THE MINERAL EQUILIBRIUM AND PROTEIN STABILITY IN MILK

A Thesis submitted to the University of Glasgow for the Degree of Doctor of Philosophy in the Faculty of Science

by

DAVID THOMAS DAVIES

August, 1957

The Hannah Dairy Research Institute, Kirkhill, Ayr.

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ACKNOWLEDGEMENT

I am very grateful for the opportunity and the facilities given to me to do this work by the Council and Director of the Hannah Dairy Research Institute. I thank especially Dr J.C.D. White for his help, guidance and encouragement throughout the study and Dr R. Waite for his advice and criticism. I also thank Dr P.S. Blackburn for the bacteriological and cell-count examination of samples and other members of the Institute Staff for assistance with the chemical analyses.

CONTENTS

Synopsis

The object of the work described in this thesis was to relate the colloidal stability of the calcium caseinate-calcium phosphate complex in milk to the chemical composition of the milk. The stability of the complex was measured in three ways: (1) by determining the strength of ethanol which, when added to an equal volume of milk, caused coagulation; (2) by determining the time required for coagulation of milk by rennet; (3) by determining the times taken by milk to coagulate when heated at 130, 140 and 150°C. The 132 milk samples examined were taken from herd bulk milk, from individual cows in different stages of lactation and also from cows with sub-clinical mastitis.

Part 1 gives a description of the milk samples and of the methods used to measure stability and to make a detailed chemical analysis of the samples. The composition of the milk samples is also given together with the changes in composition during lactation and the relationships between the concentrations of different constituents in milk.

Part 2 deals with the coagulation of milk protein by ethanol. Samples of herd bulk milk varied little in stability to ethanol but those from individual cows were coagulated by ethanol solutions ranging in strength from 66 to 90% v/v. Early lactation milk, especially colostrum was very unstable to ethanol. After the early lactation period, stability to ethanol

became greater but apart from that it was unrelated to the stage of lactation of the cow. The concentration of ionized calcium in milk appeared to be the major factor governing the strength of ethanol required to coagulate the casein complex; as the concentration of this constituent increased, the strength of ethanol causing coagulation decreased. About 60% of the variation in stability could be attributed to the variation in concentration of ionized calcium.

Part 3 describes the variation in renneting time of the samples and the relationship between renneting time and milk composition. The herd bulk milks showed only little variation in renneting time but the renneting times of the samples from individual cows varied greatly and were related to stage of lactation. In early lactation renneting times were short but they increased as lactation advanced; the increase was gradual in the mid-lactation period but became more marked towards the end of lactation. Renneting time appeared to be largely dependent on pH; as the pH of milk increased, renneting time increased in a curvilinear manner. Although milk containing a high concentration of ionized calcium coagulated quickly with rennet, milk containing average or less than average amounts of this constituent had a wide range of renneting times. The influence of calcium ion concentration on renneting time was thus secondary

to the influence of pH.

Part 4 deals with the coagulation of milk protein by heat. Within the temperature range 130 - 150°C a 10°C rise in temperature caused a threefold increase in rate of coagulation. Coagulation times of herd bulk milk varied appreciably and those of milks from individual cows varied greatly. Stage of lactation appeared to have little effect on the stability of milk to heat except for a marked instability in colostrum and a slight tendency for increased stability in late lactation milk. The instability of colostrum could be attributed to its high content of lactalbumin plus lactoglobulin but little relationship could be established between stability and chemical composition in all other samples. Coagulation times were largely independent of such factors as concentration of ionized calcium, ratio of colloidal calcium phosphate to casein and the ratio of soluble calcium plus magnesium to soluble phosphate plus citrate, all of which have been regarded as important in relation to heat stability. The results suggested that some undetermined physical or chemical property of the caseinate complex determined its stability to heat.

General Introduction

The stability of milk protein may be defined as the ability of the casein to remain in colloidal suspension and of the lactalbumin and lactoglobulin to remain in solution when milk is subjected to heat or other modifying influences. A study of the many aspects of protein stability, in addition to being of purely chemical interest, is also of practical importance. The successful manufacture of evaporated milk, condensed milk and cheese for example, is largely dependent on the behaviour of the milk proteins, especially casein. This is clearly shown by a brief consideration of some of the defects arising during the manufacture and storage of these products.

Probably the most common defect is the excessive coagulation of protein which sometimes occurs during the sterilization of evaporated milk. The coagulation is normally only incipient or limited and the resultant increase in viscosity just sufficient to prevent separation of fat during a reasonable period of storage. However, when the protein is unstable to heat, an occurrence reported to be seasonal in incidence, a hard curd forms which cannot be dispersed by shaking. Such a product is unmarketable. The problem of the age-thickening of sweetened evaporated milk (condensed milk) during storage is another example of a defect related to protein stability. For some unknown reason, changes occasionally occur in the protein which cause such an

increase in viscosity that the product can scarcely be poured from the tin.

Whereas the aim of manufacturers of evaporated and condensed milk is to make a product of the correct consistency with the minimum alteration of the protein, the efficient manufacture of cheese necessitates as complete a conversion as possible of the dispersed casein and fat to a suitably firm curd or coagulum. Many of the difficulties of cheese-making arise from a retarded or incomplete coagulation of the casein by rennet. The curd under these conditions is usually soft, contains large quantities of trapped whey and yields a product which is generally poor in flavour, texture and keeping quality.

The stability to heat of the protein in unconcentrated milk is also becoming an increasingly important factor in milk processing because of the introduction of very high temperature-short time heating to obtain sterile milk with an unimpaired flavour.

Although empirical methods of overcoming some of the difficulties in the processing of milk have been devised the underlying causes of the defects are far from being completely understood. That this should be so despite intensive research for many years is not surprising in view of the variability in the composition of milk and the complex nature of some of its constituents. Nevertheless, sufficient is now

known about casein, as it exists in milk, to enable theories to be put forward concerning its behaviour when subjected to heat, rennin and other coagulating agents. The exact role of lactalbumin and lactoglobulin in the coagulation of milk is not known but apart from colostrum, whose content of these proteins is very high, the stability of the major protein casein is thought to determine the coagulability of milk.

The present view is that the calcium caseinate in milk, or more correctly the mixture of α-, β- and γ-caseinates, is combined or associated with varying amounts of inorganic material, which is thought to be mainly tricalcium phosphate. This calcium caseinate-calcium phosphate complex or mixture is colloidally dispersed as globular micelles with diameters ranging from a few mμ to 800 mμ but with the most common diameter being about 100 mμ. According to the general theories of colloid stability, such a protein dispersion exists by virtue of the hydration of the particles and the net electrical charge they carry. A progressive decrease in either the degree of hydration or the net electrical charge causes the particles to aggregate, increase in size and eventually to appear as clots of coagulated protein. It seems probable that these changes are always the prerequisite of coagulation whatever the coagulating agent. The coagulation of casein during the processing of milk, however, is also accompanied by other changes, in

that the structure of the casein is altered before coagulation occurs. For example, coagulation of casein by heat is preceded by a certain amount of dephosphorylation, while prior to coagulation by rennin, the casein undergoes a small amount of proteolysis.

It is well known that the protein in different milks shows great variation in stability to heat, to rennin and to a protein precipitant such as ethanol, and many efforts have been made to relate the composition of milk to the stability of the protein but with only limited success. However, some evidence has been obtained that the mineral composition of milk, particularly the relative amounts of certain cations and anions, is important in controlling the stability of the casein. More recent work has suggested that the concentration of ionized calcium in milk and the amount of colloidal calcium phosphate associated with the casein may be connected with protein stability but the results are far from conclusive.

The object of the work to be described was to determine the stability of the casein complex in milk by a number of methods and by making at the same time a detailed chemical analysis of the milk, to endeavour to correlate stability and chemical composition. The ease with which the casein coagulated on addition of ethanol and rennet and on the application of heat were used as measures of its stability. These three coagulating agents were chosen since the reaction of

milk to them is of considerable practical importance. Since each involves somewhat different chemical reactions during the denaturation of the caseinate complex before coagulation, a separate section, which has its own survey of literature, has been devoted to each. Samples of raw, skim milk were used and to ensure a wide range of stability in the samples they were taken from herd bulk milk, from individual cows in different stages of lactation and also from cows with sub-clinical mastitis.

Table 1 Monthly averages for the number of lactating cows, volume of milk at a.m. milking and average stage of lactation of the herd

Month	Lactating cows	Volume of milk at a.m. milking (gal.)	Stage of lactation of herd (days)
January	32	57	99
February	33	47	113
March	48	76	120
April	48	71	139
May	52	77	152
June	50	67	176
July	38	39	205
August	29	30	206
September	26	25	186
October	31	35	136
November	34	50	88
December	35	50	89

Part 1

Description of samples

The samples, which numbered 132, were obtained from the Hannah Institute herd of pedigree Ayrshire cows. Most of the samples (104) were from individual cows some of which were sampled several times in one lactation, and the remainder were samples of the herd bulk milk. To obtain milk of varying composition samples were taken from cows differing in age and stage of lactation and some were taken from cows with sub-clinical mastitis. All samples from individual cows were taken from their complete morning milking and the herd bulk samples were obtained by mixing representative samples from 10 gallon cans containing the complete morning yield of milk from the herd. The volume of each sample taken for analysis was about 1500 ml.

Herd bulk milk Samples of the herd bulk milk were taken each month over a period of one year. A total of 28 samples was obtained. Where several samples were taken in one month, the composition and stability of each were found to be similar and the results were therefore averaged. In this way, one set of values for each month was obtained for the composition and stability of the herd bulk milks. At each sampling of the herd, a note was taken of the number of lactating cows, the stage of lactation of each and the total volume of milk, and the mean values calculated for each month (Table 1). The table

shows that the bulk milk from November to February was provided mainly by cows in the first third of their lactations and that during July, August and September it came largely from cows that were well advanced in lactation. The cows were turned out to grass in early May and were returned to the byre in late October.

Early lactation milk The 15 samples of early lactation milk were obtained from 13 cows which had been milking for 10 days or less. The samples were divided into two groups designated colostrum and post-colostrum milk. The colostrum samples were generally easily recognised by their physical appearance, but in doubtful cases chemical composition, e.g. lactose content, was used as a basis for their separation from the post-colostrum milks. The colostrum samples were obtained from cows which had been milking for an average of 4 days and the post-colostrum samples from cows which had been milking for an average of 8 days.

Mid-lactation milk The 55 samples in this group were from 27 cows whose stage of lactation varied from 16 to 281 days after calving; they represented the period excluding the first 5% and the last 10% of lactation. Thirty-one of the samples came from 6 cows which were being studied at intervals during their lactation. All the samples were from cows yielding more than 10 lb. at the morning milking on the day on which the samples were collected.

Late lactation milk The 15 samples in this group were

from 13 cows in the last 10% of their lactations. The cows had been in milk for periods ranging from 185 to 336 days. The yield of milk at the time of sampling was less than 6 lb. in eleven instances and for the remaining four it varied from 6.5 to 8.25 lb.

Mastitis milk The 19 samples of mastitis milk were obtained from 13 cows suffering from sub-clinical mastitis. The evidence that these cows had sub-clinical mastitis was provided by the Veterinary Pathologist at the Institute, Dr P.S. Blackburn, M.R.C.V.S., who determined the differential cell count of the milks by a technique recently described by Blackburn, Laing & Malcolm (1955) and also their bacterial content and by these two criteria, he adjudged the cows to be suffering from sub-clinical mastitis. Seventeen of the samples were from cows in mid-lactation, but the remaining two were from two cows in early lactation and the chemical composition of these milks appeared to be related more to stage of lactation than to the effect of sub-clinical mastitis.

All the 104 samples of milk from individual cows used in this experiment were examined for cell and bacterial content by Dr Blackburn to ensure that none from cows with sub-clinical mastitis was included among the normal samples.

Methods

Preparation of samples for analysis

Preliminary studies showed that the removal of

fat from milk had an insignificant effect on the stability of the milk protein as measured by its rate of coagulation by heat and rennet and by the strength of ethanol required to cause coagulation. These observations are in agreement with those of Holm, Deysher & Evans (1923), Webb & Holm (1932) and Mitamura (1937). Since the removal of fat simplified the analysis of samples by dispensing with the determination of lipid P in partitioning the phosphorus in milk, it was decided to use skim milk for all stability tests and chemical analyses.

The fat was separated by centrifuging the whole milk for 30 min. at a force of 1000 x g. A hole was made in the separated layer of fat, a glass tube passed through and the skim milk removed by suction. Most of the sediment on the bottom of the centrifuge bottles after centrifuging was removed with the skim milk even although its exclusion did not alter the concentrations of casein, calcium and phosphorus in the milk. The sediment was presumably cellular debris; Hostettler, Rychener & Künzle (1949) have shown that casein does not separate at a centrifugal force of 1000 x g. Skim milk obtained by this procedure contained about 0.1% fat as determined by the Röse-Gottlieb method and, as a check, all samples used were tested by the Gerber method to ensure that this level was not exceeded.

Protein stability tests

(1) Ethanol The stability of the milk protein to ethanol was determined by finding the strength of

ethanol which, when added to an equal volume of milk, caused the formation of clots. Thirteen aqueous solutions of ethanol were used covering the range 66 to 90% v/v ethanol in 2% intervals.

A volume of 2 ml. of milk was pipetted into a test-tube, 2 ml. of 90% ethanol added, and the liquids mixed by gentle inversion. The mixture was poured into a glass petri dish and examined for the presence of clots. The test was repeated using the other ethanol solutions, in order of decreasing strength, until coagulation did not occur. The strength of the weakest ethanol solution that caused coagulation was recorded.

(2) Rennet The stability of milk protein to rennet was determined by measuring the time required for clots to appear in milk to which a small volume of a dilute rennet solution had been added. The method used was that described by Berridge (1952). The strength of the stock rennet solution, which was stored at 4°C was checked at frequent intervals by measuring the renneting time of a 'standard milk', prepared by reconstituting a sample of dried skim milk powder in a solution of calcium chloride as recommended by Berridge; it did not alter during the period of the experiment. The renneting time of each milk was measured to the nearest second and recorded to the nearest tenth of a minute. The conditions of the test were such that milk of average composition coagulated in approximately 4 min. The renneting time of each sample was determined in triplicate; variation from the mean was only a few seconds.

(3) Heat The stability of milk protein to heat was measured by determining the time required for clots

to appear in milk heated at 130, 140 and 150°C. Temperatures of this order were necessary to cause coagulation in a reasonable time. The time required for coagulation was determined at three different temperatures to enable the temperature coefficient of the coagulation reaction to be calculated. The procedure was briefly as follows:

A volume of 1.5 ml. of milk was pipetted into a thick-walled 'Pyrex' tube about 12 cm. long and 3.5 ml. in capacity. The tube was closed with a 'Silicone' rubber stopper and attached horizontally to a metal carriage in such a way that the stopper could not be forced from the tube. The carriage was then immersed in liquid paraffin maintained at one of the prescribed temperatures in a bath equipped with a stirrer, electric heaters controlled by a contact thermometer and a 'Sunvic' relay, and an accurate thermometer. At the moment of immersion of the tube, a stop-watch was started. The carriage was given a gentle rocking motion at a constant speed, so that the milk flowed slowly backward and forward from one end of the tube to the other. When clots were seen in the milk, the watch was stopped and the time required for coagulation recorded to the nearest 0.1 of a minute.

The coagulation time of each sample was determined in duplicate at each temperature. When the coagulation time was short (10 min. or less), variation from the mean was negligible. When the coagulation time was longer, the variation from the mean was usually within $\pm$ 0.5 min. and when very long, or when the coagulation point was difficult to detect, the variation was of the order of $\pm$ 1 min.

Similar techniques for assessing the stability of milk to heat have been used by Miller & Sommer (1940) and Cole & Tarassuk (1946).

Chemical analysis of samples

Sufficient evidence abounds in the literature

to show that in studying the effect of milk composition on protein stability, more than an analysis for the major constituents in milk is required. The possibility of the importance of the relative amounts of various mineral and protein fractions made it necessary to take these into account. Hence, in addition to analyzing for the major constituents the calcium, magnesium, phosphorus and citrate were partitioned into their colloidal and soluble fractions and the nitrogen into various protein and non-protein fractions. Values for sodium, potassium and chloride were also obtained and the pH and titratable acidity determined.

The major constituents in each milk sample were determined as follows: - water (British Standard 1741: 1951, Method 2), protein (total nitrogen x 6.38, micro-Kjeldahl method of Hiller, Plazin & Van Slyke (1948)), lactose (by Rowland's modification (1948) of the method of Hinton & Macara (1927)), and ash (British Standard 1741: 1951).

The ash was dissolved in dilute acid and the solution analysed for sodium and potassium by flame photometry. These sodium and potassium estimations were done by Mr R.C. Voss of the Spectrographic Unit at the West of Scotland Agricultural College and by Mr N. Strachan of the Director's Laboratory at the Hannah Institute. Chloride was estimated by the method of Davies (1932). pH was measured with a glass electrode and titratable acidity determined as described in British Standard 1741: 1951.

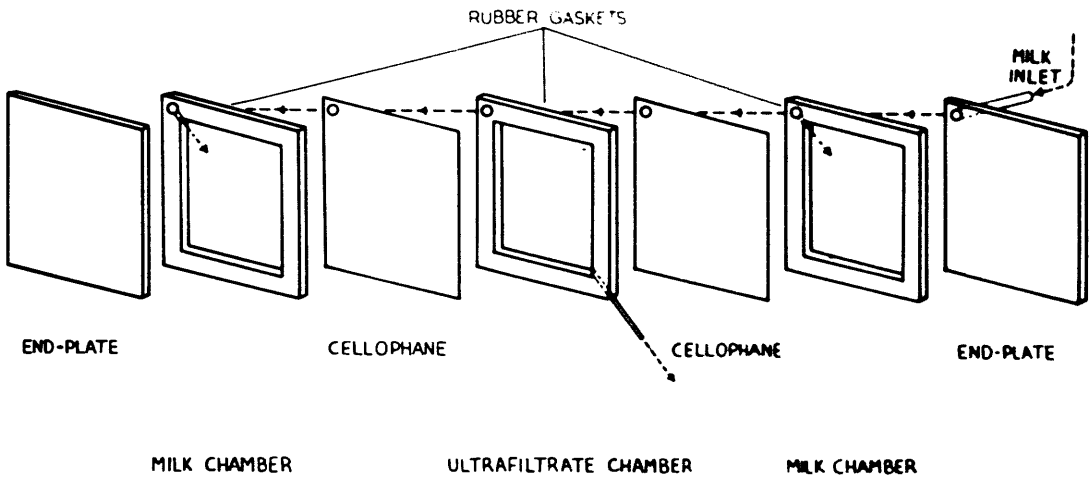

Fig. 1 Diagram of an ultrafiltration apparatus with two milk chambers and one ultrafiltrate chamber.

The nitrogen in the milk was partitioned into casein N, lactalbumin plus lactoglobulin N, proteose-peptone N and non-protein N by Rowland's method (1938) except that tungstic acid was used to prepare the filtrate containing non-protein nitrogen. The concentration of nitrogen in the various filtrates was determined by the micro-Kjeldahl method of Hiller, Plazin & Van Slyke (1948).

The preparation of milk ultrafiltrate To partition the calcium, magnesium, phosphate and citrate in the samples it was necessary to separate the fractions of these constituents in the colloidal and aqueous phases of milk. This was done by ultrafiltration of milk through 'Cellophane' in the apparatus illustrated in Fig. 1, which is an improved model of that described by Clark (1951). The apparatus consisted of five 'Neoprene' rubber gaskets, 1/8 in. thick (15 x 15 cm. external dimensions) separated by four sheets of 'Cellophane' (grade P.T. 300, washed and superficially dried), the assembly being clamped between two metal end-plates. (For convenience, Fig. 1 shows an apparatus consisting of three gaskets only). Milk was forced into the apparatus under a pressure of 15 Lb./sq.in. of nitrogen and entered each milk chamber through a narrow stainless steel tube. From the milk chambers, the aqueous phase of the milk passed through the 'Cellophane' into the ultrafiltrate chambers which contained a square of 'Perspex' (1/10 in. thick) flanked on either side by a sheet of rayon-base filter paper having mainly unidirectional

Table 2 The concentrations of milk constituents in successive 10 ml. fractions of ultrafiltrate

Ultrafiltrate fractions	Soluble calcium	Soluble ionized calcium	Soluble magnesium	Soluble phosphorus	Soluble citrate
		mg./100 g. milk			
1	33.5	12.8	7.5	47.5	123
2	32.8	11.9	7.5	47.8	127
3	32.8	11.8	7.4	47.8	126
4	32.7	11.8	7.5	48.0	131
5	32.8	11.5	7.5	48.5	133
6	32.8	11.6	7.4	48.4	130
7	32.8	11.5	7.5	48.5	132

fibres. The 'Perspex' supported the sheets of 'Cellophane' and the paper absorbed the ultrafiltrate and facilitated its drainage to the base of the chamber, from where it drained through a narrow stainless steel tube. The internal dimensions of the rubber gaskets were 11 x 11 cm. so that the four sheets of 'Cellophane' had a combined filtering area of 484 sq.cm. With this apparatus and using 400 ml. milk, about 25 ml. of ultrafiltrate were obtained per hour.

Since the pores of the washed 'Cellophane' contained water, and since there might be a slight initial adsorption of cations or anions by the 'Cellophane', it was essential to establish what volume of ultrafiltrate had to be collected before its composition became constant. Table 2 shows typical values for the concentrations of milk constituents in successive 10 ml. fractions of ultrafiltrate. The fractions following the first two were reasonably constant in composition. The practice adopted was therefore to discard the first 20 ml. of ultrafiltrate and retain the next 50 ml. for analysis. It was found that the composition of ultrafiltrate so collected was independent, within reasonable limits, of the volume of milk, the thickness of the 'Cellophane' and the pressure used, even although variation of each of the last two factors caused appreciable variation in the rate of filtration. Each ultrafiltrate was analysed for its water and nitrogen content. The latter indicated that the

Table 3 Mean values (10 samples) for some soluble constituents in milk obtained by ultrafiltration and dialysis

Constituent	Ultrafiltration (U)	Dialysis (D)	U/D x 100
	mg./100 g. milk		
Total soluble calcium	36.1	39.9	91 ± 2.5
Soluble ionized calcium	11.0	12.7	87 ± 2.0
Total soluble magnesium	7.8	7.8	100 ± 2.5
Total soluble phosphorus	38.0	39.7	96 ± 1.5
Inorganic soluble phosphorus	30.9	33.0	94 ± 2.0
Total soluble citric acid	160	168	95 ± 3.0

ultrafiltrate contained virtually no nitrogen other than non-protein nitrogen.

The concentrations of constituents in the ultrafiltrate were determined as mg. per 100 g. of ultrafiltrate and were converted to mg. per 100 g. of milk by multiplying by the factor $\left(\frac{100 - \% \text{ total solids in milk}}{100 - \% \text{ total solids in ultrafiltrate}}\right)$, which was usually about 0.965. This calculation compensated for the difference in the weight of water in equal weights of milk and ultrafiltrate but ignored the small amount of bound water in milk.

Another method of estimating the concentrations of the soluble constituents of milk is by dialysis, and results obtained by this method were compared with those obtained by ultrafiltration. Milk dialysate was prepared by placing 1 volume of water in a length of 'Visking' seamless 'Cellophane' tube and immersing the tube in 50 volumes of milk for 48 hr. at 4°C. Table 3 shows the mean values for a number of soluble constituents obtained by ultrafiltration and dialyses of ten milks. The results were calculated per 100 g. of milk by making allowances for differences in the water content of the milk and the two sera and also, in the case of dialysate, by allowing for the dilution of 50 volumes of milk with one volume of water. These results show that ultrafiltration gave slightly lower values for most of the soluble constituents. However, for any constituent the ultrafiltrate value was, within the limits of experimental error, approximately a constant percentage

of the dialysate value. Also the differences between the values obtained by the two methods were small compared with the natural variation in the concentrations of the soluble constituents. Since both procedures can be criticised on theoretical grounds, it was impossible to decide which results were likely to be the more accurate and consequently, ultrafiltration was preferred because of its rapidity.

The preparation of trichloroacetic acid-milk filtrate

Total calcium and total magnesium in milk were determined in the filtrate after precipitation of milk protein with trichloroacetic acid. This filtrate was also used for the determination of total citric acid, total acid-soluble phosphorus, and inorganic acid-soluble phosphorus. The trichloroacetic acid (TCA) filtrate was prepared as follows:-

A volume of 20 ml. of milk was diluted to 100 ml. in a graduated flask with a 15% w/v solution of trichloroacetic acid (A.R.). The stoppered flask was shaken vigorously for a few seconds and left for 30 min. The mixture was then filtered through a Whatman No. 40 paper and the filtrate collected.

The values thus obtained (mg. per 100 g. milk) were multiplied by a factor of 0.996 to compensate for the volume occupied by the precipitated protein in the graduated flask (Rowland, 1938). The use of TCA filtrate for the estimation of the total calcium and magnesium in milk was advocated by Sanders (1931) as an alternative to the more laborious procedure of ashing milk, making a solution of the ash and analysing the solution. The total calcium and magnesium contents of milks using the TCA filtrate and ash solution were found to be identical.

The partition and estimation of phosphorus To determine the phosphorus partition in the milk samples five estimations were made as follows:-

1. Total phosphorus in milk; i.e. colloidal inorganic P + casein P + soluble inorganic P + ester P
2. Total acid-soluble phosphorus; i.e. colloidal inorganic P + soluble inorganic P + ester P
3. Inorganic acid-soluble phosphorus; i.e. colloidal inorganic P + soluble inorganic P
4. Total soluble phosphorus; i.e. soluble inorganic P + ester P
5. Soluble inorganic P

From these estimations the concentrations of the four main types or classes of phosphorus were calculated thus:-

$$\text{Casein P} = 1 - 2$$

$$\text{Colloidal inorganic P} = 3 - 5 \text{ (or } 2 - 4)$$

$$\text{Soluble inorganic P} = 5$$

$$\text{Ester P} = 2 - 3 \text{ (or } 4 - 5)$$

The two methods of calculating the concentrations of colloidal inorganic P and ester P gave similar results.

Estimations 1, 2 and 4 were made respectively on samples of milk, TCA filtrate and ultrafiltrate by digestion with a mixture of perchloric acid (60% v/v) and hydrogen peroxide to destroy all organic material and to convert all forms of phosphate to orthophosphate. The phosphorus in the diluted digests was then estimated colorimetrically as molybdenum blue, formed by the addition of the reducing agent amidol (2:4 diaminophenol hydrochloride) and ammonium molybdate. The digestion and colorimetric

procedures were based on those of Allen (1940).

Estimations 3 and 5 were made on samples of TCA filtrate and ultrafiltrate respectively, but without the digestion stage. Molybdenum blue was formed directly in the diluted filtrates by adding perchloric acid, amidol and ammonium molybdate. In these two estimations it was essential that there was no liberation of phosphorus from the ester phosphorus compounds present. Since there was greater likelihood of this happening in the presence of TCA, estimation 3 was made immediately the TCA filtrate was obtained. That no hydrolysis of ester phosphorus compounds occurred in estimation 3 was shown by preliminary studies on several milks using two procedures in which special precautions are taken to prevent hydrolysis. In one, the milk was extracted for 5 min. with TCA cooled to $4^{o}C$ (Basu & Mukherjee, 1943) and in the other a buffer mixture of sodium acetate and acetic acid saturated with ammonium sulphate was used to precipitate the protein in milk and the mixture filtered after 5 min. (Lowry & Lopez, 1946). In the latter method the molybdenum blue was formed at the relatively high pH of 4, with ascorbic acid as the reducing agent. Both of these methods gave the same results as the procedure used in this investigation. By using the procedure of Lowry & Lopez with ultrafiltrate it was also shown that no hydrolysis of ester phosphorus compounds took place during estimation 5.

The partition and estimation of calcium To determine

the calcium partition in the milk samples, three estimations were first made:-

1. Total calcium in milk: i.e. colloidal inorganic Ca + caseinate Ca + soluble unionized Ca + soluble ionized Ca

2. Total soluble calcium: i.e. soluble unionized Ca + soluble ionized Ca

3. Soluble ionized calcium

From these estimations, values for the following fractions were calculated:-

Colloidal inorganic + caseinate Ca = 1 - 2

Soluble unionized Ca = 2 - 3

Soluble ionized Ca = 3

Estimations 1 and 2 were made respectively on TCA filtrate and ultrafiltrate by precipitating the calcium as oxalate and titrating the washed precipitate with potassium permanganate in acid solution. The technique was based on the micro method of the Association of Official Agricultural Chemists (1945). Estimation 3 was made by the method described by Smeets & Seekles (1952), Seekles & Smeets (1954) and Smeets (1955) using ultrafiltrate. This method is based on the measurement of the colour change which takes place when a solution of murexide (ammonium purpurate) is added to a solution containing ionized calcium. The extent to which the red-violet colour of the ammonium purpurate changes to the orange-yellow of calcium purpurate is related to the concentration of ionized calcium within certain pH limits. The colour intensity of the mixture is measured at a wavelength of 470 mμ, where the difference

between the spectral absorptions of the two purpurates is greatest. A calibration curve is prepared using a series of standard solutions of calcium chloride with the same ionic strength (μ = 0.07) and the same pH (6.7) as milk ultrafiltrate.

