor growth reported by Becker *et al.* (1957) for weanling pigs given 20 and 30 % cheese whey powder in partial replacement of a maize-soyabean oil meal diet. There was more general agreement with their results for feeding cheese whey powder in semi-purified diets to finishing pigs. However, inasmuch as the pigs on treatment 3 in the present trials were significantly superior to the controls in terms of growth rate and food economy, casein whey powder appeared to give relatively better results under these particular conditions than those reported for cheese whey powder.

The occurrence of some scouring in the present trials, particularly by pigs fed the 2 diets containing most whey powder, contrasted with the absence of scouring noted in the earlier trial (Dunkin, 1958) when condensed cheese whey was given in conjunction with basal meal allowances similar to those used in the present trial. On the other hand, an average incidence of scouring of 7 and 10 % for pigs given the two highest whey powder diets (which contained 58 % whey powder at 50 lb liveweight increasing to 75 and 88 % respectively at 110 lb liveweight) probably represented a lower incidence of scouring than the 'marked diarrhoea' reported by Becker *et al.* (1957) when pigs weighing 85 lb initially were hand-fed to appetite a semi-purified diet containing 60 % cheese whey powder. If, as these authors have suggested, the mineral composition of the diet may influence the response to high levels of whey intake, differences in the mineral content and balance of casein and cheese wheys and in the remaining constituents of the respective diets may have contributed to the differences in the degree of scouring that occurred both between the earlier condensed whey trial and the present casein whey trials and between the latter and those of Becker *et al.* (1957).

In considering the average incidence of scouring for the various treatment groups, the marked variation between individual pigs and between blocks of animals should not be overlooked. This variation, which has also been noted among pigs fed liquid whey (Dunkin, 1959), is perhaps a somewhat similar phenomenon to the variable ability of dogs and rats to adapt themselves to high lactose diets, which was reported by several authors in work reviewed by Fischer & Sutton (1949).

In trial 1 the slow growth of the control pigs and the absence of any decline in growth rate with increasing whey treatment level up to about 110 lb liveweight contrasted both with the results obtained in trial 2 and also with those of the earlier trial with condensed cheese whey (Dunkin, 1958). There was no indication of possible causes of this difference. However, if the barley meal used in trial 1 had been of inferior quality, such an effect would have operated differentially, the control group being penalized the most severely. In spite of the better performance of the control pigs on the second occasion, replacement of some or all of the barley meal by whey powder on an estimated dry-matter basis significantly reduced the amount of food

required for pigs to grow from 48–49 lb to 110 lb in both trials. However, the results of the second trial indicated that while the highest whey powder ration and the control diet gave comparable growth rates, some decline in rate and economy of gains would be likely relative to those obtainable with a basal meal allowance of 2 lb daily (treatment 3).

The results from trial 1 indicated that from about 110 lb liveweight onwards, growth rate and efficiency of food utilization were affected adversely by high levels of whey powder. Beyond this stage, apparently, whey powder cannot form more than about 55 % of the ration without impairing the rate and economy of weight gains to some extent.

Consideration of the overall results from both trials suggests that the best performance is most likely to be obtained when the amount of whey powder fed is limited to about 40 % of the total food consumed, as in treatment 3.

In view of the frequent association between high intake of whey and scouring it was surprising that from 80 to 90 lb liveweight onwards scarcely any scouring occurred even though the proportions of whey powder in the diets were still rising. However it was at about this stage that some of the pigs on treatments 1 and 2 started to pass abnormally dry dung or urine from which deposits separated. From these observations it seems that the supply of water was barely sufficient for the elimination of the large quantities of inorganic salts associated with the high intakes of whey powder by these groups, and that as much water as possible was being removed from the food residues for this purpose; this, in turn, was countering any laxative tendency arising from the high intake of lactose.

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Lactic casein whey powder in rations for pigs

II. A comparison of un-neutralized and neutralized lactic casein whey powders with barley meal in all-meal rations for growing pigs

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Summary. From 50 to 140 lb liveweight pigs were given mixtures containing 25 or 50 % of either un-neutralized or neutralized lactic casein whey powders in partial replacement of barley meal in a control ration.

Those animals that were given rations containing 25 and 50 % un-neutralized whey powder and 25 % neutralized whey grew significantly faster and more efficiently up to 110 lb liveweight than the controls and those given the ration containing 50 % neutralized whey powder. For the overall trial period, only differences between the last-named group and each of the 3 other groups that received whey powder were statistically significant.

Water intake increased with level of whey powder in the diet and was significantly greater at both levels with the mixtures containing the neutralized powder than with the other diets.

Carcass measurements did not indicate any significant difference in treatment.

Both whey powders were sticky and unpleasant to handle, the neutralized material being worst in this respect.

In trials with growing pigs previously reported (Dunkin, 1963), rations containing un-neutralized lactic casein whey powder and supplemented with different fixed daily amounts of meat meal and barley meal permitted slightly faster growth than the control mixture up to 110 lb liveweight. However, from 110 to 140 lb liveweight, rations containing less than 2 lb meal daily resulted in significantly slower growth than the control ration.

During 1960–61 at least one New Zealand dairy company produced considerable quantities of casein whey powder that had been neutralized with caustic soda. This treatment resulted in the addition of about 3 % by weight of sodium to the material, and since the high content of soluble salts is thought to be a possible limiting factor to the nutritive value of whey (Daniel & Harvey, 1947; Wegelin, 1952), information was required as to how the neutralized whey powder compared in feeding value with the un-neutralized product.

A trial was made during the winter of 1961 to compare the relative feeding values of the two casein whey powders and to obtain further information on the value of casein whey powder relative to that of barley meal in all-meal rations.

EXPERIMENTAL

Experimental design and animals used. The pigs were drawn from 4 first-cross (Large White × Berkshire) litters which had been weaned at 3 weeks of age. Five experimental blocks, each consisting of 5 litter-mates matched for weight, were selected at 8 weeks of age. The allotment of treatments among the members of each block was at random, except for the restriction that each treatment group contained 3 females and 2 castrated males.

Table 1. *The pre-experimental and experimental meal mixtures*

		Treatment				
		(1)	(2)	(3)	(4)	(5)
			Un-neutralized casein whey powder		Neutralized casein whey powder	
Treatment	Pre-experimental A %	Control %	25 %	50 %	25 %	50 %
Dried buttermilk	15	7½	7½	7½	7½	7½
Meat meal	—	7½	7½	7½	7½	7½
Barley meal	85	85	60	35	60	35
Un-neutralized casein whey powder	—	—	25	50	—	—
Neutralized casein whey powder	—	—	—	—	25	50
	100	100	100	100	100	100
Steamed bone flour lb/100 lb	1	1	0·5	—	0·5	—
Vitamin A and D_3 supplement, oz/100 lb*	1	1	1	1	1	1

* Stated to contain 10000 i.u. vitamin A and 2000 i.u. vitamin D_3 per g.

Experimental treatments and feeding. The 5 treatments consisted of a control mixture and 4 mixtures which contained either 25 % or 50 % of un-neutralized or neutralized casein whey powder in place of an equivalent amount of barley meal in the control mixture.

Details of these five meal mixtures are given in Table 1 and the percentage composition of samples of the foods in Table 2. Daily meal allowances were regulated according to the feeding scale given in Table 3 which was the same as the 'B' scale suggested by Woodman (1960) and used in the earlier trial (Dunkin, 1963).

At 8 weeks of age, the experimental animals (average weight 41 lb) were placed in individual pens in the same fattening house as that used for the earlier trials (Dunkin, 1963). From 7 weeks old the pigs had been given mixture A (see Table 1). This mixture continued to be given, in amounts indicated by the feeding scale, until each pig reached a weight of 49–51 lb, when the appropriate experimental meal mixture was introduced. The change-over was completed after 1 week.

The meal for each pig was weighed each morning and water was added in the ratio of 2 parts by weight of water to 1 part meal. Approximately half of the stirred slop was given at the morning feed and the remainder in the afternoon. After the latter

feed had been tipped into the troughs, 1 lb of water was added to each bucket and any residual meal was washed into the trough.

Because of the high soluble salt content of the whey powders, all the pigs were provided with an unrestricted supply of drinking water in separate troughs. Recorded quantities of water were added as necessary and residues were weighed twice weekly. The pigs were weighed once weekly. In order to obtain comparable weights, all drinking water was removed at 5.00 p.m. on the day before and the troughs were not replenished until after the pigs had been weighed at 6.30 a.m.

Table 2. *The percentage composition of the feeds*

Food	Dry matter	Crude protein	Ether extract	N-free extract	Crude fibre	Total ash	Acidity (lactic acid equiv.)	Calcium	Phosphorus	Manganese, ppm
Barley meal	86·2	11·3	1·1	65·3	4·0	3·5	—	0·03	0·24	20·0
Dried buttermilk	95·4	35·9	8·2*	43·5	—	7·8	—	0·94	0·82	1·9
Meat meal	91·8	61·1	15·0	—	—	15·7	—	2·86	2·02	21·2
Un-neutralized casein whey powder	87·1	12·5	0·9	63·1	—	10·6	7·02	1·59	0·95	1·8
Neutralized casein whey powder	89·5	15·0	1·0	58·5	—	15·0	1·62	1·82	0·91	1·9
Bone flour	96·6	—	—	—	—	—	—	23·5	11·6	5·6

* Fat content determined by Gerber test.

Table 3. *Feeding scale*

Liveweight, lb	Amount of meal given, lb/day	Liveweight, lb	Amount of meal given, lb/day
40	2·1	120	4·6
60	2·9	140	5·1
80	3·5	160	5·6
100	4·2		

Throughout the trial, daily observations were made on the consistency of the dung voided by each pig.

Carcass measurements. The pigs were despatched to the factory on a fixed day each week at the first opportunity after they had reached 140 lb liveweight. Their last feed was given at 4.00 p.m. on the day before despatch and at this time the drinking water was removed and weighed.

The following morning the pigs were weighed at 6.30 a.m. and despatched to the factory. They were slaughtered between 10 and 11 a.m. With the exception of 3 pigs the carcasses were not singed. The eviscerated carcasses were sawn or chopped down the mid-line and placed in a chiller room for approximately 3 h, after which they were taken out and measured.

Measurements were taken from both sides with the carcasses hanging, and the readings averaged. The sites of measurement of back fat thickness at the shoulder, loin (mid-back) and rump, and of belly thickness were the same as those used by Hammond & Murray (1937). Length of side was measured from the anterior edge of the symphysis pubis bone to the vascular impression in the anterior edge of the first rib.

RESULTS

Physical properties of mixtures containing whey powder. All the 4 mixtures containing whey powder 'caked' and were sticky and unpleasant to handle. Both mixtures containing 25% whey powder set into a solid mass within a week or so even when stored in plastic bags. The mixtures containing 50% whey powder did not set during storage in plastic bags but quickly caked on the surface when exposed to the air. The neutralized whey powder was appreciably more hygroscopic than the un-neutralized powder. As a result the mixture containing 25% neutralized powder caked more than the one containing twice as much un-neutralized powder. However, the caked meals quickly formed a normal gruel or slop when water was added to them.

Table 4. *The number of pigs in each treatment group that exhibited bandiness of the hind legs at 70–90 lb liveweight*

	Treatment				
	1	2	3	4	5
		Un-neutralized casein whey powder		Neutralized casein whey powder	
Degree of abnormality	Control	25%	50%	25%	50%
Slightly bandy	2*	—	1	1	—
Bandy	—	3	—	1	—
Very bandy	—	1	—	—	2
Total	2	4	1	2	2

* Showed at 140 lb liveweight only.

Health. At 70–90 lb liveweight several pigs given the whey powder diets showed signs of bandiness of the hind legs to a varying degree. Subsequently a gradual recovery took place and by 140 lb liveweight the condition was no longer apparent except in two of the most severely affected pigs. One of these showed improvement, but the other (in the treatment 5 group) became progressively worse and at 103 lb liveweight lost its appetite and had to be culled. Whereas no leg abnormalities were present in any of the control pigs in the early stages of the trial, 2 animals showed very slight signs of bandiness at 140 lb liveweight. The incidence and degree of bandiness among the 5 treatment groups are shown in Table 4.

The general health of the pigs was good. Two pigs on treatment 3 were off their food for 2 days but were treated with sulphamezathine and soon recovered.

Very little scouring occurred and no pig scoured on more than a total of 3 days during the course of the trial. The total number of 'scour' observations for treatment groups 1–5 were 2, 0, 4, 1 and 9, respectively.

Growth rate, food and water consumption and food conversion efficiency. The results for growth performance and food and water consumption are presented in Table 5. In order to enable treatment comparisons to be made at different stages of growth, the results were tabulated from the start of the experiment (49–51 lb) to 110 lb liveweight, and from 110 to 140 lb liveweight as well as for the overall trial period.

Missing plot values were calculated for the treatment 5 pig which had to be withdrawn from the trial at 103 lb liveweight.

The pigs on treatments 2, 3 and 4 took significantly less time and consumed significantly less meal to grow from approximately 50 to 110 lb than either the controls or the pigs on treatment 5. The small differences between groups from 110 to 140 lb liveweight were not significant.

Table 5. *Summary of growth and food results*

	Treatment (T)						
	1	2	3	4	5		
		Un-neutralized casein whey powder		Neutralized casein whey powder			
	Control	25 %	50 %	25 %	50 %	Standard error of means	Significance of difference
No of pigs	5	5	5	5	5†	—	—
Start–110 lb liveweight							
Initial liveweight, lb	49·8	50·1	49·5	49·5	49·9	—	—
No. of days	54·6	49·2	50·8	50·2	55·6	1·04	T2, 3, 4 < T5**; T2 < T1**; T3, 4 < T1*
Average daily gain, lb	1·10	1·22	1·19	1·21	1·08	—	—
Total food consumed, lb	180·6	164·1	169·3	166·6	185·3	3·53	T2, 3, 4 < T5**; T2 < T1**; T3, 4 < T1*
110–140 lb liveweight							
No. of days	20·8	22·2	22·0	21·4	22·8	0·74	N.S.
Average daily gain, lb	1·44	1·35	1·36	1·40	1·32	—	—
Total food consumed, lb	97·3	103·3	100·5	99·7	106·3	3·32	N.S.
Start–140 lb liveweight							
No. of days	75·4	71·4	72·8	71·6	78·4	1·51	T2, 4 < T5**; T3 < T5*
Average daily gain, lb	1·20	1·26	1·24	1·26	1·15	—	—
Total food consumed, lb	277·8	267·3	269·8	266·2	291·6	5·99	T4 < T5**; T2, 3 < T5*;
Lb food eonsumed/lb gain	3·08	2·97	2·98	2·94	3·24	—	—
Total water consumed, gal	76·4	79·3	115·6	99·8	175·5	7·0	T1, 2, 3, 4 < T5**; T1, 2 < T3** T1 < T4*

† Includes missing plot values for one pig.
* $P < 0{\cdot}05$.
** $P < 0{\cdot}01$.

For the overall trial period, growth and food economy were significantly better for treatments 2, 3 and 4 than for treatment 5 (50 % neutralized whey powder). The treatment 1 group (controls) was intermediate.

Mean water intake was increased significantly and progressively with the amount of whey powder in the diet, with the exception that the difference between treatments 1 and 2 just failed to reach the 5 % level of significance. At both feeding levels pigs fed the neutralized whey powder consumed appreciably more water than those fed the un-neutralized powder ($P < 0{\cdot}01$).

Carcass measurements. The mean killing-out percentages and carcass measurements for the treatment groups are presented in Table 6.

Analysis of the results failed to show any significant regression of the variables measured on deadweight. No significant treatment differences were found in respect of any of the variables measured. There was a slight indication that the pigs given neutralized whey powder, especially at the 50 % level, had thinner backfat in the mid-back region and over the 'rump' than those on the remaining treatments.

Table 6. *Treatment means and standard errors of means for carcass data*

	Treatment						
		Un-neutralized casein whey powder		Neutralized casein whey powder		Standard error of means	Significance of difference
	Control	25 %	50 %	25 %	50 %		
No. of pigs	5	5	5	5	5*		
Cold dead weight, lb†	106·0	106·4	104·6	108·8	107·0	—	—
Killing-out percentage	74·0	75·2	73·5	75·1	74·7	0·33	N.S.
Length of side, mm	704	706	715	717	714	6·01	N.S.
Backfat thickness, mm:							
Max. shoulder	36·3	33·1	33·8	33·4	35·3	2·2	N.S.
Min. mid-back	18·6	18·5	19·3	17·5	15·3	1·9	N.S.
Av. of 3 rump measurements	22·1	21·9	22·4	21·0	18·9	1·8	N.S.
Thickness of belly, mm‡	26·0	25·9	26·0	25·7	26·5	1·0	N.S.

* Missing values calculated for one pig.
† Hot weight less 6 %.
‡ Average of three measurements per side.

DISCUSSION

A comparison of the incidence of leg abnormalities with the growth data did not suggest that growth was adversely affected, except in the pig that had to be culled and, even then, it gained normally until its appetite became affected 10–14 days before removal from the trial.

The results confirmed previous findings (Dunkin, 1963) that, at least until approximately 100 lb liveweight, rations containing appreciable amounts of casein whey powder resulted in faster and more efficient growth than the control diet. It was of interest that these results were obtained even when the control diet contained additional milk protein as provided by the inclusion of $7\frac{1}{2}$ % dried buttermilk. It seemed, therefore, that at this stage of growth the pigs were able to utilize the nutrients from the casein whey powder more efficiently than those from an equivalent weight of barley meal.

The very low incidence of scouring in the present trial was similar to the results obtained previously and again contrasted with the incidence rate of diarrhoea noted by Becker, Terrill, Jensen & Hanson (1957) at Illinois, when finishing pigs were hand-fed to appetite semi-purified diets containing 40 and 60 % cheese whey powder. In the latter experiment, food intake and growth rate declined substantially at the 60 % whey powder level. Similar adverse effects occurred when whey powder

formed as little as 20 % of a maize-soyabean oil meal ration fed *ad lib.* to weanling pigs.

The fact that in our trials high levels of casein whey powder produced less marked adverse effects than those obtained in the experiments of Becker *et al.* (1957) may have been due to qualitative differences between cheese and casein whey powders or between the basal rations employed. However, the fact that in the Illinois experiments whey powder was assessed in relation to a higher plane of energy nutrition may also have been a contributory factor.

No reason can be given for the bandiness which occurred in some pigs. While its low incidence among the control pigs suggested that the condition was related in some way to the casein whey powder diets, the incidence among pigs given the various casein whey powder rations did not conform to any obvious pattern. The calcium and phosphorus contents of the 4 casein whey powder mixtures were all in excess of the levels recommended by the National Research Council, U.S.A. (1959), whereas the calcium content of the control diet was slightly less than the recommended level.

The dietary levels of manganese varied from 18 ppm in the control diet to 8–13 ppm in the casein whey powder diets. It is not clear from the literature whether these levels were adequate. Grummer, Bentley, Phillips & Bohstedt (1950) found that increased growth occurred when a ration containing 12 ppm manganese was supplemented with additional manganese, but that no response was obtained when the basal ration contained 20 ppm manganese. No skeletal abnormalities were reported for the animals given the low manganese diets. On the other hand, Plumlee, Thrasher, Beeson, Andrews & Parker (1956) reported the appearance of skeletal abnormalities and other deficiency symptoms when pigs were given a ration containing 0·5 ppm manganese from an early age, and also in 1 experiment out of 3 when pigs from 35 to 43 lb liveweight were fed rations containing 1·0–3·4 ppm manganese. However, in none of these 4 experiments, in which the experimental levels of manganese were much lower than those in Grummer *et al.* (1950), was growth rate affected significantly.

It appears, therefore, that a marginal manganese deficiency may have been responsible for the leg abnormalities that occurred in the present trial but the evidence is inconclusive.

The physical properties of both the un-neutralized and neutralized casein whey powders left much to be desired. Their extreme stickiness on exposure to air would render impracticable their inclusion in concentrate mixtures in quantities in excess of 10–15 %.

The neutralized powder was even more hygroscopic than the un-neutralized product and in view of the significantly poorer results obtained in terms of rate and efficiency of gains when it formed 50 % of the ration, it was clearly inferior to the un-neutralized powder as a concentrate for pigs.

It is concluded that in an all-meal ration lactic casein whey powder compares favourably in feed value with barley meal and can form at least 50 % of the diet for pigs from approximately 50 lb to 100 lb liveweight: from this stage of growth onwards a slightly lower level may be desirable. However, such high levels are unlikely to be used in practice unless the physical properties of the powder can be improved.

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Fatty acid composition of sow's colostrum, milk and body fat as determined by gas-liquid chromatography*

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Summary. As determined by gas-liquid chromatography, the mean fatty acid composition (weight percentages of total fatty acids) of milk fat from sows fed a diet to meet U.S. N.R.C. nutrient requirements was: oleic, 35·3; palmitic, 30·3; linoleic, 13·0; palmitoleic, 9·9; stearic, 4·0; myristic, 3·3; linolenic, 2·5; unidentified 0·7 and 0·5, presumably *n*-odd chain and branched fatty acids; lauric, 0·3; and capric, 0·2. The corresponding fatty acid composition of colostrum fat was: oleic, 41·7; palmitic, 22·5; linoleic, 20·9; palmitoleic, 5·0; stearic, 5·7; myristic, 1·4; linolenic, 2·4; and unidentified acids, 0·3 and 0·1. Dietary fat increased fat levels in the milk and influenced fatty acid composition of the milk fat. Backfat resembled colostrum fat more than milk fat.

Cow's milk fat contains several minor fatty acids which have straight chains of odd numbers of C-atoms or odd and even numbers of C-atoms in branched chains (Shorland & Hansen, 1957). They occur in quantities of less than 2% of the total fatty acids. On gas-liquid chromatograms of the methyl esters these minor constituents appear as peaks between those of myristic, palmitic and stearic acid methyl esters (Smith, 1961). Herb, Magidman, Luddy & Riemenschneider (1962) have recently identified 27 minor fatty acids in a cow's milk, including a homologous series of mono-unsaturated *n*-acids with odd numbers of C-atoms from 15:1 to 23:1.

A recent review by von Neuhaus (1961) summarizes studies that have been conducted since the 19th century on the composition of sow's milk. Among the more extensive investigations which have included fat analyses of sow's milk are those of Braude *et al.* (1947), Bowland, Grummer, Phillips & Bohstedt (1949*a*, *b*) and Perrin (1954).

Despite the numerous reports dealing with fat levels of sow's milk, few have dealt with the fatty acid composition of the fat, and all were published before gas-liquid chromatographic analysis of fatty acids had been developed. A partial analysis of the fatty acids in sow's milk fat was given by Laxa (1931), and a more complete analysis of one sample of sow's milk fat was reported by de la Mare & Shorland (1944). Sheffy, Phillips, Dymsza, Grummer & Bohstedt (1952) determined iodine and saponification number of sow's milk fat, colostrum fat and lard, and concluded that the fatty acid composition of sow's milk fat and lard are similar.

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The results presented here compare the fatty acid composition of fat in sow's colostrum with that in milk later in lactation. Limited data are given on fatty acid composition of backfat from some of the same sows and of commercial lard.

EXPERIMENTAL

Milk samples between the 4th and 5th weeks of lactation and colostrum samples at 5–12 h after farrowing were obtained from Yorkshire or Lacombe × Yorkshire cross sows. Milk let-down was induced by injection of 10 i.u. of oxytocin (Pitocin, Parke-Davis and Co.) (Bowland, Grummer, Phillips & Bohstedt, 1949*c*). The colostrum and milk were obtained by milking the entire udder after the young pigs had been removed from the sow for at least 2 h. Fat samples were taken by biopsy from the mid-back region of 2 sows of Lacombe × Yorkshire breeding, one receiving the standard ration and one receiving the high fat, high protein ration.

A standard lactation diet based on barley, wheat, oats and a mixed protein supplement of soyabean meal, meat scrap, linseed meal and fishmeal and containing 4·2% crude fat as determined by extraction with n-hexane, was formulated to meet National Research Council Nutrient Requirements for Swine (Beeson, Crampton, Cunha, Ellis & Luecke, 1949). This diet was fed to the 5 sows from which milk was obtained and to 5 sows from which colostrum was obtained. Colostrum and milk were obtained from the same sow in 4 of the 5 cases. Three sows from which milk samples were obtained were fed a diet containing 15% stabilized tallow and 18% protein, but otherwise based on the same ingredients as the standard diet. These latter sows were restricted in feed intake to a level 20% below the other sows to equalize digestible energy and protein intake. All sows had been on similar diets from the time that they were 40 lb in weight and had no access to pasture from the time of birth.

Table 1. *Factors for the correction of peak areas of fatty acid methyl esters, retention volumes of methyl esters relative to methyl myristate*

Fatty acid ester	Correction factor	Relative retention volume
Caprylic	0·81	0·33
Capric	0·86	0·44
Lauric	0·91	0·64
Myristic	0·96	1·00
Unidentified	— *	1·19, 1·26, 1·44
Palmitic	1·01	1·64
Palmitoleic	— *	1·87
Unidentified	— *	2·08, 2·53
Stearic	1·06	2·70
Oleic	1·10	3·13
Linolic	1·14	3·69
Linolenic	1·20	4·62

* No correction factors were used for palmitoleic and the unidentified fatty acids.

The milk fat was extracted from the milk with a 50/50 (v/v) mixture of ethyl ether and petroleum ether. Methyl esters of the fatty acids were prepared as outlined by deMan (1961). The pentane solution of the methyl esters was injected directly on to the column of the gas chromatograph. The instrument was a Wilkens Aerograph

Table 2. *The fatty acid composition (as % w/w of total fatty acids) in sow's colostrum, milk and backfat and in commercial lard and stabilized tallow as determined by gas-liquid chromatography; also gross composition of sow's colostrum and milk (where applicable means and standard deviations are given)*

		Milk		Backfat			
	Colostrum std. diet, 5 pigs	Std. diet, 5 pigs	High fat, high protein diet, 3 pigs	Std. diet, 1 pig	High fat, high protein diet, 1 pig	Commercial lard	Stabilized tallow*
Fatty acid, weight %							
Caprylic	—	—	—	—	—	—	0·1
Capric	—	0·2 (0)	0·1 (0·05)	—	—	0·1	0·1
Lauric	—	0·3 (0)	0·2 (0)	—	—	0·1	0·2
Myristic	1·4 (0·13)	3·3 (0·55)	2·9 (0·02)	1·0	1·2	1·5	2·8
Unidentified	0·1 (0·07)	0·5 (0·10)	0·7 (0·17)†	—	—	—	1·4‡
Palmitic	22·5 (1·10)	30·3 (3·00)	22·6 (0·90)	23·7	20·4	24·6	25·1
Palmitoleic	5·0 (0·15)	9·9 (1·60)	7·2 (0·33)	2·8	4·3	2·7	3·7
Unidentified	0·3 (0·09)	0·7 (0·22)†	1·2 (0·14)†	0·3	1·2†	0·2	1·8‡
Stearic	5·7 (0·76)	4·0 (0·56)	4·8 (0·20)	14·6	11·0	13·1	18·1
Oleic	41·7 (5·60)	35·3 (4·50)	48·6 (2·00)	44·4	52·7	46·4	44·5
Linoleic	20·9 (5·80)	13·0 (0·60)	9·3 (1·90)	13·2	9·2	11·3	2·0
Linolenic	2·4 (1·11)	2·5 (0·46)	2·4 (0·62)	—	—	—	0·2
Gross composition, %							
Total solids	18·1 (2·50)	18·3 (0·20)	20·5 (0·90)	—	—	—	—
Fat	5·7 (1·32)	6·2 (0·25)	8·4 (0·90)	—	—	—	—
Solids-not-fat	12·4 (1·20)	12·1 (0·20)	12·1 (0·20)	—	—	—	—
Nitrogen	1·2 (0·23)	0·87 (0·05)	0·94 (0·08)	—	—	—	—
Protein	7·7 (1·50)	5·8 (0·39)	6·0 (0·51)	—	—	—	—

* Stabilized tallow which was fed at a level of 15% in the high fat ration.
† Combined figures for 2 peaks in some of the samples.
‡ Combined figures for 2 peaks.

model 110 with thermal conductivity detector. The column was 10 ft long, of 1/4 in O.D. stainless steel, packed with 20% diethyleneglycol succinate on 50–80 mesh firebrick. The column was held at 205 °C and the helium flow rate was 50 ml/min. The filament current was 250 mA. The areas under the peaks were measured by planimeter and converted to weight percentage methyl ester by using correction factors established with pure compounds under identical conditions. These correction factors are listed in Table 1 and are almost identical with those determined by Smith (1961) under similar conditions.

Fat and total solids contents of the milks were measured by the Mojonnier method, nitrogen by the Kjeldahl method. Protein was calculated from Kjeldahl nitrogen content by using the factor 6·38.

RESULTS AND DISCUSSION

The gross composition of the 5 milk samples from sows fed the standard diet averaged 18·3% total solids, 6·2% fat, 12·1% solids-not-fat and 5·8% protein (Table 2). There was an average of 8·4% fat in the milk of the 3 sows receiving 15% supplemental tallow in their diets but solids-not-fat and protein levels were similar to those in milk of the control group. Willett & Maruyama (1946) have reported that the fat content of sow's milk was increased to 10% when a diet containing high levels of garbage (27·1% ether extractable material in the diet) was fed as compared to 6% milk fat for other sows receiving a standard diet containing 2·8% ether extractable material.

The colostrum contained a higher level of protein than the milk samples which is in agreement with previous observations, e.g. Bowland *et al.* (1949*a*, *b*). Fat content of the colostrum was variable but on the average it was lower than milk fat levels. The colostrum samples were more variable in overall gross composition than were the milk samples. The results of colostrum and milk analyses were within the range of the averages reported in the literature (von Neuhaus, 1961).

The milk fat from the sows receiving the high fat diet contained on the average 13·3% more oleic, 7·7% less palmitic, 3·7% less linoleic and 2·77% less palmitoleic acid than the milk fat from the sows fed the standard diet. In the milk from the sows receiving 15% stabilized tallow, lauric and myristic acids were also lower, although these 2 fatty acids were not of major quantitative significance in the milk fat. There was also more of the unidentified acids with retention volumes between palmitoleic and stearic. The retention volumes are listed in Table 1. The stabilized tallow used in this diet (Table 2) contained 1·8% of its fatty acids with the same peaks. Also the concentration of these minor unidentified acids in the backfat from a sow receiving 15% tallow in her diet was high.

The fatty acid composition of colostrum fat differed in several respects from that of milk fat. Colostrum fat contained no capric and lauric acids and only small amounts of myristic and palmitoleic acids. In colostrum fat the unidentified fatty acids were present in lower concentrations than in the milk fat. The C_{18} fatty acid content of colostrum fat was relatively high (70%). This contrasts with milk fat obtained from the sows on the standard diet which contained less than 55% of C_{18} fatty acids. The high level of linoleic acid (20·9%) in colostrum fat was the most marked difference in any single fatty acid when compared with milk fat.

The fatty acid composition of the sow's backfat differed quite markedly from that of milk fat and to a lesser extent from colostrum fat. Backfat lacked capric and lauric acids, although traces of these acids were found in commercial lard. According to Maynard & Loosli (1962) lard contains traces of caprylic, capric and lauric acids. However, de la Mare & Shorland (1944) found no steam-volatile fatty acids (capric and lauric) in a sample of pig's backfat. These results are similar in most respects to those obtained in the present study. The backfat and milk fat from a sow given the diet containing 15 % of tallow showed the same differences in fatty acid composition, i.e. lower in palmitic and linoleic and higher in oleic and in unidentified minor fatty acids.

Minor unidentified fatty acids with retention volumes between those of myristic and palmitic acids and between palmitoleic and stearic acids were present in the colostrum and milk fats, As it was not always possible to judge the number of these minor fatty acids, they have been listed together in two groups. The backfats of the two sows from which samples were taken contained no minor fatty acids with retention volumes between those of myristic and palmitic acids but did contain those with the higher retention volumes. The fatty acid composition of the tallow used in these feeding experiments is also listed in Table 2. It contained both groups of minor fatty acids and also an unusually high proportion of oleic acid.

As with ruminants, the composition of the milk fat of the sow is more complex than the depot fat. This applies to the shorter chain fatty acids as well as to the unidentified groups. It is also evident from the results with milk from sows fed stabilized tallow, that some of the unidentified fatty acids may be of dietary origin.

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Occurrence of variants sensitive to agglutinins and to lactoperoxidase in a lactenin-resistant strain of *Streptococcus lactis*

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Summary. A strain of *Streptococcus lactis*, C_{10}, known to be resistant to lactenins, was found to be composed of 47% resistant cells, 10% cells sensitive to lactoperoxidase and 43% cells sensitive to both agglutinins and lactoperoxidase. By subculturing a strain composed of 100% resistant cells in different conditions of medium, temperature and incubation time, it was possible to obtain cultures composed of resistant and sensitive cells in various proportions. These findings help to explain how, after numerous subculturings in autoclaved milk, a resistant strain may become sensitive to the natural inhibitors of milk.

When strains of lactic acid bacteria are subcultured daily in autoclaved milk they often lose all or part of their activity when reintroduced into raw milk. This loss of activity which results from an inhibition by the natural anti-bacterial substances of milk (lactenins) has been particularly demonstrated by Jago & Swinbourne (1959).

We have confirmed these observations and shown that *Streptococcus lactis* C_{10}, a 'resistant strain' which develops equally well in both raw and heated milk, loses after a few weeks of subculturing in autoclaved milk its ability to acidify raw milk but not reconstituted spray-dried milk: it becomes 'sensitive' to lactenins. If subculturing in autoclaved milk is continued, the strain gradually loses all activity in both reconstituted and raw milk. On the other hand, another resistant strain of *Str. lactis*, C_2, showed no sign of sensitivity to lactenins after 12 months of subculturing in autoclaved milk.

We were interested in investigating the cause of a resistant strain becoming sensitive. It could be assumed, as suggested by Jago & Swinbourne (1959), that in a strain of resistant bacteria there would appear, after successive subculturings in autoclaved milk, sensitive variants which finally predominate through a mechanism of selection as yet unknown.

We therefore tried to find out whether a resistant strain such as C_{10} contained, after several culturings, a certain proportion of sensitive cells and whether, in that case, the percentage of sensitive cells in the total population varied when the strain was subcultured in different media at various temperatures.

An examination was also made of another resistant strain C_2 and of two strains, one (strain 760) sensitive to agglutinins and the other (strain 972) sensitive to both agglutinins and lactoperoxidase.

METHODS

Strains

The resistant strains C_{10} and C_2 (*Str. lactis*) were received as freeze-dried cultures from Dr J. Czulak of the Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia. The sensitive strains 760 and 972 (*Str. cremoris*) were kindly provided as milk cultures by Dr J. Tramer (United Dairies Ltd., London); they were then freeze-dried in our laboratory.

Isolations

To study the relative proportion of resistant and sensitive cells in a given culture, a suitable dilution was inoculated on the surface of a plate of glucose agar (Lab-Lemco, 5 g; Difco yeast extract, 3 g; Evans peptone, 15 g; K_2HPO_4, 5 g; $NaHPO_4.12H_2O$, 5 g; glucose, 4 g; agar, 20 g; water 1 l; pH 7·15).

One hundred colonies were picked at random and transferred to glucose broth. After incubation for 20 h at 30 °C the cultures obtained were kept at 4 °C until examined.

Determination of sensitivity to agglutinins and to lactoperoxidase

After incubation for 18 h the cultures to be tested were diluted ten thousandfold and 0·3 ml samples of the diluted culture were inoculated simultaneously into:

(*a*) 3 ml of whey obtained by clotting raw skim-milk at 37 °C with rennet (0·5 ml of commercial rennet per 1 ml of milk) and sterilizing by filtration on an EKS Carlson filter.