The only important modifications made to the method as published were in the preparation of the standard solutions. A more alkaline buffer solution was found necessary to achieve the correct pH and the amount of magnesium in the salt solution used to obtain the correct ionic strength was reduced to allow for the fact that only about one quarter of the soluble magnesium in milk is ionized (van Kreveld & van Minnen, 1955).

To complete the partition of the calcium it was necessary to obtain a value for the concentration of either colloidal inorganic calcium or caseinate calcium. The first alternative was adopted. The concentration of colloidal inorganic calcium in milk can be calculated from the concentration of colloidal inorganic phosphorus provided the composition of the colloidal calcium phosphate in milk is known. The latter information was obtained by the method of Pyne & Ryan (1950). This method determines the fraction of the colloidal inorganic phosphorus present as $Ca_3(PO_4)_2$ and the fraction present as $CaHPO_4$. The concentrations of colloidal tricalcium and dicalcium phosphate in the sample of milk can then be calculated and hence the concentration of calcium in colloidal inorganic form. The concentration of caseinate calcium

was then obtained by subtracting the value for colloidal inorganic calcium from the value for total colloidal calcium (1 - 2).

The basis of Pyne & Ryan's method is to add potassium oxalate to milk, and by back-titration with acid to the original pH, to measure the alkalinity produced by the conversion of colloidal tricalcium and dicalcium phosphates to the corresponding potassium salts. A correction is applied for the contribution made to the developed alkalinity by the reaction of potassium oxalate with the soluble calcium salts and the calcium caseinate. From a knowledge of the concentration of colloidal inorganic phosphorus, and the ratio of $H_2PO_4^-$ to $HPO_4^=$ at the pH of the milk (obtained from Sorenson's table of phosphate buffers), it is possible to calculate the proportions of the colloidal inorganic phosphorus in the tricalcic and dicalcic forms.

<u>The partition and estimation of magnesium</u> Magnesium probably exists in milk in forms similar to those in which calcium exists but the lack of a suitable method for determining ionized magnesium made it impossible to achieve a complete partition. This was not considered a serious drawback since the total amount of magnesium in milk is only about 12 mg. per 100 g. of milk and therefore the amounts occurring in the different forms are accordingly relatively small. The following estimations were made:-

1. Total magnesium in milk: i.e. total colloidal Mg + total soluble Mg
2. Total soluble magnesium

The concentration of total colloidal magnesium was obtained by difference.

Estimation 1 was made on the supernatant liquid after the precipitation of calcium oxalate in TCA filtrate and estimation 2 on the supernatant liquid after the precipitation of calcium oxalate in ultrafiltrate. In each estimation, the magnesium was precipitated as $MgNH_4PO_4 .6H_2O$ by the addition of diammonium hydrogen phosphate and ammonia, taking the precaution suggested by Bushill, Lampitt & Filmer (1937) to prevent the simultaneous precipitation of $Mg(NH_4)_4(PO_4)_2$. The precipitate was washed with the solution recommended by Michaels, Anderson, Margen & Kinsell (1949), dried and dissolved in perchloric acid (60% v/v). The phosphorus in this solution was estimated in the same way as already described in the partition of phosphorus and converted to the equivalent amount of magnesium by multiplying by the factor 0.784.

The partition and estimation of citric acid Most of the citric acid in milk is present in the aqueous phase, but a small amount is associated with the casein in the dispersed phase (de Kadt & van Minnen, 1943; Eilers, Saal & van der Waarden, 1947; Verma & Sommer, 1950). It is therefore possible to partition the citric acid into soluble and colloidal fractions. Two estimations were made as follows:-

1. Total citric acid in milk: i.e. colloidal citric acid + soluble citric acid
2. Soluble citric acid

The concentration of colloidal citric acid was obtained by difference.

Estimation 1 was made on TCA filtrate and estimation 2 on ultrafiltrate. The method used was that of Saffran & Denstedt (1948) in which the intensity of the yellow colour formed by the interaction of citric acid, acetic anhydride and pyridine at 60°C is proportional to the concentration of the citric acid. Accurate and reproducible results were obtained only under carefully controlled conditions i.e. a constant temperature during colour development, the use of anhydrous pyridine and acetic anhydride, and the use of freshly prepared standard solutions of citric acid for the calibration curves.

All the analytical methods were tested for reproducibility, and where possible for precision, by estimating known amounts of the various elements and compounds. Many alternative procedures to those described were examined but they were rejected as unsatisfactory. Those finally adopted were considered the best available. Each estimation was made in duplicate and the mean value used in relating composition to protein stability.

Table 4 Composition of fat-free, herd bulk milk (12 samples)

	Mean (g./100g. milk)	Range (g./100g. milk)	Standard deviation
Total solids	9.07	8.82 - 9.30	0.14
Protein (total N x 6.38)	3.30	3.04 - 3.54	0.16
Lactose (anhydride)	4.70	4.50 - 4.91	0.13
Ash	0.76	0.73 - 0.78	0.02
pH	6.72	6.65 - 6.84	0.08
Titratable acidity (ml. 0.1N NaOH/100g. milk)	15.5	12.2 - 17.1	0.17
	(mg./100g. milk)	(mg./100g. milk)	
Total calcium	117.7	110.9 - 120.3	2.5
Total magnesium	12.1	11.4 - 13.0	0.6
Total citric acid	176	166 - 192	9
Total phosphorus	95.1	79.8 - 101.7	6.1
Sodium	58	47 - 77	10
Potassium	154	113 - 171	14
Chloride	104.5	89.8 - 127.0	11.4
Nitrogen fractions			
Casein N	402.3	380.0 - 424.2	15.6
Lactalbumin + lactoglobulin N	65.9	55.6 - 73.9	5.9
Proteose-peptone N	24.6	19.4 - 30.6	4.0
Non-protein N	23.8	18.5 - 33.0	4.6
Calcium fractions			
Colloidal inorganic Ca	49.7	41.8 - 54.0	3.2
Caseinate Ca	31.4	28.9 - 33.9	1.7
Soluble unionized Ca	25.3	21.9 - 28.8	1.9
Soluble ionized Ca	11.4	10.5 - 12.8	0.7
Magnesium fractions			
Colloidal Mg	4.3	3.8 - 4.5	0.2
Soluble Mg	7.8	7.0 - 8.5	0.4
Citric acid fractions			
Colloidal citrate	19	15 - 22	2.6
Soluble citrate	158	143 - 175	11
Phosphorus fractions			
Colloidal inorganic P	29.3	24.9 - 31.1	1.6
Casein P	21.5	18.7 - 23.0	1.3
Soluble inorganic P	33.6	27.0 - 38.9	3.7
Ester P	10.6	7.7 - 13.1	1.7
	(mg./g. casein)	(mg./g. casein)	
Caseinate Ca	12.2	11.1 - 13.9	0.8
Casein P	8.4	7.7 - 8.9	0.4
Colloidal inorganic Ca	19.3	17.1 - 20.3	0.9
Colloidal inorganic P	11.4	10.2 - 12.2	0.6
Tricalcium phosphate	39.1	33.3 - 47.1	4.6
Dicalcium phosphate	16.1	9.1 - 20.6	4.0
Tri-plus dicalcium phosphate	55.2	49.0 - 58.9	2.7
Colloidal Mg	1.7	1.5 - 1.8	0.1
Colloidal citrate	7.3	5.5 - 8.4	0.9

Chemical composition of the milk samples

As previously stated, the fat was removed from all samples of milk and the chemical analyses and protein stability tests made on the separated milk. All results therefore apply to skim milk and not to whole milk. The fat content of all the original samples was, however, determined by the Gerber method, and the mean fat contents of the herd bulk, early lactation, mid-lactation, late lactation and mastitis milks were respectively 3.68, 3.80, 3.46, 4.12 and 3.52%.

Herd bulk milk (12 samples) The mean values, ranges and standard deviations of the individual values for the concentrations of the constituents of the herd bulk milks are given in Table 4. This table also shows the concentrations of the colloidal constituents expressed as mg. per g. of casein. The twelve samples were fairly similar in composition as indicated by the small standard deviations. However, there were appreciable differences between the minimum and maximum concentrations of most constituents and these could be attributed to the fluctuations in the composition of the herd bulk milk caused by the wide extremes of the average stage of lactation of the herd, namely 88 to 206 days.

The mean values for the various constituents, when calculated per 100 g. of milk or per g. of casein, agreed reasonably well with those reported in the literature. The mean value for the concentration of the ionized calcium (11.4 mg. per

Table 5 Composition of fat-free early lactation milk (15 samples) with the mean value for each constituent expressed as a percentage of the corresponding bulk mean value

Constituent	Colostrum group (7 samples) Mean (g./100g.milk)	Range (g./100g.milk)	Mean as % of bulk mean	Post-colostrum group (8 samples) Mean (g./100g.milk)	Range (g./100g.milk)	Mean as % of bulk mean
Total solids	9.82	8.90 - 11.56	108	9.92	9.54 - 10.17	109
Protein (total N x 6.38)	4.39	3.48 - 6.37	133	3.98	3.67 - 4.45	121
Lactose (anhydride)	4.20	3.82 - 4.46	89	4.78	4.50 - 5.09	102
Ash	0.85	0.80 - 0.89	112	0.82	0.76 - 0.89	108
pH	6.53	6.41 - 6.62	97	6.55	6.47 - 6.69	98
Titratable acidity (ml. 0.1N NaOH/100g. milk)	21.5	19.0 - 25.0	139	21.6	20.2 - 22.9	140
	(mg./100g.milk)	(mg./100g.milk)		(mg./100g.milk)	(mg./100g.milk)	
Total calcium	130.6	116.4 - 150.7	111	134.5	124.9 - 141.7	114
Total magnesium	13.6	12.2 - 15.4	112	13.0	11.7 - 14.8	107
Total citric acid	176	126 - 236	100	185	145 - 238	105
Total phosphorus	113.7	104.0 - 124.8	120	117.5	107.4 - 127.1	124
Sodium	67	46 - 78	116	57	42 - 78	98
Potassium	160	146 - 173	104	164	145 - 182	107
Chloride	127.7	110.0 - 151.6	122	84.6	67.4 - 104.2	81
Nitrogen fractions						
Casein N	461.0	382.7 - 529.0	115	489.0	458.2 - 530.8	122
Lactalbumin + lactoglobulin N	163.4	102.8 - 442.1	248	87.9	72.5 - 113.8	133
Proteose-peptone N	28.4	14.8 - 48.1	115	19.2	14.1 - 28.0	78
Non-protein N	35.2	27.2 - 44.1	148	27.1	24.9 - 32.0	114
Calcium fractions						
Colloidal inorganic Ca	44.7	34.8 - 56.8	90	51.0	42.7 - 61.3	103
Caseinate Ca	35.5	28.6 - 42.2	113	36.4	32.6 - 39.2	116
Soluble unionized Ca	33.4	26.8 - 41.8	132	31.5	25.3 - 37.7	124
Soluble ionized Ca	17.0	15.1 - 20.1	149	15.6	13.1 - 17.7	137
Magnesium fractions						
Colloidal Mg	4.4	3.5 - 5.8	102	4.3	3.7 - 4.8	100
Soluble Mg	9.2	8.0 - 10.9	118	8.8	7.7 - 10.4	113
Citric acid fractions						
Colloidal citrate	18	11 - 25	95	18	10 - 25	95
Soluble citrate	158	109 - 216	100	167	122 - 216	106
Phosphorus fractions						
Colloidal inorganic P	27.2	20.4 - 34.6	93	29.1	23.2 - 34.9	99
Casein P	24.3	20.3 - 28.6	113	24.8	22.4 - 26.9	115
Soluble inorganic P	31.7	26.9 - 38.2	94	37.1	33.0 - 41.3	110
Ester P	30.5	25.0 - 41.2	288	26.5	24.6 - 29.6	250
	(mg./g.casein)	(mg./g. casein)		(mg./g. casein)	(mg./g. casein)	
Caseinate Ca	12.2	9.9 - 16.0	100	11.7	10.9 - 12.9	96
Casein P	8.3	7.6 - 9.4	99	8.0	6.9 - 8.7	95
Colloidal inorganic Ca	15.4	10.9 - 21.5	80	16.4	12.9 - 20.1	85
Colloidal inorganic P	9.4	6.4 - 13.1	82	9.3	7.0 - 11.4	82
Tricalcium phosphate	28.0	16.6 - 38.7	72	37.0	29.4 - 45.4	95
Dicalcium phosphate	16.8	8.4 - 24.9	104	8.6	2.1 - 12.8	53
Tri-plus dicalcium phosphate	44.8	31.0 - 62.4	81	45.6	34.8 - 55.9	83
Colloidal Mg	1.5	1.1 - 1.8	88	1.4	1.2 - 1.6	82
Colloidal citrate	6.2	3.1 - 9.5	85	5.8	3.6 - 7.6	79

100 g. milk) was very similar to that obtained by Seekles & Smeets (1954) whose method was used. Another technique for determining the concentration of ionized calcium in milk has been developed by van Kreveld & van Minnen (1955) and Christianson, Jenness & Coulter (1954) which gives a slightly lower average value of 8 - 9 mg. per 100 g. of milk. Boulet & Rose (1954) state that a high value for calcium ion concentration is inherent in the method of Seekles & Smeets. The mean values, per g. of casein, for caseinate calcium and for colloidal calcium phosphate are similar to the corresponding values that can be calculated from the data of Ramsdell & Whittier (1944).

Early lactation milk (15 samples) The mean compositions of the two groups of early lactation milk are shown in Table 5 together with the ranges of values. The standard deviations were not calculated because the number of samples in each group was small, but the mean values have been expressed as percentages of the corresponding bulk means for comparison. There was considerable variation in the composition of the colostrum samples but all differed from the herd bulk milk in being richer in most constituents, especially ester phosphorus, lactalbumin plus lactoglobulin, soluble ionized calcium and non-protein nitrogen. The high average concentration of proteose-peptone was caused by two samples (41.6 and 48.1 mg. of proteose-peptone N per 100 g. milk); the average value for this constituent in the other five samples was 21.8 mg.

Table 6 Composition of fat-free, mid-lactation milk (55 samples)

Constituent	Mean (g./100g. milk)	Range (g./100g. milk)	Standard deviation	Mean as % of bulk milk
Total solids	9.07	8.35 - 9.97	0.34	100
Protein (total N x 6.38)	3.30	2.63 - 3.83	0.26	100
Lactose (anhydride)	4.74	4.40 - 5.12	0.18	101
Ash	0.75	0.66 - 0.83	0.04	99
pH	6.73	6.53 - 6.86	0.08	100
Titratable acidity (ml. 0.1N NaOH/100g. milk)	16.0	11.5 - 20.0	2.34	103
	(mg./100g. milk)	(mg./100g. milk)		
Total calcium	115.3	91.7 - 136.3	10.4	98
Total magnesium	11.8	9.6 - 14.7	1.3	98
Total citric acid	166	108 - 230	28	94
Total phosphorus	95.2	69.0 - 119.6	12.8	100
Sodium	56	32 - 80	12	97
Potassium	154	123 - 176	14	100
Chloride	103.5	59.7 - 149.8	16.8	99
Nitrogen fractions				
Casein N	404.5	309.4 - 493.1	36.2	101
Lactalbumin + lactoglobulin N	64.5	43.4 - 82.5	8.8	98
Proteose-peptone N	23.7	13.8 - 40.5	5.9	96
Non-protein N	23.9	17.6 - 32.4	4.0	100
Calcium fractions				
Colloidal inorganic Ca	47.7	27.5 - 64.2	9.0	96
Caseinate Ca	30.4	23.0 - 37.5	3.6	97
Soluble unionized Ca	25.6	16.2 - 39.0	5.2	101
Soluble ionized Ca	11.6	9.2 - 14.0	1.2	102
Magnesium fractions				
Colloidal Mg	4.1	2.7 - 5.8	0.7	95
Soluble Mg	7.7	5.9 - 9.6	0.9	99
Citric acid fractions				
Colloidal citrate	17	1 - 57	9	90
Soluble citrate	149	94 - 201	27	94
Phosphorus fractions				
Colloidal inorganic P	27.9	16.1 - 38.1	5.1	95
Casein P	21.4	16.7 - 26.5	2.3	100
Soluble inorganic P	35.5	20.3 - 52.4	7.0	106
Ester P	10.5	2.9 - 19.5	3.9	99
	(mg./g.casein)	(mg./g. casein)		
Caseinate Ca	11.9	9.0 - 16.1	1.3	98
Casein phosphorus	8.3	7.0 - 10.2	0.6	99
Colloidal inorganic Ca	18.6	11.4 - 25.4	3.4	96
Colloidal inorganic P	10.9	6.5 - 12.3	2.0	96
Tricalcium phosphate	38.1	17.8 - 54.7	8.2	98
Dicalcium phosphate	14.5	0.0 - 25.9	5.9	90
Tri-plus dicalcium phosphate	52.6	32.3 - 71.8	9.7	95
Colloidal Mg	1.6	1.1 - 2.1	0.7	94
Colloidal citrate	6.7	0.3 - 24.9	3.9	92

per 100 g. milk corresponding to 89% of the herd bulk milk mean. In these samples therefore, colostrum like post-colostrum milk, tended to be low in proteose-peptone.

The concentrations of colloidal inorganic calcium and colloidal inorganic phosphorus were lower than in the herd bulk milk. This difference, coupled with the increase in the casein content of colostrum, meant that there was an appreciable decrease in the average amount of colloidal calcium phosphate associated with one gram of casein; the decrease appeared to be mainly in the tricalcium phosphate fraction. This decrease was probably linked with the lower pH values of the colostrum samples. There were also decreases in the average amounts of colloidal magnesium and citrate associated with the casein.

The post-colostrum samples differed from the herd bulk milks in much the same way as the colostrum samples, but to a lesser degree. The concentrations of ester phosphorus, soluble ionized calcium and lactalbumin plus lactoglobulin were still appreciably above the herd bulk levels, but the average concentration of lactalbumin plus lactoglobulin was only about half that of the colostrum samples. The higher average concentration of casein in colostrum compared with the herd bulk milk was maintained, and even exceeded, in the post-colostrum milks. As in the colostrum samples, the amount of colloidal material associated with one gram of casein was less than in the herd bulk milks, and in this respect

Table 7 Composition of fat-free, late lactation milk (15 samples) with the mean value for each constituent expressed as a percentage of the corresponding bulk mean value

Constituent	Mean (g./100g. milk)	Range (g./100 g.milk)	Standard deviation	Mean as % of bulk mean
Total solids	9.00	7.78 - 10.42	0.77	99
Protein (total N x 6.38)	3.87	3.25 - 4.94	0.49	117
Lactose (anhydride)	4.02	3.37 - 4.43	0.34	86
Ash	0.80	0.71 - 0.90	0.05	105
pH	6.98	6.78 - 7.41	0.18	104
Titratable acidity (ml. 0.1N NaOH/100g. milk)	9.7	3.9 - 14.9	2.8	63
	(mg./100g. milk)	(mg./100g. milk)		
Total calcium	126.1	98.3 - 174.6	22.6	107
Total magnesium	12.8	10.6 - 16.6	1.5	106
Total citric acid	154	108 - 200	27.2	88
Total phosphorus	84.4	68.2 - 104.8	12.0	89
Sodium	110	74 - 152	22	190
Potassium	103	84 - 120	10	67
Chloride	161.7	122.1 - 215.1	23.1	155
Nitrogen fractions				
Casein N	442.4	358.6 - 553.0	58.1	110
Lactalbumin + lactoglobulin N	90.1	49.6 - 116.0	18.6	137
Proteose-peptone N	44.3	22.2 - 63.7	9.7	180
Non-protein N	29.5	15.7 - 42.2	7.9	124
Calcium fractions				
Colloidal inorganic Ca	58.3	40.7 - 91.2	15.0	117
Caseinate Ca	36.4	24.0 - 53.1	7.7	116
Soluble unionized Ca	20.3	13.0 - 29.3	4.2	80
Soluble ionized Ca	11.1	8.8 - 14.6	1.6	97
Magnesium fractions				
Colloidal Mg	5.0	4.0 - 6.2	0.6	116
Soluble Mg	7.8	6.2 - 10.4	1.0	100
Citric acid fractions				
Colloidal citrate	20	9 - 41	8.9	105
Soluble citrate	134	86 - 172	25.7	85
Phosphorus fractions				
Colloidal inorganic P	34.6	24.9 - 53.9	8.7	118
Casein P	22.7	18.7 - 28.3	2.9	106
Soluble inorganic P	21.2	6.6 - 40.5	7.9	63
Ester P	5.8	2.0 - 10.2	2.3	55
	(mg./g. casein)	(mg./g. casein)		
Caseinate Ca	12.8	10.3 - 15.1	1.7	105
Casein P	8.1	7.2 - 9.1	0.2	96
Colloidal inorganic Ca	20.6	14.6 - 25.8	3.7	107
Colloidal inorganic P	12.1	9.0 - 15.3	2.1	106
Tricalcium phosphate	42.2	25.9 - 56.7	9.5	108
Dicalcium phosphate	16.0	5.3 - 25.6	6.3	99
Tri-plus dicalcium phosphate	58.2	42.5 - 73.5	10.3	105
Colloidal Mg	1.8	1.3 - 2.3	0.2	106
Colloidal citrate	7.0	3.1 - 11.5	2.6	96

there was little difference between colostrum and post-colostrum milk except that in the latter, the decrease in colloidal calcium phosphate seemed to be mainly in the dicalcium phosphate fraction. The concentration of lactose in the post-colostrum milks reached a normal level and there were compensatory decreases in the concentrations of sodium and chloride. The pH values and titratable acidities of both the early lactation groups were very similar and showed the higher acidity of these samples compared with the herd bulk milks.

Mid-lactation milk (55 samples) As would be expected, the mean composition of the mid-lactation samples was similar to that of the herd bulk milks but the variation in composition between individual samples was much greater (Table 6). The considerable variation in the composition of these samples could be attributed to the fact that, although designated as mid-lactation milk they came from 27 cows whose stage of lactation ranged from 16 to 281 days after calving. The composition of the casein complex in these milks showed much variation, especially in the amount and composition of the colloidal calcium phosphate and in the amount of citrate associated with 1 g. of casein.

Late lactation milk (15 samples) The mean composition of these milks (Table 7) differed considerably from the mean composition of the herd bulk milks. On average, the late lactation milks contained more protein, especially proteose-peptone and lactalbumin

Table 8 Composition of fat-free milk from cows with sub-clinical mastitis (17 samples) with the mean value for each constituent expressed as a percentage of the corresponding bulk mean value

Constituent	Mean (g./100g.milk)	Range (g./100g.milk)	Standard deviation	Mean as % of bulk mean
Total solids	8.63	8.27 - 9.20	0.93	95
Protein (total N x 6.38)	3.27	2.69 - 3.82	0.33	99
Lactose (anhydride)	4.28	3.82 - 4.54	0.20	91
Ash	0.77	0.73 - 0.82	0.03	101
pH	6.87	6.65 - 7.10	0.13	102
Titratable acidity (ml. 0.1N NaOH/100g.milk)	12.3	8.3 - 14.9	1.91	80
	(mg./100g.milk)	(mg./100g.milk)		
Total calcium	115.4	103.1 - 138.4	9.2	98
Total magnesium	11.7	9.6 - 14.0	1.4	97
Total citric acid	160	125 - 194	21	91
Total phosphorus	84.6	74.0 - 94.8	5.6	89
Sodium	78	59 - 98	12	135
Potassium	138	116 - 160	14	90
Chloride	141.0	120.5 - 163.7	11.7	135
Nitrogen fractions				
Casein N	371.8	313.0 - 445.0	38.6	92
Lactalbumin + lactoglobulin N	77.6	58.8 - 91.7	10.5	118
Proteose-peptone N	38.3	21.1 - 53.9	8.4	156
Non-protein N	24.3	15.8 - 33.1	4.2	102
Calcium fractions				
Colloidal inorganic Ca	48.7	40.4 - 62.7	5.8	98
Caseinate Ca	29.9	21.8 - 38.7	4.4	95
Soluble unionized Ca	24.1	18.5 - 28.9	3.1	95
Soluble ionized Ca	11.6	9.7 - 13.5	1.2	102
Magnesium fractions				
Colloidal Mg	4.2	3.2 - 5.2	0.6	98
Soluble Mg	7.6	6.3 - 9.1	1.0	97
Citric acid fractions				
Colloidal citrate	17	10 - 32	7	90
Soluble citrate	143	107 - 182	19	91
Phosphorus fractions				
Colloidal inorganic P	29.6	24.2 - 37.8	3.8	101
Casein P	20.3	17.3 - 23.1	1.6	94
Soluble inorganic P	27.9	20.6 - 35.4	4.1	83
Ester P	6.8	4.4 - 9.6	1.5	64
	(mg./g.casein)	(mg./g. casein)		
Caseinate calcium	12.8	9.9 - 15.6	1.6	105
Casein P	8.6	7.6 - 9.3	0.5	102
Colloidal inorganic Ca	20.8	18.4 - 24.5	1.9	108
Colloidal inorganic P	12.5	11.2 - 14.8	0.9	110
Tricalcium phosphate	40.1	29.4 - 61.8	8.5	103
Dicalcium phosphate	19.4	4.2 - 31.5	6.6	120
Tri-plus dicalcium phosphate	59.6	53.4 - 70.5	4.8	108
Colloidal Mg	1.8	1.3 - 2.1	0.2	106
Colloidal citrate	7.1	4.3 - 11.9	2.4	97

plus lactoglobulin, and less lactose and had a higher pH and a much lower titratable acidity than the herd bulk milks. They were also richer in sodium and chloride but poorer in potassium. The concentrations of the soluble fractions of calcium, citrate and especially of phosphorus were low while the concentrations of the colloidal fractions of these constituents and of magnesium were high in the late lactation milks. The casein in these milks had an increased amount of calcium, inorganic phosphorus and magnesium associated with it despite the greater concentration of the casein.

Mastitis milk (19 samples) In compiling Table 8, the results from two of the 19 samples from cows with sub-clinical mastitis were omitted. This was done because these samples came from two cows which had been lactating for only 6 and 9 days respectively. The composition of their milk was therefore more typical of early lactation than of mastitis milk.

Table 8 shows that the mastitis milks were low in total solids mainly as a result of a low lactose content. To compensate osmotically for this, the concentrations of sodium and chloride were high. The nitrogen partition in these milks was appreciably different from that of the herd bulk milks; the average concentration of casein was low and that of the serum proteins high, especially proteose-peptone. The mastitis milks contained less citric acid and phosphorus than the herd bulk milks. The decrease in the latter constituent was mainly in its soluble

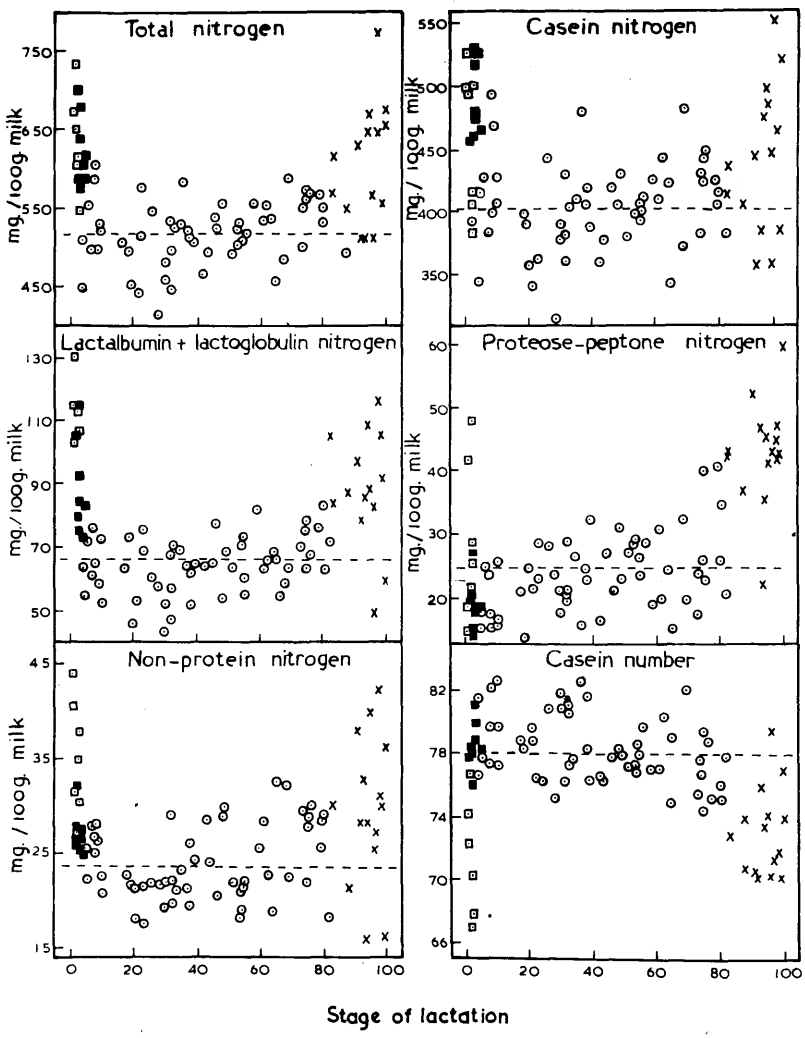

Fig. 2 The relationships of concentration of total nitrogen and of its components, and casein number to stage of lactation, expressed as a percentage of total lactation, in milks from individual cows (--- mean value for herd bulk milk, ⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, x late lactation milk).

fractions whereas the concentrations of both colloidal and soluble citric acid were low. The mean values for colloidal inorganic calcium and phosphorus were similar to those of the herd bulk milk and the low values for caseinate calcium and casein phosphorus were mainly the result of the decreased amount of casein present. The average composition of the casein complex in the mastitis milks was similar to that in the late lactation milks.

The effect of stage of lactation on the composition of the milk samples The results in Tables 5, 6 and 7, show that stage of lactation has a considerable effect on milk composition and the relationship between these two factors will now be examined in greater detail. For this purpose, the mastitis milks were excluded and only the results obtained from 85 samples of milk, from 36 cows, considered. Composition and stage of lactation were compared by plotting the concentration of each constituent in each milk against stage of lactation expressed as a percentage of total lactation. The relationships of greatest interest are shown in Figs. 2, 3 and 4.

The relationships between the concentrations of the nitrogen fractions and stage of lactation shown in Fig. 2 call for little comment since they are already well known. They illustrate the extent of the variations from the mid-lactation range of values that occur in the first 5% and the last 10% of the lactation period. The concentrations of total nitrogen and of each fraction, except proteose-

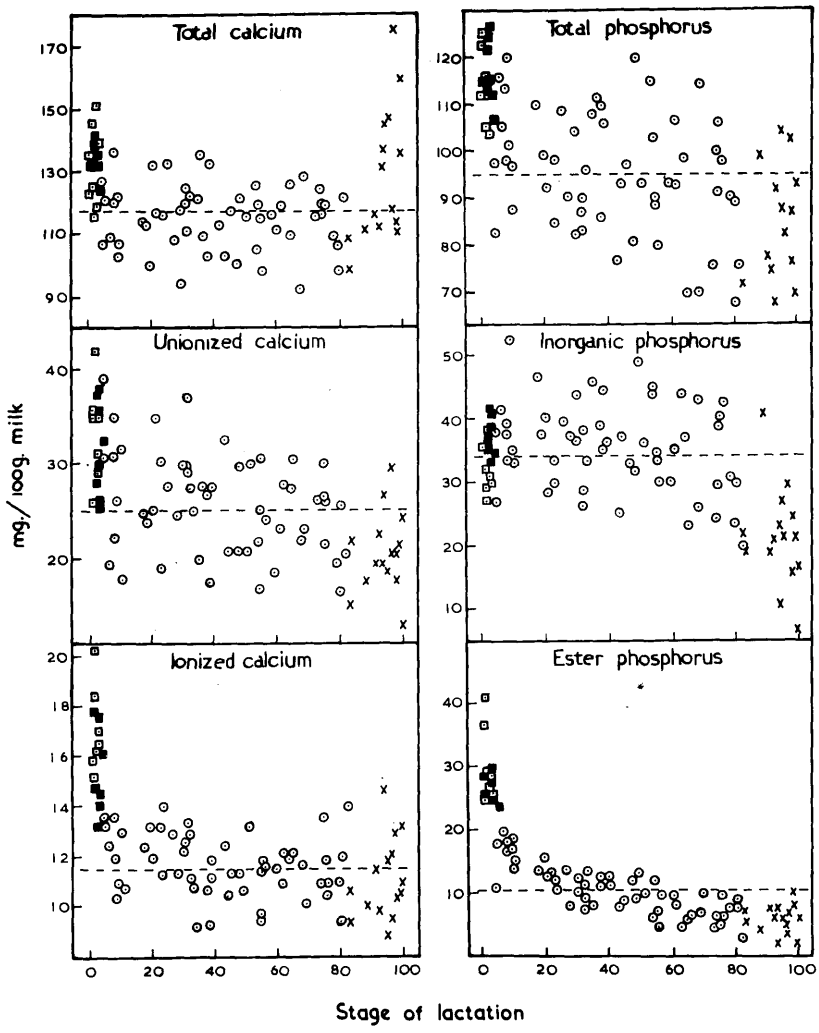

Fig. 3 The relationship of concentrations of total calcium, total phosphorus and of the soluble fractions of these constituents to stage of lactation, expressed as a percentage of total lactation, in milks from individual cows (--- mean value for herd bulk milk, ⊡ colostrum, ■ post-colostrum, ⊙ mid-lactation milk, x late lactation milk).

peptone N, were high at the beginning of lactation but fell rapidly to the level for bulk milk within the first 5% of the lactation period. Thereafter the concentration of each fraction slowly increased, and then rapidly increased in the last 10% or so of lactation to much the same high level as at the beginning of lactation. Although the concentration of casein was high in early and late lactation, the concentrations of the other nitrogen fractions, especially lactalbumin plus lactoglobulin, were then proportionately even higher with the result that the casein number (casein N/total N x 100) tended to be low in these periods.

Fig. 3 illustrates how the concentrations of the total and soluble fractions of calcium and phosphorus change with stage of lactation. The concentration of total calcium was above the herd bulk average at the beginning of lactation but decreased rapidly to normal levels. Towards the end of lactation the concentration of total calcium increased in some milks. The levels of both soluble fractions of calcium were high at the beginning of lactation but thereafter they showed little relationship with stage of lactation apart from a tendency for the unionized fraction to decrease in late lactation. There was much variation in the concentration of total phosphorus during the lactation period but there was a gradual decrease from high values in early lactation to lower values in late lactation. The concentration of soluble inorganic phosphorus was not related to stage

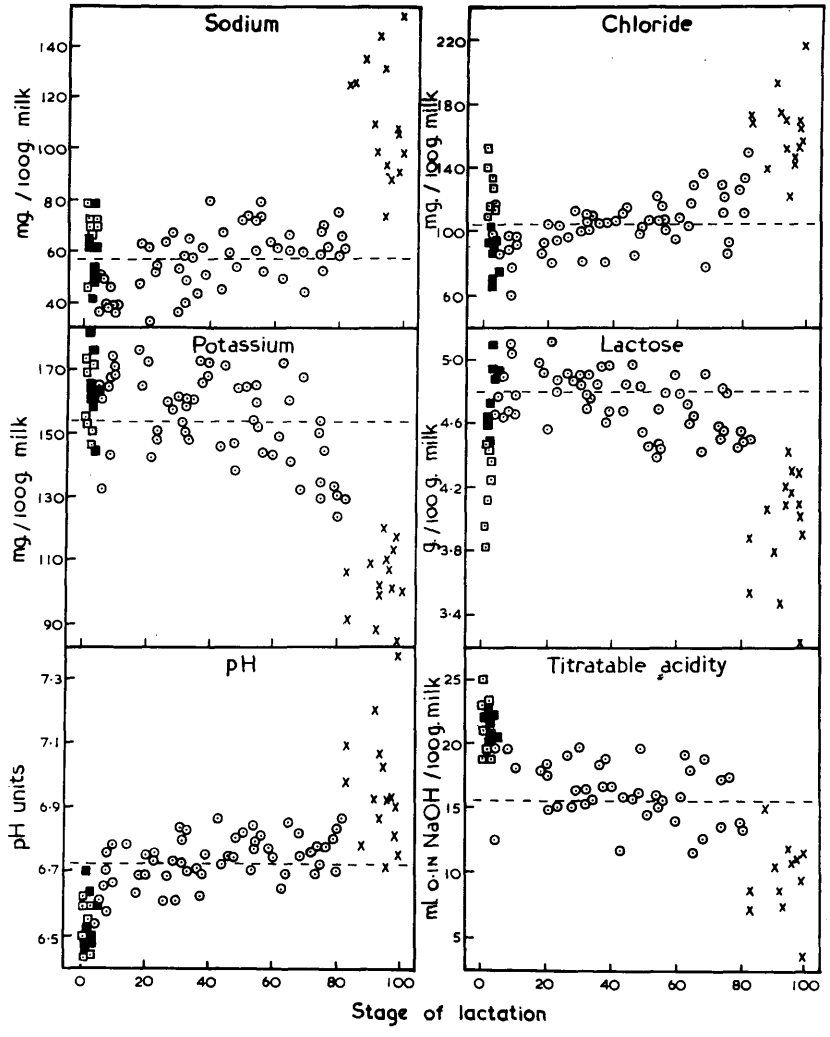

Fig. 4 The relationship of concentrations of sodium, chloride, potassium and lactose, and pH and titratable acidity to stage of lactation, in milks from individual cows (--- mean value for herd bulk milk, ⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, x late lactation milk).

of lactation except for a marked decrease in late lactation. The amount of ester phosphorus in the samples was closely related to stage of lactation in an inverse curvilinear manner. In early lactation, the concentration was as much as four times the herd bulk average but decreased rapidly and then more gradually throughout the remainder of the lactation period.

The concentrations of colloidal inorganic calcium and phosphorus when expressed per 100 g. of milk or per 1 g. of casein, showed much irregular variation during most of the lactation period, but minimum and maximum values occurred in early and late lactation respectively. The variations in caseinate calcium and casein phosphorus (per g. casein), and in colloidal magnesium and citrate, showed little relation to stage of lactation. Also values for total magnesium and citric acid, and for the soluble fractions of these constituents, were in general unrelated to stage of lactation.

The variations in values for sodium, chloride, potassium, lactose and acidity with stage of lactation are shown in Fig. 4. Sodium and chloride showed parallel changes. The concentrations of both tended to be above average at the beginning of lactation, rapidly decreased to a minimum level, then gradually increased until near the end of lactation when there was a rapid increase to high levels. The changes in lactose concentration during lactation were the opposite of the changes in concentrations

of sodium and chloride, and were similar to the changes in potassium concentration, except that the concentration of the latter was above and not below average in early lactation. The variations in the concentrations of these four soluble constituents show how a constant osmotic pressure is maintained in milk; changes in sodium and chloride content were balanced by opposing changes in potassium and lactose content.

Fig. 4 shows also that pH was low at the beginning of lactation, but increased rapidly to a normal level. Thereafter values increased very slowly until near the end of lactation, when values increased rapidly. The changes in titratable acidity, as expected, were the reverse of the changes in pH.

The variation in concentration of constituents at any particular stage in lactation (Figs. 2, 3 & 4) was due to the individual variation in composition with stage of lactation of the milk from the 36 cows. However, the results from individual cows sampled at intervals throughout their lactation defined more precisely and confirmed the changing pattern of composition illustrated in Figs. 2, 3 and 4. The results from these cows showed also that in the milk from some of them, the total magnesium content varied with stage of lactation in the same way as total calcium and that soluble magnesium and soluble citrate decreased as lactation progressed. With the other individuals these relationships were not evident. The differences in the milk from individual cows

Table 9 Relationships between the pH of milk and the concentrations of certain milk constituents

Relationship with pH	
Inversely related	Directly related
Titratable acidity	Colloidal inorganic calcium
	Colloidal inorganic phosphorus
Total soluble calcium	Colloidal calcium phosphate
Soluble unionized calcium	
Soluble ionized calcium	Caseinate calcium
Soluble magnesium	Proteose-peptone nitrogen
Soluble citric acid	
	Sodium
Total phosphorus	Chloride
Soluble inorganic phosphorus	
Ester phosphorus	
Potassium	
Lactose	

probably explains the lack of relationship between the concentrations of the fractions of magnesium and of citric acid and stage of lactation when the results from the 36 cows were considered collectively.

Interrelationships of milk constituents From the lactational changes in milk composition just described it can be seen that there were relationships between the concentrations of certain milk constituents. For example, it was clear that there was a direct relationship between the concentrations of sodium and chloride and that both were inversely related to lactose concentration. The method used to determine further interrelationships was to plot the concentration of each milk constituent against pH for all samples from individual cows, except those from cows with sub-clinical mastitis. Inspection of the scatter diagrams led to the conclusions in Table 9. The most interesting feature of the relationships was the influence of acidity on the relative amounts of soluble and colloidal calcium and phosphorus in milk. When the pH was low (high acidity), the ratio of soluble calcium and phosphorus to colloidal calcium and phosphorus was greater than when the pH was high. The relationships, apart from the one between pH and titratable acidity were not very close.

In general, constituents whose concentrations were related to pH in the same way were directly related and those related to pH in opposite ways were inversely related. For example, there were direct relationships between the concentrations of

soluble ionized calcium and total soluble calcium and between those of sodium and chloride, and there was an inverse relationship between the concentrations of soluble ionized calcium and sodium.

The results obtained on the effect of stage of lactation on milk composition, and the interrelationships of milk constituents were used in the three sections which follow to assist in interpreting the results when examining the effect of milk composition on protein stability.

Part 2

The Coagulation of Milk Protein by Ethanol

Introduction

When ethanol solutions of increasing strength are added, volume for volume, to a series of samples of the same milk, a strength of ethanol is ultimately reached which causes the immediate formation of clots. These clots consist of partially denatured casein, still associated with calcium and calcium phosphate, with possibly a small amount of contaminating lactalbumin and lactoglobulin (Sutermeister & Browne, 1939). As already mentioned, casein sols coagulate when either the degree of hydration or the net negative charge on the micelles is sufficiently reduced. The coagulation of the casein in milk by ethanol can therefore most probably be attributed to the neutralisation of the charge on the partially dehydrated casein by calcium and magnesium ions, which, by virtue of their concentration and divalency have greater coagulating power than the other cations present (McBain, 1950). Since the extent of dehydration and the concentration of divalent cations necessary for coagulation are in all likelihood inversely related, one would expect milks containing large concentrations of calcium and magnesium ions to be coagulated by weaker ethanol solutions than milks containing low concentrations of these cations. There is also the possibility that the strength of ethanol required to coagulate milk may be influenced by the initial degree of hydration of the casein but there is

no evidence on the variability of this factor from milk to milk.

Other factors which may influence the susceptibility of casein to coagulate with ethanol are the concentration of the casein, the proportion of the α, β- and γ-fractions, the composition of the calcium caseinate-calcium phosphate complex and the average size of the casein-complex micelles. Also the phosphate and citrate in milk, through their influence on the ionic conditions surrounding the casein micelles, may modify the neutralizing power of the calcium and magnesium ions: if that is so, the balance between the concentrations of cations and anions will be more important in relation to coagulation than simply the concentrations of cations.

The only comprehensive study that has been published on the relationship between the chemical composition of milk and its stability to ethanol is that of Mitamura (1937). He compared the composition and ethanol stability of 2,704 milk samples, of which 927 were from Ayrshire cows. From his results for all the samples he found that the average strength of ethanol required to coagulate an equal volume of milk was approximately 80% v/v; the majority of samples coagulated within the range 75 to 82% ethanol but the overall range was 66 to 94%. Mitamura also studied the effect of stage of lactation on stability to ethanol by examining the milk from 15 Ayrshire cows throughout two lactations. Up to 7 to 28 days after calving, the milk was very unstable to ethanol, but

thereafter it gradually became more stable and then remained at a fairly constant level of stability, which was specific for each cow. Even in the mid-lactation period, however, instances of unstable milk occurred. Towards the end of lactation, the milk from some cows became more stable to ethanol but the general tendency was towards the secretion of less stable milk.

Mitamura found that these changes in ethanol stability with stage of lactation could be related to changes in the chemical composition of the milk; unstable milks contained more soluble calcium, soluble magnesium and chloride but less soluble inorganic phosphorus than milks stable to ethanol. The important influence of the divalent cations on ethanol stability was confirmed by adding neutral salts to milk, when it was found that stability was inversely related to the concentration of the added cations. The pH and concentrations of fat and of colloidal calcium and phosphorus, on the other hand, were not related to the stability of the protein to ethanol. Feeding various salts to cows did not alter the chemical composition or stability to ethanol of the milk but a diet of fermented soya-bean cake did cause the milk to be temporarily unstable.

The few other investigations dealing with the stability of milk to ethanol are largely concerned with the effect of adding various salts to milk. Sommer & Binney (1923) found that milk which was stable to 75% v/v ethanol was coagulated by this strength of ethanol when 0.2 ml. of 0.25M solutions of either

calcium acetate or magnesium chloride was added to 25 ml. of the milk. On the other hand, up to 0.9 ml. of 0.25M solutions of potassium chloride, dipotassium phosphate and sodium citrate could be added without lessening the stability of the milk. Addition of dipotassium phosphate or sodium citrate to milk, which had previously been made unstable to ethanol by adding calcium acetate or magnesium chloride, counteracted the destabilizing influence of the cations; these results were considered to show the influence of salt-balance on stability to ethanol. These authors found moreover that the milk of three cows was made unstable to ethanol by feeding calcium carbonate even although the total calcium content of the milk from each cow was not altered. They found also that although developed acidity made milk unstable to ethanol, there was no relationship between stability to ethanol and titratable acidity.

Seekles & Smeets (1947) suggested that the 'Utrecht abnormality' of milk, i.e. an instability of the protein to heat and ethanol, prevalent in the Netherlands about 1930, was probably caused by a high concentration of soluble ionized calcium and was not related to the total calcium content. They thought that this abnormality may have been caused by certain diets, or excessive feeding of calcium carbonate to prevent milk-fever, and they demonstrated that it could be cured by feeding or injecting sodium citrate. They pointed out also that Sommer & Binney (1923), when investigating the influence of added

salts on ethanol stability, did not take into account the changes in pH caused by the added salts. Seekles & Smeets found that milk, to which 7 m-equiv. of calcium chloride per litre had been added, coagulated when mixed with an equal volume of 70% ethanol, but when at the same time the pH was kept constant by adding sodium hydroxide, 12 to 14 m-equiv. of calcium chloride were required. They showed also that by adding alkali to lower the concentration of hydrogen and calcium ions, or by adding anions which formed insoluble or weakly dissociated compounds with calcium, e.g. citrate, fluoride, oxalate or phosphate, the stability of milk to ethanol was increased. Eilers (1945) also found that milk was made less stable to ethanol by adding acids or calcium salts but was made more stable by adding bases and salts whose anions formed weakly dissociated calcium compounds.

It is difficult to say whether the diet of cows or the feeding of salts has any influence on the stability of their milk; the reports of Mitamura, Sommer & Binney and Seekles & Smeets are conflicting and inconclusive on this topic. The investigation of Sommer & Binney suggests that instability induced by the ingestion of calcium salts is not caused by an increase in the total calcium content of the milk but by a change in the partition of calcium. Several other reports support the view that diet does have some influence on the stability of milk to ethanol. For example, Echenique & Suarez (1935) and Echenique (1937) found that when cows eat plants unusually

rich in calcium, the milk is unstable to ethanol although normal in acidity. Also, Hughes & Ellison (1949) and Jones-Evans (1949) quote a personal communication from Scarlett & Blomfield stating that cows grazing on land which has a high calcium content often secrete milk which is unstable to ethanol. Rowlands, Barkworth, Hosking & Kempthorne (1950) report that only one of 618 fresh bulk milks examined coagulated with 68% ethanol, but with higher concentrations of ethanol, the incidence of ethanol - unstable samples rose sharply, especially in milk from cows in a limestone district. These authors emphasize that a wide-scale survey is necessary to establish the frequency distribution of the stability of fresh bulk milks to different concentrations of ethanol before an ethanol concentration greater than 68% can be recommended as a measure of the end-point of keeping quality.

From the preceding theoretical considerations and review of published work, it would appear that the most important factors governing the stability of casein to ethanol are the concentrations in milk of the divalent cations, calcium and magesium. According to van Kreveld and van Minnen (1955), milk contains about 4.5 times as much ionized calcium as ionized magnesium and therefore the concentration of calcium ions is probably the most important single factor. Some evidence for this hypothesis has been obtained by Smeets (1952), Seekles & Smeets (1954) and Boogaerdt (1954) for milk abnormally sensitive

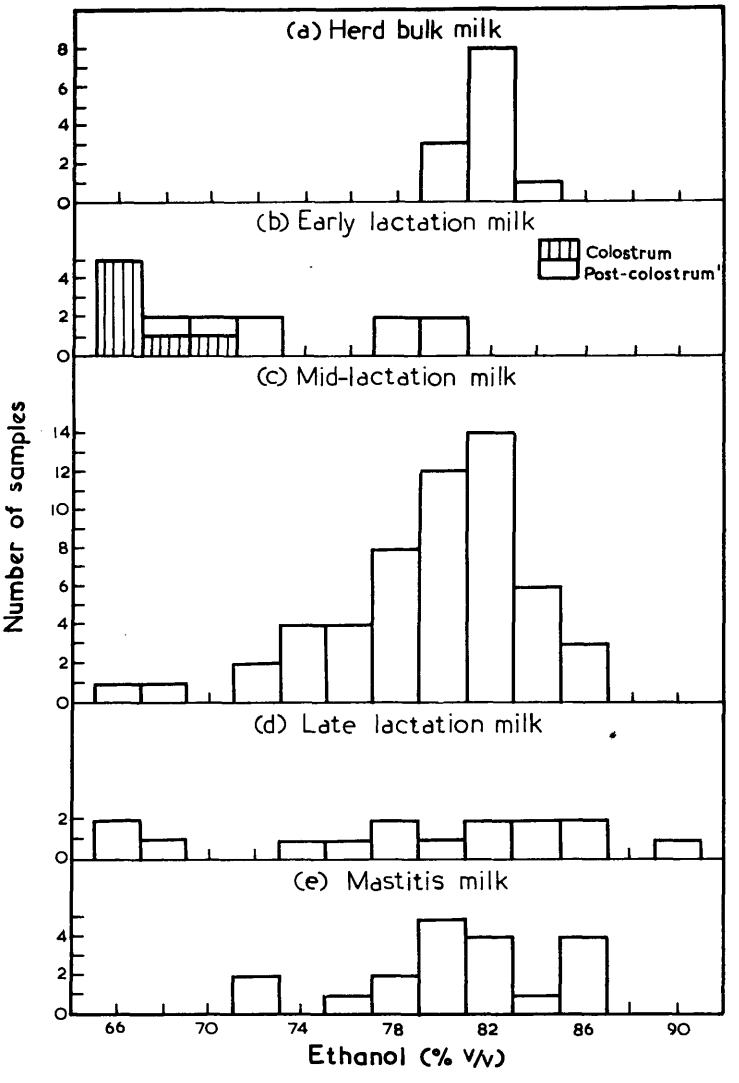

Fig. 5 The distribution of samples in relation to strength of ethanol required for coagulation in each of the five groups of milks.

to ethanol but no really thorough study has been made of the relationship between the detailed composition of milk and ethanol stability. And apart from the work of Mitamura, no account has been taken of the concentration and composition of the calcium caseinate-calcium phosphate complex in milk in relation to its coagulation by ethanol.

In the investigation now to be described bulk milk from a herd of Ayrshire cows, and milks from individual cows in various stages of lactation and from some cows with sub-clinical mastitis were analysed in detail (p. 11) and tested for their stability to ethanol (p. 9) and the relationship between milk composition and ethanol stability studied.

Results

Range of stability of milk protein to ethanol

A wide range of stability to ethanol was found in the milks examined; some were coagulated by the weakest solution used, 66% v/v ethanol, while one milk required 90% v/v ethanol for coagulation. Each of the other eleven ethanol solutions was necessary to classify the milks of intermediate stability.

Herd bulk milk(12 samples) Fig. 5a shows that the herd bulk milks were very similar in stability to ethanol. The range of ethanol solutions required for coagulation was only 80 to 84% v/v, with the average being 82% v/v. The three samples least stable to ethanol were obtained in September, October and November, when many newly calved cows were entering the herd, while the most stable sample was obtained

in May, when the cows were turned out to pasture after stall feeding during the winter.

Early lactation milk (15 samples) Most of the milks in this group were very unstable to ethanol; eleven coagulated with 66 to 72% v/v ethanol and four with 78 to 80% v/v (Fig. 5b) and the average for the group as a whole was 70% v/v ethanol. Five of the seven colostrum samples were coagulated by 66% v/v ethanol. The rapidity and completeness of coagulation in these samples suggested that they would have probably coagulated with an even weaker solution of ethanol.

Mid-lactation milk (55 samples) In this group, 48 of the samples were coagulated by solutions of ethanol varying from 74 to 84% v/v (Fig. 5c). Of the seven samples outside this range, the two coagulated by 66 and 68% v/v ethanol came from the same cow, and the two samples coagulated by 72% v/v ethanol came from another cow. Also, two of the three samples most stable to ethanol came from one cow. The average strength of ethanol required for the coagulation of milks in this group was 80% v/v, which, as expected, was very similar to the average for the herd bulk milks.

Late lactation milk (15 samples) The late lactation samples showed no definite trend in behaviour to ethanol, almost the whole range of ethanol stability being covered (Fig. 5d). The two most unstable milks in the group came from the same cow which provided the two least stable samples in the mid-lactation group. An interesting feature of the late lactation

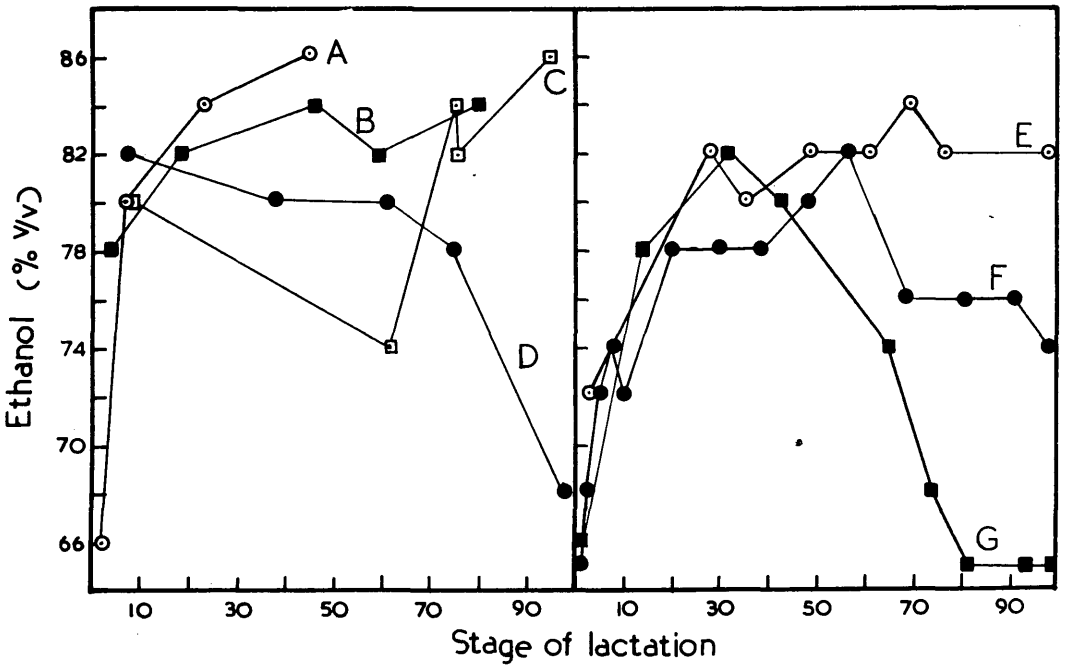

Fig. 6 The relationship between stage of lactation, expressed as a percentage of total lactation, and strength of ethanol required for coagulation in milks from seven cows (cows A and E developed sub-clinical mastitis).

milks was that the coagulum formed with ethanol was usually 'weaker' and more easily dispersed by shaking than that from milks in the herd bulk, early lactation and mid-lactation groups.

Mastitis milk (19 samples) The range of ethanol solutions required to coagulate the mastitis milks was 72 to 86% v/v (Fig. 5e). Most of the samples gave the same 'weak' type of coagulum as the late lactation milks.

Stability to ethanol in relation to stage of lactation

The foregoing survey of the range of stability to ethanol showed that in early lactation, milk was very unstable and that in the mid-lactation period stability was greater but variable, coagulation occurring with ethanol solutions ranging from 74 to 84% v/v for the majority of samples.

The relationship between stage of lactation and ethanol stability in milks of seven cows sampled at intervals during their lactations is shown in Fig. 6. It can be seen that in mid- and late lactation there was considerable individual variation in stability. This variability no doubt accounted for the differences in stability to ethanol seen in Figs. 5c and 5d.

Relationship between chemical composition of milk and stability of milk protein to ethanol

Herd bulk milk The similarity in stability to ethanol (Fig. 5a) of the herd bulk milks undoubtedly resulted from their similarity in chemical composition (Table 4). The samples differed appreciably only in sodium, potassium and chloride contents and it would appear

therefore that these constituents were of little importance in controlling stability.

Although the similarity in chemical composition and in stability to ethanol of the bulk samples made it impossible to say which were the important factors governing stability, the results provided a useful standard for comparison with other types of milk and established 82% v/v ethanol as the strength required for the coagulation of milk of average composition.

Early lactation milk The majority of samples of early lactation milk were coagulated by much weaker ethanol solutions than the herd bulk milks (Fig. 5), and this difference in stability was associated with marked differences in chemical composition (Table 5). The differences in composition between the early lactation and herd bulk milks have been described in detail on p. 25 and were most marked with respect to the concentrations of ester phosphorus, lactalbumin plus lactoglobulin nitrogen and soluble ionized calcium, the early lactation milks being richer than bulk milk in all three constituents. The early lactation milks also had lower pH values and higher concentrations of soluble magnesium, soluble unionized calcium and non-protein nitrogen than the herd bulk milks.