(*b*) 3 ml of whey prepared in a similar way but using milk which had been heated at 90 °C for 30 min and to which 0·02 % (w/v) $CaCl_2$ had been added to favour clotting. 3 ml of the same whey as (*b*) to which was added 1 % of a crude preparation of lactoperoxide obtained by adsorbing lactoperoxidase from whey on resin IRC-50 (XE-64) and eluting with K_2HPO_4 according to the method of Morrison, Hamilton & Stotz (1957) and Auclair & Portmann (1959).

After incubation for 18 h at 30 °C the whey cultures (*a*) were examined for agglutination. Cultures showing agglutination visible to the naked eye were considered to be derived from colonies of cells sensitive to agglutinins.

The pH's of the remaining cultures were measured using a glass electrode. A strain was considered to be sensitive to lactoperoxidase when the culture in heated whey plus lactoperoxidase was markedly inhibited compared with the culture in heated whey containing no lactoperoxidase: a difference of 0·8 pH unit was considered as significant, the difference generally observed for the sensitive strains being 1·5–2 units.

RESULTS

Strain C_{10}

The first culture (A) of the resistant strain C_{10} obtained in glucose broth directly from the freeze-dried culture was found to contain 47 % of cells which were resistant to lactenins, 10 % of cells sensitive to lactoperoxidase only and 43 % of cells sensitive to both agglutinins and lactoperoxidase (Table 1).

From one of the resistant cultures obtained in this way (culture B), 100 colonies were isolated and studied as above. Isolations were made also from one of the cultures sensitive to lactoperoxidase (culture C) and from one of the cultures sensitive to both agglutinins and lactoperoxidase (culture D). The results (Table 1) show that, even in the first subculture of a colony of resistant cells (B), 1 % of the cells were sensitive to lactoperoxidase and in a culture derived from a single colony sensitive to lactoperoxidase (C), 4 % of the cells were sensitive both to agglutinins and to lactoperoxidase. All cells of culture D, derived from a colony sensitive to both inhibitors, were sensitive to both inhibitors.

Table 1. *Percentage of cells resistant or sensitive to lactenins in different cultures of* Streptococcus lactis C_{10}

Cultures	Resistant cells	Cells sensitive to lactoperoxidase	Cells sensitive to both agglutinins and lactoperoxidase	Cells growing slowly in whey
A	47	10	43	0
B	99	1	0	0
C	0	96	4	0
D	0	0	100	0
B_1	98	1	0	1
B_2	100	0	0	0
B_3*	100	0	0	0
B_4*	35	40	0	25
B_5	0	0	100	0
B_6	69	24	0	7
B_7*	90	10	0	0
B_8	98	2	0	0

* 20 colonies tested instead of 100.

A Original culture.

B Resistant culture isolated from A.

C Culture isolated from A and sensitive to lactoperoxidase.

D Culture isolated from A and sensitive to both agglutinins and lactoperoxidase.

B_1, B_2, etc. Resistant cultures isolated from B and subcultured under the following conditions:

B_1 16 weekly subcultures in autoclaved litmus milk (1 % inoculum), the cultures being stored at 4 °C *before* incubation at 30 °C for 16 h.

B_2 28 weekly subcultures in autoclaved litmus milk (10 % inoculum) the cultures being stored at 4 °C *after* incubation at 30 °C for 6 h.

B_3 32 twice-weekly subcultures under conditions of B_2.

B_4 28 weekly subcultures in glucose broth (10 % inoculum), the cultures being stored at 4 °C after incubation at 30 °C for 6 h.

B_5 200 twice-daily subcultures in autoclaved litmus milk (1 % inoculum), the cultures being incubated at 30 °C for 8 or 16 h, and stored at 4 °C during week-ends before incubation.

B_6 100 daily subcultures in autoclaved litmus milk (1 % inoculum), the cultures being incubated at 20 °C for 24 h and stored at 4 °C during week-ends before incubation.

B_7 32 twice-weekly subcultures in autoclaved litmus milk (10 % inoculum) the cultures being stored at 4 °C after incubation at 20 °C for 16 h.

B_8 Culture in autoclaved litmus milk (1 % inoculum) stored 3 months at 4 °C before incubation at 30 °C for 16 h.

One of the resistant cultures, derived from B, was then subcultured under different conditions and the sensitivity of the final subcultures to lactenins was tested (Table 1).

It can be seen that cultures B_1, B_2 and B_3, which were subcultured once or twice per week in autoclaved litmus milk, still had at the end of 4 months nearly 100 %

of resistant cells. On the other hand, culture B_4, which was subcultured weekly 28 times in glucose broth, contained a high proportion of cells sensitive to lactoperoxidase (40 %) and of cells growing slowly in whey (25 %). The latter are probably cells which have lost their ability to ferment lactose after continuous subculturing in glucose broth.

Culture B_5, which was subcultured 200 times in 4 months (twice a day in autoclaved litmus milk) was constituted entirely of cells sensitive both to agglutinins and to lactoperoxidase.

Cultures B_6 and B_7, maintained in autoclaved milk at 20 °C, still had a majority of resistant cells after 4 months, but they contained a high proportion of cells sensitive to lactoperoxidase; the greater the number of subculturings, the higher was this proportion.

Finally culture B_8, kept in the refrigerator without incubation, was still almost entirely resistant after 3 months (98 % of resistant cells), like the original culture B.

In none of the cultures studied were any cells found sensitive to agglutinins which were not also sensitive to lactoperoxidase.

Strains C_2, 760 *and* 972

An examination of a culture of the resistant *Str. lactis* C_2 showed that it contained 100 % of resistant cells. Similarly the agglutinin and lactoperoxidase sensitive *Str. cremoris* 972 was found to contain 100 % of cells sensitive to both inhibitors. On the other hand, although the agglutinin-sensitive *Str. cremoris* 760 contained 100 % of cells sensitive to agglutinins, 48 % were also sensitive to lactoperoxidase.

DISCUSSION

The presence, in a bacterial strain resistant to lactenins (lactoperoxidase and agglutinins), of some cells which after isolation produce cultures sensitive to these inhibitors, may explain how, after numerous subculturings in autoclaved milk, a resistant strain may become sensitive. In the case of the resistant strain C_{10}, variants sensitive to lactoperoxidase could be detected in the first subculture from a resistant colony. Variants sensitive to both agglutinins and lactoperoxidase only appeared later, after a certain number of subculturings.

On the other hand, it is interesting to note that weekly subculturing of a resistant strain B_2 (100 % of resistant cells) for 4 months in litmus milk, keeping the clotted cultures in the refrigerator between each subculturing, did not modify the characteristics of resistance of this strain. However, by subculturing the same strain twice a day for 4 months, a culture was obtained consisting entirely of sensitive cells (B_5).

The method of subculturing, and also the culture medium used, have therefore a great influence on the characteristics of sensitivity of the cultures derived from a strain originally resistant (B).

In the cultures derived from the resistant strain C_{10}, none was observed which was sensitive to agglutinins only. However, a few inactive cultures (cultures unable to ferment lactose) were obtained particularly after subculturing for 7 months in glucose broth.

The ease with which strain C_{10} gives variants sensitive to lactoperoxidase and to agglutinins is certainly one of the characteristics of this strain. On the other hand, it was observed that the other resistant strain, C_2, consisting of 100% of resistant cells, was still resistant after several months of subculturing.

Examination of the two sensitive strains 760 and 972 showed that neither of them contained resistant cells. However, strain 760, considered to be sensitive only to agglutinins (Wright & Tramer, 1957; Auclair & Portmann, 1959), contained cells sensitive both to agglutinins and to lactoperoxidase. As for strain 972, known to be sensitive to both agglutinins and lactoperoxidase (Wright & Tramer, 1958; Auclair & Portmann, 1959; Portmann & Auclair, 1959), it contained 100% of cells sensitive to both inhibitors. The question arises whether a strain such as the latter differs from the cultures derived from C_{10} which presented the same characteristics of sensitivity. It has been observed in a previous study (Portmann & Auclair, 1959) that the sensitive strains 760 and 972 were inhibited by different agglutinins. By using the method of adsorption of agglutinins then employed we have been able to show that the cultures derived from C_{10} and sensitive to agglutinins (and at the same time to lactoperoxidase), belong to an antigen group different from the 5 groups already described (Portmann & Auclair, 1959) and in particular from 972 and 760. It appears that a specific antigen has been unmasked on the surface of the cells of strain C_{10} during subculturing in autoclaved milk, an antigen whose homologous antibody exists normally in cow's milk.

In the case of strain C_{10} no differences in biochemical characteristics or phage sensitivity were found between the resistant cultures, the cultures sensitive to lactoperoxidase and the cultures sensitive to both agglutinins and lactoperoxidase. They all had the typical biochemical characteristics of *Str. lactis* and were sensitive to the same phages.

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Transduction in *Streptococcus lactis*

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Summary. Transduction experiments were carried out using recipient strain *Streptococcus lactis* C_2S^s (streptomycin-sensitive) and virulent phages prepared on donor strain *Str. lactis* C_2S^r (streptomycin-resistant). At a high multiplicity of infection of 10, transduction to streptomycin resistance occurred at a frequency approximately 1 in 30 surviving cells and the recombinant character exhibited a delayed expression. Transduction did not occur when phages propagated on *Str. lactis* C_2S^s were used or when phage antiserum was added to the transducing phage suspension. The transducing phage manifested an altered lytic cycle following passage through the *Str. lactis* C_2S^r donor which rendered it incapable of reinfecting the donor but did not alter its virulence for the *Str. lactis* C_2S^s recipient.

Recent studies of enteric and other bacteria have produced evidence of the occurrence of culture variation as a consequence of genetic exchange. While several types of exchange have been described (Jacob & Wollman, 1961), it is likely that transduction, i.e. the bacteriophage-mediated transfer between bacteria of deoxyribonucleic acid (DNA), plays an important role in causing variations in mixed cultures of strains of lactic streptococci. Such a phenomenon has not previously been reported in lactic acid bacteria. Indeed, the only Gram-positive bacteria which have been reported to undergo transduction are *Bacillus subtilis* (Thorne, 1961), *B. licheniformis* (Thorne, pers. comm.) and *Staphylococcus aureus* (Ritz & Baldwin, 1958; Morse, 1959).

It is the purpose of this paper to report the transduction of streptomycin resistance in *Str. lactis* and to describe some of the properties of this transducing system; a preliminary report has already been made (Sandine, Elliker, Allen & Brown, 1962).

Methods

Bacterial cultures

Str. lactis prototroph strain C_2, obtained from the stock culture collection maintained in the Department of Microbiology at Oregon State University, was used as the recipient. It was sensitive to 250 μg/ml of streptomycin and was therefore designated C_2S^s; a mutant (C_2S^r) of this strain isolated by Brown (1962) and resistant to 2000 μg/ml of streptomycin served as donor in the transduction of streptomycin resistance.

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Cultures were maintained by weekly transfer of a 1% inoculum into the lactic broth of Elliker, Anderson & Hannesson (1956) containing only glucose as the added sugar (LG broth); incubation was at 30 °C for 18–20 h. In preparation for each transduction experiment, cultures were maintained in the log phase of growth by transferring 4·5 h-old cultures into fresh, sterile LG broth. *Str. lactis* C_2S^r and C_2S^s were periodically screened for reversion to streptomycin sensitivity and mutation to antibiotic resistance, respectively, by inoculation into LG broth containing streptomycin at final concentrations of 250, 500, 1000 and 2000 μg/ml.

Preparation and assay of phage suspensions

Bacteriophage c_2, capable of infecting both donor and recipient organisms, was obtained from Dr E. B. Collins, University of California at Davis. Transducing phage lysates were prepared on *Str. lactis* C_2S^r and control lysates were prepared on *Str. lactis* C_2S^s. One ml of the appropriate phage preparation was delivered into a sterile 18 × 150 mm test tube, to which was added 0·5 ml of the appropriate sensitive indicator bacterium in the log growth phase, and the mixture was incubated for 15 min. The mixture was then inoculated into 50 ml of LG broth containing 2×10^{-3}M-$CaCl_2$ and incubated at 30 °C for 4 h. In a second 50 ml control flask of broth, 0·5 ml of the sensitive host bacterium was substituted for the phage mixture. After 4 h, the phage-containing culture was lysed, in contrast to the uninfected control culture. The lysate was adjusted to pH 7 and found in repeated trials to contain approximately 10^8 plaque-forming units (pfu) per ml when assayed by the overlay method (Adams, 1959) using a semi-solid agar seeded with 10^7 host cells.

Concentration of phage suspensions

Since a direct proportionality exists between the number of transductants and amount of phage (Morse, 1959), the transducing-phage preparations were concentrated; 50 ml portions of lysate were poured into dialysis tubing ($12 \times \frac{3}{4}$ in) which had been previously boiled for 10 min in a 2·0% (w/v) solution of ethylenediaminetetra-acetic acid. The tubing was then submerged in 50 g of polyethylene glycol 4000 (Union Carbide Chemical Co., Charleston, N. Va.) and the lysate was permitted to concentrate by dialysis over a period of approximately 3 h at room temperature. The concentrated lysate was filtered through a sterile millipore filter with an average pore diameter of 0·3μ and the filtrate assayed for pfu. Preparations exhibiting at least 10^9 pfu/ml were used as transducing lysates. They were streaked on LG agar to ensure sterility.

Transduction technique

A modification of the procedure of Lennox (1955) was used to effect transduction. Recipient culture, grown in 40 ml of LG broth until in the log phase of growth and containing approximately 4×10^9 cells, was centrifuged at 12000 rev/min in a Sorvall Model SS-1 centrifuge for 20 min. The pellet was resuspended in 2 ml of LG broth to provide approximately 2×10^9 cells/ml. A 0·2 ml sample of the suspension, containing approximately 4×10^8 cells, was combined with 1·8 ml of the transducing phage containing about 10^9 pfu/ml; this provided a final phage to host ratio of

approximately 10:1. In experiments where a phage to host ratio of 1:1 was desired, the phage lysates were used without concentration.

As a control to measure the frequency of spontaneous mutation to antibiotic resistance, LG broth was substituted for the phage lysate in another tube. Also in order to establish the necessity that transducing phage be prepared on the streptomycin-resistant strain to bring about the transfer of streptomycin resistance, phage lysate prepared on the *Str. lactis* C_2S^s recipient strain was substituted for transducing phage in a third tube.

Following a 20 min period at 30 °C, the transduction mixtures were centrifuged, washed twice in 5 ml portions of LG broth, and finally suspended in 2 ml of the same medium. The bacterial kill by virulent phages was estimated by comparing bacterial plate counts before and after phage infection.

Preliminary screening for streptomycin resistance was conducted in broth. Samples (0·2 ml) from each of the control and experimental suspensions were inoculated directly into 1 tube of LG broth and 6 tubes of the same medium supplemented with 1000 μg/ml of streptomycin. In quantitative experiments, an agar medium was substituted for the broth; samples (0·2 ml) from the control and experimental suspensions were spread over the surface of previously prepared LG agar plates. The plates were incubated for 5 days at 30 °C then replicas prepared (Lederberg & Lederberg, 1951) on LG agar plates containing 250 μg/ml of streptomycin. The replica plates were incubated at 30 °C for 5 days and observed for the development of colonies.

Colonies growing on the replica plates were picked into LG broth. Following incubation for 24 h at 30 °C, transfers were made into the same broth containing 250 μg/ml of streptomycin. From these mature cultures (18 h), 0·01-ml samples were removed with a loop, stained and examined microscopically. Gram-positive streptococci, morphologically and culturally indistinguishable from *Str. lactis*, were observed.

Antiserum preparation

Antiserum was prepared in rabbits by intravenous injection of phage c_2, which had been collected by high-speed centrifugation (144000 ***g***), washed once with a solution containing 0·15 M-NaCl and 0·001 M-$MgSO_4$, and re-suspended to a concentration of about 10^{12} pfu/ml. Each of 4 rabbits was injected with 0·5 ml of the phage suspension and injections were repeated every second day for 2 weeks. Sera were collected a week after the last injection.

Deoxyribonuclease preparation

A stock solution of sterile deoxyribonuclease (DNase) containing 45000 Dornase units/mg (California Corporation for Biochemical Research) was prepared by dissolving 200 mg of the enzyme in 10 ml of distilled water and filtering through a millipore filter with an average pore diameter of 0·3 μ. This stock solution was diluted 1:10 with sterile distilled water to obtain a concentration of 2 mg/ml.

RESULTS

Effect of high and low multiplicities of infection

Transduction of streptomycin resistance by virulent phage c_2 was examined using as the recipient *Str. lactis* C_2S^s, which has never been observed to mutate spontaneously to antibiotic resistance. Recombinants which were screened in broth containing 1000 μg/ml of streptomycin immediately following transduction were unable to grow. However, those which were allowed a period of time for delayed expression in LG broth grew on repeated transfer in broth containing 1000 μg/ml of streptomycin. From results of a typical experiment in Table 1, it may be seen that a low multiplicity of infection (moi—the ratio of number of phage particles to number of bacteria) proved ineffective, while a high moi resulted in the production of recombinant progeny. The frequency of transduction at a high moi was at least 1 in 10^3 surviving cells.

Table 1. *Effect of low and high multiplicity of infection (moi) on the transduction of streptomycin resistance from* Str. lactis C_2S^r *to* Str. lactis C_2S^s *mediated by phage* c_2

	Experiment I. moi = 1	Experiment II. moi = 10
Phage titre (pfu/ml)	5×10^9	1×10^9
No. of surviving cells/ml	1×10^3	1×10^3
Growth of survivors in Lg broth containing 10^3 units/ml of streptomycin	No	Yes
Minimum frequency of transduction/ml*	0	$1/10^3$

* Controls without added phages or with phages propagated on *Str. lactis* C_2S^s revealed no growth in streptomycin-containing broth.

Table 2. *Frequency of transduction of streptomycin resistance mediated by phage* c_2 *propagated* Str. lactis C_2S^r *and used to infect* Str. lactis C_2S^s *as revealed by the replica plating technique*

		Host for phage propagation	
	Control (no phage)	*Str. lactis* C_2S^s	*Str. lactis* C_2S^r
Phage titre, pfu/ml	0	1×10^9	1×10^9
No. of surviving cells/ml	1×10^9	1×10^2	8×10^2
Mean no. of colonies/ master plate	Uncountable	50	186
Mean no. of transductants/ replica plate	0	0	6*
Frequency of transduction	0	0	1/27†

* 6 colonies/0·2 ml of transduction mixture.
† Based on 800 survivors/ml.

Frequency of transduction

The frequency of transduction was determined by plating the surviving cells on LG agar, and replica plating on agar containing 250 μg/ml of streptomycin. Scoring the plates after 5 days of incubation at 30 °C for recombinant clones indicated 1 in 27 survivors were transduced; typical results appear in Table 2.

Experiments with antiserum and deoxyribonuclease (DNase)

To ensure that the recombinational events resulting in the transfer of streptomycin resistance were phage mediated and not caused by DNA present in the phage lysate, experiments were done using phage antiserum and DNase. The antiserum inhibited plaque formation (Table 3) and was tested for its effect on the ability of phage lysates to produce recombinants. Table 4 shows the inability of antiserum-treated phage to produce recombinants while phage pretreated with pre-immune serum exhibited a recombinational frequency approximately equal to that obtained with an untreated C_2S^r phage suspension. Control samples without phage produced no recombinants, indicating that none of the cells mutated. When phage suspension prepared on *Str. lactis* C_2S^s was substituted for one prepared on *Str. lactis* C_2S^r, no recombinants were observed, indicating that the genetic exchange was only mediated by the latter preparation.

Results of DNase experiments are also shown in Table 4. The inability of the enzyme to lower the recombinational frequency is evident.

Table 3. *Effect of pre-immune and phage antiserum on plaque formation by phage c_2 assayed on* Str. lactis C_2S^s

Dilution of phage	Plaques/plate	
	With pre-immune serum	With antiserum
10^{-2}	Confluent lysis	> 300
10^{-3}	Confluent lysis	30
10^{-4}	Confluent lysis	3
10^{-5}	Confluent lysis	0
10^{-6}	Near confluent lysis	0
10^{-7}	> 1000	0
10^{-8}	8	0

One ml of pre-immune serum or antiserum plus 1 ml of phage suspension prepared on *Str. lactis* C_2S^r was held 20 min at 30 °C. The samples were then diluted in LG broth and plated with *Str. lactis* C_2S^s in a semi-solid overlay on prehardened solid LG agar base.

Table 4. *The effect of DNase, phage antiserum and source of phage on transduction of streptomycin resistance in* Str. lactis

c_2S^r phage suspension, ml	LG broth, ml	Normal serum, ml	Phage antiserum, ml	DNase solution (2 mg/ml), ml	c_2S^s phage suspension, ml	No. of recombinants/ml of transduction mixture
0	2·0	0	0	0	0	0
1·0	1·0	0	0	0	0	26
1·0	0	1·0	0	0	0	30
1·0	0	0	1·0	0	0	0
1·0	0	0	0	1·0	0	31
0	1·0	0	0	0	1·0	0

Cells (0·2 ml of 4-h culture) of *Str. lactis* C_2S^s were incubated for 30 min at 30 °C with the constituents given in the table. Samples were plated on LG agar, incubated for 5 days at 30 °C and replica plated on solid LG agar plates containing 250 μg/ml of streptomycin sulphate. The c_2S^r phage (10^{10} pfu/ml) was propagated on *Str. lactis* C_2S^r; c_2S^s phage (10^{10} pfu/ml) was propagated on *Str. lactis* C_2S^s.

Lytic cycle of the transducing phage

During the course of this study it was noted that the c_2 phage exhibited an altered lytic cycle following propagation on the *Str. lactis* C_2S^r donor. This phage, when propagated in LG broth, would repeatedly lyse the *Str. lactis* C_2S^s strain, yielding a phage lysate containing about 10^8 pfu/ml. The phage particles present in such a culture lysate also were able to lyse the *Str. lactis* C_2S^r strain, resulting in lysate of equally high titre. The phage particles produced in this latter propagation, however, were incapable of lysing a second time the *Str. lactis* C_2S^r host from which they were originally obtained; also, plaques were not produced on this indicator in semi-solid overlay. However, this altered phage was capable of lysing the original *Str. lactis* C_2S^s strain, but with production of only about 10^6 pfu/ml. The particles formed in the recycling of the *Str. lactis* C_2S^r phage through *Str. lactis* C_2S^s host were found again to be capable of maturation on the *Str. lactis* C_2S^r victim. Reasons for the inability of the c_2 phage to propagate on the streptomycin-resistant host are not clear, and this phenomenon is being studied further.

DISCUSSION AND CONCLUSIONS

The demonstration of phage-mediated genetic exchange in *Str. lactis* brings to light another factor which may contribute to variability in mixed strain lactic acid starter cultures. The prevalence of bacteriophages for lactic streptococci provides ample opportunity for transduction to occur under commercial conditions, especially since this process has been found to occur also in milk (Allen, Sandine & Elliker, unpublished data). It is likely that any character which cells express may be conferred upon other cells by this exchange; for example, transduction of a nutritional marker (tryptophan independence) in *Str. lactis* has been shown recently (Sandine *et al.* 1962). Consequently, it may be seen how a normal starter culture may assume, in only a few transfers, an undesirable flavour or body characteristic. This would be especially true if transduction under natural conditions occurs at the high frequency observed in this study.

The occurrence of lysogenic lactic streptococci (Reiter, 1949; Sandine *et al.* 1962), which spontaneously release phages that may infect other strains in a mixed strain culture, establishes a condition for culture variation which seems impossible to prevent at the present time. While precautions may be exercised to prevent introduction of phages from the outside environment, or the adsorption and proliferation of these phages once they do gain entry (United States Department of Agriculture, 1962), it would appear that little can be done to prevent transduction by phages released from a lysogenic cell. One possible approach to this problem, however, which is being used in the United States, is to avoid the transfer of mother cultures; lyophilized cultures are used to inoculate as much as 5 gal of milk, thus providing sufficient mature culture to inoculate 500–1000 gal of bulk starter, cultured buttermilk, or sour cream. When such lyophilized mixed strain starter cultures are used, the decrease in number of transfer operations in the dairy provides additional insurance against phage contamination.

The existence of lysogenic lactic streptococci provides opportunity for another

genetic exchange phenomenon to occur, namely phage conversion. This process, like transduction, is phage mediated. In this case, the DNA of the phage particle is integrated with that of the lysogenic cell, which, as a result, exhibits new characteristics. This so-called conversion, as exemplified by acquisition of a new character upon lysogenization, is a well-documented contributor to bacterial variation (Freeman, 1951; Groman, 1960), but has not yet been shown to occur in lactic acid bacteria.

Recently, Møller-Madsen (1962) reported transformation of the ability to produce a malty aroma between strains of *Str. lactis*; DNA isolated from aroma positive strains, when added to milk cultures of aroma negative strains, conferred upon them the ability to produce a malty aroma. Autolysis and lysis of cells by antibiotics, phages and lysozyme-like substances may permit such a process to occur. However, whether this method of genetic exchange plays a significant role in starter culture variation under plant conditions remains to be determined.

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The detection of volatile components of milk by gas-liquid chromatography and its possible application in assessing keeping quality and flavour

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Summary. The volatile components of milk have been isolated in a form suitable for application to a gas chromatograph, using the simple rapid technique of entraining them in a gas stream and trapping in a syringe barrel cooled in liquid nitrogen.

The souring process in milk which occurred during incubation at 22 °C was followed by observing the differences between chromatograms of samples taken at intervals. Onset of souring was detected with a sensitivity similar to that obtained by measurement of pH, but the chromatographic method had the advantage of being independent of the initial pH and buffering power of the milk.

With suitable chromatographic apparatus the total analysis time including the preparation of the sample could be about 3 min. Modifications are discussed which would increase the speed of analysis, and thus form a basis for the development of a practical method of assessing the odour of milk before processing.

The advent of gas-liquid chromatography has opened a new chapter in flavour research. It has so far revealed the chemical complexity of various aromas, but, in general, the identification of all the separated components has not been completed and their contribution to flavour has not been assessed. In less demanding situations the detailed analytical data obtained by gas chromatography, and the ease with which the technique can be automated, have combined to make it a powerful method of controlling chemical processes (Bartle, 1960). Its use for such purposes received a new impetus with the introduction of fast analysis capillary columns requiring only seconds for their operation (Scott & Cumming, 1960).

Until now the detection of off-flavours in milk, as received on the dairy platform, has been a subjective process based on smell. Where undesirable bacteriological changes are suspected more tests, e.g. reduction of resazurin, clot on boiling, bacterial counts, are employed, but these are time-consuming and often provide inadequate information as to the cause of the defect. Analysis of the volatile components of milk by gas chromatography should give objective and detailed information concerning their amount and identity. Also, since food taints and frequently bacterial products are of a volatile nature, both defects should be detectable in one operation.

The testing of all milk received at the dairy would be practicable only if it could be done as rapidly as cans are handled. However, the swifter the test the more

difficult it becomes to operate manually. The automatic nature of gas chromatographic analysis makes it, therefore, highly suited for tests of this kind.

Previous attempts to apply gas chromatography to the examination of milk have been concerned mainly with feed taints (Wynn, Brunner & Trout, 1960) and flavours resulting from processing (Patton, 1961), although a recent investigation dealt with the normal flavour of milk and cream (Wong & Patton, 1962). In nearly all instances distillation and extraction procedures have been used to concentrate the volatile substances and, as far as possible, to remove water from the sample. Such methods are laborious and introduce the possibility of changing the composition of the sample by heat treatment (Lawrence, 1963) or selective extraction. Also, a multiplicity of techniques increases the danger of contamination, which is a real hazard when dealing with substances like milk which have a low flavour level and when the more sensitive detectors are being employed.

To avoid these difficulties Mackay, Lang & Berdick (1959), using the sensitive argon detector, have shown that the vapour above bananas in a closed vessel can be successfully analysed by direct injection of a 5–10 ml vapour sample. A relatively high flavour level is required for this technique to be successful. In addition, the high sample volume diminishes column efficiency. Nevertheless, a recent attempt to apply this technique to milk has succeeded in demonstrating changes in chromatograms as oxidative off-flavours developed (Jennings, Viljhalmsson & Dunkley, 1962). However, the very high sensitivity of detection required enhanced the danger of contamination and necessitated the preparation of samples outside the laboratory. In order to obtain meaningful results by this technique it is clear that the vapour sample must be in equilibrium with the milk, a requirement which would be difficult to meet if milk in churns were under test. Also, although the composition of the equilibrium vapour is related to the composition of the volatile substances in the milk, it is relatively more concentrated in the components with high volatility. For these reasons it would be preferable if the milk itself were examined.

With the objective of a practical test for milk flavour quality in view, it seemed desirable to seek a method of preparing the volatile components from milk which was simple and quick in operation, involved no treatment with solvents, and which provided a concentrated sample suitable for analysis on a gas chromatograph.

To assess the technique which was developed, its ability to follow the souring of milk was determined. A preliminary report of the results obtained has already been published (Mabbitt, 1963).

MATERIALS AND METHODS

Milk. The raw milk used was mixed morning and evening milk held at 40 °F from the Institute's Experimental Dairy. When received its evening's component was 17 h old and the morning's component 3 h old. It was sterilized as required by steaming for $\frac{1}{2}$ h in 100 ml quantities on 3 successive days.

Inoculation. Streptococcus lactis (N.C.D.O. 712) was grown overnight at 22 °C in Yeastrel–glucose broth, and 1 ml of a 10^{-7} dilution of the culture was added aseptically to 100 ml of the sterile milk. Bacterial growth was followed by plating dilutions of the milk on Yeastrel–glucose agar and counting colonies after 3-days' incubation at 30 °C.

Chromatography. A Pye Chromatograph with an argon detector containing ^{90}Sr as radioactive source was used. In order to cope with aqueous samples the separating column was made up of two portions (Swoboda, 1960) consisting of a 6-in length containing 20 % diglycerol on Celite (100–200 mesh) attached to a $3\frac{1}{2}$-ft length containing 10 % polyethylene glycol on Celite. Samples were injected via a silicone rubber septum at the top of the pre-column. The column was operated at an argon pressure of 5 lb/in^2 giving a flow rate of 30 ml/min.

EXPERIMENTAL AND RESULTS

Initial attempts to isolate the volatile substances from milk employed conventional distillation and extraction techniques. The results were useful only in that they emphasized the dangers of contamination from solvents, lubricated joints in apparatus, and large glass surfaces. Also, the strong adsorptive capacity of glass surfaces for milk odours was very apparent and suggested that the surface area of glass apparatus should be kept at a minimum.

If the concentration of volatile components in the equilibrium vapour over milk was sufficiently high to permit direct injection of vapour samples on to a reasonably sensitive chromatograph, many of these difficulties would be avoided. However, in our hands such samples gave very weak chromatograms, even when the instrument was run at its highest sensitivity. Consequently a rapid method of concentrating the volatile components of milk was sought, which required only simple equipment with a minimum of joints and a small area of glass surface.

Removal of the volatile components of milk by gas entrainment seemed likely to be the simplest method and at the same time to cause minimum change in composition, but its successful use was dependent on a sufficiently pure gas supply and a simple efficient method of trapping the entrained vapours. Since liquid nitrogen was used as a cooling agent for the trap, it was convenient to use nitrogen as the entraining gas (argon is solid at the temperature of liquid nitrogen). Its purity was found to be satisfactory after it had passed through a 30×3 cm column packed with Linde Molecular Sieve 5A (British Drug Houses Ltd., England).

When the design of a suitable trap was considered two conflicting requirements were apparent. The trap had to be of low total volume, otherwise the vapour sample presented to the column would be undesirably large. On the other hand, a trap of small bore tubing was quickly blocked by ice. A combination of wide and narrow bore tubing seemed therefore essential, the trap being arranged so that most of the water from the gas stream was condensed in the wide part of the trap. Nevertheless, if a trap based on these ideas were of the conventional single or multiple U construction and if, after trapping and warming, the sample of volatile substances was blown directly on to the column (Swoboda, 1962), there was danger of transferring liquid water. One solution was to warm the closed trap to a suitable temperature and transfer a vapour sample to the chromatograph by syringe (Wong & Patton, 1962), but this technique seemed cumbersome and unsuited to automation.

An alternative method was devised which used a syringe barrel as the trap itself. For this purpose a 1-ml Agla syringe barrel fitted with a glass capillary tip was found to be suitable. It was connected to an apparatus for entraining the volatile

components of milk, as shown in Fig. 1. All the flexible joints in the apparatus were made with silicone rubber to diminish the danger of contamination.

The following procedure was used to obtain a sample of the volatile components from milk. The sintered glass plate was first moistened with water and the nitrogen pressure adjusted to a predetermined value, just insufficient to overcome the resistance of the plate to gas flow. A 2 ml milk sample pipetted on to the plate now

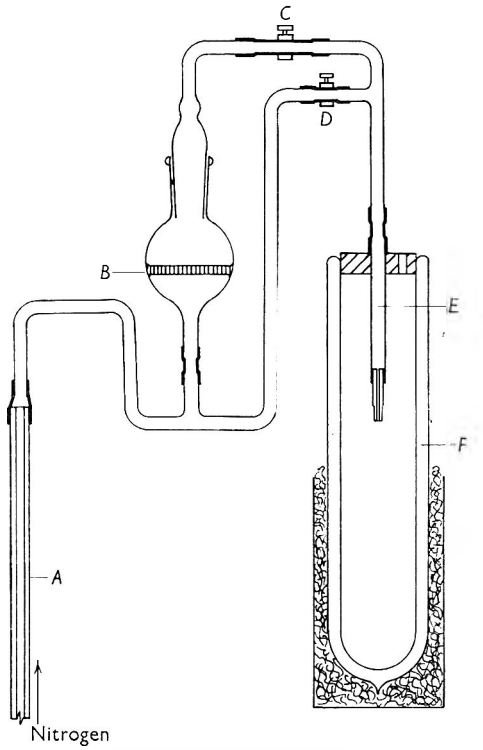

Fig. 1. Apparatus used to trap the volatile components of milk. *A*, Rotameter; *B*, sintered glass disk; *C*, and *D*, screw clips; *E*, Agla syringe barrel; *F*, Dewar flask.

stayed on the surface with minimum tendency to run down into the pores of the sintered glass. It formed a layer about 2 mm thick. Clip *C* was now closed and clip *D* opened so that the syringe barrel could be purged with gas as its tip was lowered to the required level below the surface of liquid nitrogen contained in the Dewar flask, *F*. Clip *D* was then closed and clip *C* opened, and the nitrogen pressure increased to overcome the resistance of the sintered plate and to establish a flow rate through the milk of 80 ml/min. After a suitable period of bubbling (1–10 min) the gas flow was stopped, the syringe barrel disconnected and the barrel plunger inserted into the barrel as far as possible; this position was usually just short of the ice line in the barrel. A hypodermic needle with the point embedded in a small piece of silicone rubber was now held ready, the syringe was rapidly removed from the cooling agent, and the needle with one end sealed was fitted to the barrel tip. The temperature of the assembly was rapidly raised to room temperature (an increase in volume of about 50 % occurs) and the vapour sample (about 1 ml) injected on to the

chromatograph by piercing in one movement the silicone rubber which sealed the needle and the silicone rubber septum at the injection port of the column.