Of these compositional differences the increased concentrations of soluble ionized calcium and soluble magnesium and the lower pH values seemed most likely to be the causes of the instability of the early lactation samples. Other possible factors

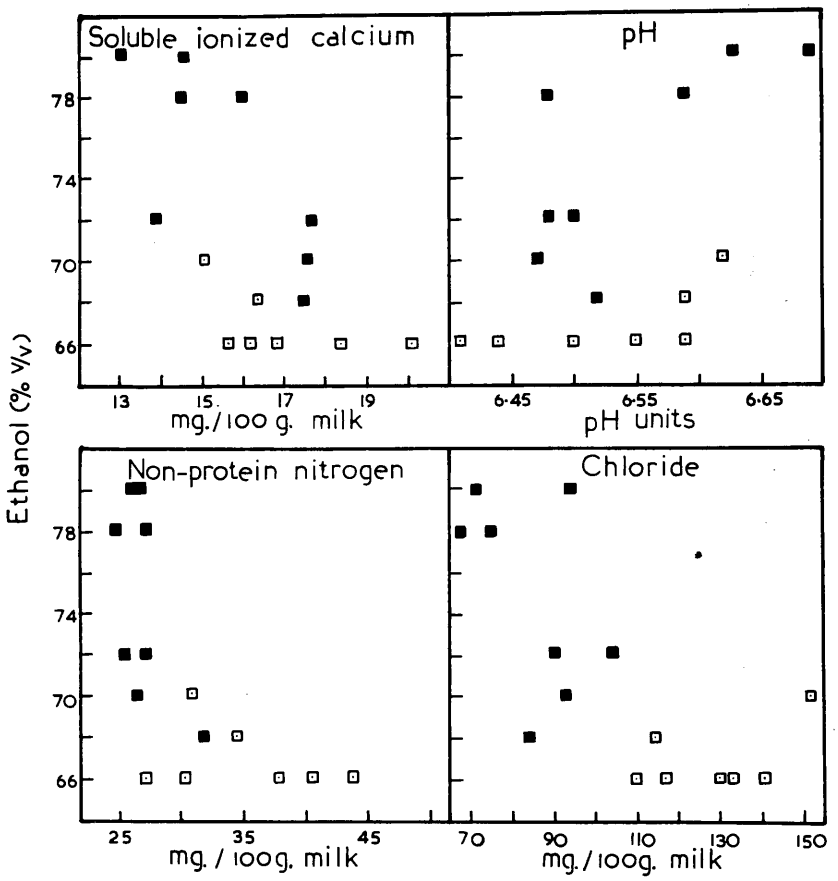

Fig. 7 The relationship of pH and concentrations of soluble ionized calcium, non-protein nitrogen and chloride to strength of ethanol required for coagulation in early lactation milks(⊡ colostrum, ■ post-colostrum milk).

contributing to the instability were the high ratio of lactalbumin plus lactoglobulin to casein, and the smaller amount of calcium phosphate in the caseinate-phosphate complex in early lactation milk.

When the stability and chemical composition of individual samples of early lactation milk were compared, however, only moderately close relationships were found. This may have been due partly to the fact that several of the colostrum samples would undoubtedly have been coagulated by equal volumes of ethanol solutions weaker than 66% v/v. Nevertheless, as is shown in Fig. 7, stability to ethanol tended to increase as concentration of soluble ionized calcium decreased and pH values increased; these relationships are in agreement with the theory relating to factors governing the stability of casein sols. The concentrations of non-protein nitrogen and chloride tended to be inversely related to the concentration of ethanol required for coagulation but there is no obvious reason for regarding these two constituents as being of importance in relation to ethanol stability. These two relationships were due most probably to the coincidental changes in concentration of soluble ionized calcium and pH in early lactation (cf Figs. 2, 3 & 4). It seems probable therefore that the stability to ethanol of the early lactation milks was largely governed by the concentration of soluble ionized calcium and by pH.

Mid-lactation milk As this group contained milks of widely differing stability to ethanol (Fig. 5c) and

Table 10 The average composition of groups of mid-lactation milks (fat-free) arranged in order of increasing stability to ethanol

Ethanol solution causing coagulation (% v/v)	72	74	76	78	80	82	84	86
No. of samples	2	4	4	8	12	14	6	3
				g./100 g. milk				
Total solids	9.08	9.10	8.89	8.95	9.02	9.16	9.35	9.03
Protein (total N x 6.38)	3.28	3.42	3.26	3.22	3.20	3.32	3.49	3.34
Lactose (anhydride)	4.72	4.69	4.61	4.69	4.82	4.79	4.82	4.57
Ash	0.75	0.75	0.74	0.74	0.75	0.77	0.74	0.80
pH	6.60	6.72	6.70	6.71	6.72	6.76	6.76	6.75
Titratable acidity (ml. 0.1N NaOH/100 g. milk)	18.9	16.0	16.0	16.1	16.3	16.4	16.0	16.1
				mg./100 g. milk				
Total calcium	107.1	117.1	108.4	111.0	115.4	119.4	115.0	117.4
Total magnesium	12.3	12.5	11.5	12.2	11.5	12.0	10.8	12.5
Total citric acid	189	188	173	169	170	170	141	133
Total phosphorus	92.9	89.1	85.2	93.8	95.7	98.5	99.6	108.9
Sodium	36	51	57	58	54	61	50	63
Potassium	166	158	144	153	154	156	149	159
Chloride	89.6	108.1	117.4	110.6	100.0	97.6	91.2	111.3
Nitrogen fractions								
Casein N	421.8	427.6	397.4	394.7	392.3	407.0	426.7	404.0
Lactalbumin + lactoglobulin N	53.4	63.7	60.5	61.7	60.7	66.8	72.2	71.1
Proteose-peptone N	15.9	18.7	27.2	25.8	24.5	22.8	24.1	28.1
Non-protein N	23.1	25.5	25.5	23.1	24.1	24.4	23.5	20.7
Calcium fractions								
Colloidal inorganic Ca	36.1	41.5	41.1	42.0	47.9	52.5	53.6	58.9
Caseinate Ca	27.1	32.9	29.3	29.4	29.2	31.3	32.9	30.1
Soluble unionized Ca	31.0	30.1	26.0	26.3	26.4	26.1	20.5	19.1
Soluble ionized Ca	13.1	12.7	12.0	11.8	11.8	11.5	10.6	9.3
Magnesium fractions								
Colloidal Mg	3.9	4.2	3.6	4.1	3.9	4.3	4.1	4.9
Soluble Mg	8.5	8.3	7.9	8.1	7.6	7.7	6.7	7.5
Citric acid fractions								
Colloidal citrate	19	14	10	14	22	15	20	22
Soluble citrate	170	174	163	155	148	155	121	111
Phosphorus fractions								
Colloidal inorganic P	21.2	24.6	23.9	25.6	28.1	29.4	30.5	34.6
Casein P	20.3	23.1	20.4	21.4	20.5	21.6	22.7	20.8
Soluble inorganic P	35.6	32.4	32.8	35.7	36.3	36.0	35.9	45.0
Ester P	15.8	9.0	8.1	11.1	10.8	11.5	10.6	8.6
				mg./g. casein				
Caseinate Ca	10.0	12.2	11.5	11.8	11.7	12.0	11.9	11.7
Casein P	7.5	8.5	8.0	8.6	8.2	8.4	8.4	8.1
Colloidal inorganic Ca	13.4	15.3	16.2	16.8	19.4	20.2	19.1	22.8
Colloidal inorganic P	7.9	9.1	9.4	10.2	11.2	11.4	11.2	13.4
Tricalcium phosphate	27.4	30.7	34.3	32.6	39.7	43.2	37.5	46.9
Dicalcium phosphate	10.6	13.1	11.2	15.7	15.2	13.6	17.1	17.8
Tri-plus dicalcium phosphate	38.0	43.8	45.5	48.2	54.9	56.8	54.6	64.7
Colloidal Mg	1.4	1.5	1.4	1.6	1.5	1.6	1.5	1.9
Colloidal citrate	7.0	5.0	3.9	5.6	9.2	6.0	7.4	6.1

of varying chemical composition (Table 6), it provided greater possibility of relating the two factors. Chemical composition and ethanol stability were first compared by dividing the samples into sub-groups, each containing milks coagulated by the same strength of ethanol, and calculating the mean composition of the milks in each sub-group (Table 10). Since the two sub-groups most sensitive to ethanol (66 and 68% v/v) each contained only one sample whose composition might not be typical of unstable milks, they were omitted from Table 10. Comparison of the mean chemical composition of the samples in the other sub-groups showed that the concentration of several constituents progressively increased or decreased as stability to ethanol increased.

As stability to ethanol increased, total citric acid decreased, the main change occurring in the soluble fraction. At the same time total phosphorus increased mainly as a result of an increase in colloidal inorganic phosphorus. Although the total calcium content was practically the same for each sub-group, as stability increased, the concentrations of unionized and ionized soluble calcium decreased, while the concentration of colloidal inorganic calcium increased. Since colloidal inorganic calcium and colloidal inorganic phosphorus are associated in milk as colloidal calcium phosphate, and as most of the soluble unionized calcium and soluble citrate are probably in the form of calcium citrate, the changes in the concentrations of calcium, phosphorus and

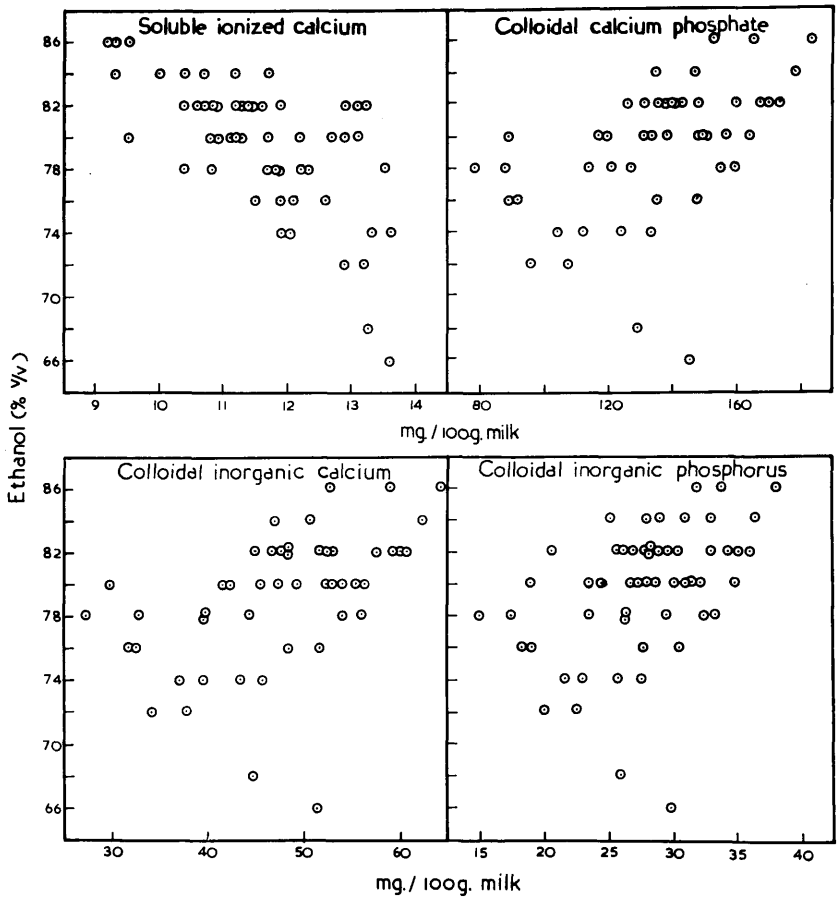

Fig. 8 The relationship of concentrations of soluble ionized calcium, colloidal calcium phosphate, colloidal inorganic calcium and colloidal inorganic phosphorus to strength of ethanol required for coagulation in mid-lactation milks.

citrate meant that as stability to ethanol increased, the concentration of colloidal calcium phosphate increased and the concentration of soluble calcium citrate decreased. The concentration of calcium phosphate also showed an increase with increasing stability, when expressed as mg./lg. casein.

Although the concentrations of total protein in the sub-groups were similar, those more stable to ethanol were richer in lactalbumin plus lactoglobulin and had a higher proportion of these proteins to the coagulable protein, casein. The more stable sub-groups also had slightly higher pH values than the less stable groups.

Since the concentration of none of the other milk constituents appeared to be related to stability, it was concluded tentatively from Table 10 that the factors which might determine the stability of milk to ethanol were pH and the concentrations of soluble ionized calcium, colloidal calcium phosphate, soluble calcium citrate and lactalbumin plus lactoglobulin, and also the proportion of these proteins to casein.

The method of relating composition and ethanol stability used above did not take into account the variation in composition of the samples within each sub-group. The second step in examining the results was therefore to plot the concentration of all the constituents listed in Table 10, in each sample against the corresponding strength of ethanol required for coagulation. The best relationships obtained by this method are shown in Figs. 8 and 9. Fig. 8

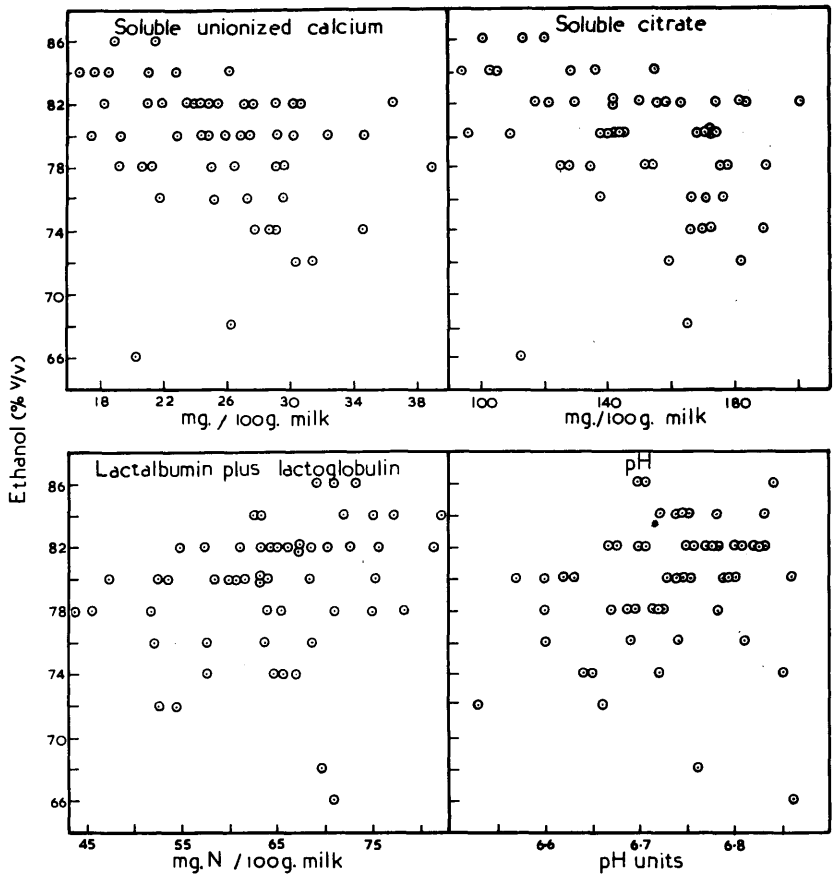

Fig. 9 The relationship of pH and concentrations of soluble unionized calcium, soluble citrate and lactalbumin plus lactoglobulin nitrogen to strength of ethanol required for coagulation in mid-lactation milks.

confirms that there was an inverse relationship between the concentration of soluble ionized calcium and ethanol stability. The other diagrams in Fig. 8 also confirm the conclusion from Table 10 that as stability to ethanol increased so did the concentration of colloidal calcium phosphate, and naturally so did the concentrations of its components, colloidal inorganic calcium and phosphorus.

There were theoretical difficulties in calculating the concentration of soluble calcium citrate in the samples, but since Fig. 9 shows that the concentrations of soluble unionized calcium and soluble citrate both tended to increase as ethanol stability decreased, it is very probable that the concentration of calcium citrate was likewise inversely related to ethanol stability. The two lower diagrams in Fig. 9 show that there was a slight tendency for milks more stable to ethanol to have a higher pH value and to contain more lactalbumin plus lactoglobulin. There was also a slight suggestion of a positive relationship between the ratio of lactalbumin plus lactoglobulin to casein and the strength of ethanol required for coagulation. Of the remaining constituents listed in Table 10, soluble magnesium was the only factor that appeared to be related to ethanol stability; the inverse relationship in this case was poor.

It can be seen from Figs. 8 and 9 that the composition of the two least stable milks, coagulated by 66 and 68% v/v ethanol respectively and omitted from Table 10, was related to stability in the same way as that of the other samples, only with respect

to soluble ionized calcium. This suggested that the concentration of soluble ionized calcium in the mid-lactation samples was probably more important in controlling stability than the concentration of the other constituents, and that the relationships of the latter with stability resulted only from their relation to soluble ionized calcium (see Table 9). The relationship between concentration of soluble ionized calcium and stability to ethanol is the best of those illustrated in Figs. 8 and 9. The correlation coefficient (r) was -0.64 (significant at 0.001 level); r^2 is thus equal to 0.41 indicating that the concentration of soluble ionized calcium accounted for about 40% of the total variability in stability to ethanol. Further statistical analyses kindly made by Dr D.J. Finney, F.R.S. showed that the relationships between the concentrations of the other constituents and stability (Figs. 8 and 9) were due mainly to the relationships between the levels of these constituents and that of soluble ionized calcium in the samples, as suggested above.

Late lactation milk The composition of the late lactation milks (Table 7), described on p. 27, differed from that of the herd bulk and mid-lactation milks in many respects. The differences in composition, however, unlike those of early lactation milk, were not associated with any marked general stability or instability to ethanol (Fig. 5d). The late lactation milks were generally richer in sodium, chloride and in the fractions of non-casein nitrogen and contained

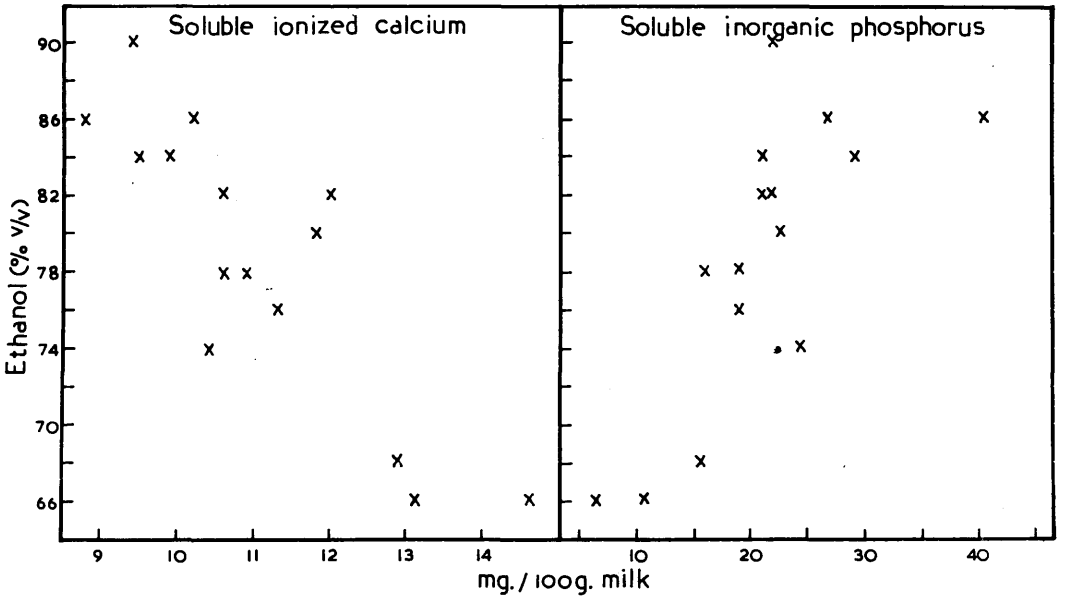

Fig. 10 The relationship of concentrations of soluble ionized calcium and soluble inorganic phosphorus to strength of ethanol required for coagulation in late lactation milks.

smaller amounts of lactose, potassium, soluble inorganic and ester phosphorus than the mid-lactation samples, yet the range of stability of both types of milk was similar. It would appear therefore that none of these constituents was an important factor in controlling the stability of milk to ethanol. It was also noteworthy that the higher pH values and the lower titratable acidities of the late lactation samples did not result in a high stability to ethanol. The fact that the two most unstable samples had pH values greater than 7.0 suggested also that acidity alone, was not an important factor in determining the concentration of ethanol required to coagulate the late lactation milks. The only two constituents which had similar means and ranges of values in the groups of late and mid-lactation milks were soluble ionized calcium and soluble magnesium. It therefore seemed reasonable to conclude that the similar ranges of stability to ethanol of the milks of these two groups, resulted from their similar content of soluble ionized calcium, and possibly also of soluble magnesium.

When the concentrations of the chemical constituents (Table 7) in the individual late lactation milks were plotted against the corresponding concentration of ethanol required for coagulation, the only relationships found were those shown in Fig. 10. Concentration of soluble ionized calcium, as with early and mid-lactation milks, was inversely related to the strength of ethanol required to produce coagulation. The relationship between the

concentration of soluble inorganic phosphorus and stability to ethanol was probably of little significance since it was not found in any of the other groups of milks and may have resulted from the tendency for the concentrations of soluble inorganic phosphorus and soluble ionized calcium to be inversely related in late lactation milks. Soluble magnesium and pH were not related to ethanol stability in these samples.

Thus the results for the late lactation milks confirmed the finding from the early and mid-lactation groups that the concentration of soluble ionized calcium was an important factor in controlling the stability of casein to ethanol.

Mastitis milk The samples of milk from cows with sub-clinical mastitis varied appreciably in stability to ethanol (Fig. 5e) but showed no definite trend towards either increased or decreased stability compared with the herd bulk milks despite the fact that the two groups differed considerably in chemical composition (Table 8). The compositional differences were similar in some respects to those between herd bulk and late lactation milks, e.g. the average values in mastitis milk for pH, sodium, chloride and the non-casein nitrogen fractions were higher and the values for lactose, citric acid and soluble phosphorus were lower than the corresponding herd bulk averages. It could be concluded therefore that these abnormalities in composition of the mastitis samples had little effect on their stability to ethanol. The fact that

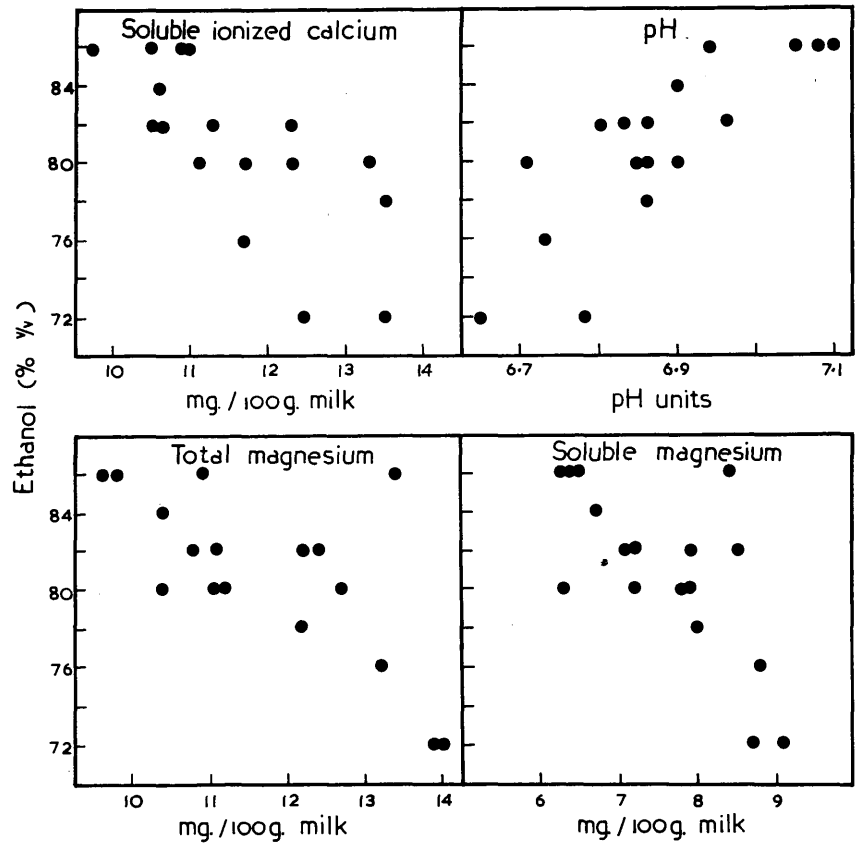

Fig. 11 The relationship of pH and concentrations of soluble ionized calcium, total magnesium and soluble magnesium to strength of ethanol required for coagulation in milks from cows with sub-clinical mastitis.

the average concentration and range of concentration of soluble ionized calcium and soluble magnesium in the mastitis milks were similar to the corresponding values for the mid- and late lactation samples, suggested that these constituents, in this group also, were probably important in controlling stability to ethanol.

This was confirmed by plotting the concentrations of all constituents listed in Table 8, in each mastitis milk, against the strength of ethanol required for coagulation. The closest relationships found are shown in Fig. 11. The concentrations of soluble ionized calcium, total magnesium and soluble magnesium were inversely related to stability whereas pH was directly related. The latter relationship, as in the early and mid-lactation groups, could probably be attributed to the inverse relationship between pH and concentration of soluble ionized calcium in milk (Table 9). The concentrations of sodium and chloride tended to be higher, and the concentration of potassium to be lower in the more stable mastitis samples and vice versa but since these relationships were poor and the opposite of those found in some of the other groups of milks, they added to the general evidence that the concentrations of the monovalent ions in milk were unimportant in relation to stability to ethanol.

The 'loose' type of coagulum formed in many of the mastitis milks may have been caused by their lower content of casein but it should be noted that

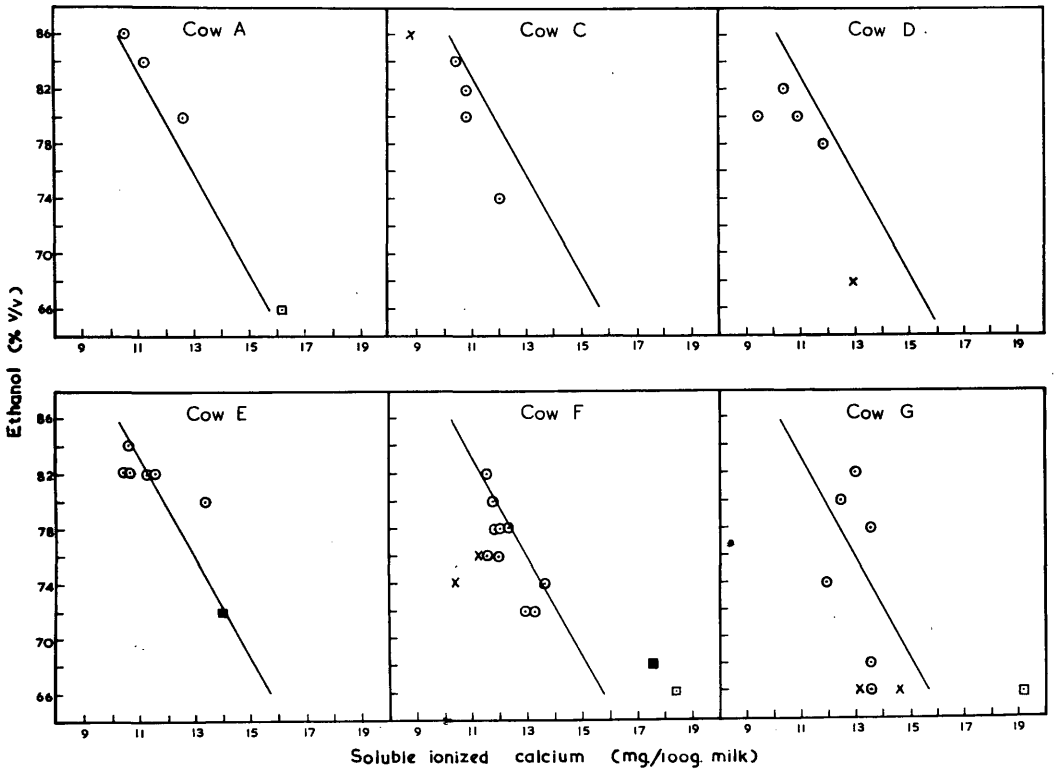

Fig. 12 The relationship of concentration of soluble ionized calcium to strength of ethanol required for coagulation in milks from six cows (—— regression line for relationship between concentration of soluble ionized calcium and strength of ethanol required for coagulation calculated from results for all samples from individual cows Fig. 13 , ⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, x late lactation milk).

a similar type of coagulum was obtained in late lactation milks whose casein content was above normal.

It was concluded that even in this group of abnormal, mastitis milks the chief constituent controlling stability to ethanol appeared to be soluble ionized calcium, augmented possibly by magnesium ions, thus confirming once again the theoretical prediction.

Milk from cows sampled several times during lactation

The absence of close relationships between certain chemical constituents and stability to ethanol in the groups of milks referred to above may have resulted from the fact that the samples came from many cows and would be likely to show considerable individual variation. To find the extent of this individual variation, comparison was made between chemical composition and stability to ethanol in milks collected at intervals from each of six cows. The number of samples from each cow was not large but the results (Fig. 12) confirmed that the concentration of soluble ionized calcium was, in general, inversely related to stability. However, Fig. 12 shows also that for one cow (G), the relationship was not close and that the concentration of soluble ionized calcium associated with a particular stability level varied slightly from cow to cow.