The efficiency of the system for trapping volatile substances was first studied by fitting an injection port in the line connecting the milk vessel and syringe barrel, and injecting a 0·5 ml sample of acetone vapour diluted in nitrogen (about 1 μg acetone). After passing gas at 80 ml/min for 2 min the acetone condensed in the syringe barrel was then injected on to the chromatograph, using the technique described above, and the integrated peak was compared with that obtained by direct injection of an identical sample of the same vapour mixture. During these trials the relative position of the syringe barrel, the Dewar flask and the liquid nitrogen level was found to be of some importance. The best results were obtained when the maximum length of barrel was below the rubber bung of the Dewar flask and when the tip of the barrel was about 1 in below the surface of the liquid nitrogen (Fig. 1). Also the gas flow rate had an important influence, a maximum recovery being achieved in the range 80–120 ml/min. Under optimal conditions the recovery of acetone was $57 \pm 6\%$ in 8 determinations based on a mean value derived from 8 controls in which acetone vapour was injected directly on to the chromatograph. A recovery of about 60% was repeatedly obtained in numerous isolated checks. The accuracy with which 0·4 ml of vapour containing acetone could be injected on to the column using the Agla syringe was not better than $\pm 6\%$, so that variations in the recovery figure were attributed mainly to inaccuracies at the injection stage. This was confirmed by tests which showed that no detectable losses occurred during freezing and thawing of the syringe barrel.

In order to assess the degree of concentration effected from aqueous solutions an acetone solution in water was prepared, whose equilibrium vapour contained sufficient acetone to give a measurable chromatogram when injected directly on to the chromatograph. This was then compared with the result obtained by bubbling a 2 ml sample under the optimum conditions described. From the results of such experiments it was deduced that about a 50-fold concentration was achieved.

That significant concentrations were being obtained was further confirmed when the technique was applied to milk; chromatograms of milk, which were very superior to those obtained by direct injection of the vapour in equilibrium with milk, were consistently produced.

The sensitivity of the method for following the natural souring of milk was tested by incubating portions of raw milk at 22 °C for different periods, and preparing chromatograms at the end of the incubation time. Chromatograms of one such series obtained with the separating column at 65 °C are presented in Fig. 2. The most striking feature was the progressive increase in peak 4. A change in the size of this peak was apparent in the 26 h sample when no change in pH was recorded. It later became clear that the component represented by this peak was, in fact, mainly produced by the growth of coliform bacteria which also contributed to peaks 1 and 2.

Since the early peaks were incompletely separated when the chromatogram was run at 65 °C, the temperature of the column was lowered to 45 °C to increase the resolution. Also, to facilitate interpretation of the results, the souring of milk was brought about by inoculating sterile milk with a pure culture of *Str. lactis*. For this purpose milk was steam-sterilized in screw-capped 4-oz bottles. This procedure

increased the volatile content of the milk and the chromatogram was consequently undesirably large even before growth of *Str. lactis* had begun (Fig. 3). During growth of the culture chromatograms were prepared at about hourly intervals and a selection of them are reproduced in Fig. 3. All chromatograms contained at least 5 easily recognized peaks which have been designated A, C, D, E, F, in order of their emergence from the column. However, in chromatograms prepared during the later

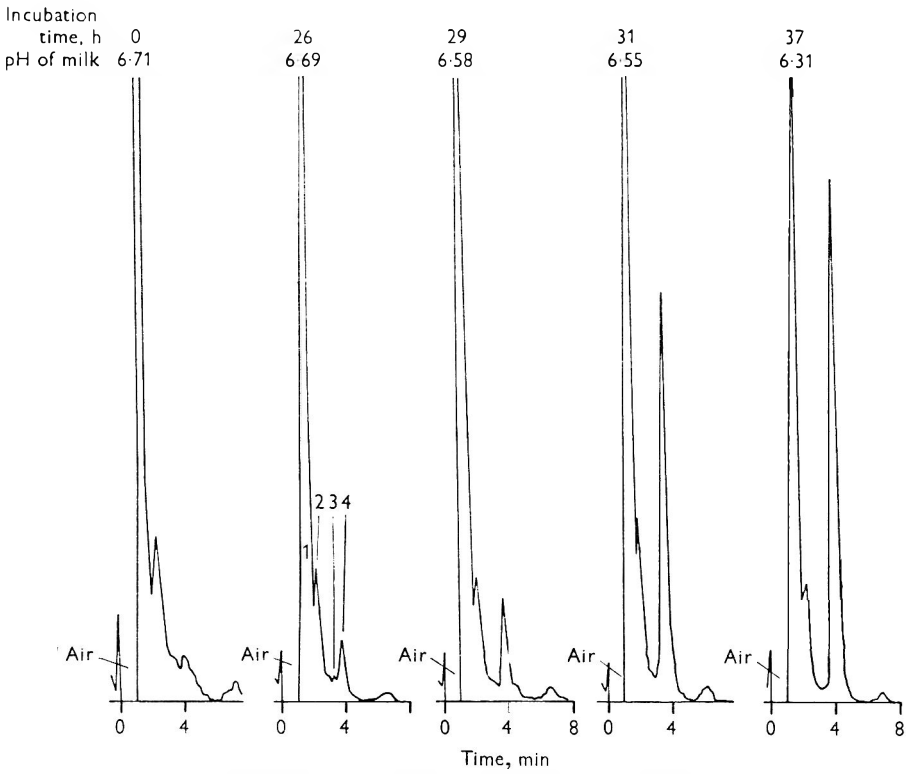

Fig. 2. Gas chromatograms of raw milk held for different times at 22 °C. Column, 10 % polyethylene glycol on 80–100 mesh Celite with a pre-column of 20 % diglycerol on 80–100 mesh Celite; temperature of column, 65 °C.

stages of incubation, an inflexion was observed in the first peak which, although it has been lost in the reproduction of Fig. 3, was clearly visible in the original trace. Thus the first peak contained at least 2 components, called A and B.

The increase in size of the first peak as incubation continued appeared to be due mainly to an increase in peak B. Because of the poor resolution the area of this peak could not be estimated and so, in order to obtain a quantitative measurement of the changes in the chromatogram, the areas of peak (A + B) and peak E were measured and their ratio calculated. The value peak (A + B)/peak E increased rapidly from 0·25 to 1·40 as the pH of the milk decreased and could be easily detected (ratio = 0·62) when a fall of 0·15 pH units was noted at the first sampling time (19 h).

In an experiment to confirm these results milk was steam-sterilized in 4-oz bottles fitted with cotton plugs which, after sterilization, were replaced aseptically with sterile screw caps. The volatile content of the milk after this treatment was found to

be very low and changes in chromatograms due to growth of bacteria were more easily detected (Fig. 4). After inoculation and incubation of the milk a small increase in peak B was observed even before a pH change could be detected ($17\frac{1}{2}$ h). This increase became pronounced when the pH of the culture had fallen 0·1 unit

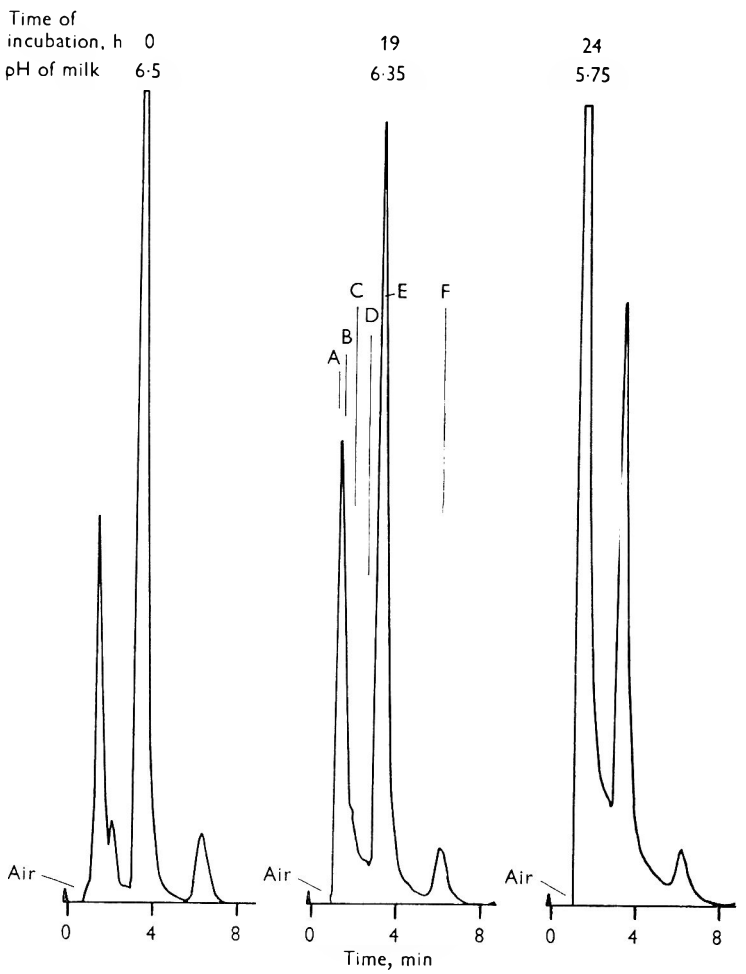

Fig. 3. Gas chromatograms of samples of pure cultures of *Str. lactis* in milk after incubation for different times at 22 °C. Column, 10 % polyethylene glycol on 80–100 mesh Celite with precolumn, 20 % diglycerol on 80–100 mesh Celite; temperature, 45 °C; argon pressure 5 lb/in²; flow rate, 30 ml/min.

and continued with growth of the culture. As in the previous experiment, the growth of the culture was also accompanied by a relative decrease in peak E, so that a rapid increase in the ratio peak B/peak E was observed (Fig. 5). In addition, however, an increase in peak F occurred in this experiment. The total volatile content of the culture, as measured by the total area under the curve of the chromatogram, also generally increased as souring progressed, the rate of increase being most rapid when the pH of the culture was falling quickly, i.e. when bacterial activity was at its height (Fig. 5).

A fall in total volatile content was noted from the 22 to 24-h sample when the culture was clotted, but no explanation for this is available.

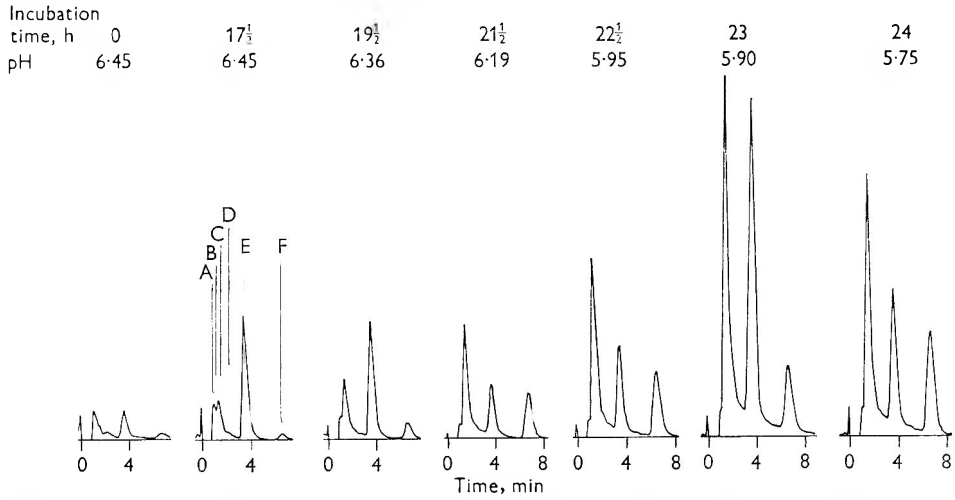

Fig. 4. Gas chromatograms of samples of milk inoculated with *Str. lactis* and incubated for various times at 22 °C. Column conditions as described under Fig. 3.

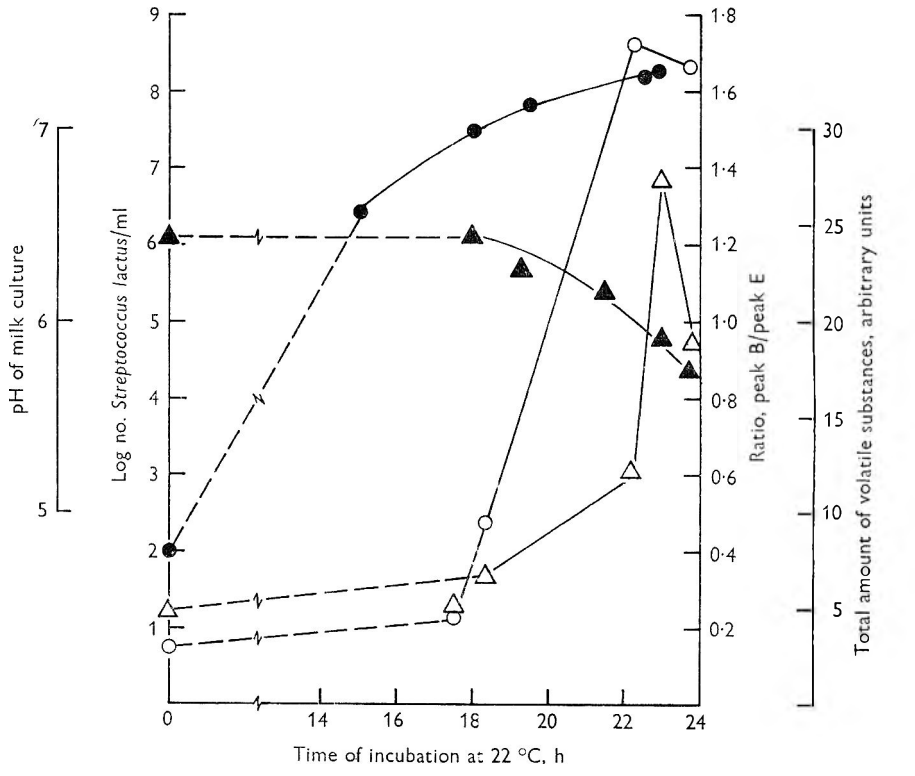

Fig. 5. Changes in volatile components, pH and bacterial count of milk during growth of *Str. lactis*. △, total volatile content; ○, Ratio of chromatogram peaks A and E; ▲, pH; ●, number of bacteria.

DISCUSSION

The recovery figure of about 60 %, obtained when using the syringe barrel for trapping about 1 μg of acetone, is not unexpected considering the amount of material involved for, although the area of glass surface was kept as small as possible, it was found that about 10 % of the volatile substances would be lost by adsorption. Entrainment of solid aggregates of condensable material which are blown out of the cold syringe barrel will contribute to the remaining loss. Modifications in the design of the syringe, for example an increase in length of the capillary tip, might minimize this effect.

The decision to use 2 ml samples of milk was determined by the diameter chosen for the sintered glass plate. The layer of milk which resulted was just sufficient to allow efficient bubbling at the gas flow rate used. Since the volatile material was present in excess of requirements (2 ml contained sufficient volatile material for about 5 chromatograms) divergences from the standard routine, e.g. alteration of time bubbling or of gas flow rate, or unavoidable changes in conditions which effect the efficiency of bubbling, such as a partial blocking of the sintered glass plate, would have a corresponding effect on the total quantity of volatile components trapped in the syringe. These factors may contribute to the occasional unexpected size of peaks obtained (e.g. Fig. 4, 24 h). For this reason the use of a smaller quantity of milk and the removal of a greater proportion of its volatile content would be better. An apparatus smaller in size but similar in design to the one described has been used. Preliminary trials have indicated that 0·2 ml of milk would give satisfactory results.

The concentration method described requires approximately 3 min between sampling the milk and injecting the sample of volatile components on to the chromatogram column. Using high-speed chromatographic methods, and bearing in mind that the compounds involved are of high volatility, it is reasonable to assume that the chromatogram would take only a few seconds to develop. Thus the overall time for analysis is potentially of the order of 3 min per sample. This is still too slow for use as a practical test for milk flavour quality on the dairy platform, where churns may be handled at rates of 1 per 30 sec or less. To achieve a similar speed in analysis it is clear that the concentration step must be eliminated. This could be done by applying a sample of vapour which has been equilibrated with the milk in which case a very sensitive method of detection (Jennings *et al.* 1962), and a very rapid method of preparing a vapour sample, would be required. Alternatively, since about 0·1 ml milk contains sufficient volatile material for one chromatogram, a relatively small improvement in the sensitivity of detection would allow the use of such a small sample that direct injection of liquid milk on to the column would be possible, if a flame ionization detector which is relatively insensitive to water vapour were employed. The resultant elimination of the concentration step would be of particular importance in removing variations in recovery of the volatile components, and in decreasing the analysis time per sample.

No attempt has been made to identify the compounds from milk represented by the peaks of the chromatograms. Of the fermentation products of *Streptococcus lactis* about 95 % (w/w) is lactic acid, which is insufficiently volatile to appear in quantity in the trapped sample, and about 3·5 % (w/w) is made up of acetic and

formic acids which would be lost on the chromatograph column because of adsorption. Thus only about 1% (w/w) of the total fermentation products are available for detection. They include ethanol, diacetyl, acetoin, 2, 3-butanediol (Platt & Forster, 1958), acetone and acetaldehyde (Harvey, 1960). Peak B whose increase was most marked during growth of the culture could not, by its position, be any of these substances. Peak F, however, which also increased during souring, had a position identical with butanone and peak E with acetone.

The quantitative changes in chromatogram as measured by the ratio of peaks B and E showed a close relationship with the changes in pH, as would be expected if the substance represented by peak B were a metabolic product of the culture. In the last experiment the number of bacteria had reached $2{\cdot}9 \times 10^7$/ml before detectable chromatogram changes were observed. For heterofermentative bacteria, Punch, Olson & Thomas (1961) reported that depending on species 2×10^6–2×10^8 organisms/ml of milk are required before definite off-flavours can be detected organoleptically. Since these bacteria would be expected to produce higher concentrations of volatile substances than the homofermentative *Str. lactis*, it is probable that the gas chromatographic method will be even more sensitive for detecting off-flavours due to bacteria other than *Str. lactis*. That it is capable of detecting a product of the growth of coliform bacteria has already been shown.

Although, unlike the pH test, the chromatographic method is independent of the variable buffering power of different milks, it is possible that the varying flavour levels of different milks may complicate the interpretation of chromatograms. It may also prevent the use of a measurement of total volatile substance as an indicator of flavour quality. Nevertheless, it is clear (Fig. 4) that such an indicator gives a good measure of bacterial growth in any one milk, at least when the flavour level of the fresh milk is low.

The examination of a large number of milk samples by the gas chromatographic techniques is required to settle these points. It would be desirable also to try analyses on columns suitable for the separation of acidic and especially basic substances since the latter are common products of undesirable bacteria.

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The oxidative stability of butterfat extracted from Cheddar cheese

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SUMMARY. Cheeses collected monthly for 6 months from 6 factories in the same district were stored for a period of 12 months. At intervals the cheeses were sampled and the fats extracted for oxidation tests. There was no increase in peroxide value, no decrease in induction period and only a slight increase in free fat acidity during storage of the cheeses.

Cheddar cheese, when placed on the retail market within 7–8 months of manufacture, is generally of good quality and free from major faults. Cheese stored for longer periods, however, occasionally develops the objectionable defect known as tallowy discoloration. This is due primarily to entry of air through slits which extend to the rind of the cheese. Oxidation occurs in the fat on both sides of the slit, with consequent bleaching of the carotene and production of tallowy flavours (Riddet, Whitehead, Robertson & Harkness, 1961).

It has been shown recently that in the presence of oxygen all cheddar cheese is liable to oxidation during long storage (Robertson, 1959). However, accelerating factors due to bacterial action, such as the formation of slits or, possibly, the production of a fat-oxidizing enzyme system could predispose the cheese to oxidation (Riddet *et al.* 1961). The intrinsic tendency of the cheese fat to oxidize may also be a contributing factor. The resistance to oxidation, which will depend on the composition of the fat and on the composition and content of natural antioxidants in it, could alter during the ripening of the cheese. Conceivably also it could vary with such factors as breed of cow, type of pasture, and season of the year.

Work in this Institute, with a modified Schaal oven test for fat stability, had indicated that there were variations in the resistance to oxidation of fat from cheese from factories in the same district, and that the least stable fats were from factories with the greatest incidence of discoloration in the stored cheese (Robertson, 1957). The present paper describes a detailed examination of the oxidative resistance of the fat from cheese made at different factories and stored for 12 months.

MATERIALS AND METHODS

Source of the cheese samples

One 80 lb export cheese was forwarded to the Institute each month for 6 months (October 1957–March 1958) from each of 6 factories in the Wairarapa district of the

North Island of New Zealand. The cheeses, all of which were approximately 14 days old on arrival at the Institute, were placed in the Institute curing-room and stored for 12 months at 45 °F.

Sampling

Each cheese was sampled at 14 days and at 2, 4, 6, 8, 10 and 12 months after the date of manufacture. The samples were obtained with a cheese trier and the top $\frac{3}{8}$ in of each plug of cheese was discarded.

Extraction of fat

A 60-g sample of cheese was passed through a small grater. The grated cheese was heated in a 100-ml centrifuge tube in a water-bath at 50–55 °C for 20–30 min and then centrifuged. The fat was recovered by decantation and filtered at 50 °C.

Oxidation and acid value tests

(*a*) *Peroxide value.* The peroxide value of the freshly filtered fat was estimated by the method of Loftus Hills & Thiel (1946) using a Bausch and Lomb 'Spectronic 20' colorimeter to measure the intensity of colour at 515 mμ. All peroxide values were recorded in m-equiv. of oxygen/kg of fat.

(*b*) *Induction period.* The induction periods of the cheese fats were estimated by the A.O.M. test. The procedure recommended by the American Oil Chemists' Society (Newby, 1957) was followed except that the amount of fat was reduced from 20 to 5 ml with a corresponding reduction in the flow of air to each tube. The induction period was reckoned as the number of hours for the fat to lose almost completely its yellow colour. This end-point corresponded to an average peroxide value (estimated by the Wheeler test) of 20 and, as was found by Stebnitz & Sommer (1937) for butterfat, it marked the beginning of a very rapid increase in peroxide value.

(*c*) *Free fat acidity.* Filtered fat (0·25 g) was dissolved in 5 ml of a 1 : 1 mixture of light petroleum and rectified spirits. The solution was titrated to phenolphthalein with 0·025 N-alcoholic potash. Results were recorded as free fat acidity, i.e. as ml of N-alkali/kg of fat. Since this estimation was not commenced until late in the investigation there were no results for fat acidity in the cheeses at 2 weeks and few results at 2 and 4 months after manufacture.

RESULTS

Fats from cheese at 14 days after manufacture

The peroxide values were low and the induction periods were high (Table 1), and both were of the same order as for butterfat from fresh butter. The tests indicated that there had not been more than slight oxidation of the fat either during manufacture of the cheese, or during the period of storage in the factory curing-room.

Effect of month of manufacture. Mean monthly values for the induction periods of the fats from the cheese at 14 days after manufacture (Table 1) were similar, with the exception of that for February cheese which was significantly higher ($P < 0{\cdot}05$), than for the other months. Since the cheeses made during this month were inadvertently exposed to a higher temperature during transit from the factories to the

Institute than were the remainder of the cheeses, it seems likely that the difference is attributable to the effect of the resultant higher biological activity in the cheeses rather than to seasonal difference in the properties of the butterfat.

Effect of factory of origin. The mean peroxide values of the fats from the 14-day-old cheeses from the separate factories were uniformly low (Table 2), but the induction periods of the fats from factories E and F were slightly lower than those of the fats from factories A, B, C and D. The difference in induction period for factory F was statistically significant ($P < 0{\cdot}05$).

Table 1. *Mean and range of results at 2 weeks after manufacture for peroxide value and induction period of the fats from all cheeses made in the same month*

Month of manufacture	Peroxide value, m.equiv. O_2/kg		Induction period, h	
	Mean	Range of results	Mean	Range of results
October	0·13	0·10–0·17	17·5	15·3–18·5
November	0·11	0·08–0·15	18·7	16·7–20·7
December	0·14	0·11–0·20	17·8	15·6–18·9
January	0·12	0·06–0·17	17·4	15·1–19·3
February	0·12	0·07–0·18	21·4	20·0–22·0
March	0·10	0·08–0·13	18·1	17·0–19·4
Standard error of mean	—	—	±0·4	—
Least sig. diff. at 5% level	—	—	1·2	—
1% level	—	—	1·6	—

Fats from cheese after storage

The mean results for the peroxide values and induction periods of the fats from cheeses from each of the factories after 4, 8 and 12 months' storage showed only slight changes (Table 2), indicating that oxidation of the fat in the cheeses did not occur during the 12-month experimental period. There was actually a slight rise in the induction period of the fat with increase in period of storage. This could have been due to carry over of traces of antioxidant in the form of protein decomposition products during extraction of the fat for analysis. The quantity of these products could be expected to increase as the cheeses ripened.

The free fat acidity of fat from cheese at 14 days after manufacture is usually within the range 0·8–1·0. In the present investigation, results for a small number of cheeses at 2, 4, and 6 months after manufacture, together with the results for all cheeses after 8 and 12 months (Table 2), indicate that there was a fairly regular rise in acidity of the fat as the cheeses matured. The changes in free fat acidity were similar for the cheeses from different factories. The values are in close agreement with those reported by Babel & Hammer (1945) and Sheuring & Tuckey (1947) for fat from Cheddar cheese made from pasteurized milk and at the same stage of ripening.

Table 2. *Mean results* at 2 weeks and at 4, 8 and 12 months after manufacture for peroxide value, free fat acidity and induction period of the fats from all cheeses manufactured at the same factory*

	Peroxide value, m-equiv. O_2/kg				Free fat acidity, ml N acid/kg		Induction period, h			
Factory	After 2 weeks	After 4 months	After 8 months	After 12 months	After 8 months	After 12 months	After 2 months	After 4 months	After 8 months	After 12 months
A	0·13	0·15	0·09	0·11	1·7	2·0	18·5	19·5	19·9	20·4
B	0·14	0·11	0·10	0·11	1·5	1·8	19·2	20·0	20·5	20·1
C	0·09	0·12	0·12	0·09	1·9	2·2	18·8	20·0	20·2	21·1
D	0·12	0·11	0·12	0·11	1·6	1·9	19·3	19·8	19·8	20·3
E	0·15	0·12	0·13	0·10	1·8	2·1	18·0	18·3	18·2	18·4
F	0·14	0·14	0·08	0·11	1·8	2·0	17·0	17·9	18·1	18·3
Standard error of mean	—	—	—	—	—	—	±0·4	—	—	±0·6
Least sig. diff. at										
5 % level	—	—	—	—	—	—	1·2	—	—	1·7
1 % level	—	—	—	—	—	—	1·6	—	—	2·4
All factories	0·12	0·12	0·11	0·11	1·7	2·0	18·5	19·3	19·5	19·8

* Mean results after 2, 6 and 10 months of storage are not included since they differ only slightly from those after 4, 8 and 12 months of storage.

DISCUSSION

The absence of any evidence of oxidation of fat in the experimental cheese is in agreement with the generally accepted theory that, owing to the presence of large numbers of bacteria with their demand for oxygen, oxidation of the fat does not take place in cheese.

The differences between the average induction periods of fats from cheeses manufactured at different factories were small in comparison with the duration of the induction period itself. Thus it may be concluded that although there were slight variations in the susceptibility of the fat to oxidation they were too small to provide an explanation for variations in the tendency of cheeses to show tallowy discoloration.

The author wishes to thank Dr F. H. McDowall, Chief Chemist of this Institute, for advice and criticism; Mr A. C. Glenday, Applied Mathematics Laboratory, Department of Scientific and Industrial Research, for advice on the statistical analysis; and the managers of the 6 factories for their co-operation in supplying the cheeses.

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The effect of preparative conditions on the composition of the κ-casein complex

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Summary. The effects of changes in the conditions of preparation on the composition of the κ-casein complex have been studied and a method of preparation is suggested which embodies the optimal conditions. The varied conditions include temperature of precipitation of the acid casein, the pH, temperature, $CaCl_2$ concentration and duration of the $CaCl_2$ treatment, and the conditions of centrifuging. Changes in composition caused by alcohol fractionation are reported and the results are discussed from the point of view of κ-casein as a complex of three or more proteins.

In an earlier paper (Hill, 1963) a method was given for the chromatographic separation of β-casein from crude κ-casein in conditions which caused little alteration of the composition of the κ-casein complex itself. In that paper, it was pointed out that little was known of the effects of different conditions of preparation on this composition, because the nature of κ-casein as a complex, and not a single protein, had not been previously appreciated.

The separation of the κ-casein from the α-κ complex is commonly achieved by the addition of $CaCl_2$ until its concentration is about 0·25 molar (Waugh & Von Hippel, 1956). However, somewhat different conditions of temperature and $CaCl_2$ concentration have been favoured by different workers, and a study of the effect of these and other preparative conditions on the yield and composition of the κ-casein appears necessary.

The probable composition of the κ-casein complex has been discussed by Beeby (1963), and his concepts have formed the basis for comparing the compositions of the κ-caseins produced by various treatments. Rennin releases from κ-casein a sialic-acid-containing glycopeptide which is similar to that released from whole casein (Nitschmann & Beeby, 1960). Since the action of rennin on whole casein releases all the sialic-acid-containing material (Hill, unpublished) it is probable that all the sialic acid of whole casein is located in the κ-casein fraction. Particular attention has therefore been given to ensuring that as much as possible of the sialic acid originally present in the whole casein is retained in the κ-casein.

Materials and Methods

Caseins were prepared from fresh raw milk which, like the protein solutions, was preserved by thymol during storage. The water was deionized by an Elgastat column, or was distilled from glass. The diethylaminoethyl cellulose used for chromatography was Whatman DE.50, and fractions were collected on an LKB fraction collector having a UV absorptiometer which recorded transmission at 253 mμ. Crude κ-caseins were purified by the method of Hill (1963). The Sephadex used for gel filtration was medium grade G 25 supplied by Paramacia, Uppsala, Sweden, and the technique used was that of Porath & Flodin (1959).

Nitrogen was determined by semi-micro Kjeldahl distillation, and sialic acid by the method of Warren (1959). Cystine was estimated by the method of Allison & Cecil (1958), with phenyl-mercury acetate as the titrant. In converting weights of nitrogen to protein weights, the factors used were 6·7 for κ-casein and 6·4 for whole casein.

Starch gel electrophoresis was performed in the manner suggested by Wake & Baldwin (1961). All chemicals were of reagent grade.

RESULTS

The preparation of acid casein

Casein micelles freed of serum proteins and whey have a sialic acid content usually in the range 0·5–0·6 % (Hill, unpublished), but during acid precipitation this content is reduced to about 0·4 % (Table 1). Since this loss might be temperature dependent, a comparison of the sialic acid content of acid casein precipitated at two different temperatures was made. For one preparation, skim-milk was cooled to 3 °C, the pH adjusted to 4·7 with N-HCl, and the sample slowly warmed until precipitation occurred. The precipitate was washed, macerated in water and redissolved with N-NaOH to a pH ≯ 7·5. It was then reprecipitated and redissolved as described above. Another portion of the skim-milk was treated similarly, except that the acid was added at 30 °C on each occasion. The sialic acid content of the casein prepared by the addition of acid at 30 °C (0·46 %) was not significantly different from that of the casein prepared by acidification at 3 °C (0·43 %). The simpler method at 30 °C was therefore used to prepare the sodium caseinates for the tests described in this paper.

The preparation of crude κ-caseins

In preparing crude κ-casein the conditions that may be varied include pH, temperature, concentration of Ca^{++} and of other ions, the duration of treatment and the conditions of centrifuging. It is evident that a complete study based on a statistically planned variation of all these factors at a number of levels could not be attempted. The plan adopted was to change one factor at a time in order to find a set of optimum conditions for the recovery of sialic acid in the κ-casein and then to make a limited survey of the effect of relatively small changes in the factors near the combined optimum. The results in Table 1 do not represent all the tests, but sufficient have been given to show the relevant effects. The κ-caseins in this table have sialic acid contents in the range 0·98–2·08 %. The lower sialic acid contents could be caused

either by loss of the sialic acid-containing component in the course of preparation, or by contamination with α- or β-caseins. In either case the preparative treatment would be considered unsatisfactory.

Table 1. *Effect of preparative treatment of κ-casein on its sialic acid and cystine contents*

	$CaCl_2$ treatment				Centrifuge			Na caseinate			Chromatographed κ-casein	
								Crude κ-casein	S–S g protein/			S–S g protein/
mp. °C	pH	$CaCl_2$ M	NaCl M	Time h	10^{-3} g	Time h	Temp. °C	NANA* %	mole cystine	NANA %	NANA %	mole cystine
3	7	0·25	—	1	35	½	30	0·40	—	1·39	—	—
30	7	0·25	—	1	35	½	30	0·40	—	1·48	—	—
3	7	0·25	—	1	35	½	30	0·42	—	1·15	—	—
30	7	0·25	—	1	35	½	30	0·42	—	1·41	—	—
30	6–7	0·25	—	1	35	½	30	0·42	—	1·43	—	—
30	6–7	0·08	0·40	1	35	½	30	0·42	—	1·30	—	—
30	6	0·08	—	1	35	½	30	0·42	—	0·91	—	—
30	6	0·17	—	1	35	½	30	0·42	—	1·06	—	—
30	6	0·25	—	1	35	½	30	0·42	—	0·98	—	—
30	8–7	0·25	—	1	35	½	30	0·42	—	1·06	—	—
30	7	0·08	—	1	35	½	30	0·42	—	1·12	—	—
30	7	0·17	—	1	35	½	30	0·42	—	1·29	—	—
30	7	0·25	—	1	35	½	30	0·42	—	1·35	—	—
37	7	0·08	—	1	35	½	30	0·42	—	1·13	—	—
37	7	0·17	—	1	35	½	30	0·42	—	1·45	—	—
37	7	0·25	—	1	35	½	30	0·42	—	1·38	—	—
37	7	0·33	—	1	35	½	30	0·42	—	1·42	—	—
2	7	0·2	—	½	60	1	30	0·42	—	1·22	—	—
2	7	0·2	—	½	35	½	30	0·42	—	1·45	1·7	37300
6	7	0·3	—	1	35	½	30	0·42	—	1·6	1·7	41500
2	7	0·2	—	1	35	½	35	0·42	—	1·6–1·7	1·88	38600‡
2	7	0·2	—	1	35	½	35	0·42	—	1·39	1·62	45100†
2	7	0·2	—	1	9	½	35	0·37	87400	1·33	1·48	38000
2	7	0·2	—	1	35	½	35	0·46	97000	—	2·08	36000
2	7	0·2	—	1	35	½	37	0·50	75200	—	1·92	40000
2	7	0·2	—	1	35	½	40	0·34	96000	—	1·95	48000
2	7	0·2	—	1	35	½	42	0·42	—	—	1·85	44000

* NANA, *N*-acetyl neuramic acid (sialic acid).
† $CaCl_2$ treatment done at 3% protein concentration.
‡ Estimated from relative peak areas on chromatogram.

Temperature of $CaCl_2$ treatment

The effect of changes of temperature of the $CaCl_2$ treatment is shown in Table 1. At constant $CaCl_2$ concentration (e.g. 0·25 M) the percentage of sialic acid in the crude κ-casein changed from 1·15 for a treatment at 3 °C to 1·6–1·7 for one at 42 °C (tests 3, 7, 16, 21). Test 21 was not strictly comparable with the others since the $CaCl_2$ concentration was different, but the difference was not sufficient to affect the conclusion (cf. tests 12, 13 and 15, 16). Tests 3–22 were all made on a single batch of casein (stored in the frozen state at − 10 °C during the series of tests) but a similar behaviour is evident in tests involving two different caseinates (tests 1–4). No increase in the yield of sialic acid in the crude κ-casein was obtained by increasing the temperature of treatment from 42 to 46 °C (tests 20, 21).

pH *of $CaCl_2$ treatment*

Tests 4–13, Table 1, show the effect of different conditions of pH at a number of $CaCl_2$ concentrations. In test 4 the pH was kept between 6·7 and 7 as $CaCl_2$ was added, whereas in tests 5 and 6 all the $CaCl_2$ was added without adjusting the pH which fell

from 7 initially to 5·9 after the $CaCl_2$ was added. The pH was then returned to 7 with N-NaOH. Comparing tests 4 and 5, it can be seen that no advantage was gained by the continuous adjustment of the pH. When the pH was left at 6, however (tests 7, 8, 9), the proportion of sialic acid in the crude κ-casein was lower than that of the κ-casein for which the pH was returned to 7 after addition of $CaCl_2$ (tests 11, 12, 13). When the pH of the sodium caseinate solution was initially adjusted to 8 so that after $CaCl_2$ was added the final pH was 7, there was likewise a decrease in sialic acid content (cf. tests 10, 4). The procedure used for tests 5 and 6 was therefore adopted as the standard one.

$CaCl_2$ *concentration*

As $CaCl_2$ was added to the sodium caseinate solution, the pH at first dropped sharply. This continued until the $CaCl_2$ concentration was about 0·05 M, at which stage the bulk of the binding capacity of the protein for Ca appeared to be satisfied. The minimum $CaCl_2$ concentration used in these tests was therefore fixed somewhat higher at 0·08 M. Tests 4, 5 and 11–17, Table 1, show that as the $CaCl_2$ concentration increased to 0·25 M at 30 °C and pH 7, there was a corresponding increase in the sialic acid content of the crude κ-casein to about 1·40 %, while at 37 °C this value was achieved within the range 0·17–0·33 M-$CaCl_2$. In tests at 37 °C and higher temperatures a $CaCl_2$ concentration of 0·20 M was selected for normal use. Comparison of tests 6 and 11 suggests that, in addition to the effect of $CaCl_2$ concentration, there was also an effect due to the addition of NaCl.

Duration of $CaCl_2$ *treatment*

At 42 °C a 1-h treatment with $CaCl_2$ at 0·2 M was needed to obtain an optimum yield of sialic acid in the crude κ-casein, treatment for $\frac{1}{2}$ h being insufficient (tests 19, 21). After 1 h the sialic acid content in the crude κ-casein represented 80–85 % of that originally present in the sodium caseinate. As the volume of the κ-casein solution recovered after centrifuging was only 85–90 % of that of the parent sodium caseinate solution it is evident that lengthier treatment could increase yield by only a few per cent.

Conditions of centrifuging

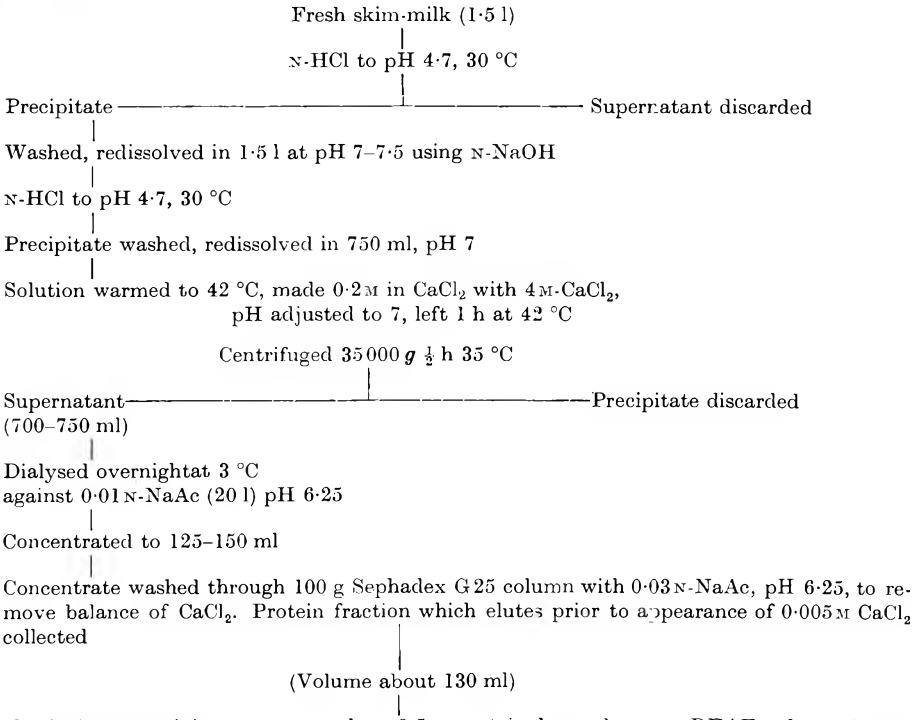
In centrifuging, it is necessary to achieve a compromise between sedimenting out finely divided β-casein, and avoiding selective loss of sialic acid-containing material if the centrifuging be excessive. As a result of a number of tests, $\frac{1}{2}$ h of centrifuging in the Servall SS 34 rotor at 35000 ***g*** and 35–40 °C was selected as the most suitable treatment. The effect of excessive or of insufficient centrifuge treatment can be seen by comparing the results of test 18 with 19 and 23 with 21. The $\frac{1}{2}$ h treatment at 35000 ***g*** need not be the most suitable, if the other experimental conditions be changed. For example, the concentration of the sodium caseinate solutions was usually 5–6 %; a change in the protein concentration with consequent change of viscosity of the solution would necessitate a different centrifuge treatment for best results (cf. tests 21, 22).

Sialic acid and cystine content of sodium caseinate

The last four κ-caseins listed in Table 1 were made from different milks, which yielded sodium caseinates of different sialic acid content. The preparative conditions were the optimum ones derived from the earlier experiments (test 21), and the κ-caseins were all purified chromatographically. The sialic acid contents of the purified κ-caseins were reasonably similar (tests 21, and 24–27). The variations which occurred did not bear any apparent relation to the variations in sialic acid content of the original sodium caseinates, except of course that the sialic acid content of the sodium caseinate fixed the maximum yield of κ-casein obtainable. Likewise no direct relation between the cystine content of the purified κ-caseins and that of the sodium caseinates could be found.

Scheme for preparation of κ-casein

The scheme below sets out briefly the conditions thought to be optimal for preparing κ-casein.



A volume of solution containing not more than 3·5 g protein layered on to DEAE column (50 g). Elution system as in Hill (1963) with increase in volume to suit larger column.

Purified κ-casein collected (500–600 ml), dialysed overnight with stirring against large excess 0·5 g/l, NaCl 3 °C.
Freeze dried. Yield 2·5–3 g.

The alcohol fractionation of κ-casein

A method currently in use for preparation of κ-casein (McKenzie & Wake, 1961) relies upon fractionation in 50 % ethanol for the purification of the crude κ-casein, and studies were therefore made to ascertain if this treatment caused any change in

the composition of the κ-casein complex. Table 2 gives the cystine and sialic acid co tents of the alcohol fractionated caseins, and the conditions of preparation. The fir preparation was made according to the method of McKenzie & Wake (1961) usi 50 % ethanol, but since the yield could be increased considerably by fractionation fro 60 % ethanol, this concentration was used in the subsequent preparations. The co positions of the precipitates in Table 2 are those resulting from one alcohol fractio ation only as this is sufficient to show clearly the resulting changes in compositio Excluding preparations 3 and 4, the alcohol fractionated κ-caseins showed sialic ac contents not greatly different from those of preparations 24–27 (Table 1), but t cystine contents were much higher. The cystine content of the chromatograph κ-caseins averaged about 1 mole/40000 g protein, but that of the alcohol fractionat ones averaged about 1 mole/24000 g.

Table 2. *Preparative treatments and compositions of alcohol fractionated κ-caseins*

		Composition of starting material		Treatment			Composition precipitate	
Prep. no.	Starting material	NANA, %	S–S g protein/ mole cystine	Alcohol, %	Temp., °C	pH	NANA, %	g mol
1	Crude κ-casein (McKenzie & Wake)	—	—	50	20–25	7·2	2·11	
2	Crude κ-casein	1·36	—	60	20–25	6·5	1·91	
3	Crude κ-casein freeze-dried	1·3	—	60	3	6·5	1·3	
4	Crude κ-casein freeze-dried	1·3	—	60	20–25	6·5	1·3	
5	Crude κ-casein freeze-dried	1·3	—	60	33	6·5	1·81	
6	κ-casein chromatographed	1·85	44000	60	30	6·5	1·92	
7	κ-casein chromatographed	1·64	41800	60	30	6·5	2·54	
8	Crude κ-casein	—	—	60	30	6·5	2·05	
9*	Crude κ-casein	—	—	60*	30	6·5	1·75	

* Preparation 9 was dissolved at pH 3 prior to addition of alcohol, and was precipitated at pH 6·5

The results in Table 2 also show that when freeze-dried κ-casein was used as t starting material, a higher temperature was required to make the alcohol fractio ation effective and that there was a temperature below which fractionation did n occur (preparations 3–5, Table 2).

Starch gel electrophoresis patterns of the two types of κ-casein are given in Plate These show that one of the components of the chromatographed κ-casein w absent, or nearly so, in the pattern of alcohol fractionated κ-casein. This compone of the chromatographed κ-casein has a mobility different from that of β-casein, is shown in Plate 1C in which the pattern of a mixture of α- and β-, and chromat graphed κ-caseins is given. The band in the pattern of the chromatographed casein appears just behind that of the β-casein, and just resolved from it. The patte of the alcohol fractionated κ-casein is similar to that given by Wake & Baldw

(1961). Finally, comparison of the pattern of the chromatographed κ-casein with that of sodium caseinate showed that the preparative treatments were effective in removing all but traces of α- and β-caseins.

DISCUSSION

The method of preparation of κ-casein given above was developed with the following aims:

(1) To make the preparative procedure as rapid as possible, without undue sacrifice of yield or purity.

(2) To obtain a product of defined composition containing as much as possible of sialic acid originally present in the whole casein.

The first requirement assumes a special importance because of the relative ease of degradation of the κ-casein. The method suggested can be completed up to the stage of freeze-drying in 2 days, and gives a yield of several grams of purified product from 1·5 l of milk. This is about 60 % of that theoretically possible.

The second requirement arises from the nature of κ-casein as a complex, in which the proportions of the constituents may be altered considerably by preparative treatments. Beeby (1963) has suggested from chemical and other evidence that κ-casein is a complex of three proteins, each of molecular weight of about 16000. One of these was postulated to contain all the sialic acid in κ-casein, another all the cystine and the third to contain neither cystine nor sialic acid. These suggestions are supported by the fact that the ratio of sialic acid to cystine may be changed considerably as a result of alcohol fractionation, although the actual sialic acid contents of chromatographed and alcohol fractionated κ-caseins are quite similar. Since the alcohol treatment does not break covalent bonds, this change of ratio implies that the sialic acid and the cystine are located in different components of the complex.

Accepting 16000 as the molecular weight of each of the components of the complex the results of Beeby (1963) show that one of these components should have a sialic acid content of about 5·5 %. As chromatographed κ-casein contains approximately only 1 mole of cystine/40000 g, the cystine content of the cystine containing component should be 1 mole/16000 g. In equal combination, these three postulated components would give a complex of molecular weight 48000 containing about 2 % of sialic acid and 1 mole of cystine per 48000 g. The composition of preparation 26, Table 1, is close to this idealized one and the compositions of the chromatographed κ-caseins suggest that they are composed of approximately equal proportions of the three components. On the other hand, the cystine and sialic acid contents of the alcohol fractionated κ-caseins suggest that they are complexes of approximately one sialic acid containing component and two units of the cystine containing fraction.

This interpretation is supported by the starch gel electrophoresis patterns in Plate 1, which show in the chromatographed κ-casein an additional component not present in the alcohol fractionated casein. It is further supported by the results of Dumas (1961) who showed that κ-casein prepared according to the method of McKenzie & Wake (1961) could be separated into two fractions on chromatography in urea, one being eluted only by NaOH and containing about 40 % of the original material. In the present work, chromatography in similar conditions was performed

and from analysis of the fractions for cystine and sialic acid, it could be inferred that the fraction eluted in the NaOH contained cystine, but little or no sialic acid. The cystine content was inferred by difference because the strong alkali required to elute this fraction destroyed the cystine. Attempts to elute this fraction with other reagents failed, so that direct proof of the nature of this fraction was not possible.

The difference in composition of the two types of κ-casein is reflected in difference in their behaviour; the alcohol fractionated κ-casein dissolves with more difficulty and is less effective in stabilizing α-casein in the presence of calcium (Neelin, Rose & Tessier, 1962). This suggests that the component containing neither sialic acid nor cystine is a necessary part of the κ-casein for its casein stabilizing role in milk.

The interpretations given above are based on a minimum of three components of the κ-casein complex. The actual situation may be more complicated. There may be more than three components, the components may have genetically controlled variants and the combining proportions need not be simple integral ones. In milk, the manner in which the components are combined may be altered in the presence of α- and β-caseins. In order to resolve these questions the preparation of pure κ-casein components and a study of their interactions is needed. Because of the strength of these interactions, the task of preparing pure components is proving to be a difficult one.

The authors wish to acknowledge with pleasure the assistance of Dr N. Snow in performing the starch gel electrophoresis.

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EXPLANATION OF PLATE

Plate 1

Starch gel electrophoresis patterns of caseins. A, Chromatographed κ-casein, Prep. 24, Table 1; B, mixture of (mainly) α- and β-caseins—alcohol fraction A of Hipp *et al.* (1952); C, mixture of A and B; D, alcohol fractionated κ-casein, Prep. 1, Table 2; E, sodium caseinate.

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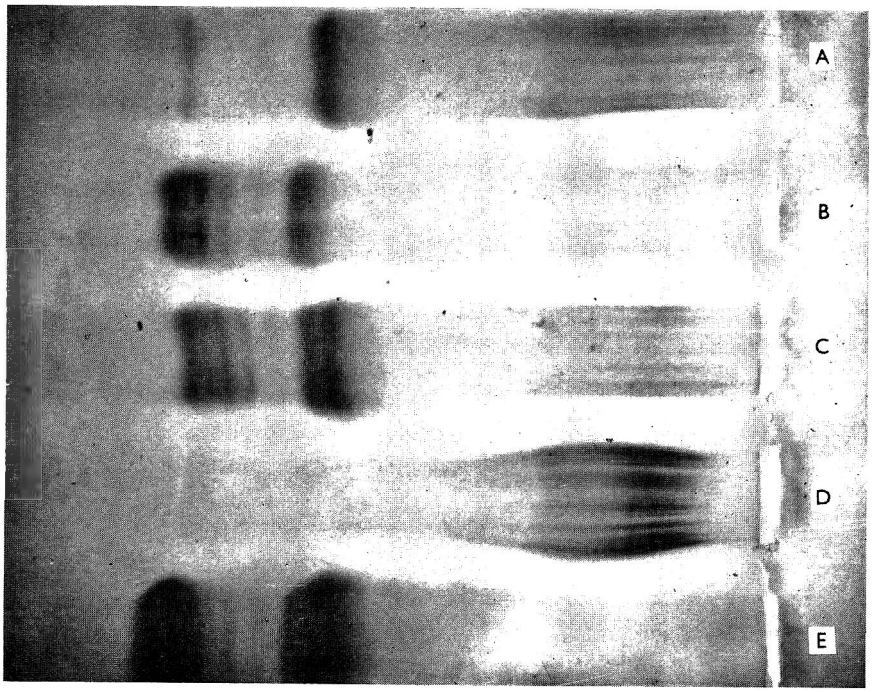
A
B
C
D
E

A simple method for detecting an early stage in coagulation of rennetted milk

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Summary. An apparatus is described which measures at 10–15 sec intervals the viscosity of rennetted milk taken from a cheese vat. The first signs of an increase in this viscosity mark what is at present the earliest measurable stage in the process of coagulation. Coagulation times so determined agreed fairly closely with the observations of a skilled cheese-maker and, for a given milk, were inversely proportional to rennet concentration over quite a wide range. Clotting times obtained by the method of Berridge (1952) for standardizing rennets came 2 or 3 min after those determined by the new method and were linearly related but not proportional to inverse rennet concentration.

Burnett & Scott Blair (1963) have recently described a torsiometer which, suspended in the milk in a cheese vat after rennetting, measures the increasing rigidity of the curd until it is time to cut. Alternatively, it will signal the cheese-maker as soon as a pre-selected firmness is reached. The instrument has the advantage that both the amount of strain and the rate of straining of the curd are kept constant during the test, and this is important because the rigidity modulus is not independent of these factors. An examination of many curves for normal setting curds and also, using a different method, for coagulating cow's blood, has shown that, for both systems, good straight lines are generally obtained when the logarithm of the increasing rigidity modulus is plotted against the reciprocal of the time which has elapsed since the time (t_c) when the first traces of coagulation appeared (see Scott Blair & Burnett, 1963, in which the significance of this plot is discussed).

The observation of the very earliest evidence of coagulation, which is essential for this method of plotting, cannot be made with the torsiometer and, in the case of milk, the time zero (t_c) was originally defined by the careful observations of a skilled cheese-maker. His procedure is to allow a drop of water to fall on to the surface of the milk and to look for a little spot or 'crater' which forms once coagulation has started. Although this change occurs some 5 min before the torsiometer shows any rigidity, the equation represented by the log-reciprocal plot fits the data best when a time is used for the zero about 50 sec later than the cheese-maker's t_c.

It seemed advantageous, however, to devise an objective method for measuring t_c which would not depend on the subjective judgement of a highly trained expert. First, a method was tried in which a little milk taken from the vat dripped through

a vertical glass capillary tube, the time taken (about 12 sec) for each drop to form being recorded. The onset of coagulation was marked by a slowing down of drop formation. The values of t_c obtained in this way agreed fairly well with those given by the cheese-maker but the 'end-point' was not sharp enough to be satisfactory. Moreover, the drop-time depends on changes in several physical properties (viscosity, surface tension, contact angles) which makes the process needlessly complicated.

The method to be described here measures only a viscosity and its variation, if any, with shear rate. The test is exceedingly simple and involves no expensive equipment; and the values of t_c which it gives agree closely with the cheese-maker's assessments, as well as generally providing a correct time zero for the log-reciprocal plot. It seems possible that, apart from its use in relation to the log-reciprocal equation, the method might well be of value in the cheese factory and in the laboratory for other purposes.

EXPERIMENTAL METHOD

The apparatus is shown in Fig. 1.

The glass tube A, about 40 cm long and 0·7 cm bore is surmounted by a funnel top. The tube is supported vertically, the lower end passing through a rubber bung into a collecting beaker. The bottom of the tube is connected by a rubber sleeve to a capillary C, 7 cm long, 0·04 cm bore, the lower end of which is suspended 1–2 mm above the bottom of the beaker. A second tube D keeps the pressure in the beaker at the atmospheric level. A scale is etched on, or attached to, A starting at a reading of 10 cm which marks this height above the level of the bottom of the capillary. The whole apparatus stands in a large measuring cylinder which is partly lagged (leaving a window for observing the scale and thermometer) and filled with water at the same temperature as that of the cheese vat. In our experiments, the temperature used was 30 °C, and this temperature did not drop appreciably during the short course of the experiment.

About 5 min before the time when the milk is expected to show the first signs of coagulation, 15 ml. are removed from the vat in a pipette and run into the funnel top of the apparatus. After a minute or two the milk will have run through the capillary to form a pool in the beaker into which the capillary dips, and the top of the column of milk will have appeared on the scale. During the next 5 or 6 min, scale readings (h) are taken every 10 or 15 sec using a stop-watch or metronome, and afterwards the logarithms of h are plotted against time (semi-log paper may be used but is slightly less accurate).

For a fluid of constant viscosity, the plot of log h against time would be linear. There are various factors which would tend to produce curvilinearity for rennetted milk. First, milk itself is not a quite true (Newtonian) fluid, but the effect of this deviation shows itself only at very low rates of shear and in this experiment readings were not taken at heads of less than 10 cm. Secondly, it is known that the first action of rennet is to cause a fall in the viscosity of milk (Scott Blair & Oosthuizen, 1961, 1962). This effect becomes just observable with the present apparatus if 2 % sodium caseinate is used instead of milk and quite marked with 6 % caseinate, but is not sufficient to show when rennetted milk is used.

A third source of curvilinearity would be failure to correct the head for the rise in

level of milk in the beaker, but in so short an experiment this effect is negligible. Finally, as soon as coagulation starts, there will of course be a genuine increase in viscosity, soon followed by rigidity.

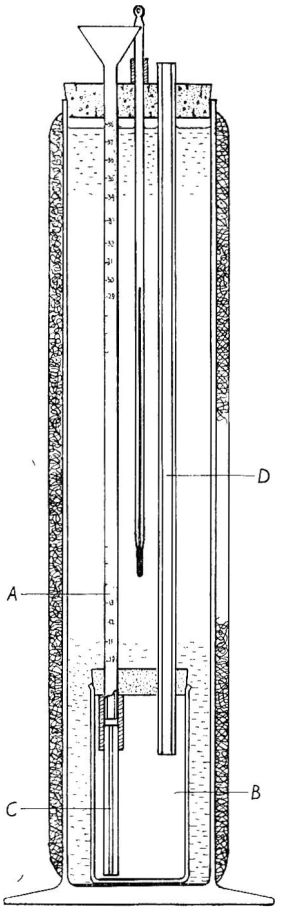

Fig. 1. Apparatus for detecting an early stage in coagulation of rennetted milk. *A*, Graduated glass tube; *B*, beaker; *C*, glass capillary tube; *D*, outlet to atmosphere.

EXPERIMENTAL RESULTS

Fig 2 shows three curves for rennetted milk and, for comparison, one for unrennetted milk. The upper parts of all these curves are remarkably linear and with rennetted milk it is not difficult to define t_c, the time when the points start to diverge from the straight line, to within about a quarter of a minute. Fig. 3 shows curves for 2 and 6 % sodium caseinate (solutions prepared as described by Scott Blair & Oosthuizen (1962)). Since the slope of these curves is an inverse measure of viscosity, it is clear that there is a general downward trend in the slope of the curves indicating a marked fall in viscosity for 6 % and a trace of a fall for 2 % sodium caseinate with of course no subsequent rise, since no calcium is present.

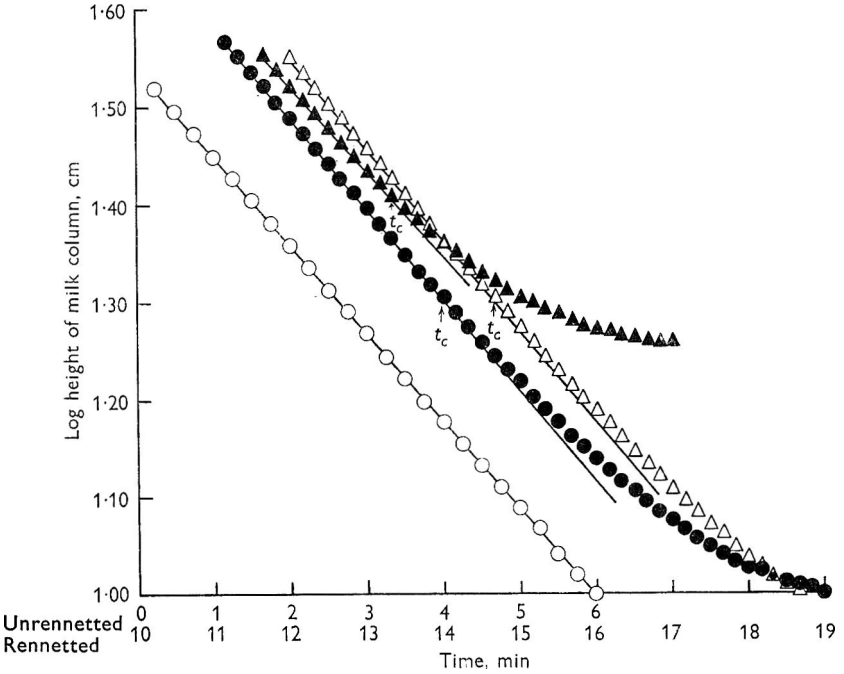

Fig. 2. Flow characteristics of rennetted and unrennetted milk as determined by the falling column method. ●, △, ▲, rennetted milk; ○, unrennetted milk; t_c, time at which first sign of coagulation can be detected.

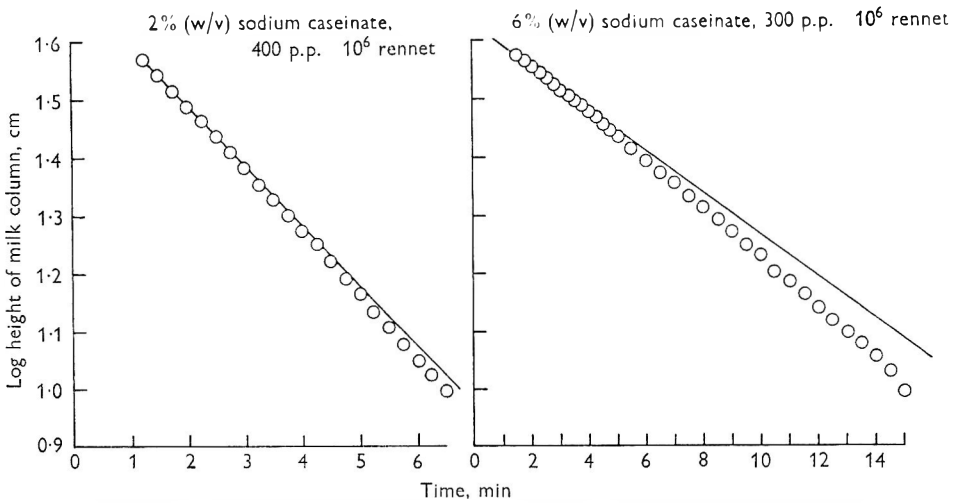

Fig. 3. Fall in viscosity of 2 and 6% sodium caseinate solutions after rennetting as determined by the falling column method.

A comparison of values of t_c derived by different methods

During the course of some weeks, observations were made on the coagulation of milk in small cheese vats. Three different 'coagulation times' were recorded: t_1, representing the first signs of coagulation observed by the cheese-maker using the

Table 1. *A comparison of coagulation times (min) of rennetted milk obtained by cheese-maker's observation (t_1), by falling column method (t_2), and from the best fit for the log-reciprocal plot (t_3)*

	February						March								April
Date	14	19	20	27	28(*a*)	28(*b*)	5(*a*)	5(*b*)	6	12	20	21	26	28	4
t_1	13¼	13	12¼	12¾	12¾	12¾	13	13¼	13	13	12¾	13¼	13	13	12¼
t_2	13¾	13½	14	13½	13¼	13¾	13¾	14	13¾	14½	13¾	13¾	14	14	13½
t_3	13¼	13½	14¼	13¾	14	14½	13¾	14½	13¾	14¾	14	14	14¼	14¼	13
t_2-t_1	½	½	1¾	¾	½	1	¾	¾	¾	1½	1	½	1	1	1¼
															av. ¾

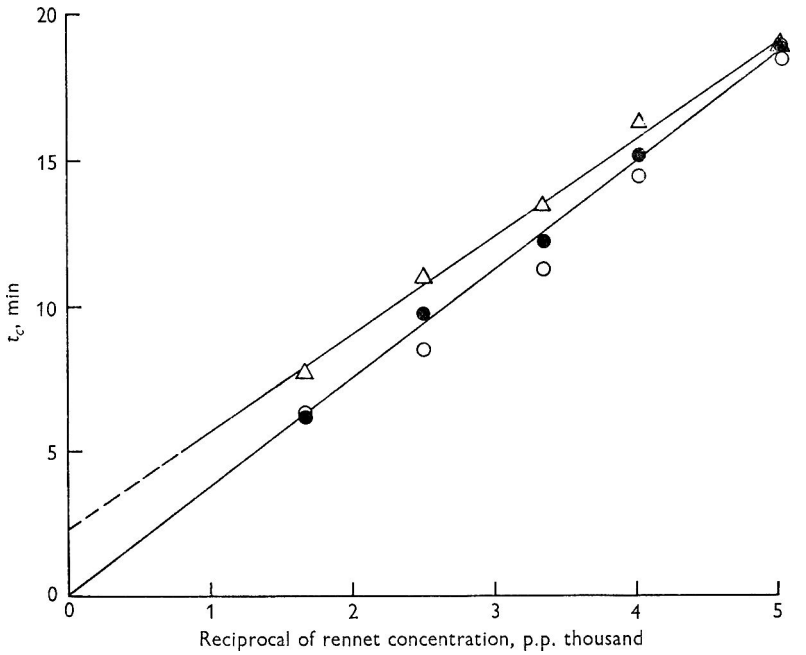

Fig. 4. A comparison at different rennet concentrations of t_c obtained from cheese-maker's assessment or by falling column method with clotting times determined by the Berridge test. ○, Cheese-maker's assessment; ● falling column method; △, Berridge test.

water-drop method; t_2, the time when a definite increase in viscosity appeared in the curves from the falling column apparatus; and t_3, the time which gave the best straight line when taken as time-zero for the log-reciprocal plot. This last time can be determined either by an algebraic method or by approximations from trial-and-error graphs. The accuracy of the two methods is about the same. None of these values can be determined to better than about a quarter of a minute, but their comparison as shown in Table 1 is interesting. It is clear that whereas t_2 and t_3 did not differ appreciably the cheese-maker's estimate generally comes somewhere about 50 sec before the other two.

Comparison with Berridge's coagulation test

Berridge (1952) has described a useful test for assaying rennet, which 'requires the formation, under reproducible conditions of a thin flowing film of milk'. A test

tube containing the rennetted milk is rotated in a water-bath in which it is set at an angle of 30°, and the end-point is observed when the coagulating milk forms a thin opaque film on the wall of the test tube. It is not necessary for standardizing rennets to detect the very earliest stages of coagulation and no claim was made that this test had such an objective. Nevertheless, it is of interest to compare the Berridge t_r with that derived from the method described in this paper.

For this purpose, small samples from the same milk were made up in the laboratory with different amounts of rennet and simultaneously falling column and Berridge tests were done by different observers. The Berridge-test observer also attempted quite independently to record the start of coagulation in a beaker using the cheese-maker's drop method. This was admittedly difficult to do in a beaker but the results, though less accurate, did not differ very much from the falling column figures. If anything, the 'drop' t_c came slightly sooner. The values of t_c for all three tests from one of these experiments are shown plotted against the reciprocal of the rennet concentration in Fig. 4. It will be seen that the values of t_c determined both by the new method and as assessed by the cheese-maker's subjective test were inversely proportional to rennet concentration within the limits of error. The Berridge points come a few minutes later and the curve, though linear, makes a significant intercept on the time axis. Theoretically, there should be some intercept on this axis, since at very high rennet concentrations the coagulation would not start instantaneously, but, in view of the scale of the graph, there is no reason why the intercept should be at all large. It is in any case an extrapolated intercept, i.e. the proportionality is not claimed to hold up to infinite concentration.

DISCUSSION

Little is yet known about the physical changes which take place during the earliest stages of coagulation of milk by rennet. It is known that the macropeptides produced by the action of rennin on κ-casein diminish the viscosity of the casein by screening electric charges and so decreasing the electro-viscous effects (Scott Blair & Oosthuizen, 1963), though the mechanism of this process is not yet fully understood. It seems certain, however, that it is these electrical changes which make coagulation possible in the presence of calcium ions. At first the casein micelles presumably form clusters and as soon as these become chain-like (Hostettler & Imhof, 1952), there will be a considerable rise in viscosity as well as the appearance of viscous anomalies, i.e. dependence of viscosity on shear-rate. It will not then be long before the 'jelly precursors' (as Loeb, 1921, called them) form and join together into a very soft gel structure which will have rigidity, after which, as more and more bonds are formed, the rigidity will progressively increase (Scott Blair & Burnett, 1963). Unpublished laboratory experiments have shown that once coagulation has started the various stages follow extremely quickly.

It is not clear just exactly what the cheese-maker observes when he watches the drop of water on the surface of the milk. If there is really a little 'crater' formed, this would suggest at least a trace of local rigidity; yet it seems as if this observation is made slightly earlier than the time when an increase in viscosity can first be observed with the falling column method with reasonable certainty. Either a

rise in viscosity or the beginnings of non-Newtonian behaviour, i.e. an increase in viscosity as the head falls, would produce divergence from linearity, but it is more likely that it is the former that is observed since the latter would not be appreciable at this stage at the prevailing rates of shear.

The end-point of the falling column test is generally taken from the first experimental point to lie above an extrapolated straight line, drawn carefully through the earlier points, by an amount greater than the very small, apparently random variations of these earlier points. A suspicion of non-linearity can sometimes be noticed, however, some 50 sec earlier than this and it is this earlier time that corresponds most closely to the cheese-maker's t_c. The process of bond formation described by the equation represented by the log-reciprocal plot, however, may start a little later, when a quite definite increase in viscosity is observable. In general, this slightly later zero gives the best linearity for the earliest stages of the log-reciprocal plot: for all but the earliest stages the differences are too small to show on the graphs.

It must also be remembered that to measure a viscosity the liquid must be flowing, if only very slowly. The curd at rest in the vat may at a certain time have a very small but finite rigidity, whereas the sample in the falling-column apparatus may still show only a rise in viscosity. The very soft gel structure as it is first formed is easily destroyed by shearing and may well be thixotropic.

A possible further explanation is that the equation represented by the log-reciprocal plot does not fit the data perfectly. Dr W. H. Thomas (pers. comm.) has given us an algebraic method for calculating t_c for any stretch of the coagulation curve assuming the log-reciprocal relation to hold. These values have been calculated for quite a number of experiments over different time ranges and, though the data are hardly accurate enough for certainty, there is some suggestion that the values of t_c which fit the earliest part (and the latest part) of the curve are slightly higher than the average values for the whole run. Because of the non-linearity of the log-reciprocal paper, curvature tends to be much more conspicuous at short times so that the value of t_c selected to give the best straight line is biased towards the earlier part of the experiment. If a good average t_c could be calculated for the whole curve, it might prove to be very close to the cheese-maker's value. Work is in progress to increase the accuracy of the scale readings on the falling column and it is hoped to be able to determine the 'breakaway point' with greater precision.

Since none of these methods for determining t_c can be more precise than about a quarter of a minute, the rapid sequence of rheological changes culminating in the increase in rigidity of a solid gel can be followed by existing techniques with no very great precision. It is believed, however, that the falling column method gives the best objective value of t_c available for practical purposes.

We are indebted to Miss H. Chapman for much help and encouragement and to Mr H. McIntyre for assessing for us the start of coagulation of the curds, as well as for much other co-operation.

After the above paper was accepted for publication, our attention was drawn to an article by Krieger, I. M. & Maron, S. H. (1951), *J. Colloid Sci.* **6**, 528. These authors

used as a viscometer an instrument consisting of a series of bulbs of known volume placed vertically one above another along a tube, connected at the bottom to a vertical capillary. In a later paper, Maron, S. H., Krieger, I. M. & Sisko, A. W. (1954), *J. appl. Phys.* **25**, 971, used a complicated apparatus consisting essentially of a falling column of mercury or a lighter liquid, to derive both Newtonian and non-Newtonian liquids upwards through a vertical capillary, and obtained curves not unlike our own. But in neither case was the instrument used to study coagulation. Though we were not familiar with this work when we wrote our paper, these instruments bear sufficient resemblance to our own for us to feel that some reference should be made to them.

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A comparison of hay and silage for milk production

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Summary. In a series of 3 trials there was little or no difference in milk yield when cows were fed the same quantity of dry matter in the form of well-preserved hay or silage from the same sward.

A higher milk yield was obtained from silage made from grass at an early stage of maturity than from silage or hay made from the same crop 5 weeks later.