The examination of the milks from the individual cows showed also that none of the other milk constituents could be consistently closely related to stability.

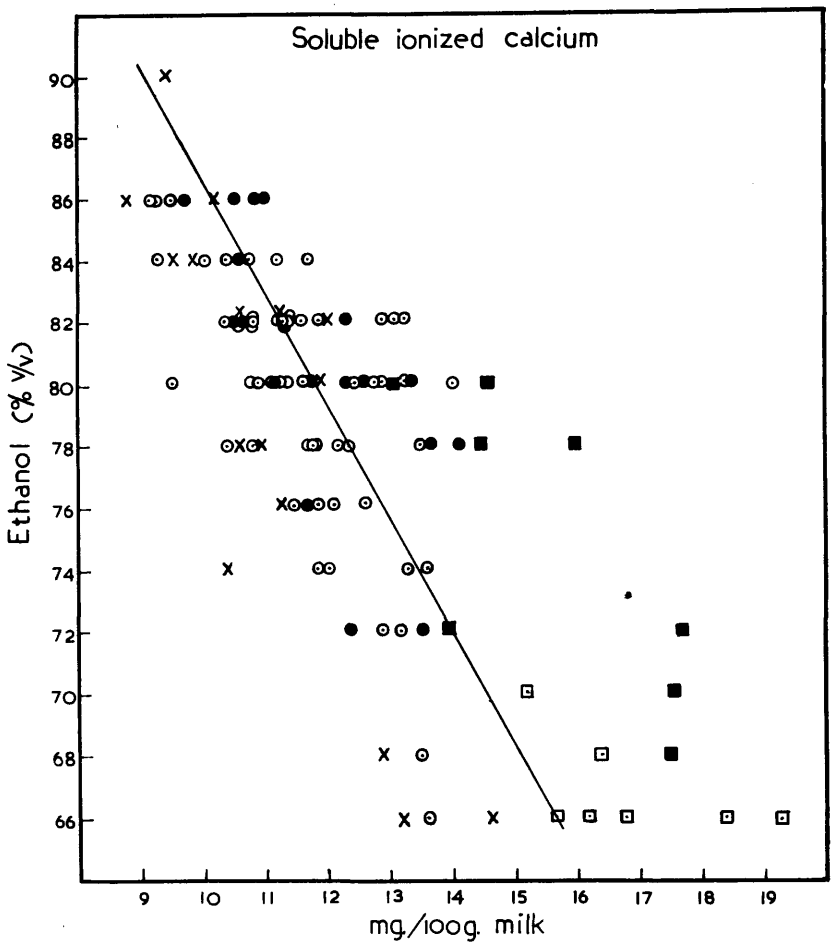

Fig. 13 The relationship of concentration of soluble ionized calcium to strength of ethanol required for coagulation in milks from individual cows in different stages of lactation and from cows with sub-clinical mastitis (—— calculated regression line, ⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, X late lactation milk, ● mastitis milk).

Comparison of chemical composition and stability to ethanol of all milk samples from individual cows

It has been shown that in all the groups of milks there was an inverse relationship between the concentration of soluble ionized calcium and stability to ethanol. To find the extent to which these two variables in all samples would fit a common regression line, all values for the concentration of soluble ionized calcium and strength of ethanol required for coagulation were compared (Fig. 13). With the exception of some of the values for the colostrum and post-colostrum samples, most of the values fitted the regression line reasonably well. The correlation coefficient was -0.76 (significant at 0.001 level) which means that about 60% of the total variability in stability to ethanol could be attributed to the concentration of soluble ionized calcium. The slope of the line indicated that, on average, an increase or decrease of 1 mg. of soluble ionized calcium per 100 g. of milk would cause the strength of ethanol required for coagulation to decrease or increase by 4% v/v.

Although the correlation coefficient would probably have been greater if the interval between the strengths of ethanol used in measuring the stability of the milks had been less than 2% v/v, and if weaker ethanol solutions had been used to test the colostrum samples, there seems little doubt that soluble ionized calcium was not the only factor influencing stability. It appeared that the effect

of other factors was most pronounced in the post-colostrum milks since these samples were considerably more stable than their content of soluble ionized calcium would have suggested. Although the type of correlation shown in Fig. 13 is of little use in predicting the stability to ethanol of the milk of an individual cow from its content of soluble ionized calcium, it could be used to predict the stability of a bulk milk. For example, the regression line indicates that a milk containing 11.3 mg. per 100 g. milk of soluble ionized calcium would be coagulated by 82% v/v ethanol, a relationship which agrees with the average data for the herd bulk milk, i.e. 11.4 mg. soluble ionized calcium per 100 g. milk and 82% v/v ethanol required for coagulation.

When the results for all samples were considered collectively it was also found that the concentrations of soluble unionized calcium and soluble magnesium tended to be inversely related, and pH tended to be directly related to ethanol stability. The relationship between soluble unionized calcium and stability most probably resulted from the interrelationship of the two soluble calcium fractions (Table 9) while that between soluble magnesium and stability suggested that the ionized fraction of magnesium augmented the controlling influence of calcium ions. It was noteworthy in relation to pH that although for the majority of samples there was a direct relationship with stability, there were a few samples in which a

high pH was associated with a marked instability. These samples, however, were rich in soluble ionized calcium, which suggested again that the concentration of this constituent was more important than pH in controlling stability to ethanol.

The concentration of casein in the milk samples was not related to ethanol stability and since the amount of colloidal mineral material combined or associated with the casein in the calcium caseinate-calcium phosphate complex was small, it followed that the concentration of the complex was also unrelated to stability. However, since the amount of some of the mineral constituents associated with casein (expressed as mg./g. casein) varied appreciably in the milks of individual cows (Tables 5, 6, 7 & 8), there was the possibility that the composition, as distinct from the concentration of the caseinate-phosphate complex might affect its stability to ethanol. There was, however, no evidence that the proportions of caseinate calcium, casein phosphorus, magnesium and citric acid in the casein complex were related to its stability to ethanol. There was appreciable variation in the amount and composition of the calcium phosphate associated with 1 g. of casein in the different types of milk and there was a direct relationship between the ratio of calcium phosphate to casein and ethanol stability. A closer examination of the results showed, however, that milks in which the ratio of calcium phosphate to casein varied appreciably were coagulated by the same strength of ethanol provided

Table 11 The ratio of calcium phosphate to casein and the concentration of soluble ionized calcium in milk samples coagulated by 82% v/v ethanol

Ratio (mg.calcium phosphate/ g. casein)	Concentration of soluble ionized calcium (mg./100 g. milk)
49.5	10.8
54.3	10.8
58.0	10.8
69.7	11.0
70.1	11.2

they had similar contents of soluble ionized calcium (Table 11). It appeared therefore that the calcium phosphate content of the casein complex was unimportant in determining the stability of the complex to ethanol. The direct relationship between stability and the ratio of colloidal calcium phosphate to casein, and also that between stability and the concentration of colloidal calcium phosphate in milk (Fig. 8), could probably be attributed to the interrelationship of these factors and the concentration of soluble ionized calcium in milk.

Relationship between salt-balance and stability of milk protein to ethanol

The concept of the salt-balance in milk was introduced by Sommer & Hart (1919) to account for the variation in stability to heat of different milks. Salt-balance was expressed as the ratio of total calcium plus magnesium to total phosphate plus citrate in terms of 'gram-equivalents'. Sommer & Hart stated that when the ratio was above or below unity, milk tended to be unstable to heat though most unstable milks contained an excess of calcium plus magnesium. Some subsequent experiments supported the salt-balance hypothesis (Sommer & Hart, 1922, 1926), others did not (Rogers, Deysher & Evans, 1921; Holm, Webb & Deysher, 1932), and in one the evidence was conflicting (Webb & Holm, 1932). In 1923, Sommer & Binney suggested that the salt-balance in milk was related to ethanol stability in the same way as it was related to heat stability.

It is important to realise that the apparent

importance of the salt-balance theory was established, not so much on the relative amounts of calcium, magnesium, phosphate and citrate found to occur naturally in milk, as by experiments in which the ratio was altered by adding soluble salts. In many of these experiments, no account was taken of the fact that the additions would alter the pH of the milk. Moreover, although Sommer & Hart (1919) referred to their salt-balance in terms of gram-equivalents they in fact calculated the balance in terms of gram-moles, and as pointed out by Eilers et al.(1947) they erroneously assumed that there was no tertiary phosphate present in milk.

To calculate a so-called salt-balance in non-equivalent amounts would seem wrong and an attempt was made in the present investigation to calculate a balance in terms of the equivalent amounts of soluble calcium, magnesium, phosphate and citrate in milk. It seemed reasonable to calculate a salt-balance for the aqueous phase of milk only, since all the evidence suggested that the composition of this phase controlled the stability of the casein complex to ethanol. The milliequivalent amounts of soluble calcium and magnesium were added together and from this total was subtracted the combined milliequivalent amounts of soluble phosphate and citrate; soluble phosphate was partitioned into $H_2PO_4^-$ and $HPO_4^=$ by means of Sorensen's phosphate buffer table (Britton, 1955) to enable the amount of each fraction to be calculated.

In every milk sample, the milliequivalents of

anions exceeded the milliequivalents of cations and the greater the excess of anions the more stable was milk to ethanol; this relationship was in agreement with the general theory of salt-balance. However, since there was a good inverse relationship between the excess of anions over cations and concentration of soluble ionized calcium in the samples, the relationship between salt-balance and stability to ethanol could probably be attributed to the interrelationship of salt-balance and concentration of soluble ionized calcium. There therefore seems to be no reason for continuing to regard salt-balance as a factor determining the colloidal stability of casein to ethanol when the latter can be related directly to the influence of ionized calcium.

Discussion

In the work described in this section, the average and range of strength of ethanol required to coagulate an equal volume of milk were much the same as those reported by Mitamura in 1937. Mitamura found an average of 80% v/v ethanol and an overall range of 66 to 94% for 2,704 milks from cows of several breeds, results which agree very well with the average of 80% and range of 66 to 90% obtained here with 104 samples from individual Ayrshire cows. Both investigations showed that early lactation milk, especially colostrum, was very unstable to ethanol and that as lactation progressed the milk became more stable but varied within fairly wide limits. Mitamura reported that unstable milk was more common in late lactation but this

trend was not found in the limited number of late lactation milks examined in the present investigation. The samples from cows with sub-clinical mastitis likewise showed no definite trend in stability. It was concluded therefore that after the first 5% or so of the lactation period, the stability of milk to ethanol did not appear to be closely related to stage of lactation.

Mitamura also compared chemical composition and stability to ethanol and found that unstable milks contained more soluble calcium, soluble magnesium and chloride but less soluble inorganic phosphorus than stable milks. He found also that pH, fat content and concentrations of colloidal calcium and phosphorus were not related to stability. On the whole, these observations have now been confirmed by the present work in which the more extensive chemical analyses used enabled composition and stability to be compared in more detail.

Since the calcium caseinate-calcium phosphate complex in milk is the fraction which coagulates when an equal volume of sufficiently strong ethanol solution is added, it was noteworthy that the concentration of the complex in milk was not related to its stability. It was true that unstable early lactation milk had a casein content above average but late lactation milk, which usually contained almost as much casein as colostrum, was not unstable to ethanol. However, there was some indication that the concentration of casein determined the firmness of the coagulum. The

coagulum formed by colostrum was always much firmer, and the coagulum from mastitis milk, usually deficient in casein, was frequently less firm than that from the herd bulk or mid-lactation milk. But it should be noted also that late lactation milk commonly gave a 'weak' coagulum even although the casein content of this type of milk was above average.

The composition of the caseinate-phosphate micelles appeared also to have little influence on their colloidal stability to ethanol. Although milks which were more stable to ethanol usually had a higher ratio of colloidal calcium phosphate to casein than less stable milks, there was no evidence that the proportion of calcium phosphate *per se* governed the stability of the caseinate. There was a direct relationship between the concentration of lactalbumin plus lactoglobulin, and also the ratio of these proteins to casein, and stability in the mid-lactation milks but the reverse relationships were true in the early lactation milks. Also, the post-colostrum and late lactation milks both had higher than average, and approximately equal contents of lactalbumin plus lactoglobulin and yet they differed widely in stability to ethanol. These facts, showed that the concentration of the serum proteins in milk had little influence on the stability of the casein. All the results suggested therefore that the stability of the caseinate-phosphate complex in milk to ethanol was independent of the concentration and composition of the complex and also of the other milk proteins; it was concluded

therefore that the composition of the aqueous phase was the factor controlling stability.

It was found that the stability of the casein complex was inversely related to the concentration of soluble ionized calcium whether the milk was from cows in early, mid- or late lactation or from cows with sub-clinical mastitis. The average concentrations of soluble ionized calcium in colostrum and post-colostrum milk, both unstable to ethanol, were respectively 17.0 and 15.6 mg. per 100 g. of milk whereas herd bulk milk, mid- and late lactation milk and mastitis milk, all with 'normal' average stabilities to ethanol, had mean values for soluble ionized calcium of 11.4, 11.6, 11.1 and 11.6 mg. per 100 g. of milk respectively. A very small change in the concentration of soluble ionized calcium was associated with an appreciable change in the stability of the casein to ethanol. The regression line for the relationship between concentration of soluble ionized calcium and stability (Fig. 13) indicated that an increase of 1 mg. in the content of this constituent in 100 g. of milk would decrease the strength of ethanol causing coagulation by about 4% v/v. The correlation coefficient of the relationship in Fig. 13 showed that about 60% of the total variability in stability to ethanol could be attributed to the variation in concentration of soluble ionized calcium. It was concluded therefore that the concentration of soluble ionized calcium in milk was the most important single factor determining the stability of the casein. For

some unknown reason, the post-colostrum milks were more stable than their contents of soluble ionized calcium would suggest.

Since the magnesium ion is similar to the calcium ion in its neutralising and coagulating power towards negatively charged colloids, it was probable that the concentration of magnesium ions in milk, although reported to be only about one fifth of the calcium ion concentration, was one of the factors responsible for the remaining 40 % of the variation in ethanol stability. The concentration of magnesium ions in the milks was not determined but since low stability was usually associated with a high concentration of soluble magnesium and vice versa, magnesium ions probably supplemented the influence of calcium ions in controlling the colloidal stability of the casein complex.

In some of the groups of milks pH and the concentrations of certain other constituents, e.g. soluble unionized calcium, soluble citrate, soluble inorganic phosphorus and chloride were related to stability to ethanol. These relationships, however, could probably be attributed to the interrelationships of the concentrations of these factors and that of soluble ionized calcium in milk. Since the intensity of the negative electrical charge on the casein micelles is dependent on the pH of milk, it might be expected that pH would be a factor in determining stability independent of its relationship to calcium ion concentration. The occurrence of samples with

a high pH, in which the casein micelles would be expected to be stable because of their increased negative charge, but which were unstable because of a high concentration of calcium ions, showed, however, that pH per se did not control the stability of the casein complex.

The use of the salt-balance theory of Sommer & Hart (1919) and Sommer & Binney (1923) appears to be unnecessary in explaining the variation in the stability of casein to ethanol. This variation can be explained quite simply and in accordance with the theory of colloid stability, in terms of calcium ion concentration. Although relatively high concentrations of soluble ionized calcium were found only in early lactation milk, this investigation supports the view of Smeets (1952), Seekles & Smeets (1954) and Boogaerdt (1954) that when instability to ethanol is encountered, the principal cause is an abnormally high concentration of calcium ions in the milk.

Conclusions

1. Samples of herd bulk milk were very similar in stability to ethanol. The range of ethanol solutions required to coagulate an equal volume of milk was only 80 to 84% v/v.
2. Milk samples from individual cows showed a wide variation in stability; coagulation was caused by ethanol solutions ranging in strength from 66 to 90% v/v.
3. The stability to ethanol of milk from individual cows was not related to the stage of lactation of the cow except in early lactation when there was a rapid

transition from the extreme instability of colostrum, through the post-colostrum period, to the greater stability of mid-lactation samples.

4. The nature of the coagulum produced by ethanol was variable. The coagulum produced in late lactation and mastitis milk was 'weaker' and more easily dispersed by shaking than that formed in herd bulk, early lactation and mid-lactation milk. Colostrum formed very large and firm clots.

5. The concentration and composition of the calcium caseinate-calcium phosphate complex in milk were not important in relation to its stability to ethanol.

6. The strength of ethanol required to coagulate the casein complex in an equal volume of milk was principally dependent on the concentration of soluble ionized calcium; as the concentration of this constituent increased, the strength of ethanol causing coagulation decreased. Approximately 60% of the variation in stability was related to the variation in the concentration of soluble ionized calcium.

7. The inverse relationship between the concentration of soluble magnesium and the stability of milk to ethanol suggested that magnesium ions supplemented the effect of calcium ions on stability.

8. The relationships between the concentrations of other milk constituents and stability could be attributed to the interrelationships of the amounts of these constituents and of soluble ionized calcium in milk.

9. The relationship between stability to ethanol and

concentration of soluble ionized calcium is in agreement with the general theory relating to the factors controlling the stability of a negatively charged colloid such as casein as it exists in milk.

Part 3

The Coagulation of Milk Protein by Rennet

Introduction

The addition of a very small amount of rennin to milk causes coagulation in a time which, within limits, is inversely related to the concentration of the rennin and the temperature of the milk. However, even when these two factors are kept constant at suitable levels, a wide variation is found in the times taken by different milks to coagulate and in the firmness of the coagula. Much work has been done to explain the mechanism of rennin action and to account for the different rates of coagulation of milks, but although considerable progress has been made in recent years, reviews of the literature by Berridge (1951) and Pyne (1955) show that much is still obscure.

It has been known for many years that the coagulation of milk by rennin occurs in two distinct stages. In the first or enzymic stage the rennin alters the casein to paracasein at a rate which increases with temperature over the range 0 to 37°C (Berridge, 1942). Recent investigations have shown that this reaction which is the essential prerequisite of coagulation, is a very rapid liberation of non-protein nitrogen (NPN) from α-casein only, converting it to α-paracasein (Alais, Mocquot, Nitschmann & Zahler, 1953; Higgins & Fraser, 1954; Mattenheimer & Nitschmann, 1955; Nitschmann & Keller, 1955; Nitschmann & Bohren, 1955). The rate of liberation of NPN was found to depend on the concentration of

rennin, pH of the milk and temperature; at a normal pH for milk, i.e. 6.7, the temperature coefficient ($Q_{10^{\circ}C}$) of the reaction was about 1.9 between 1 and 30°C. When rennin concentration and temperature were kept constant, the rate of formation of α-paracasein was controlled by pH; the rate increased as pH decreased from 6.7 to 5.5. According to Pyne (1953), the rate of this first stage increases also as the concentration of calcium ions increases.

In addition to the proteolytic action of rennin on α-caseinate during the enzymic stage, there is also a decrease in the viscosity of the milk which has been attributed to a reduction in the hydration and charge of the casein micelles (Söhngen, Wieringa & Pasveer, 1937; Hankinson & Palmer, 1943).

In the second or non-enzymic stage, the rennin-altered caseinate rapidly coagulates under suitable conditions. Calcium ions or other alkaline earth ions are necessary for the coagulation of α-paracasein but their exact function is not known. According to Pyne (1948, 1955) the rate of the coagulation reaction is appreciably altered by a change in calcium ion concentration of as little as 0.8 mg. in a total of 12 - 16 mg. per 100 ml. of substrate. Pyne (1955) states also that pH has little influence on the second stage but Smith & Bradley (1935) report that a decrease in pH accelerates both stages.

The instability of paracasein in the presence of calcium ions might be attributed to its increased base-binding capacity (Palmer, 1928) in conjunction

with the lesser hydration and charge of its micelles. However, the great effect of temperature on the rate of the second stage appears to rule out a simple coagulation of an unstable, negatively charged colloid by positively charged ions. Coagulation does not occur below 15°C but Berridge (1942) found that between this temperature and about 40°C the temperature coefficient of the coagulation stage was very large, namely, 1.3 - 1.6 per 1°C. Since the heat denaturation of proteins has a temperature coefficient of similar magnitude, Berridge suggested that the second stage reaction is essentially a complete or partial heat denaturation of the paracasein resulting in its coagulation. The calcium ions during the coagulation of denatured α-paracasein may act as 'bridges' so linking the paracaseinate micelles. When the concentration of calcium ions is sufficiently high, a premature precipitation of incompletely formed paracaseinate can take place which simulates normal rennin clotting (Pyne, 1955).

Although the first stage of rennin action is the same whether the substrate is calcium caseinate or calcium caseinate-calcium phosphate as in milk, the presence of colloidal calcium phosphate influences the second stage. Calcium paracaseinate-calcium phosphate forms a firm curd and the more phosphate present, the firmer the curd, but in the absence of calcium phosphate, only a flaky precipitate is obtained (Sutermeister & Browne, 1939). Pyne (1953) found also that calcium paracaseinate with associated calcium

phosphate was more sensitive to the coagulating influence of calcium ions than calcium paracaseinate alone. An explanation of the effect of calcium phosphate is suggested by the work of Hostettler & Rychener (1949) and Hostettler & Imhof (1951, 1952). They have shown that calcium caseinate-calcium phosphate is more coarsely dispersed than calcium caseinate, that in milk the larger particles of caseinate-phosphate contain more calcium phosphate than the smaller particles and that renneting time is inversely related to the particle size of the caseinate-phosphate complex. The latter finding is confirmed by the fact that homogenisation increases the particle size of the casein complex in milk (Hostettler & Imhof, 1953) and at the same time accelerates rennet coagulation (Zollikofer, 1949; Sasaki & Miyasawa, 1955). Thus it appears that calcium phosphate influences the second stage of rennin coagulation through its effect on particle size; the more phosphate associated with casein the larger is the size of the casein complex micelles and the more quickly do visible clots appear. The influence of calcium ions on the second stage may also be partly a particle-size effect; Philpot (1938) and Hostettler & Imhof (1951, 1952) have shown that the size of the caseinate-phosphate micelles in milk is directly related to the concentration of calcium ions.

A change in the amount of colloidal calcium phosphate associated with casein might also explain the change in the renneting time of milk stored at low temperatures (Bendixen, 1934; Ling, 1937; Rapp &

Calbert, 1954) and also of gently heated milk (Mattick & Hallett, 1929a; Powell & Palmer, 1935; Powell, 1936; Pyne, 1945) although the recent work of Kannan & Jenness (1956) indicates that the denaturation of lactalbumin and lactoglobulin may be partly responsible for the increased renneting time of heated milk. It should be pointed out that in unheated milk, lactalbumin and lactoglobulin take no part in rennin action and remain in the whey. A normal coagulum is now believed to consist of calcium α-paracaseinate and unchanged β- and γ- caseinates, together with the associated calcium phosphate (Cherbuliez & Baudet, 1950).

From the foregoing discussion of the mechanism of rennin action it would appear that the variation in the renneting time of different milks and in the physical nature of the curd might be attributed to variations in pH, calcium ion concentration and the ratio of colloidal calcium phosphate to casein. Another possible factor might be a variation in the proportion of α-casein in the whole casein. However, the constancy of the chemical composition of whole casein from different milks, despite considerable differences in the chemical composition of the α-, β- and γ- forms (Gordon, Semmett, Cable & Morris, 1949), suggests that variation in the proportion of α-casein is not likely in practice to be a cause of variations in renneting time. Also, Pyne (1953) is of the opinion that the amount of natural variation in the calcium phosphate content of the casein complex in

milk is insufficient to influence renneting time and that the composition of the aqueous phase, particularly the concentration of calcium ions, is the determining factor.

Many of the investigations concerned with the mechanism of rennin action were made on simple caseinate solutions and dealt with each stage separately. It now remains to consider, in the light of recent research, studies which attempt to relate the overall time taken by both the enzymic and coagulation reactions to form a coagulum, i.e. the renneting time of milk, to the chemical composition of the milk.

Holm, Webb & Deysher (1932) found there was a tendency for renneting time to be long when the total calcium content of milk was low and that there was no relationship between the salt-balance of Sommer & Hart (1919) (refered to in Pt. 2, p. 57) and renneting time. Golding, Mackintosh & Mattick (1935) also found that renneting time appeared to be inversely related to the percentage of total calcium and noted that an influx of milk from newly calved cows coincided with shorter renneting times of herd bulk milk. Ling (1937) discovered, on the other hand, that as renneting time increased so did the concentration of soluble calcium in the milk, and also that the concentration of soluble inorganic phosphorus was inversely related to renneting time. These two relationships resulted in a negative correlation between renneting time and the ratio of soluble inorganic phosphorus to soluble calcium. Ling noted

further that as renneting time increased, the concentrations of soluble organic phosphorus and soluble calcium citrate also increased. From these observations, together with the effect on renneting time of adding various calcium, citrate and phosphate salts to milk, Ling concluded that salt-balance might influence renneting time. Mocquot, Alais & Chevalier (1954) found that milk slow to coagulate with rennet had low contents of soluble and colloidal calcium, soluble and colloidal inorganic phosphorus and a high content of organic phosphorus. From centrifugal studies on normal and slow renneting milks, these workers concluded that the composition of the casein complex, as well as that of the aqueous phase, affected renneting time.

Further evidence on the effect of calcium on renneting time is available from experiments in which calcium was added to or subtracted from milk. It has been demonstrated by many workers, e.g. Parisi (1933), Smith & Bradley (1935), McDowall, Dolby & McDowell (1937), Berridge (1942), Vilegzhanin (1942) and Wakui & Kawachi (1954), that when calcium is added to milk renneting time decreases, whereas Lyman, Browne & Otting (1933) and Sasaki, Tsugo & Nakai (1955) found that milk from which 20% of the calcium had been removed, did not coagulate with rennet. The last investigation showed also that there was a progressive increase in renneting time as the amount of calcium removed increased.

The addition to milk of magnesium ions, as well

as of calcium ions, was shown by van der Waarden (1948) to decrease renneting time and increase the firmness of the curd, though Pyne (1955) states that magnesium (and strontium) has only half the accelerating effect of calcium on the coagulation stage. Berridge (1951) concluded from a review of the literature that the accelerating effect on renneting time of added divalent cations is due partly to the decrease they cause in the pH of milk, but that for calcium ions, the accelerating effect persists even when pH is kept constant. Berridge states further that trivalent cations also promote the coagulation stage of rennet action whereas monovalent cations inhibit it, though the evidence for the latter opinion is inconclusive. Wakui & Kawachi (1954) have confirmed the earlier findings of Mazé & Mazé (1941) that the addition of metals such as cobalt, copper and nickel retards the coagulation of milk by rennet; the latter authors suggest that the natural variation in the concentration of trace metals in milk may affect renneting time. The increase in renneting time of milk containing added polyvalent anions such as oxalate and citrate (van der Waarden, 1948), has been attributed to the ability of these ions to combine with calcium.

It is noteworthy that although renneting time is always altered in the expected way by addition or subtraction of calcium, no consistent relationship has been established between the naturally occurring total or soluble calcium contents of milk and renneting time. These facts suggest that it is the

partition of calcium in milk, especially the concentration of ionized calcium that is important in relation to renneting time. It would also appear from the literature that variations in the concentrations of cations in milk other than calcium, will have relatively little effect on renneting time and that the only anions of consequence will be those which can combine with calcium to give weakly ionized compounds, namely, citrate and phosphate.

Although the precise function of pH on the action of rennin on milk has only recently been revealed by the work of Nitschmann and his associates, earlier experiments had indicated the importance of pH in relation to the renneting time of milk. Sanders, Matheson & Burkey (1936) concluded, after comparing the renneting time and pH of 300 milk samples, that pH was the major factor controlling renneting time; low pH was associated with short renneting time and vice versa. They also noted that milk quick to coagulate usually formed a firm curd and that slow renneting milk usually gave a soft curd. Holm et al. (1932) noted that renneting time tended to be long when the pH of milk was high and Ling (1937) found that renneting time was short when the serum acidity of milk was high.

The addition of acid to milk has also been found to shorten renneting time (Parisi, 1933; McDowall et al., 1937; van der Waarden, 1948) and Ling (1956) quotes evidence that a slight development of acidity in milk caused more rapid coagulation with

rennet. The effect of addition of acid on curd firmness, however, has given variable results. van der Waarden noted that lowering the pH increased curd firmness whereas McDowall *et al*. found that it had little effect on the nature of the coagulum.

Further evidence on the influence of pH on renneting time is available from the work of Sommer & Matsen (1935) and Sanders *et al*.(1936). They found that milk from cows with sub-clinical mastitis usually had a long renneting time and gave a soft curd; it is well known that milk of this type often has an abnormally high pH value. Another possible explanation of the long renneting time of milk from cows with infected udders is provided by the observation of Hostettler & Imhof (1951, 1952) that the casein micelles in the milk from these cows are smaller than normal. Also, Hadley (1936) and Doan (1938) suggested that the low concentration of casein and calcium in mastitis milks might be responsible for their abnormal coagulation with rennet.

The influence of casein content on the behaviour of milk with rennet is shown by the occurrence of 'soft curd milk'. This type of milk has a normal pH, is obtained from cows free from mastitis but is invariably deficient in casein (Weisberg, Johnson & McCollum, 1933; Riddell, Caulfield & Whitnah, 1936; Doan, 1938; Henson & Miller, 1955). Sanders *et al*. (1936) believe that the concentration of casein in milk is more important in determining curd firmness than coagulation time and Anderson, Hankinson,

Plastridge & Weirether (1936) found a strong positive correlation between casein content and curd tension in both mastitis and non-mastitis milk. It therefore appears that while pH is a major factor in determining renneting time, the casein content of milk largely determines the firmness of the coagulum.