In 2 trials when hay or silage was fed *ad lib.* the dry-matter intakes of the cows were higher with hay than with silage, and there was a consequent increase in milk yield and S.N.F. content in favour of hay.

Some of the results indicated that with certain silages a depression in the S.N.F. content of the milk, due to a decrease in the casein content, could occur, although this was not associated with an inadequate energy intake.

Hay and silage are the two main conserved products fed to dairy cattle in Britain, yet relatively few comparative trials have been made in this country in which either silage or hay was the only roughage offered. Results from trials in N. America and Europe have shown that with crops cut at conventional times and fed at the same dry-matter intake, a higher milk yield is obtained with a ration based on silage than with one based on hay (Trimberger *et al.* 1955; Presthegge, 1959; Larsen, 1960). This result would be expected since silage is normally made from herbage cut at a less mature stage of growth than that made into hay, and consequently has a higher digestibility (Watson, 1948; Shepperson, 1960). When, however, the silage and hay have been made from herbage cut at the same stage of maturity, little or no difference in milk yield has been found in some trials (Turk, Morrison, Norton & Blaser, 1951; Shepherd *et al.* 1954; Huffman, Duncan, Dexter & Chance, 1954; Trimberger *et al.* 1955), but in others higher milk yields have been obtained from silage than from hay (Harwood & Wells, 1936; Pratt & Holdaway, 1943; Presthegge, 1959; Brown, 1962).

The nutritive value of a roughage, however, is not the only factor determining output of animal products; the intake of roughage is also important. The voluntary intake of a roughage and its dry-matter content appear to be related. For example, a higher dry-matter intake was found when cows were given free access to hay than when they were given silage (Moore, Thomas & Sykes, 1960). It has been shown also that the voluntary intake of silage dry-matter increases with increasing dry-matter content (Shepherd, Gordon & Campbell, 1953; Cooper, 1954). Increases in milk yield have been associated with a higher dry-matter intake of hay or silage (Moore *et al.* 1960), but this is not invariably so (Brown, 1960).

The 5 trials reported here were made over a period of 3 years. They were designed to give further information on the comparative value of silage and hay for milk production when they were the sole roughage offered to milking cows. In 3 of the trials (1, 2 and 3) a comparison was made of equal quantities of dry matter in the form of silage or hay, both products having been made from the same crop at the same stage of growth. In one of these trials (3), 2 silages made from the same crop at an interval of 5 weeks were compared, and in the final 2 trials (4 and 5) silage and hay made from the same crop were fed *ad lib.* to milking cows.

Two of the trials (2 and 3) have been reported briefly elsewhere (Murdoch, 1962).

METHODS AND RESULTS

Friesian cows were used in all 5 trials, and, unless otherwise stated, the trials began 4–6 weeks after the cows had calved. The digestibility of the roughages given to the cows in the trials was determined with 4 yearling steers (trial 1) or with 6 wether sheep (trials 2, 3 and 4). Milk yields were recorded daily, and two 3-day composite milk samples were taken during the final week of each experimental period.

The silage offered to the cows in all the trials was made from herbage cut with a forage harvester and receiving no other treatment. With the exception of that used in trials 4 and 5 the hay was field-cured and was made with conventional equipment. Unless otherwise stated, the silages and hays were processed satisfactorily, the pH value of the silages being in the range 3·8–4·2 and the hay having been made in good weather conditions.

Trial 1. Silage and hay were made at the same time from a meadow fescue–timothy sward, which was cut on 14–16 June, the start of cutting being delayed by unfavourable conditions for haymaking. The chemical composition and the digestibility of the roughages are given in Table 1.

Table 1. *Chemical composition and digestibility of foods* (*trial* 1)

	Dry matter, %	g/100 g dry matter				
		Crude protein	Ether extract	N-free extract	Crude Fibre	Ash
Hay	76·9	8·6	1·3	45·3	37·4	7·4
Silage	21·1	10·2	2·5	40·3	38·7	8·3
Concentrates	85·2	20·4	1·7	68·4	4·5	5·0
Digestibility coefficients:						
Hay	67·3	53·5	52·0	65·3	76·2	—
Silage	69·1	59·3	60·7	72·1	78·5	—

The silage and hay were compared in a trial with 16 cows. A simple crossover design was used with experimental periods of 3 weeks.

The mean dry-matter intake of silage was 16·1 lb and of hay 16·3 lb per cow per day. Concentrates were given at the mean rate of 17 lb per cow per day, calculated according to milk yield at the beginning of the trial, and reduced by 1½ lb per cow per day at the end of the first experimental period to adjust nutrient intake to declining milk yield. The mean intake of concentrates throughout the trial was 4·4 lb gal milk produced.

There were no significant differences in milk yield or in the fat and S.N.F. contents of the milk between the 2 groups (Table 2).

Table 2. *The average yield and composition of the milk when cows received hay and when they received silage (trial 1)*

	Hay	Silage	S.E. of means
Milk yield, lb/day	40·2	41·0	±0·43
Fat, %	3·97	3·90	±0·07
S.N.F.,%	9·02	8·99	±0·03

Trial 2. Silage and hay were made from a meadow fescue–timothy sward which was cut on 25 May.

Table 3. *Chemical composition and digestibility of foods (trial 2)*

	Dry matter, %	g/100 g dry matter				
		Crude protein	Ether extract	N-free extract	Crude fibre	Ash
Hay	79·5	11·4	2·2	47·0	30·2	9·4
Silage	22·9	12·4	3·1	40·3	32·6	11·5
Concentrates	86·2	19·6	3·5	62·3	6·7	7·9
Digestibility coefficients:						
Hay	71·5	73·6	48·1	77·9	81·1	—
Silage	71·9	73·6	58·6	71·9	81·9	—

Table 4. *The average yield and composition of the milk of cows receiving different amounts of hay and silage (trial 2)*

	Hay		Silage		S.E. of means
	20 lb	10 lb	70 lb	35 lb	
Milk yield, lb/day	31·0	26·7	31·6	27·1	±1·13
Fat, %	4·00	4·12	4·00	4·10	±0·12
S.N.F., %	8·87	8·71	8·83	8·65	±0·03
Casein N, mg/100 g milk	420	390	404	383	±7·24

The silage and hay were compared at 2 levels of intake using 8 cows in two 4 × 4 Latin square experiments, each experimental period being of 4 weeks' duration. The treatments were: (*a*) 20 lb hay/day, (*b*) 10 lb hay/day, (*c*) 70 lb silage/day and (*d*) 35 lb silage/day. The mean intake of roughage dry matter at the high and low level of intake was 16·0 and 8·0 lb/day, respectively. Concentrates were given at the mean rate of 17 lb per cow per day calculated according to milk yield at the beginning of the trial and reduced by 2 lb per cow per day at the end of each experimental period. The mean intake of concentrates at the high and low level of roughage intake was 4·5 and 5·2 lb/gal milk produced, respectively. The high level of roughage intake with concentrates provided a calculated nutrient intake equivalent to approximately 100 % of Woodman's standard for maintenance and production.

The chemical composition and digestibility of the silage and hay are given in Table 3.

The milk yield, and the S.N.F. and casein contents of the milk were significantly

lower ($P < 0{\cdot}05$) at the lower level of roughage intake (Table 4). Silage, when compared with hay at the same level of dry-matter intake, gave a slightly higher milk yield and a slightly lower S.N.F. content of the milk but these differences were not statistically significant.

Trial 3. Two silages, one from an early cut (17–18 May) and the other from a late cut (22 June), and hay (cut 22 June) were made from an S. 23 ryegrass sward. The early-cut silage was poorly fermented (pH 5·5). Some rain (0·08 in) fell on the hay shortly after it was cut.

Table 5. *Chemical composition and digestibility of foods* (*trial* 3)

	Dry matter, %	g/100 g dry matter				
		Crude protein	Ether extract	N-free extract	Crude fibre	Ash
Early-cut silage	18·1	16·7	4·9	35·2	31·9	11·3
Late-cut silage	30·1	9·3	2·3	41·5	31·9	15·0
Hay	80·9	8·0	1·7	49·2	34·3	6·8
Concentrates	86·3	19·8	2·5	64·0	5·9	7·8
Digestibility coefficients:						
Early-cut silage	73·7	73·0	72·1	66·0	82·0	—
Late-cut silage	65·8	55·1	52·2	66·8	78·8	—
Hay	58·2	44·6	28·9	59·6	62·8	—

Table 6. *The average yield and composition of the milk when cows received hay, or early-cut or late-cut silage* (*trial* 3)

	Early-cut silage	Late-cut silage	Hay	S.E. of means
Group 1				
Milk yield, lb/day	34·0	29·5	29·5	±0·45
Fat, %	4·02	4·22	4·16	±0·17
S.N.F., %	8·66	8·83	8·80	±0·06
Casein N, mg/100 g milk	368	393	398	±4·04
Group 2				
Milk yield, lb/day	21·7	18·2	19·5	±0·34
Fat, %	4·53	4·46	4·51	±0·06
S.N.F., %	8·65	8·81	8·79	±0·05
Casein N, mg/100 g milk	382	419	421	±1·15

The foods were compared in a trial with 6 cows, the trial being in the form of two 3×3 Latin squares. At the start of the trial the cows in one group had a high milk yield; the other group had a lower yield as they had calved at an earlier date. Each experimental period lasted 3 weeks.

The cows on the 3 treatments were offered 90 lb early-cut silage, 54 lb late-cut silage or 20 lb hay daily, the mean daily dry-matter intake being 14·6, 15·5 and 16·1 lb, respectively. The chemical composition and digestibility of the foods are given in Table 5. The concentrate ration was calculated according to milk yield at the beginning of the trial, and reduced by $1\frac{1}{2}$ lb per cow per day at the end of each experimental period, the mean intake of concentrates throughout the trial being 15·1 and 10·7 lb per cow per day for the high- and low-yielding groups of cows, respectively.

In both groups, the milk yield was significantly higher ($P < 0{\cdot}01$), and the S.N.F. and casein contents of the milk significantly lower ($P < 0{\cdot}05$) for the early-cut

silage than for the other two roughages (Table 6). No significant difference was found in the fat content of the milk.

Trial 4. Silage and barn-dried hay were compared in this trial, both being made from a meadow fescue–timothy sward which was cut on 9–10 May. The hay was left for 48 h in the field after cutting, and had a mean dry-matter content of 62·5 % when barn-drying began. The hay was picked up from the field with a forage harvester, and was dried in a chopped state by air being blown through it for a period of 3 days, giving a dry-matter content of 87·5 %.

The foods were compared in a simple crossover trial with 12 cows with experimental periods of 3 weeks. The roughages were given *ad lib.* into troughs to groups of cows which had continuous access to the foods. No other foods were given. The chemical analysis and dry-matter digestibility of the hay and silage are given in Table 7.

Table 7. *Chemical composition and digestibility of foods* (*trials* 4 *and* 5)

	Dry matter, %	g/100 g dry matter					Dry-matter digestibility %
		Crude protein	Ether extract	N-free extract	Crude fibre	Ash	
Silage	21·4	12·0	3·2	46·6	29·9	8·3	72·5
Hay	82·0	10·0	1·8	54·7	25·5	8·0	74·9
Concentrates	86·2	22·3	2·4	62·7	5·2	7·4	—

Table 8. *Dry-matter intake and yield and composition of milk of cows when they received silage and when they received hay* (*trial* 4)

	Silage, 20 lb D.M./day	Hay, 32 lb D.M./day	S.E. of means
Milk yield, lb/day	27·9	30·4	±0·51
Fat, %	3·78	3·74	±0·02
S.N.F., %	8·50	8·70	±0·03

The intake of dry matter was considerably higher when hay was offered than when silage was offered, and the dry-matter digestibility of the hay was also slightly higher than that of the silage, although the value for the latter had been corrected for loss of volatile substances during the oven-drying process. The cows receiving hay gave better milk yields and had a higher S.N.F. content in their milk than those receiving silage ($P < 0{\cdot}01$) (Table 8).

Trial 5. The two roughages used in trial 4 were compared at 3 levels of concentrate intake in two 3 × 3 Latin square trials, the cows again having free access to the hay and silage. Concentrates were given at the rate of 0, 2 and 4 lb/gal of milk produced, the concentrate ration being adjusted to milk yield at the beginning of the trial and decreased ½ lb per cow per day in each succeeding week. The chemical analysis of the concentrates used in the trial is given in Table 7. Each experimental period was 3 weeks in length.

The mean dry-matter intake of the hay and silage was 31·8 and 22·6 lb/day respectively. Milk yield was significantly higher ($P < 0{\cdot}05$) with both hay and silage when 2 or 4 lb concentrates/gal were given than when no concentrates were offered

(Table 9). Differences in fat content of the milk were not statistically significant, though the fat content when the food was silage without concentrates was almost significantly lower than that obtained when either of the two levels of concentrates was included in the ration. The S.N.F. content of the milk was significantly higher ($P < 0{\cdot}05$) with increasing quantities of concentrates when silage was offered to the cows, but with the hay ration only the difference between 0 and 4 lb concentrates/gal was significant ($P < 0{\cdot}05$).

Table 9. *Yield and composition of milk of cows receiving either hay or silage alone and also with two different levels of concentrates (trial 5)*

	Concentrate intake, lb/gal			
	0	2	4	S.E. of means
Silage group				
Milk yield, lb/day	37·9	45·1	48·9	±1·20
Fat, %	3·07	3·52	3·42	±0·08
S.N.F., %	8·02	8·29	8·46	±0·02
Hay group				
Milk yield, lb/day	41·4	48·1	49·8	±0·71
Fat, %	3·65	3·42	3·69	±0·19
S.N.F., %	8·51	8·73	8·95	±0·06

DISCUSSION

In 2 of the trials, in which the dry-matter intakes from silage and hay were similar and the two roughages were made from the same herbage, a slightly higher milk yield was obtained from silage, but in no instance was this difference statistically significant. As no correction was made for the loss of volatile substances in oven drying the silage, the intake of dry matter would be slightly higher than quoted, and this would probably account for the slight increase in milk yield when silage was eaten.

In all the trials silage and hay were compared at levels of nutrient intake below that recommended by Woodman, and it seems unlikely that differences between the two foods would be masked by a high plane of nutrition. With the lower roughage intake (trial 2) and the late-cut silage and hay (trial 3) it is clear from the milk yields that the nutrient intake was less than the cow's requirements, and it is noteworthy that a similar response to hay and silage occurred at this low plane of nutrition.

A relatively short experimental period of 3–4 weeks was used in the trials reported here, and it might be argued that this was insufficiently long for any marked differences in milk yield to become apparent. However, in Norwegian trials (Presthegge, 1959), where silage was found to be superior to hay, a response was obtained within a period of 3 weeks from the beginning of the experimental period.

In general, therefore, the results reported here indicate that the feeding of silage or hay, made from the same herbage, will result in similar milk yields when the dry-matter intake is the same for both roughages and when both have been well preserved.

It is of interest, however, that though no significant differences were found in the comparisons of silage and hay at the same dry-matter intakes, slight increases in milk yield when silage was fed were often associated with a slight fall in the S.N.F. and casein content of the milk. In trial 3 the feeding of early-cut silage in comparison

with late-cut silage and hay gave a pronounced increase in milk yield and a significant decrease in the S.N.F. and casein content of the milk. These results suggest that silage of high nutritional quality, in comparison with hay harvested at the usual time, may favour a higher yield of milk of lower S.N.F. content.

In trial 4 there is a clear demonstration that milk yield can be markedly affected by the stage of maturity at which the crop is cut for conservation. Results similar to this have been obtained elsewhere (Trimberger *et al.* 1955; Presthegge, 1959).

When silage and hay were offered *ad lib.* the dry-matter intake with hay was greater than that with silage. In the 2 trials there was an increase of approximately 50% in the dry-matter intake of hay compared with silage, and this increase in nutrient intake resulted in a marked increase in milk yield and S.N.F. content. Although this increase in milk production occurred it did not appear to be commensurate with the increase in nutrient intake.

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The estimation of dissolved oxygen in anhydrous milk fat

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Summary. Dissolved oxygen in anhydrous milk fat is reacted with an aqueous manganous–ferrous hydroxide suspension at 50 °C. The mixture is acidified and after separation into two layers the amount of ferric iron in the aqueous layer is estimated by iodometric titration. The procedure is relatively simple and is suitable for routine testing of milk fat.

Methods of estimation of oxygen in fats may conveniently be divided into three classes.

(1) *The direct or indirect measurement of the gas released from the melted fat.* The total volume of gas liberated i.e. oxygen plus nitrogen, is measured and not the amount of oxygen alone. Reported values for solubilities of oxygen and nitrogen in milk fat indicate, however, that about 30 % of the dissolved gas will be oxygen (Schaffer & Haller, 1943; Hills & Conochie, 1945).

Mohr & Eysank (1943) estimated the air in milk fat by heating the sample in glycerol at 90 °C and collecting the liberated gas in the graduated stem of an inverted funnel held over the dish containing the fat. It is probable that only part of the dissolved gas would be released under these conditions. Rahn & Mohr (1924) and Guthrie (1930) estimated the 'free' air obtained from melted butter under reduced pressure by measuring the effect of the expansion of the gas under vacuum. Hills & Conochie (1945) used similar apparatus to estimate the air in 'concentrated butter', but reduced the pressure from 300 to 80 mm so that more of the dissolved gas in the fat was liberated and measured. Since the sample under reduced pressure was heated only to 45 °C it seems probable that some of the gas was still retained in the fat (Schaffer & Haller, 1943).

(2) *The chemical estimation of the oxygen in the gas released from the melted fat.* Morell, Conochie & Hills (1946) passed oxygen-free nitrogen through melted milk fat and then over copper moistened with ammonia–ammonium chloride solution. The copper oxide formed was washed off with ammonia–ammonium chloride solution and the copper in solution was determined iodometrically or colorimetrically as the blue cuprammonium salt. The method appeared to be reasonably accurate but it is unsuitable for routine testing.

The present author developed a method in which the air was released from the fat under very low pressure and at a temperature of 90 °C. The liberated gas was collected in a pressure flask containing a manganous hydroxide solution. Oxygen-free nitrogen was then bubbled through the fat until the pressure was a little lower than

atmospheric. The pressure flask was detached from the apparatus and shaken for 20 min in a mechanical shaker. After adding solutions of potassium iodide and dilute sulphuric acid the liberated iodine was titrated with 0·1 N-sodium thiosulphate solution (Taylor & Alexander, 1952). Despite all precautions to prevent air leaks into the flask the amount of oxygen found during a 'blank' estimation was always rather high. The method is not suitable for routine testing of fat.

(3) *The chemical estimation of the oxygen by shaking the fat with a reagent solution.* Schulze, Lyon & Morris (1940) estimated the dissolved oxygen in hydrocarbons by shaking the liquid with alkaline manganous hydroxide solution. After separation into two layers the aqueous layer was acidified in the presence of iodide and the liberated iodine was titrated with thiosulphate. Separation under the above conditions was found by McKeown & Hibbard (1956) to be slow and unsatisfactory. They replaced the manganous solution with a manganous–ferrous hydroxide suspension and thus were able to acidify immediately after shaking so that separation into two layers was rapid and complete. The content of dissolved oxygen in the hydrocarbon was calculated from the amount of ferric iron found in the aqueous phase.

Some experiments in this laboratory indicated that McKeown & Hibbard's method might be applied to the estimation of oxygen in milk fat dissolved in air-free benzene. Further work showed that the addition of the solvent was unnecessary since the oxygen in the solvent-free melted fat reacted readily with the reagent on shaking and the mixture separated immediately into two layers on acidifying. The method was found to give consistently low results for 'blank' estimations, concordant results on the same samples of fat, and results agreeing fairly closely with those obtained by other methods.

EXPERIMENTAL

Sampling

Milk fat exposed to air was found to increase fairly rapidly in oxygen content especially if it were in a plastic semi-solid condition, in liquid form at high temperatures, or if it were melted in an open container. Thus it was essential during sampling to avoid conditions under which further absorption or solution of air could take place.

At the manufacturing plant samples were taken after the fat had been cooled but while it was still liquid. Fat stored in a sealed container at a temperature low enough to completely solidify the contents was sampled immediately after withdrawal from storage. Fat stored at higher temperatures, and therefore partly in liquid condition, was melted completely at 40–50 °C before the container was opened for sampling. After sampling, the estimation of the oxygen content of each fat was commenced without delay.

Apparatus

An Erlenmeyer flask of 150 ml capacity is used for shaking the melted fat with the reagent. The flask is fitted with a rubber stopper with two holes, one for the entry of a loose-fitting gas delivery tube, the other, slightly larger, for the introduction of pipettes for addition of reagents and melted fat. Both holes are closed with small rubber stoppers during shaking of the flask.

Reagents

Manganous sulphate solution; 22 g of $MnSO_4.4H_2O$ dissolved in water and the volume made up to 1 l.

Ferrous ammonium sulphate crystals.

Potassium hydroxide, approximately 50 % solution.

Hydrochloric acid, approximately 5N.

Potassium iodide crystals.

Sodium thiosulphate solution, 0·1N.

Procedure

Place 50 ml of manganous sulphate solution in the flask and add 3·9 g of ferrous ammonium sulphate and 2 ml of 5N-hydrochloric acid. Pass an inert gas (the author used oxygen-free nitrogen) through the solution for 10 min. If the sample of fat to be examined is in the liquid state raise the delivery tube out of the solution but continue to pass the gas into the flask. Then add 4 ml of the potassium hydroxide solution from a burette and 50 ml (43 g) of fat from a pipette. Pass the gas for a further 2–3 min then withdraw the tube and immediately stopper both openings. If the sample of fat is in the solidified state quickly slice 43 g of fat into thin pieces and add them directly to the air-free solution after withdrawing the stopper. Replace the stopper, pass the inert gas above the fat and, after 5 min, add 4 ml of the potassium hydroxide solution. Two minutes later withdraw the delivery tube and immediately stopper both openings.

Clamp the flask in a mechanical shaker so that its lower half is immersed in a water-bath maintained at 50 °C. Shake gently at first to bring the fat and reagent to the temperature of the bath, then shake vigorously for 20 min.

Carefully withdraw the stopper from the larger opening and add at once 25 ml of 5N-hydrochloric acid. Restopper the opening and shake by hand until the precipitate dissolves. Transfer the mixture to a 250 ml separating funnel, allow to stand for a few minutes then run off the aqueous layer into an Erlenmeyer titration flask. Add 25 ml of water at 50 °C to the funnel, shake with the fatty layer, and allow to separate. To the combined aqueous layers add 5 g of potassium iodide, stopper the flask and after 10 min titrate with 0·1N-thiosulphate. Replace the stopper, titrate again after 20–30 min and record the total reading.

Run an occasional 'blank', adding the reagents but not the fat, under the conditions described above. Then

dissolved O_2 content (ml at STP)/(100 g of fat)

= (sample titration − blank titration) × 0·56 × (100/wt. fat taken).

RESULTS

(1) *Reproducibility of results.* Duplicate results on samples of milk fat containing various amounts of dissolved oxygen are shown below:

Sample	Dissolved oxygen, ml at STP/100 g fat	Sample	Dissolved oxygen, ml at STP/100 g fat
1	1·7, 1·7	3	1·0, 1·0
2	1·9, 2·1	4	0·25, 0·2

(2) *Absorption of air during sampling.* It is unlikely that anhydrous milk fat manufactured from butter is entirely free from dissolved air. Even the passage of nitrogen for a considerable time through a sample of fat held in the reaction flask at 50 °C before analysis did not reduce the result for oxygen content below 0·1 ml at STP/100 g fat. Since the oxygen contents of some of the samples of fat examined were found to be as low as 0·15 ml/100 g fat, the indications are that when the fat is sampled under the conditions described above (see 'Sampling') further solution or absorption of air does not take place during sampling.

(3) *Comparison with results of other workers.* The amount of oxygen in milk fat through which air had been bubbled at 45 °C was found by the above method to be 2 3 ml at STP/100 g of fat. This is similar to the uncorrected results of Morell *et al.* (1946) of 2·3–2·45 ml for oxygen content of milk fat saturated with air at 45 °C. The solubility experiments of Schaffer & Haller (1943), however, indicate that at 45 °C 100 g of milk fat dissolved 3·2 ml (STP) of oxygen when saturated with air.

Bubbling of pure oxygen through fat at 45 °C increased the oxygen content as estimated by the present method to 9·2 ml STP/100 g fat. This is lower than the uncorrected value of 11·5 ml obtained by Morell *et al.* (1946) for fat saturated with oxygen at 45 °C and much lower than the solubility figure of 15·4 ml at STP/100 g fat reported by Schaffer & Haller (1943) for the same temperature. Since Morell *et al.* (1946) and Schaffer & Haller (1943) saturated the fat by shaking in contact with oxygen it is probable that more oxygen was dissolved than if it had been bubbled through fat in contact with air as in the present investigation.

(4) *Results for anhydrous milk fat sampled at the manufacturing plant.* Samples of fat taken at the filling line or from tins after several days' storage varied only from 0·15 to 0·25 ml of oxygen at STP/100 g of fat. McDowall (1953) reported that milk fat after passage through a vacuum dehydrator had an air content of 1 vol. %. Since this figure is equivalent to 0·3 ml of oxygen/100 g of fat there is a close agreement between it and the results of the present author.

CONCLUSION

The method gives consistent results comparable with those obtained by other more elaborate methods. Because of the simplicity of the procedure it is readily applicable as a routine method for the examination of butterfats in a commercial plant.

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The effect of pre-partum feeding of heifers on milk composition

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Summary. The effect of the level of pre-partum feeding of Friesian heifers on the yields and composition of the milk in the subsequent lactation has been investigated using 23 heifers on each of 2 treatments. For 1 treatment, over a period of 6 weeks before calving, generous grazing on good pasture was supplemented with 8 lb/cow daily of concentrates, and for the other only restricted grazing of an already sparse sward was allowed.

For the period 3–8 weeks from calving the heifers fed at the high plane pre-partum produced significantly more milk than those fed at the low plane (36·8 compared with 31·9 lb/cow daily). The solids-not-fat (s.n.f.) and fat contents of their milk were also significantly higher (8·71 compared with 8·47 %, and 3·72 compared with 3·38 %, respectively). For the period 3–14 weeks from calving the effect of milk yield was slightly greater but the effect on composition was less. For the period 3–30 weeks from calving the difference in milk yield remained significant (31·3 compared with 26·1 lb/cow daily), but the average differences in s.n.f. and fat contents over this period (8·63 compared with 8·51 %, and 3·75 compared with 3·59 %, respectively) were not significant.

The heifers fed at the higher plane increased in liveweight before calving at a rate which was highly significantly greater than that of the heifers fed at the low plane. After calving, the heifers which had been on the higher plane remained almost constant in weight during the first 12 weeks whilst the others regained some of their losses, a difference which was highly significant. This recovery was apparently made at the expense of milk yield and composition.

The effect of the level of feeding of the cow before calving on the subsequent yield and composition of milk was reviewed by Burt (1956) and Broster (1958). Since then Castle & Watson (1961) have compared the feeding of 3·3 and 1·6 cwt of concentrates during the last 5 weeks before calving, as an addition to good pasture, and found no response in milk yield. On the other hand Swanson & Hinton (1962), using 75 pairs of cows, found that the addition of 8 lb of concentrates per cow daily to a roughage ration during the last 6 weeks before calving gave an increase of 302 lb of fat-corrected milk in the first 15 weeks of lactation. There was no evidence of an effect on milk composition in either of these investigations, but in other investigations the level of feeding before calving has been shown to influence milk composition particularly in early lactation, and the following summary is confined to these papers.

In the early years of the century Eckles (1912) showed that cows 'fat' at parturition often gave a higher fat content in their milk in early lactation than 'thin' cows, the difference being more marked when a low plane of feeding was adopted after calving, so that the cow 'milked off her back'.

In an experiment to determine the effects of the addition of concentrates, or of bulky feed to rations providing energy for maintenance plus a small pregnancy allowance for 6 weeks before calving, Blaxter (1944) reported a difference of 0·19 in butterfat percentage at 4–6 weeks after calving in favour of the animals receiving extra feed before calving. This difference was not statistically significant, although it occurred when either concentrates or bulky feeds were used to increase the level of energy intake pre-partum.

Bonnier (1946), in an experiment with 9 sets of monozygous twins in which one member of each set was fed liberally and the other at a low plane throughout the rearing period, found a somewhat higher fat content for about 3 weeks after calving in the milk of the heifers from the high plane feeding.

In parallel experiments carried out over a period of 3 years at Ruakura and Palmerston North in New Zealand (Lees, McMeekan & Wallace, 1948; Campbell & Flux, 1948), the yield and composition of the milk of cattle kept pre-partum on bare pasture supplemented with a maximum of 7 or 8 lb hay/head daily were compared with those of the cows receiving much more liberal pasture with hay or silage or both *ad lib.* At both centres fat percentage was reduced in the milk of the cows sparsely fed pre-partum, and particularly for the first 3 months of lactation. In the experiment at Palmerston North there was also a slight difference in the S.N.F. percentage in favour of the more liberal pre-partum feeding, the difference disappearing by the end of the first 2 months of lactation.

In these latter experiments (Campbell & Flux, 1948) it was estimated that the animals on the low level of feeding before calving lost considerable body weight at that time while the comparable groups gained weight. In a subsequent, similar experiment with 6 sets of monozygotic twins, Flux (1950) found no effect of the level of pre-partum feeding on milk composition, but in this instance the low plane of nutrition pre-partum involved little loss of weight. In summarizing the work carried out at Palmerston North, Campbell, Flux & Patchell (1955) also referred to an experiment in which a marked reduction in S.N.F. content had been caused by under-feeding before and after calving. Patchell (1957), reporting this work in greater detail, showed that a moderate reduction in S.N.F. percentage (0·1 or 0·2 %) in the milk of Jersey cows brought about by underfeeding in the first 6 weeks of lactation was substantially increased (0·25 and 0·6 %) when the 6 weeks of underfeeding followed 10 weeks of underfeeding before calving.

Using 8 sets of monozygotic twins, Hancock (1953) compared the milk production of twins which received pre-partum, autumn-saved pasture and 30–40 lb silage each daily, with that of twin mates which were offered the same diet but with the addition of meal at the rate of 1 % of body weight daily. All the animals were fed on the same basis after calving. The supplement increased the milk yield for the first 3 months of lactation by about 10 % and increased the fat percentage for the first month of lactation by 0·17.

Wallace (1958), in a study involving 36 sets of monozygotic twins, compared the

effects of various degrees of restriction in grazing during the 6 weeks before calving on production in the first 8 weeks of lactation. Although yields were not closely related to pre-partum treatments there was evidence of a substantial decrease in fat percentage when pre-partum grazing was stringently restricted, even when grazing in the 8 weeks after calving was liberal.

In an experiment with 88 heifers Broster, Ridler & Foot (1958) found no significant difference in the fat percentage when a pre-partum intake of about 2 cwt of concentrates was compared with an intake of about ½ cwt. In the same experiment, however, a significant difference in fat percentage in favour of the lower plane of post-partum feeding was found where 5 lb concentrates/gal milk were compared with 3 lb/gal in the first 12 weeks of lactation. These results are in accord with those of Flux & Patchell (1954), who investigated the effect of underfeeding in the first 6 weeks of lactation using 14 sets of monozygotic twins and demonstrated a significant increase in fat percentage from 5·06 to 5·56 in favour of the low plane in early lactation.

It seems probable that the increased fat percentage which is sometimes induced by a low plane of feeding in early lactation may, on occasions, obscure the effect of pre-partum feeding on milk composition.

The experiment reported below was undertaken to obtain more evidence on the effect of plane of nutrition pre-partum on milk composition. The comparison was made between feeding regimes before calving at about the two extremes of energy intake likely to be experienced on dairy farms in the United Kingdom.

METHODS

The heifers used in this experiment were obtained from herds which had a history of low S.N.F. over a period of 2 or 3 years. It was considered possible that animals from these herds might be somewhat more responsive in terms of milk composition to pre-partum feeding regimes. Only herds with cattle of Friesian type were considered, and 15 suitable herds from farms spread over the southern half of England were finally selected in the summer of 1961. From each herd either 2 or 4 heifers were drawn at random from all the heifers due to calve in the following autumn. These heifers were brought on to an Institute farm about 2 months before they were expected to calve and allocated, at random within farm source, to 1 of 2 treatments. The 46 heifers collected were thus split into 2 equal groups.

The heifers allocated to the high level of feeding were allowed generous grazing on good pasture and, in addition, 8 lb of concentrates/head daily for the last 6 weeks before calving. The heifers on the low level were allowed only very restricted pasture by rationing an already sparse sward. No supplementary feed was given to this group before calving. After calving, all the heifers were managed in the same way and as a single herd. They were fed according to the same rationing scheme, and on the same feed. The diet consisted of hay and concentrates up to the end of December and then half the hay was replaced by good quality ley silage.

The heifers were rationed individually during the winter with weighed quantities of feed. The weights of feed refused were also recorded. Rationing was based on 7 lb of starch equivalent for the maintenance of an 11 cwt cow and 2·7 lb/gal of 4 % fat-corrected milk. All the animals were given the same amount of hay, or hay

and silage, sufficient to meet the maintenance requirement of the smaller animals. Concentrate cubes were fed according to the yield of 4 % fat-corrected milk during the previous 3 weeks, together with an additional maintenance allowance for the larger cows. The cubes used both before and after calving consisted of a mixture of barley 11·0, maize 1·5, bran 1·5, extracted decorticated groundnut cake 3·75, molasses 1·5, and minerals 0·75 parts by weight. The starch equivalent of this mixture was estimated as 67.

Throughout the experiment the heifers were weighed every Tuesday and Thursday after the morning milking and morning feed, except when at pasture, when they were weighed after the morning milking and before grazing. Milk samples were taken throughout lactation on 1 day each week. The mean values quoted in the results exclude the first $1\frac{1}{2}$–$2\frac{1}{2}$ weeks from calving, the period varying from heifer to heifer according to calving time in relation to day of sampling, since milk composition changes rapidly in the first 2 weeks of lactation. The mean milk yields quoted are those obtained on the days on which the milk was sampled. For each heifer a composite sample, weighted according to the milk yields at the 2 milkings on the day of sampling, was tested for fat by the Gerber method (British Standards Institution, 1955) and for total solids gravimetrically (British Standards Institution, 1951), S.N.F. percentage being obtained by difference.

RESULTS

In view of the tendency for any pre-partum feeding effects on milk composition to be restricted to the earlier part of the lactation the results for milk yield and composition have been examined for the periods 3–8 weeks, 3–4 weeks and 3–30 weeks from calving. The percentages given are means of the weighted means for each heifer.

A summary of the results is presented in Table 1. The treatment means for the first 3–8 weeks after calving show the substantial, and significant ($P < 0{\cdot}05$), difference in milk yield of 4·9 lb/cow daily in favour of the higher plane of nutrition before calving. The mean difference of 0·24 % in S.N.F. content in favour of the high plane was also significant ($P < 0{\cdot}05$) and the concomitant difference of 0·34 % in the fat content was highly significant ($P < 0{\cdot}01$). The corresponding difference in total solids content of 0·57 %, and the difference in total solids yield of 0·79 lb/cow daily, were both highly significant ($P < 0{\cdot}01$). These values represent the production of some 21 % more total solids in the first 8 weeks by the heifers receiving the higher plane of nutrition before calving.

When the results were extended to the first 3–14 weeks after calving the effect on milk yield was slightly greater but the effects on composition were decreased; the difference in S.N.F. content was now not significant ($P > 0{\cdot}05$), and the differences in fat and total solids were now significant only at the $P < 0{\cdot}05$ level. The difference in total solids production/cow daily had now risen to 0·84 lb. The results for 3–30 weeks after calving show that the difference in composition was further decreased and became not significant ($P > 0{\cdot}5$). These differences were 0·12 % for S.N.F. and 0·16 % for fat content. The difference in milk yield of 5·2 lb/cow daily remained significant ($P < 0{\cdot}05$), and total solids production was 0·71 lb/day greater with the high plane, an increase of 22 % over the other treatment.

Table 1. *Effect of pre-partum nutrition on yield and composition of milk (mean values for each treatment)*

Pre-partum nutrition	Milk yield, lb/day	S.N.F., %	Fat, %	Total solids %	Total solids Yield, lb/day
		First 3–8 weeks of testing			
High	36·8	8·71	3·72	12·42	4·57
Low	31·9	8·47	3·38	11·85	3·78
Difference (high – low)	4·9*	0·24*	0·34**	0·57**	0·79**
Standard error of difference	2·27	0·106	0·099	0·182	0·274
		First 3–14 weeks of testing			
High	35·2	8·68	3·74	12·41	4·36
Low	29·4	8·50	3·49	12·00	3·52
Difference (high – low)	5·8	0·18	0·25*	0·41*	0·84**
Standard error of difference	2·20	0·095	0·102	0·167	0·265
		First 3–30 weeks of testing			
High	31·3	8·63	3·75	12·38	3·87
Low	26·1	8·51	3·59	12·10	3·16
Difference (high – low)	5·2*	0·12	0·16	0·28	0·71**
Standard error of difference	2·07	0·088	0·087	0·159	0·253

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

Liveweight changes for the 2 treatment groups are shown in Fig. 1, which also shows the changes with time of milk yield, S.N.F. and fat percentage. Individual variation from one weighing to the next was generally small. During the period on the Institute farm before calving the heifers on the higher plane of nutrition increased by an average of 2·31 lb/head daily whilst those allowed only sparse grazing at this time lost 0·73 lb, the difference being highly significant ($P < 0{\cdot}01$).

After calving, rates of liveweight change were again calculated, excluding weighings taken in the first 2 weeks, since rumen fill may be effected at this time. Over the whole period 3–30 weeks after calving, the heifers previously on the high plane gained 0·02 lb/head daily, whereas the others gained 0·24 lb, the difference being significant ($P < 0{\cdot}02$). Most of this difference resulted from changes occurring in the first 3–14 weeks of lactation. Over this period those from the high plane of feeding remained, on average, almost constant in weight, losing less than 0·01 lb/head daily, whilst the others gained 0·58 lb, this difference being highly significant ($P < 0{\cdot}01$). For the period 14–30 weeks both groups gained slightly, those fed at the high plane by 0·03 lb/head daily and the others by 0·06 lb, a difference which was not significant ($P > 0{\cdot}05$).

There was considerable variation between individuals in liveweight change over the 28 weeks, ranging from $-0{\cdot}52$ to $+0{\cdot}73$ lb/head daily.

DISCUSSION

It is clear that the feeding regimes imposed pre-partum in this experiment were sufficiently different to bring about not only a difference in milk yield but also changes in milk composition. In this respect the results are in agreement with some of those

obtained at Palmerston North (Campbell & Flux, 1948; Campbell, Flux & Patchell, 1955) and Ruakura (Flux, 1950), and the differences in composition were again mainly in the early weeks of lactation. In terms of human food production it is noteworthy that, when yield and composition are taken into account, the differences in pre-partum feeding were responsible for a difference of about 22 % in the production of milk solids in the 3- to 30-week period.

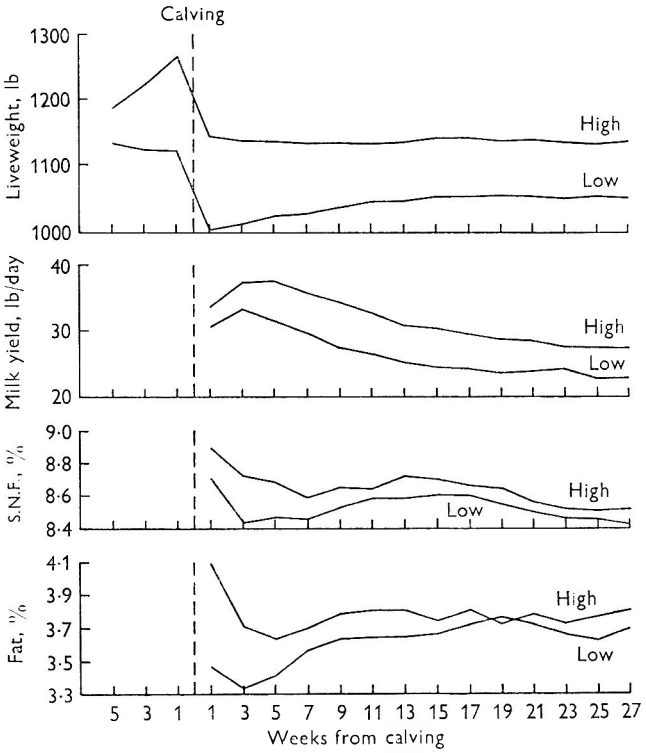

Fig. 1. Effect of pre-partum nutrition at high and low levels on the yield and composition of milk.

There is evidence in this experiment of associated changes in liveweight and milk composition. A low rate of liveweight gain, or loss of weight in late pregnancy followed by a compensating liveweight gain in early lactation, occurred in Blaxter's (1944) experiment, and loss of condition was reported in the New Zealand experiments (Lees, McMeekan & Wallace, 1948; Campbell & Flux, 1948). However, in the experiments reported by Swanson & Hinton (1962) substantial increases in liveweight gain in the 6 weeks before calving, brought about by feeding sufficient concentrates to increase milk yield significantly, had little effect on the mean fat content of milk for the whole lactation.

When the post-partum level of feeding is controlled by the yield of milk produced, as in the present experiment, it is important to differentiate between the direct effect of pre-partum feeding and the indirect effect due to changing levels of post-partum feeding adjusted to current milk production. The records of feed intake, liveweight, and milk yield and composition available from this experiment make it possible to compare the treatments on the basis of the metabolizable energy intake

against energy expenditure for fasting metabolism, milk production and liveweight change. Using values for the energy balance suggested mainly by Blaxter (1962), for the second, third and fourth weeks after calving when the milk composition differences were maximal, it was found that the greater output of energy produced in the form of milk by the heifers on the high level of feeding before calving can be accounted for by the higher energy intake supplied as a result of the higher production of milk solids in the early days of lactation. At the same time the results strongly suggest that energy required for the recovery of the liveweight of the group of heifers fed at the low plane before calving was used at the expense of milk yield and composition. In this respect our findings show some agreement with those of Patchell (1957).

It seems probable that a very low level of energy intake pre-partum is needed to produce a significant response in milk composition and less stringent restriction mainly influences milk yield. It is quite possible that decrease in the fat and S.N.F. percentage of milk in early lactation such as that obtained in this experiment is attendant on the loss in liveweight before calving and gain after calving which was clearly demonstrated in this work.

From the practical point of view raising the level of feeding before calving for the sole purpose of improving milk composition seems to be economically justified only when the existing level of pre-partum feeding is very low and a small increase in fat or S.N.F. percentage is required to meet a particular price differential. When milk yield and composition are considered in a broader context the effect of feeding before calving may become much more important. This is borne out by the increase of over 20 % in milk solids production resulting from the high, as compared with the low, level of feeding before calving.

We acknowledge the help of the Milk Marketing Board in obtaining suitable heifers for this experiment and for their support in the investigation.

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Determination of S.N.F. in milk and unsweetened condensed milk from refractive index measurements

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SUMMARY. Refractive index measurements of separated, ultracentrifuged and dialysed ultracentrifuged milk have been used to obtain values of 0·207, 0·187 and 0·140 ml/g for the specific refraction increments of the casein complex, soluble proteins and lactose, respectively. For 109 milk samples from individual Friesian cows, the standard deviation of the differences between solids-not-fat (S.N.F.) determined from refractive index and by the gravimetric method was found to be 1·8 % of the mean S.N.F. The relationship between refractive index and solids content of condensed whole milk has been studied in detail, and for condensed separated milk the predicted linear relationship between refractive index and the concentration of S.N.F. expressed as w/v has been confirmed.

Although methods based upon the measurement of refractive index have been proposed for the analysis of milk (Sacco, 1961; Prostakishin & Shkodich, 1963), and condensed milk (Menefee & Overman, 1939), little information is available on the refractive index contributions of the different constituents of milk. The recent development of continuously recording refractometers for liquids as opaque as condensed milk has lead to a renewed interest in the use of refractive index measurements for process control in dairies.

THEORETICAL

As shown by Glover & Goulden (1963), the increase in refractive index of a solution over that of the pure solvent is directly proportional to the solute concentration expressed on a w/v basis. Since the w/v concentration is the product of the density ρ and the w/w concentration c_w, the specific refraction increment r can be defined as

$$r = \frac{n - n_0}{\rho c_w} = \frac{\Delta n}{\rho c_w},$$

where n and n_0 are the refractive indices of solution and solvent measured under the same conditions. Refraction is normally an additive property, so that the refractive index increment (Δn) for a multicomponent system is given by

$$\Delta n = n - n_0 = \rho \Sigma r c_w.$$

Since refraction in an emulsion occurs at the interface of air and the continuous phase, the measured refractive index of whole milk will be that of the separated milk

fraction. If the w/w percentages of the casein complex, soluble proteins and lactose are C, S and L, and the respective specific refraction increments are r_c, r_s and r_l, the refractive index increment of milk is given by

$$100\Delta n = \rho_s (Cr_c + Sr_s + Lr_l + R), \quad (1)$$

where ρ_s is the density in g/ml of the separated milk fraction and R the refractive index contribution of the residual mineral salts. ρ_s can be calculated from the density of the whole milk (ρ_m) and the w/w fat percentage (F) by the easily deduced equation

$$\rho_s = \frac{1 - 0{\cdot}01F}{1/\rho_m - 0{\cdot}01F/\rho_f}, \quad (2)$$

ρ_f being the density of milk fat, taken as 0·93 g/ml.

Experimental data show that the fourth term of equation (1) accounts for only 11 % of Δn, so that variations in R are second order effects and can be neglected. As seen from equation 1, for Δn to be an accurate measure of S.N.F. all three r values must be equal. In such a case the value of Δn would be independent of the lactose/protein ratio. As will be shown later, these coefficients are not equal so that the natural variations in the lactose/protein ratio will be expected to lead to deviations from an exact linear relationship between Δn and S.N.F.

For condensed milks which are normally prepared from bulk milk samples, deviations due to variations in the lactose/protein ratio will be considerably less than those for milk samples from individual cows. Changes in density, particularly at high solids content will, however, play a more important role and will lead to a curvilinear relationship between Δn and S.N.F. This curvature arises from the non-linear relationship between density and w/w concentration. Although the fat does not itself influence Δn, the contribution of the fat content must be allowed for when ρ_s is calculated from the density of the whole condensed milk.

METHODS

Refractive indices were measured with a Bellingham and Stanley High Accuracy Abbé 60 refractometer, the sample temperature being controlled to $\pm 0{\cdot}2$ °C. Most measurements were made at a wavelength of 0·5893 μ using light from a sodium lamp. To determine the effects due to a change in wavelength, measurements were also made at 0·5461 μ using a mercury arc source with the appropriate filter. For milk samples, the refractive index was reproducible to $\pm 5 \times 10^{-5}$.

The specific refraction increment of lactose was evaluated from measurements of refractive index and density on solutions made up to known concentrations from 'Analar' grade lactose monohydrate. Specific refraction increments of the casein complex and soluble protein components were evaluated from refractive index, lactose (Hinton & Macara, 1927), and protein (Kjeldhal, total N × 6·38) contents of separated milk, before and after ultracentrifuging for 1 h at 80000 g to remove casein, and after dialysis of the ultracentrifuged solutions at 5 °C against distilled water for 24 h. Addition of 0·1 % sodium chloride enabled the small amount of precipitate found at the end of dialysis to be redispersed.

A series of 109 milk samples from individual Friesian cows was used to study the relationship between Δn and S.N.F. These samples had for other experiments already

been analysed for fat, S.N.F. and lactose and their total protein contents were calculated from these data, allowing 0·95% for the residual solids (Dr S. J. Rowland, pers. comm.).

Condensed milk samples were prepared by evaporation in a laboratory rotary still at a pressure of about 100 mm Hg and a temperature of about 60 °C. Their densities at 20 °C were determined with the aid of specific gravity bottles and after known dilution with water, the total solids contents were calculated from the weight loss at 105 °C.

RESULTS AND DISCUSSION

In agreement with previous measurements (Rangappa, 1948), it was found that the refractive indices of whole milk samples were exactly the same as those of the corresponding separated milks, confirming that the fat made no contribution to the refractive index. Whole milk rather than separated milk samples were therefore used for refractive index determinations, since the removal of the fat did not perceptibly improve the sharpness of the boundary observed in the refractometer eyepiece.

Table 1. *Effects of change in wavelength and temperature on refractive index* (n) *and refractive index increment* (Δn) *of milk** ($\Delta n = n_{milk} - n_{water}$)

Temp., °C	Wavelength, μ	Refractive index			
		Water	Separated milk	Condensed separated milks	
40	0·5461	1·33176	1·34718	1·36901	1·39145
40	0·5893	1·33037	1·34564	1·36727	1·38964
20	0·5893	1·33295	1·34815	1·37009	1·39295
$10^2 \times$ change in n per unit wavelength (μ) at 40 °C		3·2	3·6	4·0	4·2
$10^2 \times$ change in Δn per unit wavelength (μ) at 40 °C		—	0·4	0·8	1·0
$10^4 \times$ change in n per °C at 0·5893μ		1·29	1·25	1·41	1·65
$10^4 \times$ change in Δn per °C at 0·5893μ		—	0·04	0·12	0·36
Total solids content w/w		—	9·2	21·4	33·1

Effect of changes in wavelength and temperature on refractive index

Table 1 records the refractive indices of water, separated milk and condensed separated milk measured at two different temperatures and wavelengths. The results confirm that the refractive index increments (Δn) are less affected than single refractive index measurements by wavelength and temperature changes. Refractive index normally shows a curvilinear dependence upon both wavelength and temperature, so that the calculated increments apply only to limited wavelength and temperature ranges. Using the relationship obtained later between Δn and S.N.F., it can be calculated that for condensed milk of 33% solids content a temperature change of 1 °C results in an apparent change in S.N.F. of about 0·2%.

Specific refraction increments of milk constituents

Tables 2 and 3 record the data used and the results of the calculations of the specific refraction increments of milk constituents. The value of 0·140 mg/ml obtained for lactose is identical with that quoted by Browne & Zerban (1941), and the order of decreasing refractive index contributions lactose > proteins > residual salts is the same as that given by Rangappa (1948).

Table 2. *Data used to calculate specific refractive increments of the components of separated milk*

(λ, 0·5893 μ; temperature, 20 °C.)

	Lactose solution	Separated milk	Serum after ultra-centrifuging	Ultra-centrifuged serum after dialysis
Solids, %, w/w, gravimetric	—	8·95	6·52	0·707
Lactose (anhydrous), %, w/w, by titration	3·505	4·83	5·02	0·04
Protein (N × 6·38), %, w/w	—	3·17	0·83	0·56
Density at 20 °C, g/ml	1·011	1·034	1·03	1·003
NaCl added, %, w/v	—	—	—	0·100
$100\Delta n$*	0·496	1·520	1·020	0·127
Specific refraction increment (r), ml/g	0·140	—	—	—

* $100\Delta n$ for 0·1 % NaCl = 0·016.

Table 3. *Refractive index contributions of separated milk components*

(λ, 0·5893 μ; temperature, 20 °C; ρ, 1·03 g/ml.)

Component	Composition, C_w w/w, %	Specific refraction increment, (r) ml/g.	$\rho r C_w$	% of Δn
Casein complex	2·34	0·207	0·500	33
Soluble proteins	0·83	0·187	0·159	10
Lactose	4·83	0·140	0·695	46
Residue (by difference)	0·95	—	0·166	11
Total	8·95	—	1·520	100

The specific refraction increment of 0·207 ml/g obtained for the casein complex is outside the range of 0·17–0·19 ml/g recorded for other proteins (Stacey, 1956), and presumably arises from the presence of the casein in milk as a complex with calcium and phosphate. Possible changes in the specific refraction measurement of the casein complex due to changes in the calcium content of milk have not been investigated. McMeekin (pers. comm.) has shown that sodium caseinate has a normal protein specific refraction increment of 0·183 ml/g. The value of 0·187 ml/g obtained in the present investigation for the soluble milk proteins is in agreement with the value of 0·184 ml/g reported by Halwer, Nutting & Brice (1951) for β-lactoglobulin.

For the calculations of refractive indices of milk samples of known composition, it has been assumed that casein forms 75 % of the total protein (N × 6·38) so that the integrated specific refraction increment for all the protein can be taken as

$$(0{\cdot}75 \times 0{\cdot}207) + (0{\cdot}25 \times 0{\cdot}187) = 0{\cdot}202 \text{ ml/g.}$$

Absence of lactose-protein interaction effects

The absence of lactose-protein interaction effects was confirmed by the results shown in Table 4. Known amounts of lactose were added to dialysed separated milk containing added sodium chloride and the refractive indices were determined both by experiment and by calculation from the known concentration and specific refraction increment of lactose. The close agreement between the calculated and experimental values confirms that refractive index is a truly additive property for lactose-protein systems.

Table 4. *Effects of change in lactose/protein ratio on the refractive increment of milk*

(λ, 0·5893μ; temperature, 20 °C.)

Sample	Lactose/protein ratio	Calculated $100\Delta n$	Experimental $100\Delta n$
1	0·02	—	0·588
2	0·37	0·728	0·728
3	0·71	0·864	0·859
4	1·06	1·004	1·000
5	1·42	1·145	1·147

* Sample 1 was dialysed separated milk to which about 0 2 % sodium chloride was added.

Correlation between refractive index and S.N.F. *of milk samples from individual cows*

In the set of 109 milk samples from individual animals the lactose/protein ratio ranged from 0·7 to 1·8 about a mean value of 1·35. The regression equation for refractive index increment upon gravimetric (w/w) S.N.F. was found to be

$$100\,\Delta n = 0{\cdot}193 \times \text{S.N.F. } \% - 0{\cdot}134,$$

with a coefficient of variation of 1·8 % for the differences in S.N.F. between the two methods.

In order to confirm that variations in the lactose/protein ratio contributed to deviations from the linear relationship Δn and S.N.F., the sum of the lactose and protein refractive index contributions for all the samples were calculated from the previously obtained refractive index increments (Tables 3, 4) and the known lactose and protein contents by the formula

$$100\Delta n' = 1{\cdot}03\,(0{\cdot}202 \times \%\text{ protein} + 0{\cdot}140 \times \%\text{ lactose}).$$

Δn is the calculated refractive index increment neglecting the contribution of the mineral salts, and the density factor 1·03 enables w/w lactose and protein values to be used. When the $\Delta n'$ values were plotted against S.N.F., the scatter was reduced leading to a lower coefficient of variation of 1·5 %. This decrease in scatter was particularly apparent at high and low S.N.F. values and confirms that variations in the lactose/protein ratio are partially responsible for deviations from the linear relationship between Δn and S.N.F.

Refractive index of condensed milk

Fig. 1 records the results obtained for a series of condensed separated and condensed whole milk samples prepared from the same bulk milk supply. The experimentally determined relationship between Δn and S.N.F. w/v for condensed separated milk is identical with that calculated from the refractive index and composition of whole milk using equation (2) to obtain the densities of the separated milk fractions. Since this relationship is linear, the calculations of Glover & Goulden (1963) are further confirmed.

Both separated milk and whole milk show curvilinear relationships between Δn and w/w total solid contents, the curvature increasing at higher solids contents. Since changes in fat content of whole milk lead to corresponding changes in the total solids content but have no direct effect upon the refractive index, these results can easily be corrected for known variations in fat content. Variations in the lactose/protein ratio will affect the slope of the Δn/S.N.F. w/v line and can only be allowed for if both the S.N.F. w/v and Δn of each milk sample are known.

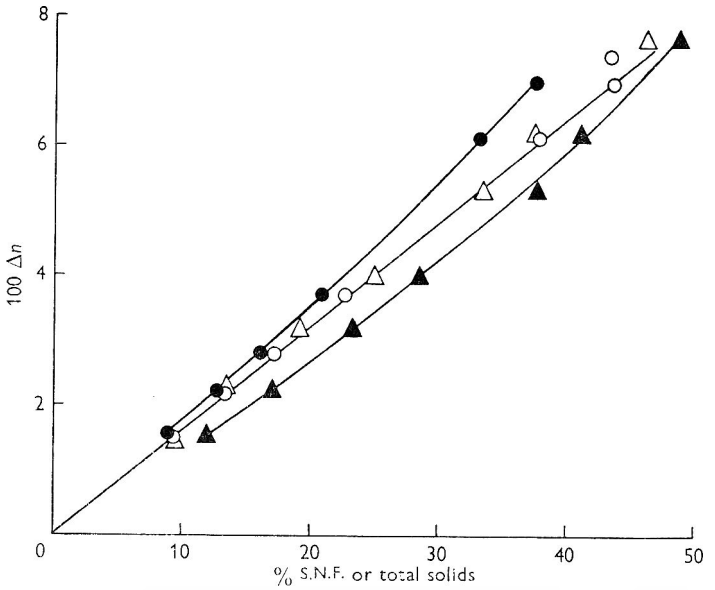

Fig. 1. Variations of refractive index increment (Δn) of condensed-separated and condensed-whole milk with the S.N.F. and total solids content. ●, condensed-separated milk (S.N.F., %, w/w); ○, condensed-separated milk (S.N.F., %, w/v); ▲, condensed-whole milk (total solids, %, w/w); △, condensed-whole milk (S.N.F., %, w/v, calculated).

CONCLUSIONS

The results obtained on milk samples from individual cows show that refractive index measurements provide a possible physical method for the determination of S.N.F. In common with several other methods, the measured physical property has different sensitivities to lactose and protein. Since the lactose/protein ratio is not constant for all samples, errors in S.N.F. are introduced due to variations in the lactose/protein ratio. The extent of these errors depends upon the nature of the sample

population and may well lead to a coefficient of variation greater than 2% when milks from different breeds of cow are included. It therefore appears that the accuracy of the refractometric method for determination of S.N.F. is of the same order as that of the hydrometric (Dr S. J. Rowland, pers. comm.) and infra red methods (Goulden, unpublished).

Since refractive index provides a rapid measure of the S.N.F. of condensed milk, it would seem that the various recording flow-through differential refractometers now becoming commercially available might well be employed as control devices in systems for the automatic control of condensed milk plants. The accuracy of control will depend upon the variations in fat and S.N.F. contents as well as the lactose/protein ratio of the milk supplies available.

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Reviews of the progress of dairy science

Section B. Cheese and butter starters

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CONTENTS

INTRODUCTION

The first attempts to control acid and flavour development in butter and cheese consisted of keeping back clean-flavoured sour milk, buttermilk or whey as starter. The recognition of the main bacterial species involved led to the separate propagation of natural cultures under laboratory conditions. Cultures obtained by such methods still form the basis of many so-called commercial or multiple-strain starters which are widely used in practice. Other mixed-strain starters appear to have been blended from pure strains of known characteristics. Butter- and cheese-makers judge such starters by taste, rate of acid development, smell and appearance, relying on past experience of what constitutes a good starter. Mixed-strain starters may remain active and preserve their characteristics for some time, but may also lose their activity rapidly according to the compatibility of their species and strains.

The use of single-strain lactic streptococcal starters consisting of special strains chosen mainly for their souring ability and uniformity of performance, as pioneered by Whitehead of New Zealand, created a split between the adherents of multiple-strain and single-strain starters both in practice and in the laboratories. Multiple-strain starters were most intensively studied by taxonomists, while single-strain starters were an ideal subject for phage workers, simply because they are artifacts creating ideal conditions for multiplication of lytic phages in the creamery.

The classification and the characterization of the strains which make up multiple-strain starters is not difficult with present laboratory methods, although the connexion between the observed characteristics and the usefulness of the bacteria as starters is as yet incomplete.

We shall attempt in this review to assess critically the case both for single-strain and multiple-strain starters and indicate where there is common ground for research.

COMPOSITION OF STARTERS

The starters to be discussed consist mainly of one or more of those species which in the 7th edition of *Bergey's Manual* (1) are classified as genus II *Streptococcus*, lactic groups, genus III *Pediococcus* and genus IV *Leuconostoc*. According to the species present, starters may be classified as follows:

I. Single-strain starters:
   (*a*) *Streptococcus lactis* or *Str. cremoris.*
   (*b*) *Str. diacetilactis.*

II. Mixed-strain starters:
   (*a*) *Str. lactis* or *Str. cremoris* or both.
   (*b*) *Str. lactis*, *Str. cremoris* and *Leuconostoc cremoris.**
   (*c*) *Str. lactis*, *Str. cremoris*, *Str. diacetilactis* and *Leuc. cremoris.*
   (*d*) *Str. lactis*, *Str. cremoris* and *Str. diacetilactis.*
   (*e*) Several strains of *Str. diacetilactis.*

Apart from these starters it is claimed that some exist consisting solely of strains of leuconostocs, which by a process of selection have acquired the ability to grow in the absence of streptococci (2,3). Starters containing pediococci are apparently not used.

Of the types of starters mentioned, types I*a*, II*a* and II*b* are especially used in English-speaking countries for the manufacture of Cheddar cheese. Type II*b* was, several years ago, the most widely used starter in Europe and the U.S.A., and in recent years it has been used especially in Holland, U.S.A. and Norway. Starters of types II*c* and II*d* were probably introduced 25–30 years ago and increased in popularity in Denmark and Sweden, and gradually replaced II*b*. In the last 1–2 years, however, types II*b* and II*c* have tended to be used rather more. Starters of types I*b* and II*e* are not normally used.

Media for selective counting and isolation

When investigating the bacterial flora of starters it is of great benefit to use a medium which helps in both the counting and isolation of the individual species, thereby giving direct information as to which type of starter is being investigated. The work of developing suitable media has, in the main, had two objectives; first, to develop a medium which will enable the aroma-producing strains *Str. diacetilactis* and *Leuc. cremoris* to be isolated and counted directly from a mixed-strain starter,

* *Leuconostoc cremoris* is used throughout the text in preference to *Leuc. citrovorum* or *Betacoccus cremoris*, see p. 422.

and secondly to develop a medium by which the other strains of streptococci can be identified and isolated.

For isolating and counting the aroma bacteria three media have been developed [4] which are known respectively as WAC 1/2 %, WACCA 1/2 % and WACCA 1 %. Basically they consist of whey agar to which 0·5 or 1 % of calcium lactate is added in order to inhibit the growth of *Str. lactis* and *Str. cremoris*. The WAC medium does not support growth of leuconostocs and if their growth is required a further 1·4 % Casamino acid and 0·1 mg Mn/l are added. To differentiate the aroma bacteria from the homofermentative streptococci the medium is made cloudy by the addition of a suspension of calcium citrate in carboxymethyl cellulose immediately before use. On this medium the aroma bacteria produce clear zones around their colonies as they ferment the citrate, and thus can be easily distinguished from the streptococci which do not ferment citrate.

For counting leuconostocs in a starter which consists solely of this organism as the aroma producer, WACCA 1 % medium is recommended with incubation at 25 °C. For starters in which the aroma bacteria consist exclusively of *Str. diacetilactis*, WAC 1/2 % medium is recommended with incubation at 30 °C. Where the starter contains both *Leuc. cremoris* and *Str. diacetilactis*, and both strains have to be counted, the use of medium WACCA 1/2 % has been found to be best as *Str. diacetilactis* is somewhat inhibited by 1 % calcium citrate. On the other hand, 1 % calcium citrate does not inhibit *Str. diacetilactis* sufficiently to separate *Str. diacetilactis* from *Leuc. cremoris*, so that in starters which contain both these strains selective counting is not possible, and differentiation must be by means of counts on WAC 1/2 % where only *Str. diacetilactis* will be counted and WACCA 1/2 % where both species produce colonies with clear zones. If the number of *Leuc. cremoris* is small in relation to the number of *Str. diacetilactis*, it is not possible to use this method. To overcome this problem 0·3 i.u. of penicillin may be added per ml of WACCA 1/2 % [5] to inhibit *Str. diacetilactis* and other streptococci. The resultant partial inhibition of *Leuc. cremoris* is not sufficient to have any significant effect on its count. Because of the difficulty of preparing these media it has been suggested [6] that whey agar and Casamino acid be replaced by commercial tomato juice agar whilst retaining the streptococcal inhibitors calcium lactate and calcium citrate. This medium, known as TJAC 1/2 %, gives the same results as WACCA 1/2 % and is more convenient to prepare if whey is not easily available. Other media incorporating tryptone or tomato juice, and sodium azide or tetracycline to inhibit the homofermentative streptococci [7, 8], have been found useful for the isolation and enumeration of leuconostocs. The inhibition of all the streptococci by these media means, however, that they cannot be used for the counting of *Str. diacetilactis*. Also selectivity for *Leuc. cremoris* appears to be somewhat less when using sodium azide than when penicillin or tetracycline is used.

With the use of calcium citrate suspension as an indicator for citrate-fermenting bacteria, of calcium citrate for selective inhibition of *Str. lactis* and *Str. cremoris*, and of suitable antibiotics for general inhibition of streptococci, easier and better methods for isolating and counting *Str. diacetilactis* and *Leuc. cremoris* in starters are now available. In addition, media have been developed within recent years for the isolation and counting of different species of homofermentative streptococci in starters. By use of the modified tetrazolium–nitrate–haemoglobin–arginine agar

of Jepsen (9) differentiation, based on colour of colony, can be made between those strains which hydrolyse arginine and those which do not possess this property (Møller-Madsen, unpublished).

Differentiation of *Str. lactis* and its variants from *Str. cremoris* can be achieved on yeast-extract tryptone agar, containing arginine (10) or citrate (5) together with 2,3,5-triphenyltetrazolium chloride. However, using this medium the Danish workers found that *Str. diacetilactis* produced grey colonies of the same size as those of *Str. lactis*.

The media discussed are diagnostic rather than specially selective, but they have contributed towards the identification of those streptococci which are normally found in starters.

Taxonomy and classification

Early work on the taxonomy of starter bacteria was concerned with classification within the genus *Streptococcus* and resulted in the recognition of the group N streptococci in 1937 (11,12). More recent work has been concerned with the separation of the various species of group N streptococci and with the differentiation of the genera *Leuconostoc* and *Pediococcus*. Numerous investigations of the biochemistry and physiology of these organisms (13-20), chromatographic analysis of the free amino acids of the cell (21,22), carbohydrate and amino acid composition of the cell walls (23,24) and nutritional studies (25,26) have helped to clarify the genera and species. Once the genera and species occurring in starters and dairy products had been established, differentiation of strains was made by phage typing, or, more recently, by serological methods.

The first species of the group N streptococci to be recognized were *Str. lactis* and *Str. cremoris*. The criteria of Sherman and his collaborators for separating these two species are still used in their original form (27-30,14,15). *Str. diacetilactis* as a third possible species was described later (31,32), and has been of considerable economic importance, particularly in Europe, because of its contribution to the aroma of dairy products. Apart from its ability to utilize citrate with the formation of CO_2, acetoin and diacetyl, *Str. diacetilactis* appears to be identical with *Str. lactis* (14,15,25,26).

Laboratory tests to distinguish *Str. diacetilactis* from *Str. lactis* have been based on the detection of acetoin and diacetyl by the Voges–Proskauer reaction (VP test). False negative results can, however, be obtained because of the reduction of acetoin to butan-2,3-diol by some bacteria. This was reported for leuconostocs (betacocci) as early as 1939 (33-35) and recently for *Str. diacetilactis* cultivated in cream (36). There is a rapid production of acetoin during the first 12 h which is followed by a rapid destruction in the next 12 h. Contaminating organisms in dairy products can also cause destruction of acetoin. Recent publications have shown that a variety of bacteria can break down diacetyl, for example Gram-negative organisms, (37) micrococci (38) and *Str. faecalis* (39). A recent report (40) indicates that *Str. lactis* and *Str. cremoris* also contain diacetyl reductase and have some destructive effect on diacetyl. Nevertheless, the VP test has been claimed to distinguish *Str. diacetilactis* from leuconostocs (41,42). According to these publications, starters which contain *Str. diacetilactis* have relatively mild reducing properties so that the starter continues to give a positive VP reaction for several days, but cultures containing leuconostocs (betacocci) have generally a greater reduction potential and give a negative VP

test by the time the milk becomes coagulated. The VP test can be of considerable value in routine investigations of starter cultures, even if it is of doubtful value when investigating the biochemical properties of an organism.

It is definitely desirable to establish whether a strain utilizes citrate (42, 43). The fact that strains of *Str. diacetilactis* have been found which contain diacetyl reductase, reducing diacetyl to acetoin may be the reason for the appearance in the literature of strains described as *Str. lactis* var. *diacetilactis* and *Str. lactis* var. *acetoinicus*, respectively (44). Some strains of *Str. lactis* and *Str. cremoris* can give a positive VP reaction and yet fail to ferment citrate. This may arise because some strains may be capable of condensing 'active' acetaldehyde to acetoin (19) as has been demonstrated for some lactobacilli (46). It has been suggested that such anomalous strains should be named *Str. lactis* var. *aromaticus* and *Str. cremoris* var. *aromaticus*, respectively (45). This is hardly appropriate as we know that under certain conditions all the species can produce unusually large amounts of products other than lactic acid (see section on carbohydrate fermentation).

Another source of confusion arises from the fact that strains of *Str. diacetilactis* can vary in their ability to ferment citrate (47). It has been repeatedly observed that a strain of *Str. diacetilactis* can even lose its ability to ferment citrate and regain it under different cultural conditions. Citrate fermentation is due to the constitutive enzyme citritase (48–50) and the failure of certain strains to ferment citrate may be caused by the loss of a citrate permease which transports the citrate across the cell barrier. Intact cells which do not possess this permease system are unable to ferment citrate although cell free extracts contain the citritase. Naturally, such strains can be mistaken for *Str. lactis*. *Str. diacetilactis* is therefore by some workers regarded as a sub-species of *Str. lactis* (32) and, indeed, in *Bergey's Manual* (1) there is no distinction made between citrate utilizing and non-utilizing strains. There are also strains of *Str. cremoris* which can ferment citrate (48, 16) and which differ from *Str. lactis* and *Str. diacetilactis* only in their response to temperature, pH and NaCl concentration.

The genera *Leuconostoc* and *Pediococcus* may also be confused but only the former is known with certainty to occur in commercial mixed starter cultures. A study of some biochemical reactions of 88 strains of leuconostocs has been made (13). The definition of the genus as given in *Bergey's Manual* (1) was accepted, except that strains forming DL-lactic acid from glucose were excluded and only strains forming D(−) lactic acid were considered to be leuconostocs. Furthermore, the genus was characterized by three tests: inactivity in litmus milk (no gas production or reduction of litmus); no production of NH_3 from arginine; and gas production from glucose in a fortified medium. The genus was subdivided into *Leuc. cremoris* (group 1), *Leuc. lactis* (group 2), *Leuc. dextranicum* (group 4) and *Leuc. mesenteroides* (groups 3, 5 and 6). The name *Leuc. cremoris* was preferred in designating the species known as *Betacoccus cremoris* (51) and in preference to *Leuc. citrovorum* which had become confused with *Pediococcus cerevisiae* (52). It appears, however, that while there are some differences between the species of leuconostocs, a clear division between them is not yet possible (14, 15).