The preceding review of the literature has indicated that such factors as pH and concentration of soluble ionized calcium have a marked effect on the coagulation of casein by rennet. It has also revealed, however, certain anomalous results when the composition of milk and renneting time were compared, e.g. the variable relationships found between renneting time and the concentrations of total and soluble calcium occurring naturally in milk. The present investigation involved a detailed chemical analysis of a series of milk samples, which was designed to include all the factors which had previously appeared to be of importance, and also the determination of the renneting time of the samples under standard conditions (p.10). By adopting this approach it was hoped to determine which of the factors in milk have the greatest effect on renneting time.

Results

Range of stability to rennet

A wide variation was found in the renneting times of the 108 milk samples examined. The range was from 1.4 to 12.9 min. and two samples did not coagulate. There was also much variation in the physical nature of the coagula. In general, milks that coagulated

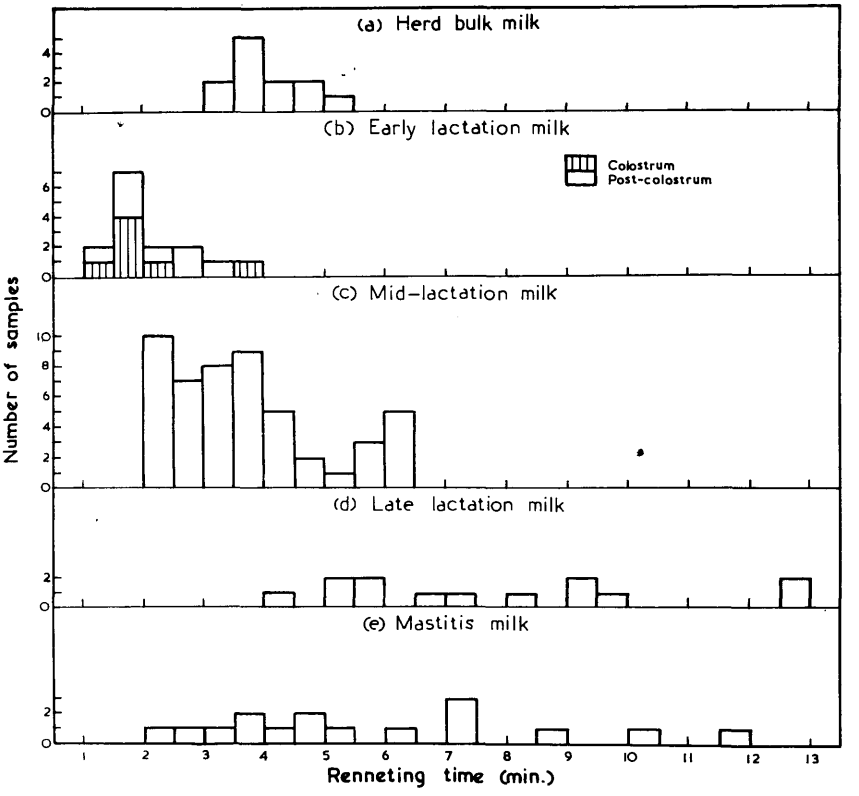

Fig.14 The distribution of samples in relation to renneting time in each of the five groups of milks.

quickly gave a firm curd and those which coagulated slowly gave a soft or loose curd.

Herd bulk milk (12 samples) The distribution of the renneting times of the herd bulk milks is shown in Fig. 14a. The average renneting time was 4.1 min. with a range of 3.2 to 5.4 min. Samples collected in late autumn, winter and early spring coagulated more rapidly than those obtained in late spring, summer and early autumn. However, this apparent seasonal effect was in all probability a lactational effect since the majority of cows in the herd calved during the winter months (see Table 1). This aspect is considered later in more detail (p. 80). All the bulk milks gave a firm curd.

Early lactation milk (15 samples) The samples in the two groups of early lactation milk, colostrum and post-colostrum, were rapidly coagulated by rennet (Fig. 14b). Nine of the fifteen samples coagulated in less than 2 min. and only two took more than 3 min. The average time taken by the seven colostrum samples to coagulate was 2.1 min. with a range of 1.4 to 3.7 min., and the average time taken by the eight post-colostrum samples was 2.2 min. with a range of 1.4 to 3.0 min. Most of the early lactation milks gave a curd which was very firm, and firmer than that formed by any of the other types of milk.

Mid-lactation milk (50 samples) The renneting time of the mid-lactation milks varied from 2.0 to 6.4 min. with an average of 3.7 min. (Fig. 14c). As expected, this average was similar to the average renneting time of the herd bulk milks (4.1 min.). Eight of the

nine mid-lactation milks which took longer than 5 min. to coagulate, came from cows whose udders had been injected with antibiotics a few days before sampling because of injury to the udder or because the milk had a rather high bacterial and cell count. There was no chemical evidence that these milks were mastitic. The curd strength of the mid-lactation milks was similar to that of the bulk samples.

Late lactation milk (15 samples) Two of the milks in this group did not coagulate and the renneting times of the remainder ranged from 4.2 to 12.9 min., with an average of 7.9 min. (Fig. 14d). Although the late lactation milks showed much variation in renneting time, the majority took longer to coagulate than the mid-lactation milks. Most of the late lactation samples formed a very soft coagulum which could easily be poured from the tube.

Mastitis milk (16 samples) Samples in this group varied greatly in renneting time; the range was 2.3 to 11.8 min. (Fig. 14e), probably reflecting to some extent a variation in the severity of infection. Eleven of the sixteen samples took longer to coagulate than the average time for the herd bulk milks. Samples with renneting times greater than 4.5 min. formed, in nearly every instance, a very soft curd but when coagulation occurred more quickly, a firm curd was obtained.

Renneting time in relation to stage of lactation

Figs. 14b, 14c and 14d indicate that renneting time tended to increase as lactation progressed and

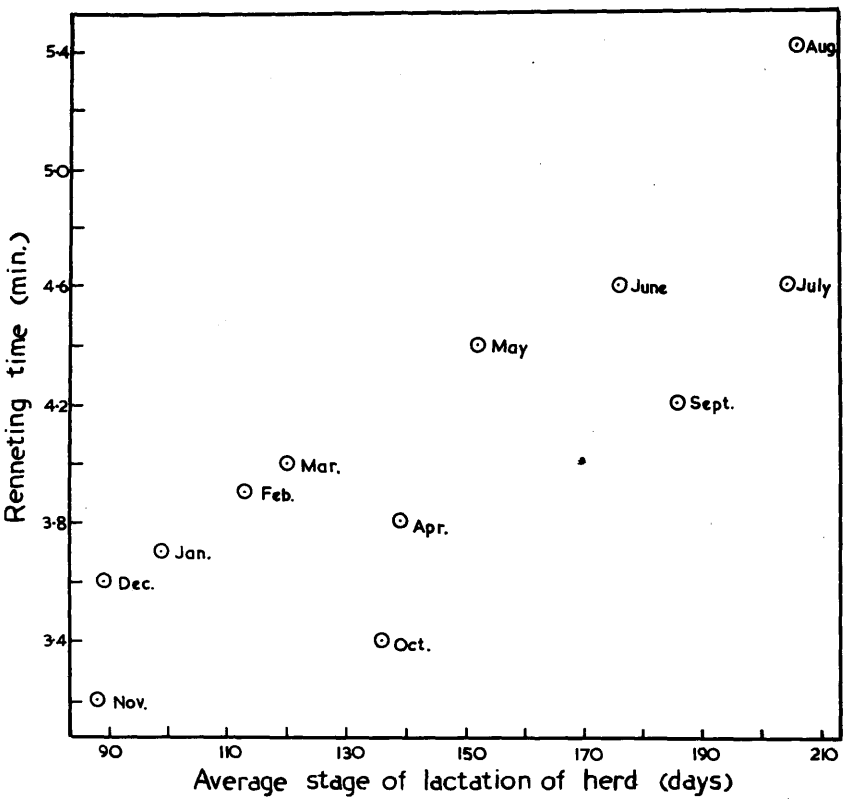

Fig. 15 The relationship between the average stage of lactation of the herd and the renneting time of the herd bulk milks.

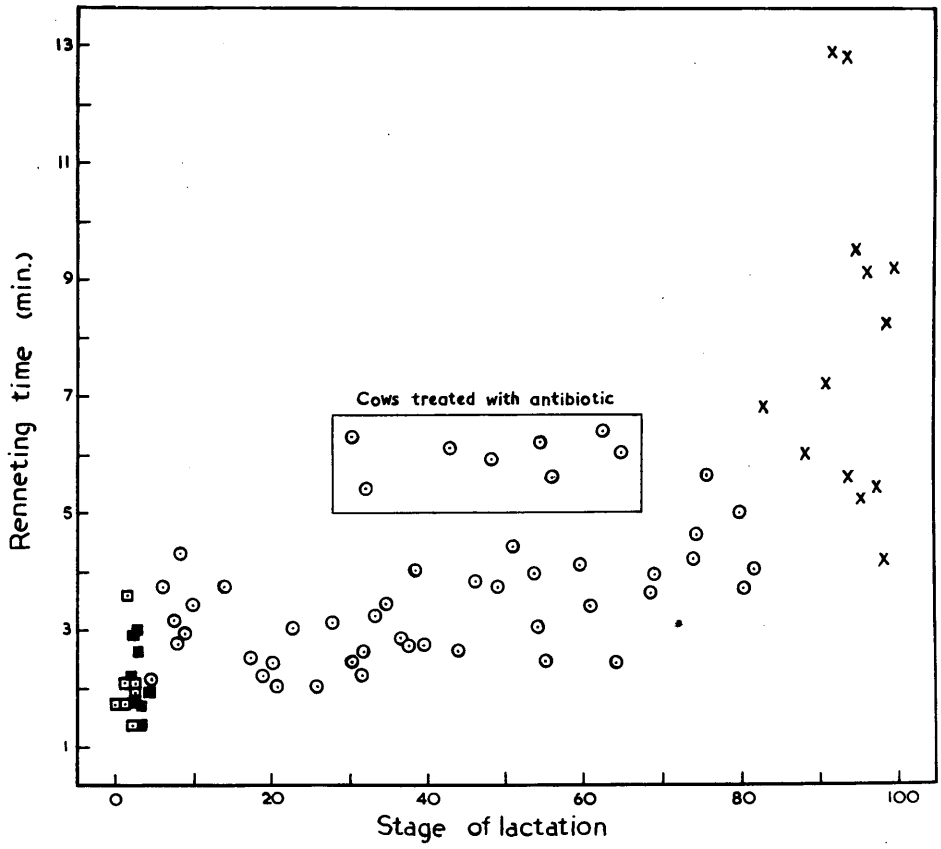

Fig. 16 The relationship between stage of lactation, expressed as a percentage of total lactation, and renneting time in milks from individual cows (⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, × late lactation milk).

this was confirmed in several ways. For example, Fig. 15 shows that, with a few exceptions, as the average stage of lactation of the herd increased, the renneting time of the herd bulk milks also increased. The extremes of renneting time, 3.2 and 5.4 min. correspond with extremes of 88 and 206 days respectively for the average stage of lactation of the herd. Fig. 15 suggests that the apparent influence of season on renneting time could be largely attributed to a lactational effect. The increase in renneting time with advancing lactation is also shown in Fig.16, where the renneting times for all samples from individual cows, excluding mastitis milks, are plotted against the corresponding stages of lactation. The increase was very gradual during the first three-quarters of the lactation period but thereafter became much greater. There was much variation from this general relationship probably because of differences in the composition of milk from individual cows at the same stage of lactation. The eight samples indicated in Fig. 16, whose renneting times appeared abnormally long for their stage in lactation, were those, which as previously mentioned, were obtained from cows treated with antibiotics (dihydro-streptomycin or aureomycin) a few days prior to sampling.

Further evidence of the relationship between stage of lactation and renneting time is given in Fig. 17 which shows the increase in renneting time of milks taken from seven cows at intervals during

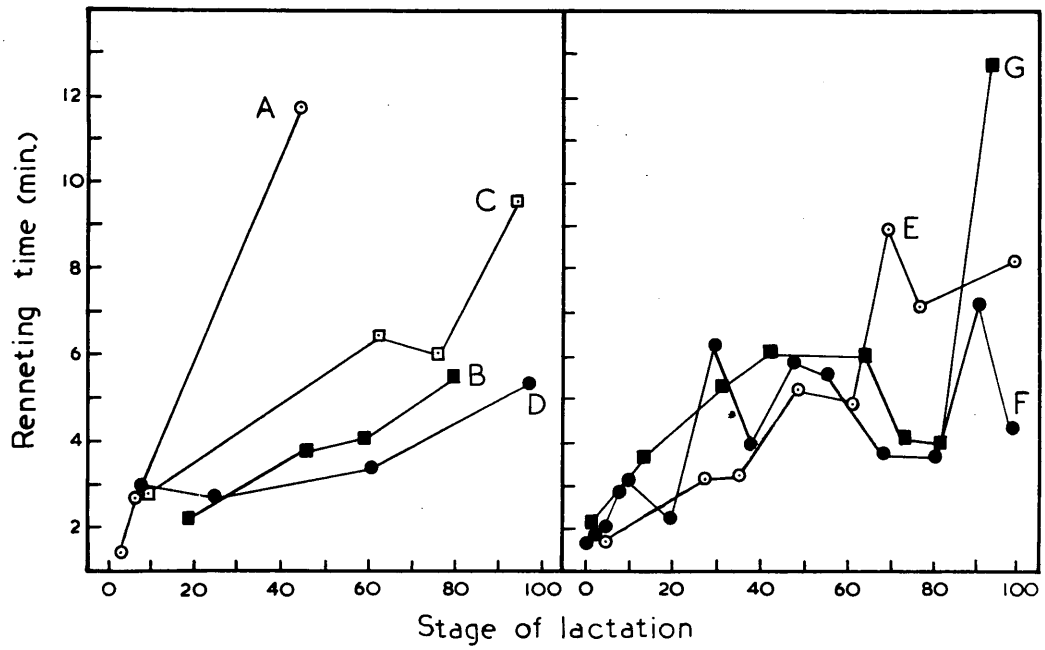

Fig. 17 The relationship between stage of lactation, expressed as a percentage of total lactation, and renneting time in milks from seven cows (cows A and E developed sub-clinical mastitis).

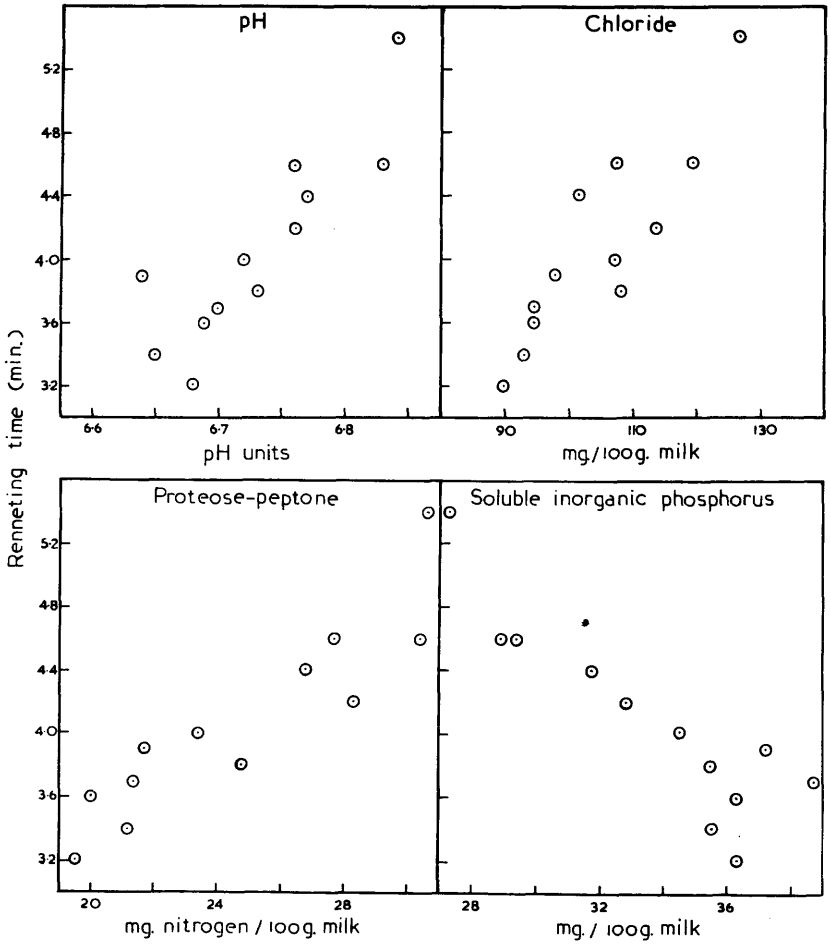

Fig. 18 The relationship of pH and concentrations of chloride, proteose-peptone nitrogen and soluble inorganic phosphorus to renneting time in herd bulk milks.

their lactation. The abnormal increases and erratic variations in the renneting times of the milk from some cows may have been due to the fact that two (A & E) developed sub-clinical mastitis early in lactation and two (F & G) were treated with antibiotic as a prophylactic measure.

Relationship between chemical composition and renneting time

Herd bulk milk The level of all constituents listed in Table 4 in each bulk milk was plotted against the corresponding renneting time; the closest relationships found are shown in Fig. 18. An increase in renneting time was associated with an increase in pH, and in the concentrations of proteose-peptone and chloride, and with a decrease in the concentration of soluble inorganic phosphorus. In addition to these relationships, values for titratable acidity, lactose, total and ester phosphorus, total soluble magnesium and soluble unionized calcium tended to be inversely related to renneting time, whilst with lactalbumin plus lactoglobulin, non-protein nitrogen and sodium the reverse relationship tended to be true. The concentration of other constituents listed in Table 4, which include soluble ionized calcium, casein and colloidal calcium phosphate were unrelated to renneting time.

The renneting times of the herd bulk milks were therefore related to their pH values in the expected way and the relationships found between other milk constituents and renneting time could probably be attributed to the interrelationships of the

concentrations of these constituents and pH (Table 9). The small variation in the concentration of soluble ionized calcium in the herd bulk milks may have been responsible for the lack of correlation between renneting time and the concentration of this constituent within the group.

Early lactation milk The majority of the early lactation milks coagulated more rapidly and formed a firmer curd than the herd bulk milks (Fig. 14). This difference in behaviour with rennet was associated with considerable differences in chemical composition (Table 5: and p.25). The most marked compositional differences were the high content and different partition of nitrogen, the high concentrations of ester phosphorus and of the soluble fractions of calcium and magnesium and the low pH of the early lactation milks. From the evidence already available on the mechanism of rennet action, the decreased stability to rennet of the early lactation samples, as a whole, could be attributed to one or more of three factors, namely, low pH, high concentration of calcium ions and high concentration of casein, though the latter probably caused the firmness of the curd rather than the rapidity of coagulation. Although the literature suggests that a decrease in the amount of colloidal calcium phosphate associated with casein would result in long renneting times and a 'weak' curd, the opposite was found to be true in this group of milks. The milks coagulated rapidly and formed a very firm curd despite the fact

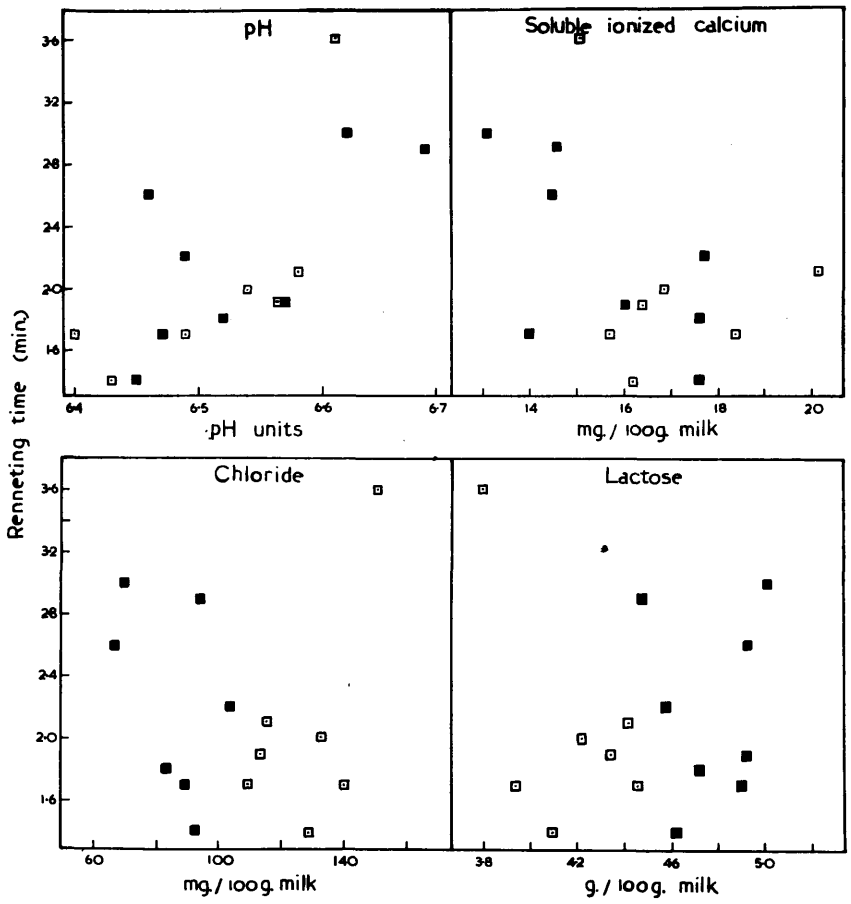

Fig. 19 The relationship of pH and concentrations of soluble ionized calcium, chloride and lactose to renneting time in early lactation milks (⊡ colostrum, ■ post-colostrum milk).

that their casein complex contained, on average, less colloidal calcium phosphate than the casein complex in milks of the other groups.

There was little close relationship between composition and renneting time within the early lactation group of milks. However, as pH increased renneting time tended to increase and there was also a tendency for longer renneting times to be associated with lower concentrations of soluble ionized calcium and chloride and higher concentrations of lactose (Fig. 19). The latter association was probably simply a result of the direct relationship between pH and lactose concentration in the early stages of lactation (Fig. 4). The weak relationship between chloride concentration and renneting time could also be attributed to the interrelation of the concentration of this constituent and pH in early lactation (Fig.4). The fact that the relationship between chloride concentration and renneting time in the early lactation group was the opposite of that in herd bulk milk suggests that the concentration of chloride in milk had no direct influence on rennet action. The concentrations of casein and colloidal calcium phosphate again appeared to be unrelated to renneting time.

Mid-lactation milk In comparing renneting time and chemical composition in the mid-lactation milks, the results for the eight samples taken from cows treated with antibiotics a few days before sampling were omitted. Most of the remaining 42 samples coagulated

Table 12 The average composition of groups of mid-lactation milks (fat-free) arranged in order of increasing renneting times

Rennet coagulation time (min.)	2.0-2.5	2.5-3.0	3.0-3.5	3.5-4.0	4.0-4.5	4.5-5.0	5.5-6.0
No. of samples	10	7	8	9	5	2	1
			g./100 g. milk				
Total solids	9.05	9.24	8.97	9.07	9.17	9.07	9.38
Protein(total N x6.38)	3.19	3.30	3.20	3.35	3.37	3.52	3.63
Lactose (anhydride)	4.80	4.85	4.73	4.67	4.73	4.55	4.80
Ash	0.77	0.78	0.76	0.75	0.75	0.75	0.78
pH	6.67	6.69	6.72	6.76	6.75	6.76	6.77
Titratable acidity (ml. 0.1N NaOH/100g. milk)	17.7	17.4	15.4	15.3	14.6	15.4	17.2
			mg./100 g. milk				
Total calcium	117.8	118.9	115.1	114.5	117.2	114.9	119.3
Total magnesium	12.1	12.3	11.9	11.3	11.8	12.6	12.9
Total citric acid	176	167	159	153	174	156	165
Total phosphorus	98.9	104.0	97.8	94.2	92.1	95.0	98.6
Sodium	54	52	58	58	57	64	62
Potassium	159	169	156	146	153	129	144
Chloride	97.1	95.2	104.8	115.8	99.9	111.4	91.8
Nitrogen fractions							
Casein N	398.1	414.9	388.9	408.1	415.4	420.4	448.6
Lactalbumin + lactoglobulin N	58.7	60.9	64.4	67.4	68.3	78.7	67.5
Proteose-peptone N	21.0	21.8	25.2	24.9	20.5	26.2	23.1
Non-protein N	21.6	23.9	23.0	24.7	24.9	25.4	30.0
Calcium fractions							
Colloidal inorganic Ca	48.5	50.9	48.9	48.4	47.0	50.5	52.5
Caseinate Ca	29.0	31.0	30.5	31.4	31.7	31.7	30.5
Soluble unionized Ca	27.7	25.9	24.6	23.0	26.2	22.7	25.5
Soluble ionized Ca	12.6	11.0	11.1	11.6	12.3	10.1	10.8
Magnesium fractions							
Colloidal Mg	4.0	4.5	4.3	4.1	3.9	4.9	5.0
Soluble Mg	8.1	7.8	7.6	7.2	8.0	7.8	7.9
Citric acid fractions							
Colloidal citrate	19	17	15	17	15	21	7
Soluble citrate	156	150	144	137	159	135	158
Phosphorus fractions							
Colloidal inorganic P	28.3	30.0	28.5	28.8	27.0	30.3	28.4
Caseinate P	21.2	21.3	20.3	21.4	22.0	22.8	26.5
Soluble inorganic P	37.4	40.2	37.3	34.2	32.6	34.4	37.4
Ester P	11.9	12.5	11.6	9.9	10.5	7.5	6.3
			mg./1 g. casein				
Caseinate Ca	11.4	11.7	12.2	12.2	12.0	11.9	10.7
Casein P	8.4	8.0	8.1	8.2	8.3	8.5	9.3
Colloidal inorganic Ca	19.2	19.4	20.0	18.5	17.7	18.9	18.4
Colloidal inorganic P	11.2	11.5	11.7	11.0	10.2	11.3	9.9
Tricalcium phosphate	39.9	39.3	42.1	36.1	38.7	36.0	47.3
Dicalcium phosphate	14.3	16.0	14.4	16.8	10.7	18.1	2.2
Tri-plus dicalcium phosphate	54.2	55.4	56.5	52.9	49.5	54.1	49.5
Colloidal Mg	1.6	1.7	1.7	1.6	1.5	1.8	1.7
Colloidal citrate	7.7	6.4	5.6	6.4	5.6	7.6	2.5

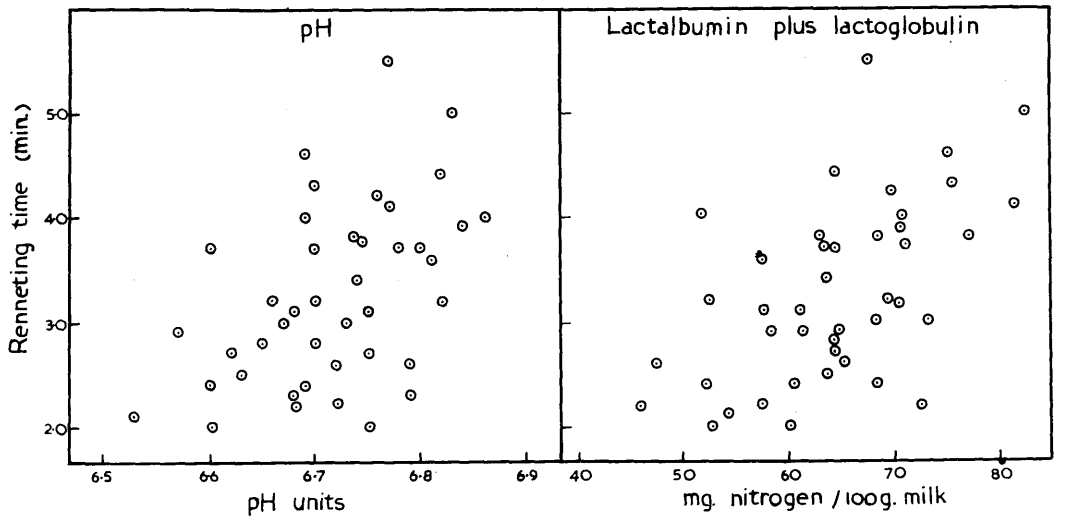

Fig. 20 The relationship of pH and concentration of lactalbumin plus lactoglobulin nitrogen to renneting time in mid-lactation milks.

within the narrow range of 2.0 to 4.5 min.

When samples with similar renneting times were grouped together and the mean chemical composition of the milks in each group calculated, it was found that as renneting time increased the mean values for pH, lactalbumin plus lactoglubulin and non-protein nitrogen increased, and those for titratable acidity decreased (Table 12). However, when the values for these, and all other constituents listed in Table 12 in each milk were compared with the corresponding renneting times, no close relationships were found. There were only poor direct relationships between pH and the concentration of lactalbumin plus lactoglobulin, and renneting time (Fig. 20) and a slight tendency for concentrations of potassium, soluble unionized calcium and soluble magnesium to decrease as renneting time increased.

Although the relationship between pH and renneting time was poorer than that with the herd bulk milk, increasing acidity was again associated with decreasing renneting time. The renneting times of the mid-lactation milks appeared to be independent of the composition and concentration of the caseinate complex and of the concentration of soluble ionized calcium.