Pediococci have been confused with leuconostocs because both are Gram-positive cocci and both are inactive in litmus milk. Some pediococci form acetoin from

glucose and some fail to form NH_3 from arginine. However, the two genera can be distinguished morphologically. In addition the pediococci produce either inactive or L(+) lactic acid from sugar, do not form gas from glucose (17–20,53), and appear to contain more asparagine in the cell wall than the leuconostocs (23). The uncertainties of differentiation between the two genera are very likely the explanation for the claims that strains of leuconostocs can be cultivated in unfortified skim-milk and therefore can be used as single-strain starters producing aroma compounds. Such strains have been considered to be intermediate between *Str. diacetilactis* and leuconostocs (2,3), but it is now clear that the organisms studied were betacocci, particularly *Betococcus cremoris* commonly found in European starters and now called *Leuc. citrovorum* or *Leuc. cremoris* (13).

When it is necessary to distinguish strains within a known species serological or phage typing can be used. Serological typing of the group N streptococci failed (54) because the strains showed such a multiplicity of reactions with rabbit antisera and this has recently been confirmed (Reiter, di Biase & Newbould, unpublished). Infusion of living organisms into the cow's udder, however, gave high concentrations of antibodies in the blood and milk, and it was found possible to divide a number of strains with known phage relationships (55) into four groups by their agglutination reactions. These groups coincided with the phage reactions, as might be expected since phage susceptibility depends on surface antigenic patterns. In this case, therefore, phage typing was not capable of subdividing serological groups as has been found with other genera and species. This is undoubtedly due to the limitations of phage typing with lytic phages. Two successful methods of subdividing serological groups have been devised. Temperate or avirulent phages (capable of lysogenization of the host) are used for typing staphylococci (56,57); and salmonellae are typed by the Vi phage typing scheme (58), which is based on adaptation of phage, now recognized to be a host-induced modification (59,60). Some workers have, in fact, used this adaptation when they were dealing with starter strains against which they had no lytic phages (61,55). However, so far no systematic approach has been made although it has been conclusively demonstrated that host-induced modifications can be produced with streptococci (61–64) (see also section on phage).

It is possible that recent methods of classification, grouping bacteria according to their overall similarity of characteristics (65,66) and utilizing chromatographic analysis of the free amino acid pool and cell wall, may resolve some of the difficulties. In this connexion it may be of interest to cite the very recent findings of Dr A. L. Davison, King's College, University of Durham (personal communication) that *Str. lactis* NCTC 6681 (Orla-Jensen, OJ) contains glycerol teichoic acid in its cell wall, but *Str. cremoris* HP and *Str. diacetilactis* DRC_2 do not. These results rather support the view that *Str. diacetilactis* and *Str. lactis* are distinct species.

On the other hand, for the routine determination of the flora of starters the tests given in Tables 1 and 2, or the selective media referred to in the previous section, are usually sufficient. A great expenditure of effort, collating very large numbers of data and analysing them statistically, may therefore be unrewarding. In any case, in investigating a large number of strains many 'atypical' strains must be accepted. It is indeed possible to find all or almost all of the combinations of properties, for instance, between typical *Str. diacetilactis*, *Str. lactis* and *Str. cremoris* (16).

Table 1. *Differentiation of* Streptococcus lactis, Str. cremoris *and* Str. diacetilactis (159)

	NH_3 from arginine	Growth at 40 °C	Growth with 4 % NaCl	Growth at pH 9·2	Acid from Maltose	Acid from Dextrin	Citritase
Str. lactis	+	+	+	+	+	+	−
Str. cremoris	−	−	−	−	±	− (+)	−
Str. diacetilactis	+	+	+	+	+	+	+

Table 2. *Differentiation of the* Streptococcus lactis *group*, Pediococcus *and* Leuconostoc

	NH_3 from arginine	Reduction of litmus	Gas from glucose	Slime from sucrose	Optical activity of lactic acid	Acid from Maltose	Acid from Dextrin
Streptococcus lactis group	±	+	±	−	Dextro	±	±
Pediococcus	±	−	−	−	Inactive or dextro	+	−
Leuconostoc	−	−	+	±	Laevo	±	±

Genetics

It is generally agreed that the function of the genetic material is to determine the specific chemical structure of enzymes, and that this occurs as a result of information contained in the deoxyribonucleic acid (DNA) of the genes which therefore control the physiological and biochemical properties of the cell. In addition to the normal transfer of DNA when a bacterial cell divides, DNA can be transferred from one cell to another in three different ways—conjugation, tranformation and transduction (67,68).

Conjugation has so far been observed only among Gram-negative organisms (enterobacteriaceae, vibrios, pseudomonads and probably azotobacters). It involves a process whereby sexually compatible strains become temporarily fused and then separate. During this process genetic material is transferred from the 'male' to the 'female' cell.

In transformation, highly polymerized DNA extracts from 'donor' cells can be taken up by intact 'recipient' cells, which acquire some of the hereditary traits that characterize the donor cells. So far there are only a few examples of successful transformation experiments with group N streptococci. Møller-Madsen & Jensen (69) (and unpublished) showed that DNA extracts from *Str. diacetilactis* and *Str. lactis* var. *maltigenes* (donors) transferred the capacity to ferment citrate and produce malty aroma to *Str. lactis*. Sandine, Elliker, Allen & Brown (70) attempted transformation of streptomycin resistance with DNA extracts of a streptomycin resistant strain to 40 strains of lactic streptococci, but failed. They succeeded, however, with the same extracts in transferring streptomycin resistance to a proven competent (transformable) strain of another species of streptococcus.

Transduction can be observed with certain strains of temperate phages which are able to incorporate during maturation part of their host's DNA and transmit it to

another bacterium where it may become incorporated into its nucleus (71). A typical example is the case of *E. coli* strain K. 12 and the phage which is linked closely to, and can thus incorporate, the gene controlling galactose fermentation. So far the only experiments on transduction with starters have been published by Sandine *et al.* (70) and Allen, Sandine & Elliker (72). These workers accomplished transduction of tryptophan independence from a strain of *Str. diacetilactis* and streptomycin resistance from a mutant of *Str. lactis* C_2 to the prototroph strain *Str. lactis* C_2, using a virulent (not temperate!) phage. This, however, appears to involve a contradiction because it seems impossible that a lysed recipient can express any transduced hereditary characteristic. In these experiments *Str. lactis* C_2 was used, which is probably similar to strain C_{10} which has been shown to contain over 10^3 phage-resistant chains/million when maintained under normal cultural conditions (73). Further, it has been found (Reiter & Oram, unpublished) that phage-resistant mutants of *Str. lactis* ML_3 and C_{10} are still capable of absorbing its phage. If it could be shown that DNA can be injected under these circumstances it would explain why a seemingly lytic phage is capable of transduction.

There is little doubt that genetic experiments will provide data of taxonomic importance and will help us to understand more fully the behaviour of the multiple strain starters, because transduction through temperate phages and even transformation through autolysis of cells may take place in nature.

Influence of starters on flavour

Butter

To estimate aroma-producing properties of a starter, a quick and accurate determination of the acetoin and diacetyl content is necessary. A distillation method has been developed (74) which achieves a 99 % yield of diacetyl. The diacetyl (1-250 μg) in the distillate is measured colorometrically, using diaminobenzidinchlorhydrate, with an accuracy of $\pm$ 1·6 %. A later method, involving direct photometric determination of acetoin and diacetyl in a sodium tungstate filtrate of the starter, is suitable for routine determination of acetoin and diacetyl (75). In addition to the method for specific determination of acetoin and diacetyl, gas chromatography can be used for investigating aromatic compounds in starters and dairy products (76, 77).

The production of acetoin and diacetyl with different starter combinations and under different propagation conditions has been studied as well as the influence of these aroma compounds on the quality of butter and buttermilk (2,3,36,42,78–81). These investigations have shown that in order to obtain good flavoured butter and buttermilk the starter should contain aroma producing bacteria. The acetoin and diacetyl content of both the starter and butter can be increased by adding lactose (82), citric acid, pyruvic acid, oxalacetic acid or by decreasing the pH to 5·0 (83) or by addition of manganese (84, 85), but it is not certain that this is always advantageous, for butter with a high diacetyl content is more susceptible to oxidation (42,5).

The acetaldehyde and acetone contents of butter have also been investigated (86) and it has been shown that the property of producing acetaldehyde by decarboxylation of pyruvic acid is a general property of *Str. lactis*, *Str. cremoris* and *Str. diacetilactis*, whereas the production of acetone is associated with individual strains.

A number of workers (42, 85–87) have found a substantial difference in the composition of the aroma components in starters which contain only *Str. diacetilactis* as aroma bacteria and in those which contain *Leuc. cremoris*. In starters which contain only *Str. diacetilactis*, acetaldehyde is always found and this may be the reason for the characteristic 'yoghurt flavour' found in such starters and in the resulting butter. Starters containing *Leuc. cremoris* produce less acetaldehyde and diacetyl and give a butter with improved taste and keeping quality. This appears to be due to a lower oxidation-reduction potential (5, 88) which results in a decreased tendency of the butter to oxidize.

The fault 'malty taste' in butter produced by *Str. lactis* var. *maltigenes* has in recent years been the object of investigations especially in U.S.A. and Scandinavia. At first the formation of acetaldehyde was thought to be responsible, but this has since been disproved (89, 93), and the malty flavour has been shown to be caused by the formation of 3-methylbutanal through transamination and decarboxylation of leucine.

The heat resistance of *Str. lactis* var. *maltigenes* and its high optimum temperature make it necessary to ensure adequate pasteurization of the milk and propagation of the starter at low temperatures in order to reduce its incidence to a low level (94, 95). Under practical conditions a starter containing 3–5 % malty flavour-producing bacteria will always give this taste defect in butter (96). In this connexion it should be noted that variants of *Str. diacetilactis* have been found which, like *Str. lactis* var. *maltigenes*, can produce malty flavour (97, 98) and therefore ought to be called *Str. diacetilactis* var. *maltigenes*.

Whilst older methods (99) were capable of detecting the malty flavour-producing streptococci in starter at a level of 1 part in 10^3 parts or higher a method has been developed based on the greater heat resistance and optimum temperature of *Str. lactis* var. *maltigenes* which, after one transfer of the culture under test, can detect 1 part of *Str. lactis* var. *maltigenes* in 10^{10} parts of normal starter (96). A further method based on chromatography of DNP hydrazones can detect the presence of 0·5 mg 3-methylbutanal in 200 g of starter or butter (99).

It has been shown (5) that the growth of *Leuc. cremoris* in a starter produces substances which reduce the aldehydes producing malty flavour so that in a starter containing 5–10 % *Leuc. cremoris* it is not possible to detect maltiness. A starter may therefore produce a malty flavour which disappears on inoculation into another milk because of a relatively rapid growth of *Leuc. cremoris*. Under normal practice propagation methods will ensure an adequate development of *Leuc. cremoris* in starters and precautionary measures guarding against infection will have an important influence in the controlling of malty flavour in dairy products.

Cheese

The influence of starter on cheese quality will only be mentioned briefly, since it has been adequately dealt with in a review concerning the flavour of Cheddar cheese (100).

During maturation hard cheeses go through a bitter stage which normally disappears later. Many investigations (101–106) have stressed that the limited proteolytic ability of some starter strains may be the reason for the frequently occurring fault in cheese known as 'bitterness'. It was concluded that in the manufacture of Cheddar cheese only those single-strain cultures which are known to produce a cheese without

bitterness should be used, or else mixed strain cultures containing non-bitter producing strains in such a proportion that the fault would be avoided.

The influence of starter on the quality of cottage and semi-soft cheeses has also been investigated (14,38,107–112), and it was found that the problem of floating curd and excessive foaming could be solved by selection of starters that were free from heterofermentative lactic acid bacteria. In the production of Cheddar cheese and semi-hard cheese, strains of leuconostocs have been shown to produce considerable amounts of gas which, depending on the type of cheese, can be either an advantage (113) or disadvantage (114). Other investigations show (115) that *Leuc. dextranicum* and *Leuc. mesenteroides* can produce an abnormally large amount of gas in Swedish cheese whereas *Leuc. cremoris* can not. *Str. diacetilactis* has also been found to be capable of developing gas in cheese (42). Regulating the aroma and flavour in creamed cottage cheese can be achieved by adding 0·2–0·5 % of a citrated whey culture of *Str. diacetilactis* (116).

Propagation

Several factors are concerned in the successful propagation of starters, and two of them, namely inhibitory substances and phage prevention, are treated later in this review.

Composition of milk

The variation frequently observed in the suitability of milk as a medium for starter bacteria has led to research into soluble N-compounds in fresh milk (117–120). It appears that although certain variations may occur in the peptide and peptone content of milk from individual cows, the variation in pooled herd milk is probably too small to explain why starters grow well in some pooled milks and not in others. To overcome variations in milk composition, it has been recommended that the starter should be grown partly or wholly in reconstituted milk powder produced in those months of the year which, through experience, have been found to be most suitable (121–123, 41). It has also been suggested that the milk should be enriched by the addition of easily accessible N-compounds, vitamins or salts, so that a better growth and souring activity can be obtained. (See section on nutrition and metabolism.) It has been suggested (124) that the addition of 0·2 % pancreas-extract to the starter milk could permit the use of freeze-dried culture for the preparation of bulk starter (125). A clear seasonal variation has been found in milk which governs the ratio between *Leuc. cremoris* and streptococci in starters. This variation can be controlled by the addition to the milk of small quantities of manganese, whereby the growth of *Leuc. cremoris* is stimulated (126, 84, 85).

The presence of colostrum, besides having an effect on the firmness of cottage cheese curd (127), also influences the ratio of *Leuc. cremoris* and streptococci in starters (5). Seasonal variations in calving may, therefore, explain the observed variations in the effect of milk on starter growth.

Starters can be protected against phage by removal of the calcium (ion exchange) or by chemical sequestration (see section on bacteriophage in cheesemaking). Some starters, in particular mixed-strain starters, were found to be less active in ion exchanged milk (128–132) than in untreated milk. It was also found that the rate of citric acid fermentation and numbers of *Leuc. cremoris* in mixed starters decreased when

propagated in decalcified or calcium sequestered milk because of the apparent calcium requirements of *Leuc. cremoris* (133). Starter containing *Leuc. cremoris* produces butter with a better quality than one containing only *Str. diacetilactis* and it is therefore recommended that, until more is known of the growth of aroma bacteria in milk with a low calcium content, such milk should not be used when preparing starters for butter manufacture.

Pasteurization of starter milk

The changes caused by pasteurization which affect the suitability of milk as a medium for starter bacteria have been intensively investigated and only a few references will be given here (134–141).

It has been generally found that starter streptococci grow a little better when milk is heated to 60–70 °C for 20–40 min, and even more so when heated to 90–120 °C for 15–60 min, but they are inhibited when heat treatment is greater than 120–130 °C for 10–30 min. Varying results, however, have been found within the temperature range 70–80 °C, some workers having found an inhibition (136) while others (142) found stimulation of growth of some strains of *Str. lactis* and *Str. cremoris*.

The stimulatory effect of the various heat-treatments has been ascribed to partial hydrolysis of the casein (143), liberation of free sulphydryl groups (144) or the formation of formate from lactose (139). The elimination of the heat-labile inhibitors (lactenins) by heat will be discussed in the section on naturally occurring inhibitors. It has also been reported that milk fortified with skim-milk powder to give a total solid content of 11 % and pasteurized in HTST apparatus gives a superior medium for starters (121). A stimulatory factor has been reported to occur in reconstituted skim-milk powder (117): some creameries regularly use skim-milk powder for bulk starter propagation, but when using milk powders the possible cumulative effect of heat on milk must be considered (145). Since cheese milk cannot be heated sufficiently to destroy inhibitors, the maintenance of starter in heat-treated (sterile) milk to which appropriate inhibitory substances have been added may be worth further study.

In investigating the effect of heat treatment on milk used for growing starter bacteria, little interest has been shown in the aroma producing bacteria. It has been found, however, that aroma production by *Leuc. cremoris* is not influenced by heat treatment of the milk (146).

Condition of propagation

The effect on the starter's properties of varying the propagation temperature and quantity of inoculum has been known for many years. During the last ten years investigations have shown how the size of inoculum and propagation temperature can be varied in order to change the ratio between aroma bacteria and acid-producing streptococci in a mixed strain starter. For starters containing *Str. diacetilactis* as the only aroma producer, a small inoculum with a high incubation temperature will relatively increase the *Str. diacetilactis* population (16, 147). When *Leuc. cremoris* is also present, a large inoculum and long incubation at low temperature increases the relative number of *Leuc. cremoris* while a small inoculum, high incubation temperature and immediate subculturing after coagulation will reduce the relative numbers (148). For starters which contain *Leuc. cremoris* as the only aroma bacteria,

a small inoculum and high incubation temperature will reduce their relative numbers (149, 42).

In two-strain cultures composed of *Str. diacetilactis* and *Str. lactis*, *Str. diacetilactis* and *Str. cremoris*, *Leuc. cremoris* and *Str. lactis*, and *Leuc. cremoris* and *Str. cremoris*, *Str. diacetilactis* is reported to show a stronger dominance over *Str. lactis* than over *Str. cremoris*, this being more pronounced at 22 °C than at 20 °C. In cultures with *Leuc. cremoris*, the streptococci dominate (150). (See also section on strain association and compatibility.)

Although intensive research into continuous propagation has been carried out in other fields of bacteriology (151), little work has been done on the continuous culture of dairy organisms. The few reports published (152–156) have been concerned with manufacture of starter culture, ripened cream and cultured milk, and results obtained are encouraging. The use of continuous propagation methods must be presumed to have certain possibilities in the manufacture of cultured milk and in continuous cheese-making processes, especially in connexion with ultra-high-temperature treated milk.

Storage and maintenance of cultures

The storage and maintenance of the bacterial cultures is an important task in the manufacture of consistently good starters. Investigations carried out have, in particular, been concerned with studying the effect of storage for several days at 5 °C on a ripened culture (157–160), deep-freeze storage (161–164) and drying carried out either by spray drying or by freeze drying (163, 165, 166) (see also the review (167)). These investigations have shown that a ripened culture can be satisfactorily stored at about 5 °C for 48 h. Better results and longer periods of storage at 5 °C can be obtained if the culture is newly coagulated or has just been inoculated. In a ripened culture containing *Leuc. cremoris* and streptococci, storage will tend to increase the number of *Leuc. cremoris*. Satisfactory storage for many years of deep-frozen and freeze-dried cultures is possible if acid production is either not particularly strong or is neutralized with fresh skim-milk or with sterile milk powder. Recommended storage temperatures for deep frozen cultures are from -18 to -25 °C and for freeze-dried cultures about 5 °C. Freeze drying is claimed to have a more destructive influence on the bacteria than deep-freeze storage. Storing the cultures in chalk milk, starch broth or on agar slope under paraffin oil is not recommended. The use of deep frozen and freeze-dried cultures may have advantages in decreasing bacterial-virus infection and in maintaining a more stable and constant starter.

Antibiotics

Because of the volume of literature dealing with the influence of antibiotics on the quality of dairy products and the recently published reviews in this field (168–171, 141), it is not proposed to deal with the subject here.

NUTRITION AND METABOLISM

Nutritional and biochemical studies on the lactic acid bacteria have contributed to the understanding of the growth and activities of starters in milk and dairy products. As it is difficult or impossible to remove single nutritional components from a

complex biological fluid such as milk, most of the investigations must be performed in synthetic and semi-synthetic media. Since, however, the enzymic constitution of a cell is influenced by its environment, care must be taken in attempting to relate results obtained in a synthetic medium to its growth and activity in milk.

Considering the complexity of synthetic media necessary to support growth of lactic acid streptococci, it is surprising that so many starter strains grow so well in milk and only a minority grows poorly. The addition of one of a variety of easily available nitrogenous substances to milk has frequently been shown to stimulate starter strains (112, 173–186), but it has not been demonstrated that proteolytic activity and starter activity are correlated. It appears that both proteolytic activity and the capacity to absorb and hydrolyse peptides are necessary before milk proteins can be utilized. A better understanding of the metabolism of starter strains will help to rationalize the selection of strains best suited to the production of particular dairy products.

Milk as a growth medium

Milk may or may not be an ideal medium for starters, but it is the stock medium for their propagation and the substrate in dairy manufacture. The natural inhibitors which are inactivated at laboratory sterilization temperatures but not by pasteurization of the manufacturing milk will be discussed later. We shall now consider how far milk is known to contain all the essential nutrients for active starter growth.

Orla-Jensen (172) clearly showed that milk supplied the essential vitamins and nitrogenous substances for many lactic acid bacteria. Lactic acid production by lactic streptococci could, however, be improved by the addition of extracts from plant and animal tissues, and of protein hydrolysates. This has been amply confirmed and it was also found that slow strains in particular benefited from easily available nitrogen sources such as liver and yeast extracts (173), peptones (174), corn steep liquor (175), pancreas extract and peptide fractions (176–180). McDonald & Husain (181–183) demonstrated that some strains utilized highly purified sodium caseinate as sole source of nitrogen while others needed in addition free amino acids. That starters are capable of hydrolysing protein to varying degrees was shown by Van der Zant & Nelson (184, 185) who isolated an endocellular proteinase and a peptidase and determined the free amino acids and peptides in milk before and after incubation of starter. Recently a particularly heat-stable extracellular proteinase was isolated (186). There is therefore no doubt that lactic acid streptococci hydrolyse protein, but there does not appear to be a relation between proteolysis and rate of lactic acid production (112). This was confirmed when 26 single-strain starters were tested for their proteolytic activity (measured by tyrosine liberation). While there was a broad trend for fast strains to be more proteolytic than slow strains many exceptions occurred. It is of interest, however, that a slow acid-producing variant of a proteolytic starter strain (174) failed to show any appreciable proteolysis (Reiter & Pickering, unpublished).

The addition of individual amino acids to milk (and also purines and pyrimidines) failed to increase appreciably the activity of any starter, but increase in the peptide content of milk (measured by formol titration) or the addition of pancreatic digest of milk increased starter activity, particularly of slow strains (135, 112). This, and other work, indicates that the preformed peptides produced by heating and drying of milk

or proliferation of the contaminant bacterial flora, or the capacity of a starter strain to form peptides from casein may be more important than the presence of free amino acids. Although the presence of both free amino acids and peptides in milk dialysates have been reported (187) this was not confirmed by Deutsch & Samuelsson (119) who found only a wide variety of amino acids. (A synthetic medium based on the proportions of amino acids found was used successfully (26).)

Synthetic media

Media and experimental conditions may influence nutritional requirements or growth antagonisms. Whether a vitamin or amino acid is essential, stimulatory, or not required, depends on the extent of deficiencies or imbalances in the basal medium (188–191, 117).

Vitamins

It has been found that single-strain starters (*Str. lactis*, *Str. diacetilactis* and *Str. cremoris*) require nicotinic acid, pantothenate and biotin (CO_2 and aspartic acid having a sparing effect) and are stimulated by pyridoxal (26). Neither thiamine, folic acid nor vitamin B_{12} are required and riboflavin only by strains of *Str. cremoris*. Isolates from commercial starters (117, 189), however, show a different and less uniform requirement for vitamins. Acetate has been found essential for lactic acid streptococci by some workers (191, 192) but in a more complete medium (26) acetate was only required under increased oxygen tension and was replaceable by α-lipoic or mevalonic acid as in the case of *Str. faecalis* (193–195). The fact that single-strain starters grew well in shallow layers of milk not containing acetate (26) suggests that α-lipoic acid may well occur in milk, although it is also possible that acetic acid is formed during the heating of lactose. Milk contains all the vitamins required, in sufficient concentration, but it may well be that starters can utilize the pyridoxamine phosphate of milk (196) better than pyridoxal as reported for *Str. faecalis* and *L. helveticus* (196, 197). A recent survey of the vitamin requirements of *Str. thermophilus* showed that riboflavin, biotin and calcium pantothenate were essential for all the strains tested; nicotinic acid was essential for most strains and stimulatory to the rest, and thiamine and pyridoxal were stimulatory (198, 199). The vitamin requirements of *Str. thermophilus* thus appear to be similar to those of *Str. cremoris*. Fluctuations in the vitamin content of milk according to season, stage of lactation, etc., occur (200) and it has been suggested that such fluctuations may affect starters (201).

Amino acids

In a medium which generally supported growth adequately, a representative collection of single-strain starters was found by single omission technique to require glutamic acid, valine, methionine, leucine, isoleucine and histidine. Aspartic acid, citrulline and ornithine were not required. In addition to the above amino acids, arginine and phenylalanine were required by some strains of *Str. lactis* and *Str. diacetilactis*. All strains of *Str. cremoris* required proline and phenylalanine, most of them required tyrosine, alanine and lysine, a few required threonine, tryptophan and arginine and one required glycine (26). Another group of workers (189) found tryptophan and arginine essential for both species, and alanine, threonine and phenyl-

alanine to be required by strains of *Str. lactis*. The amino acid requirements of *Str. thermophilus* have been shown (198,199) to be partly dependent on the presence of calcium in the medium. Glutamic acid and cystine (cysteine) were essential for all strains, histidine and tryptophan only for very few. These requirements were independent of calcium, but valine, glycine, threonine, methionine and isoleucine were essential for most strains in a calcium-free medium and inhibitory in the presence of calcium. The effect of aspartic acid, leucine, alanine, tyrosine and tryptophan appeared to vary with the calcium content of the medium.

A comparison of the requirements of *Str. lactis* (and *Str. diacetilactis*) with those of *Str. cremoris* shows that *Str. cremoris* has a more varied and exacting amino acid requirement (besides the additional requirement for riboflavin) and that the known physiological characteristics of the two species correlate with, or are confirmed by, their nutritional requirements. Too little work has been done so far on *Str. thermophilus*, *Leuc. dextranicum* and *Leuc. cremoris* to come to a firm conclusion regarding the taxonomic value of studies of their nutritional requirements. So far, however, it has been found (202) that the leuconostocs could not be differentiated by their amino acid requirements. This lends support to the view expressed in the section on taxonomy and classification that a clear division between the species in this genus has not yet been achieved. The apparent uniformity in the nutritional requirements of the single-strain starters may, however, be due to the length of time they have been cultivated under controlled conditions in the laboratory and of course their selection for a specific purpose in the first place.

Peptides

The requirements for amino acids show that starters have a limited capacity for *de novo* synthesis of amino acids, yet in view of the low amino acid content of fresh milk it is clear that the lactic acid streptococci must obtain most nitrogen from protein. As the lactic acid bacteria have only limited ability to hydrolyse proteins to amino acids, the possibility that they utilize peptides has attracted a great deal of attention (117,135,176–180). The 'strepogenin' of Woolley (203–205) from partial hydrolysates of protein was found to stimulate *L. casei*. Snell and his collaborators (206–209), studying the role of peptides in the nutrition of *L. casei* and *Str. faecalis*, recognized that the requirements for peptides stem from an imbalance among the amino acids in the medium. The excess of one amino acid inhibits the uptake of a structurally related amino acid but not that of peptides containing the related amino acid. These peptides promote growth to a greater extent than an equivalent amount of the essential free amino acid. Besides the antagonism between structurally related amino acids, these workers came to identify other circumstances which would make the peptides more utilizable. For instance, the free amino acid, but not its peptides, may be partially destroyed by reactions competitive to protein synthesis. Thus, when *Str. faecalis* is cultivated under conditions which permit activity of a decarboxylase, tyrosine is partially destroyed by conversion to tyramine and the peptide of tyrosine is then very stimulatory. The utilization of peptides implies their hydrolysis by cellular enzymes (206,209). As a result of this work it is now thought that the growth-promoting ability of peptides is dependent on their selective absorption followed by hydrolysis to essential or stimulatory amino acids.

Detailed studies with purified peptides from pancreas tissue have shown them to be stimulatory to *L. casei* (124,125,176–180) and *Str. lactis* in the presence of 'strepogenin' and recently MacLeod & Gordon (180) have begun to investigate di- and tripeptides hydrolysed by three strains of *Str. lactis* and one of *Str. cremoris*. The active compounds in pancreas extract have been identified as inosine, hypoxanthine and adenine (210). (See also requirements for nucleic acid derivatives (117).)

Amines

Kihara & Snell (211) identified certain polyamines, in particular spermine and spermidine, as growth factors for *L. casei*, and showed that crude pancreatic digests which are particularly rich in these substances gave substantially greater stimulation than digests prepared from purified casein with crystalline trypsin. Subsequently spermine and spermidine and, to a much lesser extent, putrescine and 1,3-propane diamine, were shown to be stimulatory in the presence of purified tryptic digest. (These steam volatile acid-resistant amines have been known for some time to be growth factors for *Haemophilus parainfluenzae* (212) and some other organisms.) These findings may be considered with those showing the presence in milk of rather large amounts of glycerophospho-ethanolamine and phospho-ethanolamine (46 and 83 mg/l) (119). The amines of milk may be either growth factors or for that matter inhibitors, because with increasing concentrations the stimulatory amines become toxic.

Carbohydrate metabolism

It is comparatively recently that reports of detailed investigations on the carbohydrate metabolism of starter organisms began to appear in the dairy literature; comprehensive reviews have now been written in German and English (19,213,214) and this section will therefore be kept short. For a long time it was accepted that heterofermentative organisms produce from glucose equimolar amounts of lactic acid, alcohol or acetic acid and CO_2 while homofermentative organisms convert the glucose molecule into two lactic acid molecules, producing only trace amounts of volatile acids, alcohol, fumarate and CO_2. The homofermentative organisms utilize the well-known Emden–Meyerhof pathway (EMP) for lactic acid production, analogous to muscle glycolysis. On the other hand, heterofermentative organisms do not possess the glycolytic enzymes aldolase and triosephosphate dehydrogenase of the EMP and ferment glucose by the hexose monophosphate shunt (HMP, Fig. 1) (215–218) in which glucose is metabolized via glucose monophosphate and 6-phosphogluconate to give a pentose phosphate, which is then cleaved to give D-glyceraldehyde phosphate and acetylphosphate. The first of these products is oxidized to pyruvate via the usual (EMP) glycolytic intermediates and is finally reduced to lactate. Reduction of acetylphosphate gives acetaldehyde or, on further reduction, ethanol. An alternative mechanism for the fermentation of pentose phosphate involving the enzymes transaldolase and transketolase can result in quantitative conversion of pentose to CO_2.

Gunsalus & Niven (219) were the first to show that at alkaline pH homofermentative organisms can produce, besides lactic acid, up to 40 % formate, acetate and ethanol in the ratio of 2 : 1 : 1. More sophisticated methods revealed that *Str. faecalis* produces small amounts of CO_2, glycerol, diacetyl, acetoin and 2,3-butanediol. Earlier at-

tempts to show incorporation of $^{14}CO_2$ into *Str. lactis* had failed (220) but CO_2 had been shown by several workers (221–224) to be required for initiation of growth and CO_2 produced by the HMP could well be utilized in biosynthetic processes.

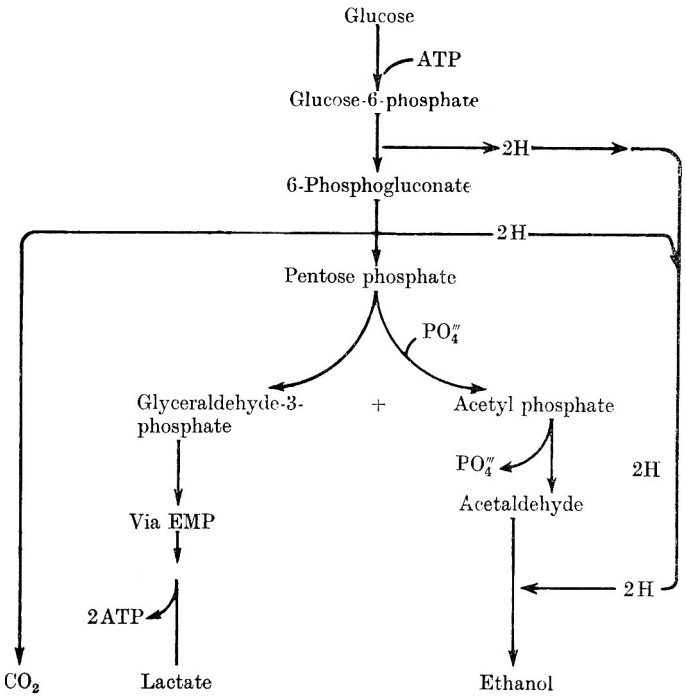

Fig. 1. Hexosemonophosphate pathway (214).

Summing up the modern conception of homo- and hetero-fermentation it appears that the lactic acid bacteria are better classified into three categories, as proposed by Buyze, Hamer & Haar (225):

(*a*) obligate homofermenters possess aldolase, but not glucose-6-phosphate dehydrogenase, nor 6-phosphogluconate dehydrogenase;

(*b*) obligate heterofermenters possess these dehydrogenases but not aldolase;

(*c*) facultative homofermenters possess aldolase and the dehydrogenases but ferment via the EMP.

Direct evidence for the involvement of either the EMP or HMP pathways in starter organisms is limited. Various enzymes of each reaction sequence have been demonstrated in cell-free extracts of one strain of *Str. lactis* by Shahani and his co-workers (226–230). The glycolytic enzymes hexokinase, aldolase and lactic dehydrogenase have been demonstrated by Shahani (personal communication), together with glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the HMP. Six cheesemaking strains (4 strains of *Str. cremoris* and 2 of *Str. lactis*) showed glucose-6-phosphate dehydrogenase activity and in all but one of the strains 6-phospho-gluconate dehydrogenase was present. This strain (HP) is presumably an obligate homofermenter (Oram & Reiter, unpublished).

Recently a comprehensive investigation of the carbohydrate metabolism of a

wide variety of lactic acid bacteria was made by van den Hamer (231) and as this work to our knowledge is only published in Dutch, some of the findings may be of particular interest. Studies with *L. casei* showed that with low glucose concentrations proportionately more glucose is passed along the HMP pathway, and that growing cells metabolize more glucose via this pathway than the glycolytic one. *Str. faecalis* and *L. delbrueckii* produced only a proportion of the total CO_2 (e.g. 20 and 2·5 %) via the HMP shunt, while the heterofermenters *L. fermenti* and *Leuc. mesenteroides* produced the whole of their CO_2 via this pathway. The route by which most of the CO_2 is produced in homofermenters appears to be unknown. It is interesting that Kandler & Busse (personal communication) found that a strain of *Str. lactis* produced 50–100 times as much $^{14}CO_2$ from glucose-1-^{14}C than from glucose-6-^{14}C. This is consistent with the fermentation of glucose via the Entner–Doudoroff pathway (Fig. 2 (214,232,233)) which may be of more quantitative importance in this organism than the HMP shunt. Glucose-6-phosphate and 6-phospho-gluconate are intermediates in both pathways, but in the former, 6-phospho-gluconate is cleaved to pyruvate and glyceraldehyde phosphate. Besides reduction to lactate, pyruvate is also decarboxylated and reduced to ethanol, $^{14}CO_2$ being produced from glucose-1-^{14}C and glucose 4-^{14}C by the Entner–Doudoroff pathway and from glucose-6-^{14}C by the HMP shunt.