Late lactation milk On the whole, the late lactation milks were coagulated more slowly by rennet than the herd bulk milks (Fig. 14d). There were many differences in the average chemical composition of these groups of milks (Table 7) but the most likely

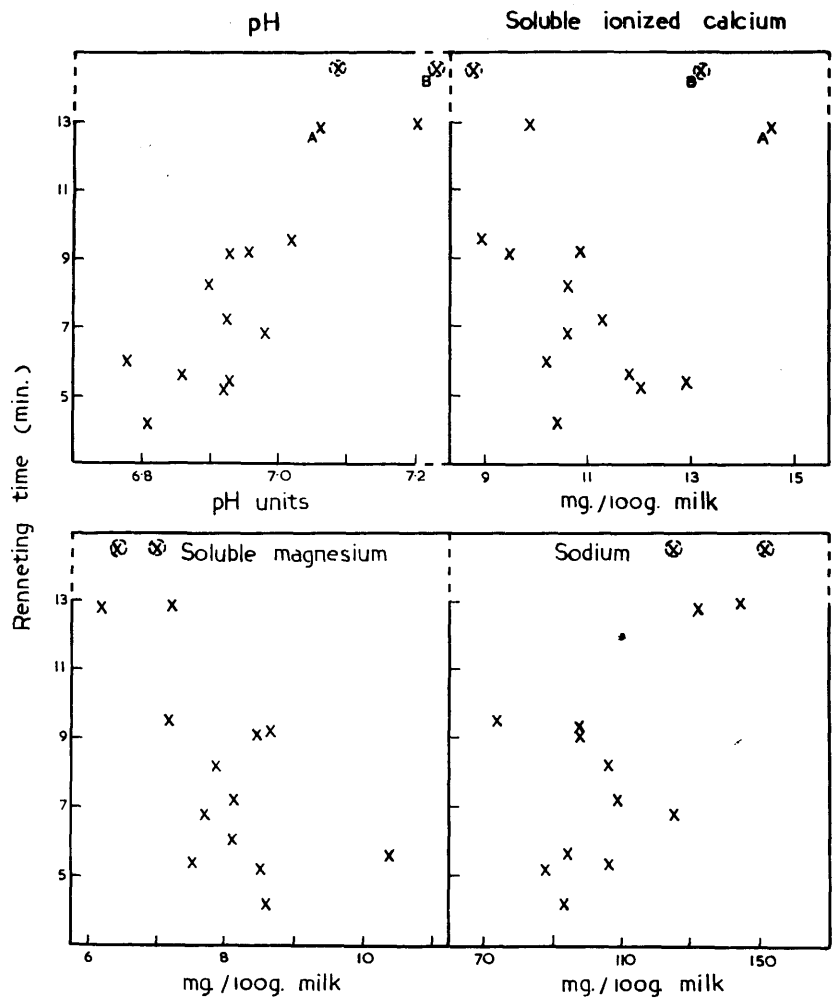

Fig. 21 The relationship of pH and concentrations of soluble ionized calcium, soluble magnesium and sodium to renneting time in late lactation milks (⊗ samples that did not coagulate).

cause of the longer renneting times of the late lactation milks was their higher pH values. The influence of this factor apparently outweighed the possible favourable effect on rennet action of the higher concentration of the calcium caseinate-calcium phosphate complex in the late lactation milks.

The within-group relationships between chemical composition and renneting time were not marked (Fig. 21) but, as in the early lactation group, values for pH tended to increase and the concentration of soluble ionized calcium tended to decrease as renneting time increased. There was also some indication that the concentration of soluble magnesium was inversely related, and the concentration of sodium directly related to renneting time. The relationship between sodium concentration and renneting time could have resulted from the direct relationship between pH and sodium concentration in late lactation milk (Fig. 4).

The two samples marked A and B in Fig. 21, which were obtained from the same cow, illustrate and help to explain some of the apparently anomalous results. Sample A took 12.8 min. to coagulate and sample B did not coagulate at all and yet each contained more soluble ionized calcium than any of the other late lactation milks. However, both these samples had high pH values, 7.06 and 7.41 respectively, which were consistent with the general relationship between pH and renneting time shown by the other late lactation milks. In these two samples, therefore, pH was considerably more important in determining rate of

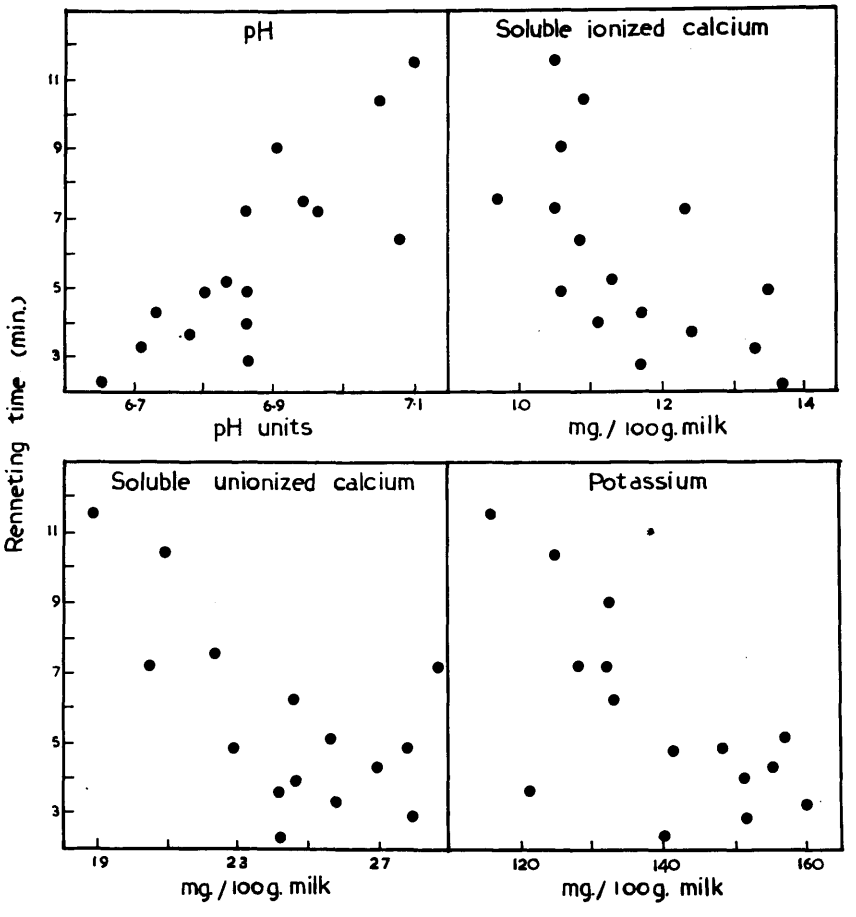

Fig. 22 The relationship of pH and concentrations of soluble ionized calcium, soluble unionized calcium and potassium to renneting time in milks from cows with sub-clinical mastitis.

coagulation than calcium ion concentration.

Mastitis milk The milk from cows with sub-clinical mastitis showed a wide range of stability to rennet (Fig. 14e), but most of the samples had a longer renneting time than the herd bulk average (4.1 min.). Of the many differences in the average composition of the mastitis and herd bulk milks (Table 8), only the higher pH values, and possibly the lower concentrations of casein in mastitis milk, seemed to be reasons for their tendency to have a greater stability to rennet.

The best within-group relationships between chemical composition and renneting time for the mastitis milks are shown in Fig. 22. Once again, as pH increased so did renneting time. Renneting time also tended to increase as the concentrations of ionized and unionized soluble calcium, and potassium decreased and there was an inverse relationship between titratable acidity and renneting time. Of the other constituents listed in Table 8, high values for the contents of total calcium, total phosphorus, soluble inorganic phosphorus, soluble citrate and lactose were usually associated with more rapid coagulation and low values with slower coagulation. The mastitis milks slow to coagulate usually contained more chloride than those with shorter renneting times, as with the herd bulk milks.

The concentrations of casein and of the other milk proteins, as well as those of the colloidal minerals, were not related to renneting time. There

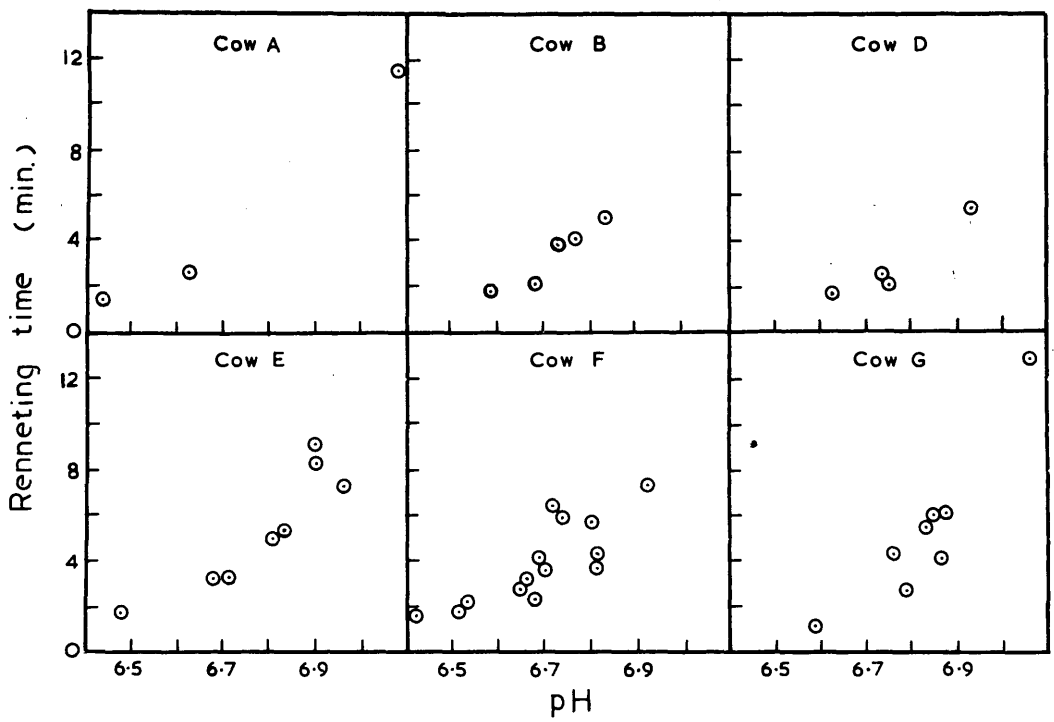

Fig. 23 The relationship of pH to renneting time in milks from six cows (cows A and E developed sub-clinical mastitis).

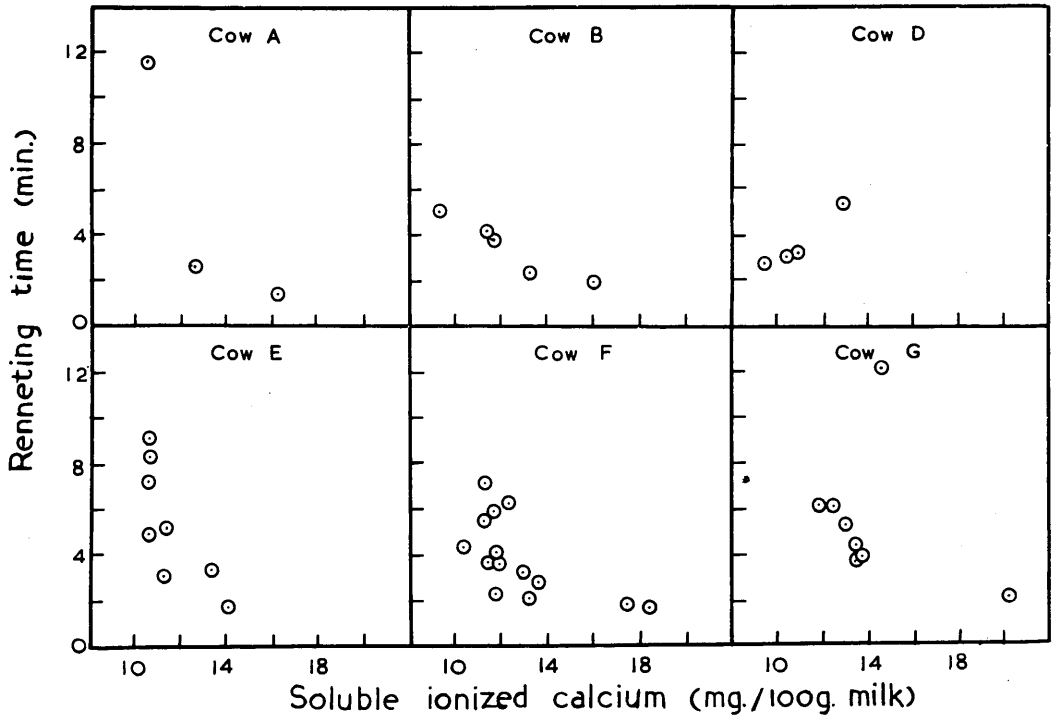

Fig. 24 The relationship of concentration of soluble ionized calcium to renneting time in milks from six cows (cows A and E developed sub-clinical mastitis).

was a tendency, also noted in some of the other groups of milks, for renneting time to increase as the casein number decreased but this relationship is unlikely to be of any significance since the non-casein proteins are not thought to play any part in rennet action.

Milk from individual cows sampled at intervals during lactation A close relationship was found between pH and renneting time in milk taken from six individual cows at intervals during their lactations; as pH increased, renneting time increased (Fig. 23). There was a tendency also for the concentration of soluble ionized calcium and renneting time to be inversely related in the milk from five of the cows; with the milk from cow D there appeared to be a direct relationship (Fig. 24). There were only four samples from cow D, however, so no definite conclusions can be drawn from the results for this cow.

In addition, increasing renneting time was usually associated with decreasing concentrations of soluble unionized calcium, soluble inorganic phosphorus, potassium and lactose and with increasing concentrations of proteose-peptone and chloride. All of these relationships, however, as previously stated, were probably consequences of the relationships between pH and the level of these constituents in milk (Table 9). The concentrations of the other milk constituents, and the composition of the casein complex, were not related to renneting time.

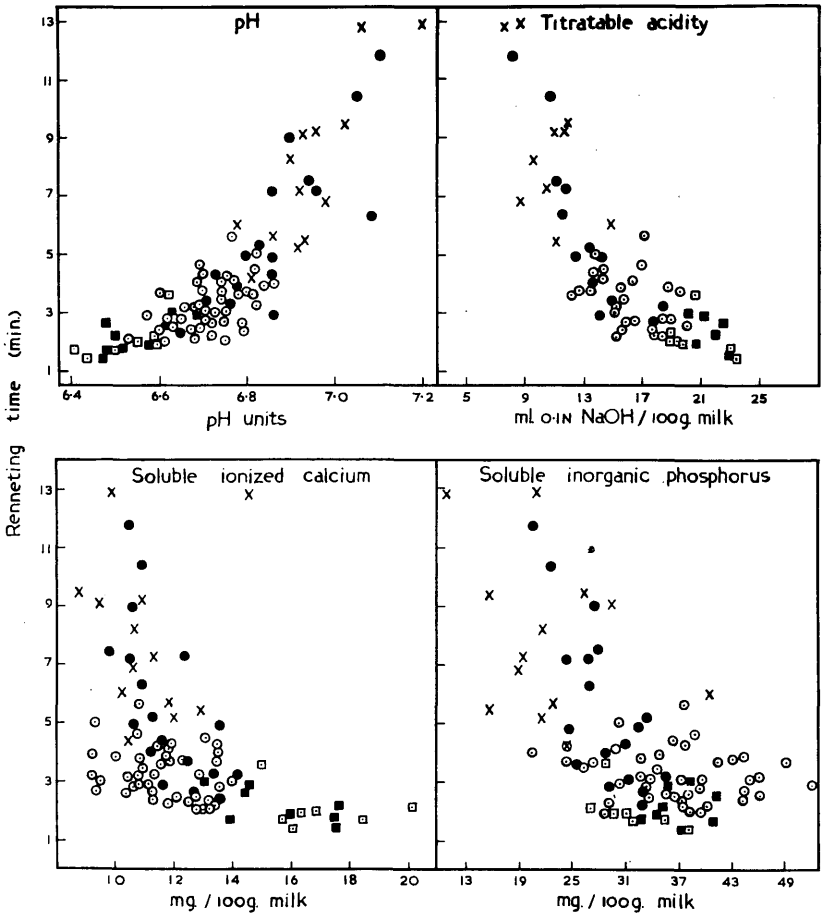

Fig. 25 The relationship of pH, titratable acidity and concentrations of soluble ionized calcium and soluble inorganic phosphorus to renneting time in milks from individual cows in different stages of lactation and from cows with sub-clinical mastitis (⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, × late lactation milk, ● mastitis milk).

Comparison of chemical composition and renneting time of all milk samples from individual cows In the different lactational sub-groups variation in some of the chemical factors which were found to be related to renneting time was relatively small, e.g. the range of pH values in mid-lactation milks was only 6.53 - 6.86. So as to increase the range of values for these factors and to examine the relationships with renneting time over the extended ranges, the results for all milks from individual cows were also collectively examined. This procedure gave the curves shown in Fig. 25. As the pH values of the milks increased from 6.4 to 7.2, there was a curvilinear increase in renneting time from about 1.5 min. to 13 min. Above pH 6.8, the increase in renneting time with increasing pH was greater than that between pH 6.4 and 6.8. The association between acidity and renneting time is shown also by the inverse curvilinear relationship between titratable acidity and renneting time (Fig. 25). This relationship was to be expected in view of the close inverse relationship which exists between pH and titratable acidity (Fig. 4).

Although high concentrations of soluble ionized calcium were invariably associated with short renneting times, when the concentration of this constituent was less than 12 mg. per 100 g. of milk, it bore little relationship to renneting time (Fig. 25).

There were also poor inverse relationships between the concentrations of soluble inorganic

phosphorus (Fig. 25), soluble unionized calcium, soluble magnesium, ester phosphorus, potassium and lactose, and renneting time. On the other hand, the concentrations of sodium, chloride and proteose-peptone tended to increase as renneting time increased. The existence of most of these relationships could probably be attributed to the interrelationships of the concentration of these milk constituents and pH (Table 9).

Renneting time was not related to the composition or to the concentration of the casein complex. Neither was there any relationship between renneting time and salt-balance, calculated as already described (p.58).

Discussion

It was evident in all the groups of milks examined, namely, herd bulk, early, middle and late lactation and mastitis milks, that renneting time increased as pH increased. Within each group, the relationship between pH and renneting time appeared to be linear but when all samples from individual cows were considered, the relationship was curvilinear (Fig. 25). This curvilinear relationship is similar to that obtained by Sanders et al. (1936) but their data show that the increase in renneting time with increasing pH became more pronounced when the pH exceeded 6.65 whereas in the present investigation a marked increase in renneting time with increased pH occurred only at pH values above 6.80. Although renneting time was more closely

related to pH than to any other property of the milks, there was an appreciable variation in renneting time at a constant pH. The analytical results obtained in this experiment did not reveal the cause of this, but neither variation in concentration of soluble ionized calcium nor variation in the calcium phosphate content of the casein complex appeared to be responsible. As there was a close inverse relationship between titratable acidity and pH there was also an inverse curvilinear relationship between titratable acidity and renneting time (Fig. 25).

That pH should have a marked effect on renneting time was expected from the information now available on each stage of the rennet coagulation of milk. Nitschmann et al. (1953 - 55) have shown that at a constant temperature and concentration of rennin, the rate of conversion of α-casein to α-paracasein is governed by pH and that as pH decreases the rate of the reaction increases. It is also known that the enzymic action of rennin ceases at a pH of about 7 or slightly above. Since it appears that the conversion of caseinate to paracaseinate must be complete or nearly so, before the typical coagulation of milk occurs (Alais et al., 1953; Pyne, 1955), it follows that renneting time will increase as pH increases. The rapid coagulation by rennet of early lactation milk, especially colostrum, and the slower coagulation of late lactation milk could therefore be attributed mainly to the respectively low and high pH values of these types of milk. The frequent occurrence of high pH in milk from cows with sub-

clinical mastitis would likewise explain the long renneting times common to mastitis milk. When milk does not coagulate with rennet, the most likely cause will be an abnormally high pH value, e.g., the two late lactation milks which failed to coagulate had pH values of 7.09 and 7.41.

The close parallelism between the changes in pH and renneting time during lactation (cf. Fig.4 & Fig. 16) was most probably the reason for the relationship found between the renneting time of the milk and the stage of lactation of the cow. A tendency for renneting time to increase as lactation progressed was evident with milk from the same cow examined periodically (Fig. 17) and also when isolated samples from a larger number of cows in different stages of lactation were examined (Fig. 16). The relationship was detectable even in herd bulk milk (Fig. 15). These results therefore confirmed the observations of Mattick & Hallett (1929b), McDowall et al. (1937) and Konovalov (1949) on the relationship between stage of lactation and renneting time. In the present experiment, minimum and maximum renneting times of 1.4 and 12.9 min. with associated pH values of 6.44 and 7.20 were obtained respectively in early and late lactation.

Although short renneting times were usually associated with high concentrations of calcium ions, over the whole range of renneting times, the relationship was not close (Fig. 25). In fact, when the concentration of soluble ionized calcium was less

than 12 mg. per 100 g. of milk, there was no relationship between the level of this constituent and renneting time. The herd bulk milks, whose calcium ion content ranged from 10.5 to 12.8 mg. per 100 g. of milk, were good examples of this lack of relationship. Pyne (1953) has stated that calcium ion concentration influences both stages of rennet action but the balance of evidence is that the concentration of this constituent primarily controls the rate of the coagulation stage. It was therefore not surprising that there was no close relationship between calcium ion concentration and renneting time, which is the sum of the times required for the independent enzymic and coagulation stages. Nevertheless, the effect of calcium ion concentration on renneting time in nearly every instance would be to supplement the effect of pH since low pH values were usually associated with high concentrations of calcium ions and vice versa. The poor relationship between calcium ion concentration and renneting time suggested that at the temperature at which the renneting time of the samples were determined, the variation in the rate of the coagulation reaction was small in relation to the variation, due to pH differences, in the rate of the enzymic reaction. The dominant influence of pH on renneting time is illustrated by samples A and B in Fig. 21. These samples, both with a high pH value, were very stable to rennet despite their content of soluble ionized calcium being above average.

When the whole range of renneting times was

considered, it was found that as renneting time increased, the concentrations of sodium, chloride and proteose-peptone increased and the concentrations of soluble inorganic phosphorus, soluble unionized calcium, soluble magnesium, ester phosphorus, potassium and lactose decreased. Each of these relationships was not close and could probably be attributed simply to the interrelation of pH and the level of these constituents in milk, but there was the possibility that some of these constituents might have a specific effect on rennet action. For example, the results of Berridge (1952), Higgins & Fraser (1954) and Wakui & Kawachi (1954) suggest that chloride may have an inhibiting effect on the rennet coagulation of milk. At first sight, the present results would seem to be in agreement with this, but the fact that the relationship between chloride concentration and renneting time in early lactation was the opposite of that during the rest of lactation suggested that the natural variation in chloride concentration was unimportant as regards renneting time. Also, magnesium ions are known to influence the second stage of rennet action in the same way as calcium ions and although this fraction of magnesium was not determined, the inverse relationship between the concentration of soluble magnesium and renneting time suggested that magnesium ions supplement the action of calcium ions. However, the magnesium ions will have much less influence on the renneting time of milk than the calcium ions since magnesium ions are only half as

effective as calcium ions in accelerating coagulation (Pyne, 1955) and their molar concentration in milk is about one-third that of calcium ions (van Kreveld & van Minnen, 1955). The absence of a relationship between renneting time and salt-balance is in agreement with the results of Holm et al. (1932).

The present results confirm the generally held view that the concentration of casein in milk is unrelated to renneting time. There was no obvious relationship between casein content and renneting time within any of the groups of milks. It is true that the early lactation milk, in which the concentration of casein was high, coagulated rapidly and that the mastitis milk, in which the concentration of casein was low, tended to be slow to coagulate, but late lactation milk also coagulated slowly despite its average casein content being almost as high as that of colostrum. The relative rates of coagulation of these three types of milk could more logically be attributed to their pH values. There was some evidence that as casein number decreased, and as the ratio of lactalbumin plus lactoglobulin to casein increased, renneting time increased but it seems probable that these relationships were the result of coincidental lactational changes in milk composition.

As has been suggested by others, the concentration of casein seemed to be related to the firmness of the coagulum formed by rennet action. Early lactation milk, rich in casein, formed a firm curd and mastitis milk, deficient in casein, formed a loose curd. On

the other hand, late lactation milk which contained almost as much casein as colostrum, also gave a loose curd. The reason for this apparent anomaly may have been the incomplete formation and coagulation of paracasein as a result of the very high pH values and the relatively low concentrations of soluble ionized calcium common in late lactation milk.

None of the colloidal mineral constituents of milk appeared to have any influence on rennet action. The concentration of calcium phosphate, whether expressed as mg. per 100 g. of milk or as mg. per 1 g. of casein, was not related to renneting time. The conclusion that the composition of the casein complex is unrelated to renneting time contradicts that of Mocquot et al. (1955) but supports the view of Pyne (1953) that the amount of natural variation in the proportion of calcium phosphate to casein is insufficient to influence renneting time.

Conclusions

1. The renneting time of samples of herd bulk milk varied within the narrow range of 3.2 to 5.4 min.
2. Milks from individual cows varied greatly in renneting time. The range was 1.4 to 12.9 min. and two samples did not coagulate.
3. The renneting time of milk from individual cows was related to the stage of lactation of the cow. In early lactation, renneting times were short and as lactation advanced there was a progressive increase

in renneting time; the increase became more pronounced towards the end of lactation. The relationship between stage of lactation and renneting time was evident also in the herd bulk milk whose renneting time increased as the average stage of lactation of the cows in the herd increased.

4. The physical nature of the coagulum formed by rennet action was variable. In general, milk which coagulated rapidly gave a firm curd and milk slow to coagulate gave a soft or loose curd.

5. The property of milk most closely related to renneting time was acidity. Over the region from highest acidity (pH 6.40) to a pH of approximately 6.80, there was a progressive increase in renneting time from 1.5 to 4 min. Above pH 6.80, increase in renneting time with increasing pH was more pronounced; a pH of 6.90 corresponded to a renneting time of approximately 6 min. and a pH of 7.20 to a renneting time of 13 min.

6. Titratable acidity was related to renneting time in a way that was opposite to that of pH.

7. Although milk containing a high concentration of soluble ionized calcium coagulated quickly with rennet, milk containing average or less than average amounts of this constituent had a wide range of renneting time. The influence of calcium ion concentration on renneting time was thus secondary to the influence of pH.

8. The conclusion that pH was the most important factor controlling the renneting time of milk is in

agreement with the recent discovery that the rate of the first stage of rennet action, i.e. the conversion of α-casein to α-paracasein, is largely dependent on pH.

9. Because of the inverse relationship between pH and concentration of soluble ionized calcium in milk, the effect of these two factors on renneting time was usually additive.

10. The weak relationships found between renneting time and the concentrations of other milk constituents could be attributed to the interrelation of the latter and pH. The inverse relationship between renneting time and the concentration of soluble magnesium, however, suggested that magnesium ions supplemented the effect of calcium ions in the coagulation stage of rennet action.

11. The concentration and composition of the calcium caseinate-calcium phosphate complex in milk were not related to renneting time.

Part 4

The Coagulation of Milk Protein by Heat

Introduction

Milk of average composition is remarkably stable to heat in that it can be boiled for several hours, provided evaporation is prevented, without any visible signs of coagulation. Eventually, however, the protein in boiling milk does coagulate, and if the temperature is increased above boiling point, the time required for clots to appear in the milk decreases logarithmically with temperature until at 150°C only a few minutes heating causes coagulation (Webb & Holm, 1932; Cole & Tarassuk, 1946). It is well known that colostrum is very sensitive to heat and rapidly coagulates at temperatures below 100°C, a fact usually attributed to the high concentrations of heat-sensitive lactalbumin and lactoglobulin in this type of milk. It is also well known that individual milks, of apparently normal composition, show great variation in the time required for coagulation at any particular temperature (Sommer & Hart, 1919; Holm, Webb & Deysher, 1932; Cole & Tarassuk, 1946; Pyne & McHenry, 1955), but the reasons for the differences in stability are still not fully explained.

Investigations concerned with coagulation of milk by heat, made prior to 1930, have been thoroughly reviewed by Allen (1932). He concluded that the two main factors which appeared to influence the heat stability of milk were the concentration of the serum proteins and salt-balance. Low stability to heat was

apparently associated with a high concentration of lactalbumin and lactoglobulin and with a marked deviation from the point of optimum balance between the concentration of calcium and magnesium on the one hand and the concentration of phosphate and citrate on the other. Allen observed also that the heat stability of fresh milk was unrelated to its pH or titratable acidity although a decrease in pH caused by natural souring or by the addition of acid reduced stability. It was believed that the stabilising effect of preheating raw milk at about 95°C for a short time on the stability of evaporated milk was caused by the denaturation of lactalbumin and lactoglobulin and also by an increase in the concentration of colloidal calcium at the expense of soluble calcium. Milk was also known to become progressively more stable to heat on dilution and progressively less stable on concentration.