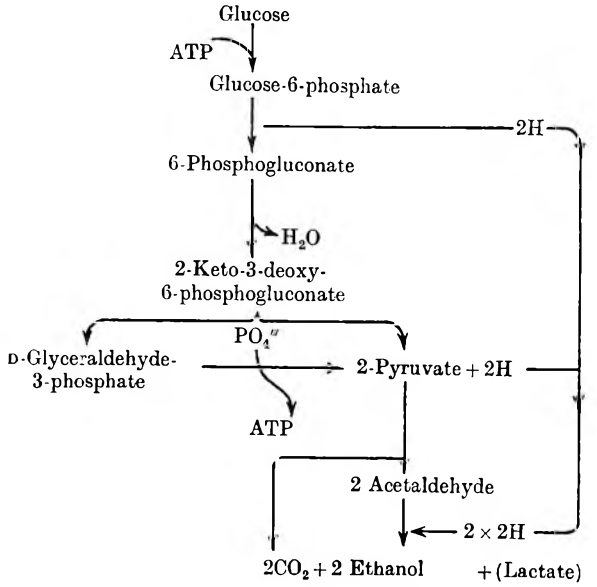

Fig. 2. The Entner–Doudoroff pathway (214).

As glucose is the usual starting material in either type of fermentation, it is of interest to examine the mechanisms involved in its production from lactose. Of the enzymes known to split lactose in other organisms, β-galactosidase, lactase and lactose dehydrogenase appear to be present in cell-free extracts of *Str. lactis* (228,229). The first two enzymes give an equimolar mixture of galactose and glucose from lactose, but lactose dehydrogenase activity results in galactose plus gluconate. The utilization of galactose in *Str. lactis* proceeds via the well known galacto–Waldenase

system (234,235). The further metabolism of gluconate was not studied: presumably if phosphorylated by a gluconate-kinase it would be fermented via the HMP shunt.

In addition to deriving energy from the lactic fermentation, some homofermenters can utilize citrate for this purpose (235–238). The pathway of citrate fermentation is believed to proceed via oxalacetate which is decarboxylated to pyruvate. Pyruvate may then be reduced to lactate or may give rise to acetoin in the usual manner. The reader is referred to the review by Kandler (19) for a detailed account of the processes involved in acetoin production.

Before citrate can be metabolized, it has to be taken up from the medium. Acetoin production by streptococci and leuconostocs occurs only when the pH of the medium is below 6·0 (239). Harvey & Collins (50) have shown that citrate uptake is enzymically mediated by an inducible transport system which is inhibited by 2,4-dinitrophenol and so, presumably, requires ATP for its continued functioning. Citrate utilization has been studied in a number of lactic acid bacteria, including some starter strains by Garvie (personal communication). Utilization of citrate from the medium was not always accompanied by the production of diacetyl and acetoin. Citrate utilization was linked with acetoin production in all strains of *Str. diacetilactis*. However, only 2 of the 6 strains of *Leuc. cremoris* that use citrate produced acetoin. Citrate was also utilized by some strains of *Str. cremoris* and by a few isolated strains of leuconostoc.

Trace metals

Comparatively little work has been done on the metal requirements of starters, probably because of the technically exacting and time-consuming methods involved. As a result of work on ion exchange of milk (240,241) it became clear that potassium and magnesium, as expected (242–244), were required in large amounts and investigations in synthetic media confirmed these findings and showed that magnesium could not be replaced by calcium (Reiter, unpublished). Pulay *et al.* (245) had observed that the sequestration of iron in milk somewhat reduced the lactic acid production of starters; under their conditions cobalt and zinc could replace iron. More recently, iron was found to be an essential trace metal, one strain of *Str. cremoris* (HP) requiring 0·01 ppm and one strain of *Str. lactis* 0·002 ppm. The requirements of another strain of *Str. lactis* (ML_3) were below the contamination level of the oxine treated medium and chromic acid treated glassware and α,α'-dipyridyl had to be added as sequestering agent to obtain no growth in the control. Vanadium (pentavalent), in appreciably higher concentrations, replaced iron. No growth responses were observed with molybdenum, cobalt, manganese, zinc and copper up to 10 ppm. In cell-free extracts, iron was found to activate aldolase, but vanadium showed no effect, which may be due to the inability of the cell-free extracts to convert it to the valency state required for activity (Reiter & Oram, unpublished).

McDonald (246) demonstrated that caseinate-utilizing lactic streptococci were stimulated by acetate and citrate and that these agents in excess or EDTA (disodium ethylenediaminetetra-acetate) caused inhibition. From this work and earlier work (192) he suggested that the response of *Str. lactis* and *Str. cremoris* to citrate and acetate may be related to their calcium and magnesium requirements. A definite calcium requirement has been shown for *L. delbrueckii* and *L. casei* (247) and *Str. thermophilus* (199,200), whereas *Leuc. mesenteroides* and *Str. faecalis* had none. The

uptake of strontium in preference to calcium by strains of lactobacilli has been recently reported, and although calcium was also taken up by the cells it did not appear to be an essential nutrient (248).

Manganese has been shown to have a specific effect on diacetyl and acetoin production by strains of leuconostoc, but not by *Str. diacetilactis*. Feeding cows on a manganese-rich diet resulted in an increased concentration of manganese in the milk and a resultant increased aroma production in butter starters (126, 84, 85). That the variation of concentration of manganese in milk is very wide (4·5–67 μg/l) has recently been shown (249). A review on trace metals in milk by Archibald (250) summarizes our knowledge to date.

NATURAL INHIBITORS OF MILK

The ability of raw milk to inhibit many bacterial species has been known since 1894 (251), but much is still to be learned of the identities and modes of action of the inhibitors. Jones and his co-workers studied the phenomenon of inhibition in detail and published their findings in a series of outstanding papers (252–254). They found that a scarlet fever streptococcus died out in raw milk after incubation for 18–24 h and a non-haemolytic streptococcus isolated from a mastitic udder failed to multiply during the first 5–6 h but then grew normally. They referred to the heat labile inhibitor(s) as lactenin, a term which has since been applied with a wider meaning. Attempts to purify and identify the lactenin by these workers and Wilson & Rosenblum (255) failed. Auclair and his co-workers (256, 257), working with strains of *Str. pyogenes*, *Str. agalactiae*, *Staph. aureus* and *Str. lactis*, suggested that these organisms were inhibited by two heat labile substances present in milk which they called lactenin 1 and lactenin 2. They also confirmed earlier findings (258) that milk contained both inhibitory substances and growth factors influencing bacterial growth.

Wright & Tramer (259), working with starters, assumed that lactenin 1 was probably the agglutinin of the fat globules but the nature of the agglutinin was not further studied. McPhillips and other workers (260–262) later established the strain specificity of the 'agglutinins' and concluded that they were antibodies occurring naturally in milk. Wright & Tramer (263) associated lactenin 2 with lactoperoxidase because of their similar sensitivity to heat, reversion of inhibition by reducing agents, etc. This was later confirmed directly by adding purified lactoperoxidase to heated milk; horseradish peroxidase was found to have no effect (137, 138). Another admittedly incomplete investigation (264) tentatively indicated that cysteine, liberated from milk protein by lactoperoxidase-resistant cells, reversed the inhibition, while susceptible strains failed to release cysteine. It has also been suggested (144) that the lethal effect of raw milk on *Str. pyogenes* was due to a deficiency of free sulphydryl groups or of denatured albumin in the milk.

Jago (265) demonstrated an inhibitory factor associated with the fat globules (not agglutinin), which could be eluted by frequent washing of the fat (266) and which also occurred in small quantities in the skim-milk fraction.

Thus the inhibitory substances of milk are certainly numerous. We shall consider them in greater detail, but to avoid confusion we shall use the term lactenin for the inhibitor observed first by Jones (252-254) and refer to the other inhibitors as agglutinins or antibodies, lactoperoxidase, and inhibitor associated with fat globules.

Lactenin

The tests for lactenin were performed by suspending the organism in raw 'sterile' milk (aseptically drawn or sterilized by ethylene oxide) and counting viable cells at intervals for 24–48 h. If the inocula were kept low (up to 10^5/ml) some species of streptococci died in 18–24 h and others showed delayed growth after a 6–8 h lag phase, but with all species large inocula resulted eventually in growth. Another assay method was to streak the organism on raw milk agar on which no intermediate results were obtained irrespective of size of the inoculum. It was found that raw milk was bactericidal against group A streptococci and some strains of groups F, G, H, K and L, but all the strains of groups B, C, D and E were resistant (255).

The inhibitory effect was only observed under aerobic conditions. Defibrinated whole blood, some peptone preparations, and thiamine at non-physiological concentrations partially or completely reversed the inhibition. The lactenin titre in normal raw milk was found to be between 1:8 and 1:16; there was little lactenin present in the colostrum, none in blood and the titre in the milk could not be increased by immunization of the cow. Lactenin was regarded as derived from the mammary tissue independent of, and unrelated to any bacterial infection. It was not dialysable, resisted trypsin digestion and was inactivated at 80 °C. It was adsorbed by charcoal, but could not be eluted for purification. Some degree of purification was, however, achieved by tryptic digestion of the whey followed by dialysis and concentration.

It had been suggested that the lactenin was an oxidase (259) because heating reduced the bactericidal power of raw milk against strains sensitive to lactenin parallel with its decreased ability to show the *p*-phenylenediamine reaction for oxidase. This was, however, disputed (255) because lactenin was not affected by cyanide, which readily inactivates oxidases. In this context it must also be pointed out that lactoperoxidase is inactivated by cyanide, which proves that lactoperoxidase cannot be identical with the lactenin originally observed (252–255).

Lactoperoxidase

Auclair and his co-workers (256,257) employed a different technique in their investigations of inhibitors in raw milk. The organisms were inoculated (1 % inocula as a rule) into separated milk fortified with glucose peptone broth, incubated for 6–8 h and the developed acidity measured. Later workers (259,137,138) tested starters in unfortified milk and from the shape of the dose-response curves the presence or absence of both inhibitory and stimulatory factors was determined. This technique thus differed markedly from the one previously described, which employed small inocula and in which the number of organisms was estimated.

The suggestion that *Str. cremoris* can be inhibited by lactoperoxidase (263) was confirmed by the finding that purified lactoperoxidase added to heated milk inhibited acid production by *Str. pyogenes*, *Str. agalactiae* and some starter strains. Most starters are, however, resistant (137,138,260–262), but give rise to sensitive mutants (137,138,268,269). These sensitive strains would, of course, be eliminated in raw milk or in milk heated at temperatures which do not inactivate the enzyme (70 °C for 20 min). That starters continuously propagated in steamed milk can become

susceptible to the inhibitors of raw milk had been shown previously (270). This phenomenon underlines the wisdom of cheese-makers who insist on cultivating starters in milk pasteurized at low temperatures similar to those used for cheese milk.

The titre of the inhibitor associated with the lactoperoxidase is of the order 1:100 when measured by diluting heated milk with raw milk and testing against a sensitive strain (263, 137, 138). This is in contrast with titres of 1:8–1:16 found for lactenin (252–255). The original lactenin which permits no growth is therefore very unlikely to be identical with the lactoperoxidase, but the delayed growth observed by the earlier workers when testing for lactenin may have been due to lactoperoxidase.

It was further suggested that lactoperoxidase may act as an inhibitor by forming an inhibitory oxidation product, probably of quinonoid structure, in the presence of hydrogen peroxide formed by starter organisms (263). Jago & Morrison (271) confirmed the role of H_2O_2 because its destruction by horseradish peroxidase or by catalase reversed the inhibition. They failed, however, to find an oxidized product. Recent work (Reiter, Pickering, Oram & Pope, unpublished) has shown that the growth of sensitive organisms and the respiration of resting cells were inhibited by lactoperoxidase in the presence of H_2O_2 and a factor which was present in minute concentrations in milk. The factor was found to be dialysable, heat and acid resistant, and absorbable on anionic exchange resin. This factor has been identified as thiocyanate (SCN^-).

It has been reported that the thiocyanate content of milk varied with season from 1 to 15 μg/ml (272). This substance may originate from thiosulphate formed by enzymes of the liver and kidney and found in urine. The enzyme rhodanese (273, 274) catalyses the 'detoxification' reaction between thiosulphate (or organic thiosulphonates) and cyanide, which is converted to thiocyanate (275, 276).

$$S_2O_3^{--} + CN^- \rightarrow SO_3^{--} + SCN^-.$$

It is perhaps significant that in 10 out of 20 samples of milk weak rhodanese activity was detected (277). Some feeds, e.g. clover, are rich in cyanide, but thiocyanate can be directly derived also from brassicae and raphani as shown by Virtanen and his collaborators (278–281). They isolated and identified two glucosides, glucobrassicin and neoglucobrassicin, which were found to be precursor substances of SCN^- and other physiologically active substances.

It may be of general biological interest that iodine uptake by the thyroid gland can be inhibited by the same system of SCN^-, animal peroxidase and H_2O_2. Milk has also been investigated for its goitrogenic activity due to SCN^- and it appears that in man it is detectable only when the level of iodine intake is very low (282–285). Thiocyanate has also been reported to be the active bactericidal substance in saliva which also contains peroxide, but so far the co-enzyme has not been identified (287). Saliva is known to contain peroxidase and it therefore seems likely that the same system operates in saliva as in milk.

Agglutinin

Agglutinins occur at low titres in raw milk throughout the lactation period but at very high titres in the colostrum (261, 262, 288). The occurrence in milk of these natural non-specific antibodies, agglutinating the non-pathogenic group N streptococci and some lactobacilli, is not surprising if one considers that a great number of the

antibodies in the blood need not necessarily arise as the result of specific infections of the animal. They can be due to other bacterial species which may share the same antigens, or they can also be formed against bacterial antigens absorbed from the intestinal canal, through the lungs, or skin (289). These antibodies tend to be present in low titre, to be difficult to remove by adsorption and to persist for long periods, just as appears to be the case with the milk antibodies (agglutinins). *Str. bovis*, a species present in large numbers in the bovine rumen, is known to share a type antigen with *Str. cremoris* (290). Thus common rumen and gut bacteria may well be the source of antigens which result in the production of antibodies active also against the lactic acid streptococci.

Agglutinable (sensitive) streptococci were observed to be carried by the rising fat globules into the cream layer (259–262, 266, 291) where it is assumed they produce little lactic acid owing to the lack of nutrients and increased O_2 tension. Non-agglutinable strains present in the milk are inhibited like sensitive strains after addition of specific rabbit antiserum. The physical removal and subsequent 'starvation' is, however, unlikely to be the only reason for inhibition. Some starters were inhibited when creaming was prevented by stirring, or when grown in skim-milk or whey in the presence of antibodies (266). It may well be that the production of antibodies and an inhibitory substance are associated in some as yet unknown way.

In this connexion the original observation of Bordet (292) in 1897 that cocci form long chains in the presence of antibodies may be pertinent. Lactic acid streptococci which appear normally as diplococci or short chains in heated milk have been observed to form long chains in raw milk (293). This is probably due to the presence of antibodies. Recent findings (294, 295) have shown that long chain formation, in the case of the group A streptococci, is due to the combination of antibody with the M protein of the cell wall. This results in the inhibition of chain fragmentation which normally takes place in the early growth phase and which is caused by an enzyme or a group of enzymes. Increased chain length due to antibodies in raw milk may well assist agglutination, but it is improbable that it can have any effect on the metabolism of the cells. Chain fragmentation can also be inhibited by the addition of surface active agents like sodium dodecyl sulphate (Teepol) or suramin (296, 297).

Inhibitor associated with fat

Agglutinated organisms and also individual chains have been observed to aggregate around the fat globules (266). The mode of adsorption and the forces involved must be of some interest, as the hypothesis of starvation of bacteria in the cream layer is not fully convincing. The question arises how the inhibitory substances associated with the fat globules and also found in the skim-milk fraction (266, 288) are associated with the inhibition of starters. Furthermore, the interrelationship between antibodies, inhibitors associated with the fat globules and those of the skim-milk fraction need further elucidation. In a very recent paper Stadhouders (298) confirmed that a small part of the inhibitory principle in raw milk is associated with the fat globules and the greater part is present in the skim-milk fraction. He found that the fractions of euglobulin, pseudo-globulin and β-lactoglobulin agglutinated a strain of *Str. cremoris* but only the euglobulin fraction showed any inhibition of acid development.

Natural inhibitors of milk—conclusions

It can be concluded that the bactericidal activity of milk originally described does not appear to be identical with the inhibition associated with lactoperoxidase. Delay in growth associated with the presence of lactoperoxidase seems to be due to an inhibitory system consisting of lactoperoxidase, H_2O_2 and thiocyanate, in which thiocyanate is oxidized to an intermediate product, the nature of which is not yet known. Agglutinins or antibodies present in raw milk have an inhibitory effect either by removing the organisms into the upper cream layer, which may cause 'starvation' of the cells, or by the physical aggregation of the organisms around the fat globules which may permit the action of an inhibitor associated with the fat globules. The nature of the inhibitor and that of the factor associated with the skim-milk fraction is not known. It will be necessary to isolate, purify and identify each of the inhibitors before it is possible to elucidate their modes of action.

In practice, the agglutinins associated with inhibition do not concern cheesemaking as, of course, the rennetting of the milk prevents the 'creaming up'. Lactoperoxidase sensitive starter strains are not as rare as first believed, but still rare enough not to be a major source of starter inhibition.

BACTERIOPHAGE IN CHEESEMAKING

The role of bacteriophage in cheesemaking has been repeatedly reviewed (299–302), and we shall therefore give only a general appreciation of the problem and treat a few specific aspects in detail.

Phage in relation to single- and multiple-strain starters

The introduction of single-strain starters for Cheddar cheesemaking created ideal conditions for the proliferation of phage. The first paper on 'the occurrence of bacteriophage in cultures of lactic streptococci' by Whitehead & Cox (303) clearly stated two objects of further research: 'From a practical point of view it is necessary to find some method of eliminating the phage or of using an organism immune to its action. The isolation of phage immune varieties seems to offer the greater promise of success...' Alas, only the first objective has been achieved, while relatively few workers have attempted to investigate phage-immune strains.

Aseptic propagation of single-strain starters has largely eliminated the hazards of cheesemaking associated with phage. The techniques used have now reached a high degree of sophistication. They include: the use of syringes for inoculating laboratory and intermediate cultures; the use of water-sealed or totally immersed bulk starter cans; separate starter rooms kept under positive pressure with filtered or sterilized air; aerosol disinfection of the air in the laboratory and cheese room; and even continuous exchange of the air in the factory (299, 304–308). Protection of the bulk starter against phage contamination can also be achieved either by removing the calcium from the starter milk by ion exchange or by sequestering it chemically (241, 128–132, 309–315), since phages of the lactic streptococci and lactobacilli require calcium for multiplication (316–321). In general, it appears that the former method is superior to the latter in suppressing phage but more starters show lowered activity in the ion exchanged milk.

The production of phage-free bulk starter was, and remains, the chief weapon

against slowness in the vat. Nevertheless, it was soon found that whey with high phage titres could spread so much phage in the air of the creamery and thus infect cheese milk and utensils, that further protective measures were necessary. Anderson & Meanwell (322, 323) first suggested a rotation of phage-unrelated starters, and this is now a well-established practice for controlling build-up of specific phages in the creamery (324–328). Whenever possible the length of the latent period and burst size of the phage (number of phage particles released per cell) ought to be considered (329). Hunter (330) found that rennetted milk could support the growth of starter in spite of phage infection, and from his observations the technique developed of adding the rennet practically simultaneously with the phage-free bulk starter (330–334). This elimination of the ripening period, together with rotation of phage-unrelated starter strains, has greatly contributed to the elimination of slowness caused by starter failure. Unfortunately some types of cheese, for example Cheshire, require very rapid acid development (3 h as against $4\frac{1}{2}$–6 h in Cheddar cheesemaking) from setting to milling, which is nearly impossible without a ripening period. Selection of starters which are only affected by phage races with a long latent period (up to 90 min) could theoretically overcome this.

When it was first shown (322, 324) that multiple-strain starters can be affected by phage, many British creameries adopted the same protective measures which had been developed for the protection of single-strain starters. Nevertheless, many cheese factories in Great Britain and practically all the continental factories maintain their starters successfully without taking any or only few precautions against phage. There are several reasons for this. Continental cheeses do not rely on extensive acid production in the cheese curd before hooping, acidity developing slowly in the unsalted curd during draining and pressing; a delay in acid production would therefore only become apparent if complete lysis of the starter occurred. This is unlikely to happen with genuine multiple-strain starters because a phage-lysed strain would be replaced by phage-unrelated or phage-resistant strains (335–339). Cheddar and Cheshire cheesemaking requires fast lactic acid production in the vat, and severe difficulties in making as well as in running the creamery are experienced if phage prevents the starter from producing lactic acid at the required rate. The scale of cheesemaking (up to 100 000 lb of cheese per day per factory), centrifugal separation of fat from whey (which can spread droplets of whey with phage titres up to 10^{10}/ml) or whey drying (phage survives in spray-dried whey (324)) help to aggravate the phage problem.

There is little doubt that scarcity of continental starters during the war years contributed to the high incidence of phage outbreaks in Great Britain. In more recent years slowness due to starter failure has notably diminished, partly because mixed starters from various sources have been freely available and partly because of the absence of a ripening period. Up to four phage cycles can take place in 2 h (which is about the longest ripening period used), raising the titre of any phage present in the milk to high levels.

Absence of starter trouble does not mean that no phage is present in a creamery. Multiple-strain starters have frequently been found to be contaminated with phage (339). A recent survey in U.S.A. showed that 74 % of samples of cultures and whey taken in factories in which production was normal were infected with phage, while 93 % of samples from factories in which starter failures regularly occurred were

infected (340). In another survey in Scotland (341) 18 of 23 active acid-producing mixed starters used regularly in 7 factories were found to be infected. Three multiple-strain starters carried phage against other multiple-strain starters, and 9 of 15 single-strain starters were lysed in 15 instances by phages present in the multiple-strain starters.

To this it must be added that factories which normally protect their starters from phage infection often employ methods which are far from perfect. The senior author had occasion to use a starter room, separated from the main factory, which was being used successfully for the preparation of mixed-strain starters but was found to be completely inadequate for the protection of a single-strain starter. After making cheese with the single-strain starter for 2 days, its homologous phage was detected in the air of the starter room. Another indication of the inadequacy of the protective methods is found in a report of a survey on contamination of starters used in cheese factories. Out of 16 starters from different creameries 7 contained lactobacilli, but of 29 mixed-strain starters kept in the laboratory none was found to be contaminated. As lactobacilli are invariably found in the air of cheese factories (342), one can assume that if a factory cannot even prevent bacterial contamination phage infection must surely take place frequently.

Incomplete measures of protection can be potentially more dangerous than absence of protection. Bulk starters are usually incubated for 16–18 h. Therefore, with an infection which is heavy enough to cause lysis, there can be sufficient time for secondary growth and cheesemaking may proceed normally. With a light infection lysis may be delayed, and if the susceptible phage-infected strain is being relied on for a major part of acid production slowness during cheesemaking will occur.

A practical example of the effect of a phage infection on multiple-strain starters is cited by Crawford & Galloway (341). A multiple-strain starter was supplied by their laboratory to a cheese factory, where it was used regularly for 4 months. Aseptic precautions were not observed; part of the bulk starter was simply set aside in milk cans, and used as inoculum. When a new starter was supplied by the laboratory, it failed completely. It was subsequently found that the factory starter now carried a phage against the same starter propagated in the laboratory.

This brings to mind the danger of the common practice of changing starters indiscriminately ('rotation') and borrowing from neighbouring factories without adequate laboratory control. In the absence of knowledge of the phage relationships, which involves continual checking for cross-phaging, a rotation or the introduction of new starters can sometimes do more harm than good. A better practice, perhaps, is to use the same starter continuously until the phage titre of the whey reaches about 10^6/ml, at which stage there is a risk of contamination of the cheese milk giving slowness in the vat even if the bulk starter is free from phage.

There are two ways in which a culture can be contaminated with phage and not be affected by it. Hunter (343) infected a pure strain with phage and found that after lysis the resistant organism regularly clotted milk, but even after many months of propagation he could still detect in the culture phage active against the original susceptible strain. When such a phage-carrying culture was plated, the isolated colonies showed no phage after propagation. That phage-carrying cultures can be freed from phage by colony re-isolation (or by exposure to phage antiserum) was reported as long ago as 1921 (344, 345). Provided infected cultures are propagated in liquid

medium a population equilibrium between resistant and sensitive cells arising by mutation will exist, and so the phage will not die out.

Temperate or avirulent phages are capable of setting up a lysogenic response in a fraction of a bacterial population. This response is not due to selection of pre-existing mutants either of the phage or the bacterium. This is in contrast to the virulent phages which lyse all the susceptible cells, thus selecting the pre-existing phage-resistant mutants. In the case of truly lysogenic organisms, each cell carries phage in the non-infective or prophage state in its nucleus and transmits it to its progeny. Each cell can potentially produce infective phage particles after induction by treatment with UV or X-rays or chemical agents, but even without induction a small proportion of cells of the order of 1 in 10^6 releases spontaneously fully infective phage particles. These are unable to attack the lysogenic cells, but destroy by lysis any susceptible mutants which have arisen in the culture (346), and can be assayed only by the use of susceptible 'indicator' strains. Furthermore, the lysogenic cell is not only immune to the phage it carries as prophage, but also against serologically related phage races. Lysogenic strains are known to occur amongst all genera of bacteria and have also been reported for lactic streptococci (317,347,348). In only one instance has lysogenization of a starter been successfully performed in the laboratory (Czulak & Naylor (348)). Undoubtedly lysogenic strains constitute the reservoir of phage in nature, as was recognized by early workers in the field (349).

The evidence available seems to show that multiple-strain starters consist of lysogenic or phage-carrying strains or both. Single-strain starters are artifacts susceptible to lytic phages and are in fact indicator strains requiring, therefore, the utmost protection.

Are multiple-strain starters therefore preferable to single-strain starters? Not necessarily so. We now have means of maintaining single-strain starters in use for long periods, as has been shown in several trials (132,309); this makes possible the selection of a strain for a particular type of cheese, which would help the cheesemaker enormously. Can we rely on multiple-strain starters to remain trouble free? Certainly not. No large-scale fermentation industry, and cheesemaking is basically a fermentation process, should let its culture change its characteristics by chance. The ideal starter would be a stable mixture of lysogenic strains (see section on association and compatibility). Lysogenic or phage-resistant single-strain starters have been tried in practice (343,348) but not enough information is as yet available. This field is a challenge to the research worker.

Calcium requirements

There are frequent references to the role of calcium in phage infection which need comment. Calcium is not required for adsorption; monovalent cations are just as effective and even in the complete absence of calcium it has never been found possible to elute phage particles after adsorption, which can therefore be regarded as irreversible. It was generally believed that calcium was required for the invasion process and injection of the DNA of the phage into the host, as was postulated for T_5 coliphage (350–352) and later for streptococcal phages (320). More recent work on coliphage T_5 by Lanny (353–355) throws a rather different light on the role of calcium. She distinguishes between two stages in the invasion process. During stage 1, phage

particles can be recovered after treatment of the culture in a Waring blender, but once stage 2 has been reached this is no longer possible. The process leading to stage 2 is called stabilization and depends for its completion on the presence of Ca. The process of DNA injection is therefore now thought to be independent of Ca.

Most of our basic assumptions on streptoccal phage have been taken from work on coliphages. Differences have, however, already been demonstrated. For instance, with T_5 coliphage calcium may be replaced by magnesium, strontium and barium (350,351) but with streptococcal phages also by manganese (320).

Phage adaptation or host-induced modification

A phenotypic modification of the properties of an organism implies a non-heritable change, usually attributable to some environmental influence. Occasionally a phage can infect and multiply in a formerly non-susceptible host if it is present in high concentration. This is called host-induced modification and is reversible. This adaptation of phages is the basis of the technique of Vi typing of staphylococci (58–60). Nichols & Hoyle (61) and Whitehead & Bush (55) used this method for typing starter strains against which they had no homologous phages. It may well be that this method could be further exploited for the typing of starter strains.

Phage lysin

Bronfenbrenner & Muckenfuss (356) recognized that phage lysates contain a 'ferment-like' substance other than phage. Recently, interest in the free enzyme of phage lysates and the enzyme contained in the tail of phages has been revived. Naylor & Czulak (348) and Murphy (357,358) found that 'halo formation' around plaques of certain phages was due to an enzyme which lysed the surrounding cells. There is no doubt that this enzyme is also responsible for the phenomenon of 'lysis from without' (359) (a cell infected by phage at high multiplicity lyses spontaneously without liberation of new phage progeny), but this has never been observed with streptococcal phages. Also the 'nascent phage' phenomenon (360–362), whereby an organism which is not affected by a phage when grown in pure culture is lysed when grown together with a strain susceptible to the phage, can be similarly explained. Phage lysin or lysozyme, the free enzyme contained in high titre-phage lysates, has been purified recently (363). It lyses viable cells of all group N streptococci and also those of the closely related group D streptococci, and its production is under genetic control of the phage. Lysis by the enzyme is prevented, however, in the presence of the homologous phage, enzyme and phage apparently competing for the same or adjacent sites on the cell wall. Lysis by phage lysin is also prevented by suramin, which has been shown to prevent phage adsorption (364).

Inhibition of a streptococcal phage by streptomycin

Brock (365) has recently shown that a streptomycin-resistant *Escherichia coli* was not affected by its homologous phage in the presence of streptomycin. This work has now been extended (366) to streptomycin-resistant streptococci (*Str. faecium*, *Str. faecalis*, *Str. liquefaciens* and *Str. zymogenes*). It is suggested that streptomycin, if present before and during adsorption, inhibits the injection of the phage DNA and, if present shortly after adsorption, inactivates the phage genome (genetic material).

Once a phage genome is established in the host the antibiotic has no effect on the multiplication of the phage. As group D streptococci have so many characteristics in common with group N streptococci, it is likely that this antibiotic has the same antiphage activity for group N. It is intriguing to speculate whether a streptomycin-resistant single-strain starter, at least as laboratory and intermediate cultures, could be protected against phage by streptomycin without affecting its activity.

STRAIN ASSOCIATION AND COMPATIBILITY

Our knowledge of the living processes in the microbial cell is almost exclusively based on pure culture studies and therefore, as yet, we know little about the factors which control the balance between different species or different strains of one species in mixed culture. It is common experience that the mixing of strains of *Str. thermophilus* and *L. bulgaricus*, *L. yoghurti* or *L. helveticus* does not necessarily result in a stable yoghurt starter. Likewise, the simple mixing of fresh isolates from a commercial mixed starter rarely leads to a mixture with the characteristics of the original starter. In an established mixture the proportions of the different organisms can be altered by changes in the incubation temperature, length of incubation or size of inoculum (see section on propagation), but it is difficult to establish a mixture with specified characteristics which remain stable.

The mixing of several strains of lactic acid streptococci invariably resulted in the emergence of a dominant strain after a varying number of propagations (367, 368). This was generally thought to be due to the production of inhibitory substances by some lactic streptococci. Milks containing such organisms, or their inhibitory products, were called 'slow acid' or 'non-acid' milks (369–371). Mattick & Hirsch (372, 373) and Oxford (374) isolated and purified from such inhibitory strains the antibiotics nisin and diplococcin. A strain may also become dominant in a culture through contamination with residual antibiotics used in therapeutic treatment of the cow, or with bacteriophage derived from outside, or through liberation of phage from lysogenic organisms.

Nevertheless, Czulak & Hammond (375) established some stable mixtures and after excluding all inhibitory strains found that blends of *Str. cremoris* with *Str. lactis* or *Str. diacetilactis* were more stable than blends of strains of *Str. cremoris*. This was not confirmed by Lightbody & Meanwell (376), who found that *Str. cremoris* always became dominant. They succeeded, however, in maintaining in approximately equal proportions two pairs of *Str. cremoris* strains and one pair of *Str. lactis* strains. These workers concluded that the biochemical reactions of individual bacterial components do not necessarily determine the final balance of organisms in a mixture. Also a number of organisms, e.g. *B. subtilis*, *E. coli*, *Ps. fluorescens*, micrococci (377–381) and more recently streptomyces (Reiter, unpublished) assist starter growth in milk by their proteolytic action on casein. These organisms occur frequently as starter contaminants and as components of milk flora. In the case of yoghurt starter it was found that *Str. thermophilus* stimulated *L. bulgaricus* by the liberation of amino acids (in particular valine), and also by lowering the pH and removing residual oxygen (382). Recently, it was observed that a fast acid-producing starter strain was markedly stimulated by a filtrate of a milk culture of a slower strain. The active compound was dialysable and could be adsorbed to Amberlite IRC (H^+) resin and eluted with

aqueous ammonia. Acid hydrolysis destroyed part of the activity, but the active compound has not been identified (383). It was also found that extracts of autolysates of *Str. lactis* and *Str. cremoris* stimulated the growth of *L. casei* and *Betacoccus cremoris*. Very little stimulation was observed when *Str. lactis* and *Str. cremoris* were grown in a medium enriched by their own cell extracts. The chemical nature of the stimulatory substance is unknown (384), but in another investigation the identity of the active compound in a lysate could be inferred. *L. bifidus* var. *Pennsylvania* only grows in synthetic medium in the presence of small amounts of human milk. The growth factor has been purified from the oligo- and polysaccharide fractions of human milk and found to consist of glucosamines such as *N*-acetylglucosamine (385). A lysate of *M. lysodeicticus*, produced with egg-white lysozyme, successfully replaced the human milk growth factor (Reiter, unpublished). (Incidentally, the concentration of this factor in cow's milk is only one-fiftieth of that in human milk.) It is now known that the simplest low molecular weight product liberated from cell walls of *M. lysodeicticus* by lysozyme is a substance with the properties of disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid (386), a cell-wall constituent which apparently *L. bifidus* var. *Pennsylvania* is unable to synthesize.

Another way in which one organism may contribute to the growth of another in mixed culture was reported by Møller-Madsen & Jensen (387). They showed that *Str. lactis* and *Str. cremoris* produced folinic acid, which does not occur in milk. It is therefore possible that *Pediococcus cerevisiae*, which is unable to grow in milk by itself, can be cultivated in milk together with *Str. lactis* or *Str. cremoris*. High numbers of pediococci, most of which require folinic acid, are found in mature cheese and it has been suggested (388,389) that these organisms play a role in the development of flavour in cheese. It would be interesting to know whether the pediococci depend on starter or other bacteria to provide folinic acid.

Nurmikko (390,391) realized that milk would be unsuitable for symbiotic studies and based his work on synthetic media. He found that *L. arabinosus* which requires phenylalanine, and *Str. faecalis* R (now known to be *Str. faecium*) which requires folic acid, failed to grow in pure cultures when these compounds were omitted from the medium, but grew well in symbiosis. What is more interesting is that a ratio of approximately 1 rod: 5 cocci was maintained whatever the initial ratio in the inoculum. The balance of these species was therefore maintained by the limiting factors secreted by the organisms themselves. When serine was omitted as well, the two organisms grew at a different ratio of rods to cocci—approximately 1:5 in the beginning of the symbiotic growth, 2:3 in the middle of the growth and 2:1 at the end of the growth. This was surprising, because neither of the organisms required serine for growth in the complete medium and it was to be expected that the omission of serine would leave the ratio unchanged. The folic acid requirement of *Str. faecalis* is, however, greatly increased in the absence of serine. It seemed likely, therefore, that *L. arabinosus* was unable to supply sufficient folic acid to meet the increased requirement of the streptococcus. A later paper (392) describes a dialysis cell with 6 compartments containing *Str. lactis*, *Str. faecalis* R, *Leuc. citrovorum*, *Leuc. mesenteroides*, *L. arabinosus* and *L. fermenti*—with folic acid, folinic acid and phenylalanine as limiting factors, permitting growth of all 6 strains in symbiosis.

Stability of mixed starters thus seems to depend on several types of interaction.

With the present stage of knowledge, the greatest stability seems to occur when each strain in the mixture is dependent on the others for at least one essential growth factor. Further work in this field is obviously required.

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