Since 1930, many experiments have been done to determine the changes that occur in milk when it is heated and their possible influence on protein coagulation and also on the relationship between the chemical composition of milk and its stability to heat. The heat-induced changes in casein are of particular importance since the colloidally dispersed casein complex is the phase principally concerned in the coagulation of milk.

Howat & Wright (1934, 1936) heated neutral calcium caseinate solutions at temperatures varying from 90 to 120°C for various times and found that there was a relatively rapid liberation of phosphorus, and also

a liberation of non-protein nitrogen from the caseinate. They noted that visible coagulation of the caseinate occurred when about 45% of its phosphorus had been liberated and that over the temperature range 90 - 115°C an increase in temperature of 10° caused the rate of both dephosphorylation and coagulation to increase three times. It was therefore concluded that dephosphorylation and coagulation were related reactions. Since the base-binding capacity of the dephosphorylated caseinate was less than that of the original caseinate, indicating that calcium was also released during heating, Howat & Wright concluded that the released calcium reduced the stability of the unaltered caseinate and especially of the dephosphorylated caseinate, and accelerated the rate of coagulation. The importance of calcium in the coagulation reaction was also suggested by the fact that solutions of casein and dephosphorylated casein containing more than sufficient calcium to combine with the casein were coagulated more rapidly by heat than when just sufficient calcium was present. The work of Torboli (1945) supports the view of Howat & Wright that the partial dephosphorylation of casein is an integral part of the reactions leading to the heat coagulation of casein in the presence of calcium.

Howat & Wright (1934) suggested that a decrease in the hydration and in the degree of dispersion of the casein micelles might also occur during heating and so affect stability. No investigations appear to have been done to verify the first suggestion and those on the second have given conflicting results.

Kometiani (1931) reported that there was an increase in the size of the casein micelles when milk was heated for 30 min. at temperatures ranging from 80 to 120°C, which led to a decrease in heat stability. Also, Burton (1956) suggested that the reversible increase in the reflectance of milk heated up to 50°C is caused by an increase in the particle size of the casein complex. On the other hand, Nichols, Baily, Holm, Greenbank & Deysher (1931) found no change in the degree of dispersion of casein when milk was heated at 95°C, and the work of Ramsdell & Hufnagel (1953) and Edmonson & Tarassuk (1956) suggests that the casein micelles become smaller when milk is heated at 88°C for 15 min. or at 100°C for 10 min. or less.

The effect of heat on the soluble or serum proteins of milk has been extensively examined (Rowland, 1933, 1937; Harland, Coulter & Jenness, 1952; Harland, Coulter, Townley & Jenness, 1955). Although lactalbumin and lactoglobulin are rapidly denatured by heat, these proteins do not appear to play any part in the coagulation of the casein complex in milk of normal composition. For example, Pyne & McHenry (1955) found that milk from which most of the lactalbumin and lactoglobulin had been removed, coagulated in exactly the same time at 120°C as the unaltered milk. Nevertheless, the high concentration of serum proteins in colostrum may be responsible for its marked instability to heat. Average figures quoted by Marsden (1953) show that colostrum contains respectively 4, 11 and 57 times as much lactalbumin,

proteose-peptone and lactoglobulin as normal milk. It is significant that the greatest increase is in the lactoglobulin fraction whose components, the immune globulins, are more rapidly denatured by heat than any other serum proteins (Wegelin, 1952; Larson & Rolleri, 1955). It appears that there is a critical concentration of lactoglobulin above which it flocculates under the action of heat and causes sympathetic coagulation of the casein micelles. Below this concentration the lactoglobulin is also denatured by heat but without causing the casein to coagulate. According to Engel & Schlag (1924) the critical concentration of lactoglobulin is about 1% and this amount is exceeded for about three days after calving.

The variation in the heat stability of milk, other than colostrum, has been attributed mainly to differences in mineral composition, largely as a result of the salt-balance theory propounded by Sommer & Hart (1919). According to this theory, there is an optimum value for the ratio of total calcium plus magnesium to total phosphate plus citrate in milk and any deviation from this value, occurring naturally or by the addition of salts, results in a decrease in the heat stability of the milk. Sommer & Hart stated that the most common cause of instability was an excess of calcium plus magnesium. Experiments in which the salt-balance theory was tested, with conflicting results, have already been mentioned (p.57). Webb & Holm (1932) studied the effect on the heat stability of milk of adding various salts and

concluded that the electrical charge on the casein micelles was more important in determining stability than salt-balance. Although this experiment was of little help in explaining the heat stability of milk in terms of chemical composition, it drew attention to the probable importance of the charge on the casein particles and the influence thereon of calcium ion concentration.

Many workers have postulated the existence of a relationship between the calcium content of milk, and especially the calcium ion concentration and heat stability. Mantovani (1938) and Cenni & Frateschi (1954) found that samples of goats' milk which were very unstable to heat contained much more calcium than normal goats' milk. Sandelin (1943) attributed the extreme instability to heat of colostrum to its high content of calcium and probably also of ionic calcium, and suggested that the formation of skin on heated milk was caused by the increased concentration of calcium ions at the surface due to evaporation. In 1947, Seekles & Smeets suggested that the cause of the 'Utrecht abnormality', i.e. the occurrence in Holland of milk unstable to heat, was an abnormally high concentration of calcium ions in the milk. Their subsequent work showed that the calcium ion content of ultrafiltrate from normal milk ranged from 8 to 14.4 mg. per 100 ml. and when the concentration exceeded about 15 mg. per 100 ml., the milk was very unstable to heat (Smeets, 1952, 1955; Smeets & Seekles, 1952; Seekles & Smeets, 1954;

Boogaerdt, 1954). Pyne & McHenry (1955) found an inverse, curvilinear relationship between the 'effective' calcium ion concentration in milk, measured by a renneting technique, and coagulation time at 130°C. They suggested also that the more colloidal calcium phosphate there was associated with casein, the less stable was the casein to heat. Pyne & McHenry concluded that if the concentration of calcium ions and the colloidal calcium phosphate content of the casein complex were high, only a moderate degree of developed acidity and casein denaturation would be needed to cause coagulation, which would then be fairly rapid. If, on the other hand, the levels of these two predetermining factors were low, a greater development of acidity and more denaturation of casein would be necessary before coagulation ensued and that coagulation would be relatively slow.

Further evidence of the probable importance of calcium in relation to heat stability has been obtained by altering the calcium partition in milk. For example, the addition of calcium salts to milk reduced stability (Webb & Holm, 1932; Sandelin, 1943; Torboli, 1945; Lust, 1952) whereas the inactivation of some of the calcium by addition of citrate or phosphate increased heat stability (Torboli, 1945; Miyabe & Higashi, 1953). Miller & Sommer (1940) found that the effect on the heat stability of milk of added calcium or calcium-complexing salts varied with the pH of the milk. When the pH was below 6.4,

milk was made more stable by the addition of phosphate and less stable by added calcium. When the pH was above 6.4, however, addition of phosphate, citrate, carbonate and large amounts of calcium lowered stability whereas small amounts of calcium increased stability. Josephson & Reeves (1947) showed that the removal of some calcium from milk increased the heat stability of evaporated milk made from it.

The reasons for the reported adverse effect of a high concentration of calcium ions and a high content of calcium phosphate in the casein complex on heat stability are not fully understood. However, in the presence of high concentrations of calcium ions only a small heat-induced reduction in the hydration of the micelles might be necessary to make the casein dispersion unstable, an effect analogous to that found in coagulation of casein by ethanol. Another possibility is suggested by the investigations of Bishov & Mitchell (1954, 1956), Ford, Ramsdell & Landsman (1955) and those of Hostettler et al., already mentioned (p. 70), which indicated that the greater the concentration of calcium ions and the amount of calcium phosphate associated with casein, the larger is the size of the casein micelles. The larger micelles might be expected to show visible aggregation more quickly than smaller micelles, whatever the reactions preceding coagulation.

Attempts have also been made to relate heat stability to the acidity of milk and in the narrow range of pH values found in mid-lactation milk little

relationship was found (Sommer & Hart, 1919, 1926; Holm, Webb & Deysher, 1932). When acid is added to milk, however, heat stability usually decreases (McInerney, 1920; Webb & Holm, 1932). Miller & Sommer (1940) found that the rate of decrease in heat stability on adding acid to milk was greatest between pH 6.4 and 6.2. This decreased stability has been attributed to a change in the ratio of $H_2PO_4^-$ to $HPO_4^=$ (Sommer & Hart, 1926). Another possible reason is an increase in the particle size of casein resulting from a decrease in the negative charge of the micelles (Küntzel & Doehner, 1940). A decrease in pH has been found moreover to increase the soluble calcium and phosphate contents of milk and to decrease the amounts of colloidal calcium phosphate and caseinate calcium (Lampitt & Bushill, 1934; Ling, 1936; Khambatta & Dastur, 1950; ter Horst, 1950; Carr, 1953), changes which may accelerate heat coagulation.

It is well known that preheating raw milk improves the colloidal stability of the casein complex in evaporated and condensed milk prepared from it but little information is available on the effect of this process on the heat stability of the original milk. Webb & Holm (1932) found that the effect of preheating on stability depended on the solids content of the milk. There was a critical solids content, which lay within the range 10.5 to 14% for the skim milks used; below 10.5% total solids, preheating at 95°C for 10 min. decreased heat stability whereas above 14% it increased heat stability. It is difficult to

explain this reduction in stability of unconcentrated milk since there is evidence that heating decreases the concentration of soluble calcium, including calcium ions (Lampitt & Bushill, 1934; Sandelin, 1945; Christianson, Jenness & Coulter, 1954). Webb & Holm (1932) observed that when preheating had an adverse effect on the heat stability, stability could not be restored by adding calcium chloride or other electrolytes. They therefore concluded that the decreased stability was not caused by changes in the mineral equilibrium and suggested that an irreversible change in the hydration of the caseinate micelles was responsible. Another explanation might be that the denaturation of lactalbumin and lactoglobulin caused by preheating reduces the stability of the casein complex in milk with a normal solids content.

The reaction between casein and lactose, which is detectable even in milk heated at only 80°C. (Grimbleby, 1954), is the cause of the brown colour that develops in more severely heated milk (Gould, 1951; Patton, 1955). It is not known whether the casein-lactose reaction normally plays a part in the heat coagulation of casein but Pyne & McHenry (1955) have shown that coagulation can take place, though more slowly, in a lactose-free milk. These authors attribute the accelerating effect of lactose to acidity derived from heat-induced reactions involving lactose, including decomposition of the lactose.

It is apparent from the literature that although the effect on heat stability of altering the

composition of milk can be fairly definitely predicted, no satisfactory relationship has been established between the natural composition of milk and stability to heat, with the possible exception of colostrum. The very high content of lactoglobulin in colostrum is the probable cause of its marked instability to heat. The balance of evidence suggests that in milk other than colostrum, the concentration of calcium ions and possibly the amount of colloidal calcium phosphate associated with the calcium caseinate, are the more important factors. That extensive investigation has been unable to relate composition and stability closely may be an indication that factors controlling heat stability are not measured solely by the estimation of the chemical constituents of milk. For example, small differences in the physico-chemical state of the casein complex may have a marked effect on its colloidal stability. However, no single experiment has yet compared the detailed composition and heat stability of a large number of milks. Such an experiment, utilizing one of the recently developed methods of estimating ionized calcium would help to verify or disprove the tentative explanations for the variability in heat stability of milk, and to eliminate the unimportant compositional factors. On the other hand, if no relationships between chemical composition and heat stability were revealed it would possibly indicate an alternative approach to the problem. These were the considerations underlying the present investigation.

Table 13 The mean and range of coagulation times at 130, 140 and 150°C of the groups of milks (fat-free) together with the mean and range of values for the temperature coefficient of coagulation between 130 and 150°C

Milk group (no.of samples)	Coagulation time (min.)						Temperature coefficient ($Q_{10°C}$)			
	Mean			Range			Mean		Range	
	130°C	140°C	150°C	130°C	140°C	150°C	130°-140°C	140°-150°C	130°-140°C	140°-150°C
Herd bulk (12)	29.9	12.3	3.2	17.2-59.0	6.8-26.2	2.6-5.1	2.4	3.4	1.9- 2.9	2.6-5.3
Colostrum (7)	2.6	1.1	0.7	0.6- 8.6	0.6- 2.4	0.5-1.2	2.0	1.5	1.1- 3.5	1.1-2.0
Post-colostrum (8)	23.0	9.8	4.0	16.2-31.8	6.9-13.9	2.7-5.6	2.3	2.5	2.3- 2.4	2.1-3.7
Mid-lactation (55)	28.1	10.6	3.2	10.6-70.3	3.5-28.9	1.7-7.2	2.8	3.2	1.9- 3.6	2.1-5.4
Late lactation (15)	45.5	14.7	3.4	15.3-86.2	3.2-35.0	1.4-5.6	3.7	4.0	1.6-11.2	2.3-6.2
Mastitis (17)	29.4	10.4	2.4	8.0-64.2	2.6-18.2	1.1-3.6	3.0	4.1	1.3- 8.6	1.8-6.3

Results

The stability of the milk samples to heat was measured, as previously described (p.10), by determining the time taken for particles of coagulated protein to appear when the samples were heated at 130, 140 and 150°C. Table 13 shows the mean and range of the coagulated times at the three temperatures for each group of milks, and also the mean and range of the values for the temperature coefficient of the coagulation reaction measured over the ranges 130-140°C and 140-150°C. Coagulation at 140°C was, on average, 2-4 times more rapid than at 130°C and at 150°C, 2-4 times more rapid than at 140°C. When the logarithm of coagulation time was plotted against temperature, a straight line relationship was found for most samples. Samples which did not show a linear relation were those whose exact point of coagulation was difficult to detect and also those which coagulated before the bath temperature was reached. Examples of the former were confined entirely to late lactation and mastitis milks and the latter to colostrum samples.

Because of the general proportionality of the coagulation times at the three temperatures for any one milk, only the coagulation times at 130°C have been used as a measure of the relative heat stabilities of the samples. The results obtained at 130°C were preferred because the coagulation times were longer, the differences between samples larger and the percentage error of timing smaller, than at the two higher temperatures. In addition, a temperature of

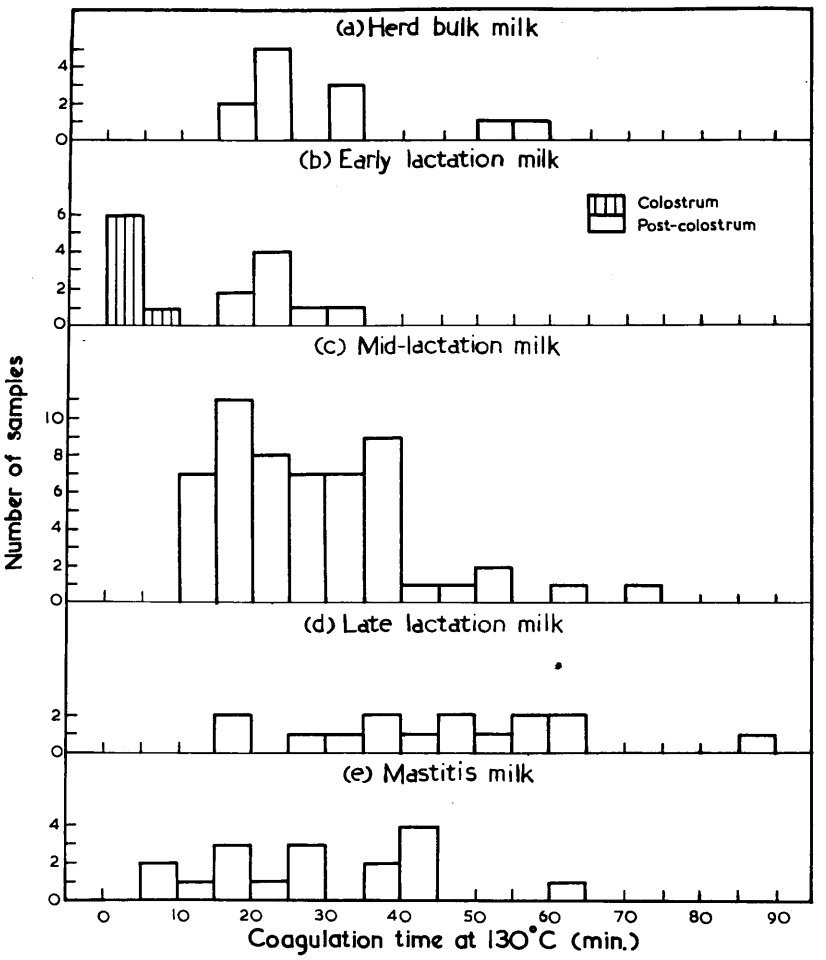

Fig. 26 The distribution of samples in relation to coagulation time at 130°C in each of the five groups of milks.

130°C is closer to the temperatures to which milk is subjected in some commercial processes.

Range of stability of milk protein to heat

Herd bulk milk (12 samples) The coagulation times at 130°C of the herd bulk milks varied between 17.2 and 59.0 min. (Fig. 26a), the average for the group being 29.9 min. Ten of the twelve samples coagulated within the range 17.2 - 33.9 min. but the other two were much more stable and took 52.5 and 59.0 min. respectively. There was some evidence that the heat stability of the herd bulk milks was related to season but, as will be shown below, this could be attributed mainly to a variation in the average stage of lactation of the herd.

Early lactation milk (15 samples) The two groups of early lactation milk differed considerably in stability to heat (Fig. 26b). The seven colostrum samples were very unstable, and with the exception of one whose coagulation time was 8.6 min., all coagulated within the range 0.6 - 2.6 min. at 130°C. The average coagulation time of the colostrum samples was 2.6 min. The eight post-colostrum samples were, however, much more stable to heat and were similar in stability to the bulk samples. Their average coagulation time at 130°C was 23.0 min. with a range of 16.2 - 31.8 min.

Mid-lactation milk (55 samples) As Fig. 26c shows, most of these samples coagulated within 10 to 40 min. at 130°C and only six took longer than 40 min. The overall range was 10.6 - 70.3 min. and the average was 28.1 min. As would be expected, the mid-lactation samples had an average coagulation time similar to

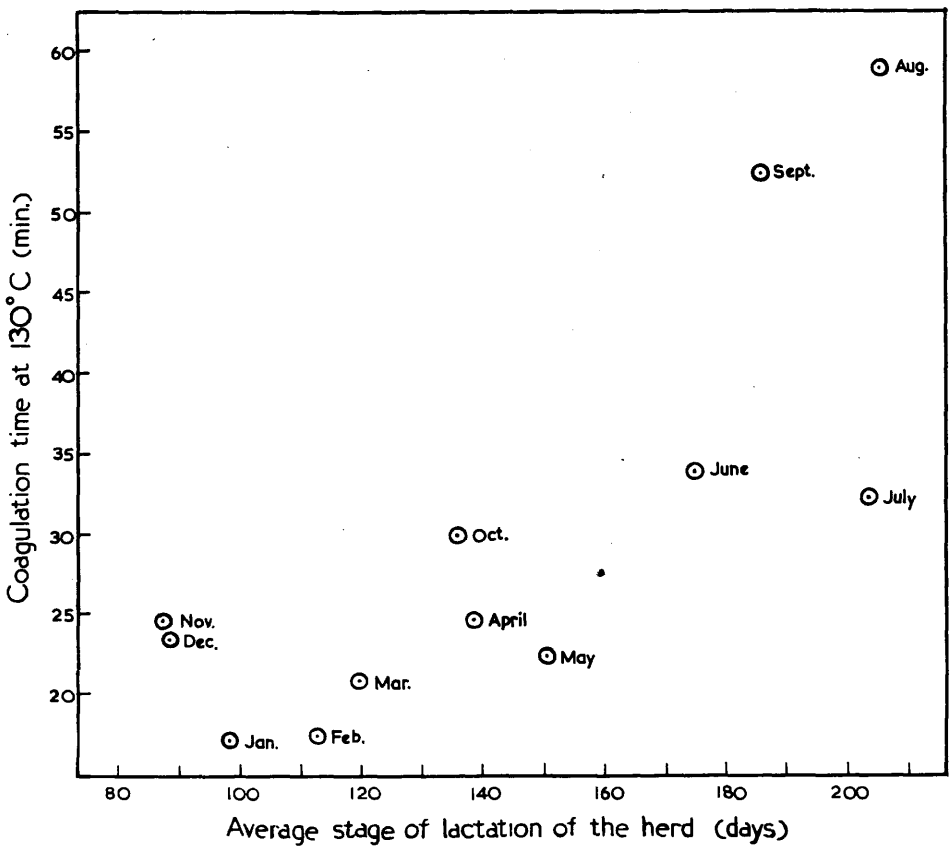

Fig. 27 The relationship between the average stage of lactation of the herd and the coagulation time at 130°C of the herd bulk milks.

that of the herd bulk milks.

Late lactation milk (15 samples) The milks in this group varied considerably in stability to heat, the range of coagulation times being 15.3 to 86.2 min. at 130°C (Fig. 26d). Since nine of the fifteen samples took longer than 40 min. to coagulate, and since the average coagulation time for all the samples was 45.5 min., it appeared that late lactation milk had a tendency to be more stable to heat than mid-lactation milk.

The detection of the exact point of coagulation of some of the late lactation milks was difficult, especially when the coagulation time was long. The clots which formed in this type of milk were usually very small and their detection was made more difficult by a thin, skin-like deposit which formed on the tube during the prolonged heating.

Mastitis milk (17 samples) The mastitis milks could not be classified as stable or unstable to heat. Their coagulation times at 130°C varied between 8.0 and 64.2 min. (Fig. 26e). There was difficulty in detecting the exact moment of coagulation of some of the mastitis samples for the same reasons as in the late lactation group.

Heat stability in relation to stage of lactation

The distribution of the coagulation times (Fig. 26) suggested that heat stability might be related to stage of lactation and this possibility was examined. Fig. 27 shows that there was a tendency for the coagulation times of the herd bulk milks to increase

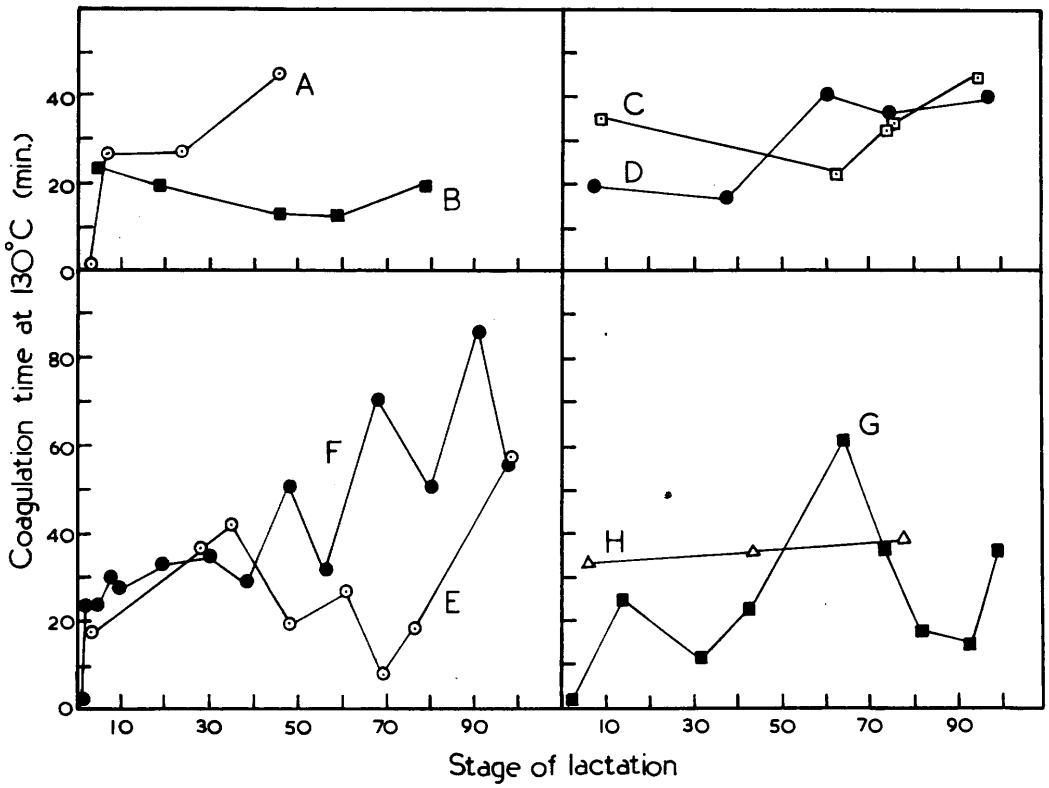

Fig. 28 The relationship between stage of lactation, expressed as a percentage of total lactation, and coagulation time at 130°C, in milks from eight cows (cows A and E developed sub-clinical mastitis).

as the average stage of lactation of the herd increased. However, when a comparison was made between the heat stability of the samples from individual cows, excluding mastitis milks, and the stage of lactation of the cows, the relationship was not close. It was true that in the first 5% of the lactation period there was a rapid increase in coagulation time from the low values for colostrum samples to the higher values for post-colostrum samples, and that there was a tendency for late lactation milks to have the longest coagulation times, but in the mid-lactation period there was no relationship between stability and stage of lactation. Stage of lactation and coagulation time were also compared by examining the milk of eight cows at intervals during their lactations. The results confirmed the general conclusions already drawn and emphasized that the variation in coagulation time with stage of lactation was very erratic and differed from cow to cow (Fig. 28).

Relationship between chemical composition of milk and stability of milk protein to heat

Herd bulk milk Although the herd bulk milks varied considerably in stability to heat (Fig. 26a), there was little relationship between stability and chemical composition within the group. The only relationships found were poor and largely dependent on the values obtained for the two most stable samples. As shown in Fig. 29, there was some indication that high stability was associated with high pH values and there were weak inverse relationships between coagulation time

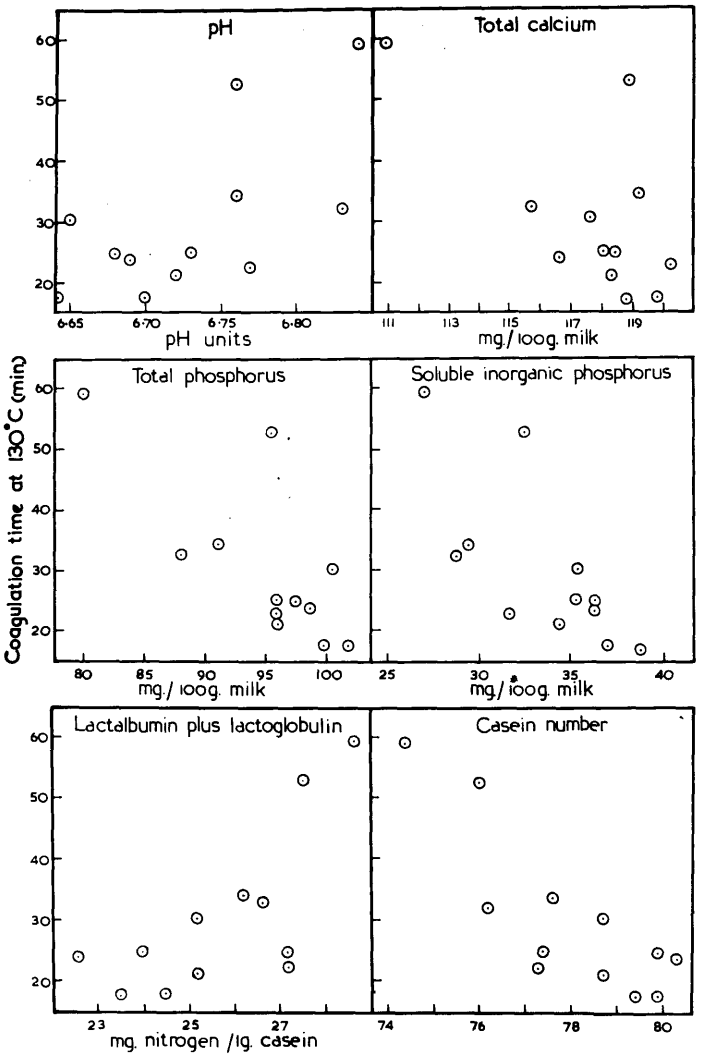

Fig. 29 The relation of pH, concentrations of total calcium, total phosphorus, soluble inorganic phosphorus and lactalbumin plus lactoglobulin nitrogen (per g. casein) and casein number to coagulation time at 130°C in herd bulk milks.

and concentrations of total calcium, total phosphorus and soluble inorganic phosphorus. There was also a tendency for long coagulation times to be associated with low titratable acidity.

There was no relationship between stability and the concentration of casein or of the serum proteins. However, it appeared that when the concentration of casein relative to the other proteins was low i.e. when the casein number was small or the ratio of lactalbumin plus lactoglobulin to casein was relatively large, the samples were more stable to heat (Fig. 29).

There was no relationship between coagulation time and either calcium ion concentration or the composition of the caseinate complex, both of which varied little within the group of herd bulk milks.

Early lactation milk Reasons for the difference in stability between the two groups of early lactation milk were suggested from a comparison of the average chemical composition of these two types of milk and that of the herd bulk milks (Table 5). The most probable cause of the marked instability of the colostrum samples was the very high concentration of heat-sensitive serum proteins; the samples contained, on average, about 2.5 times as much lactalbumin plus lactoglobulin as the herd bulk milks. The colostrum samples were also rich in soluble ionized calcium containing, on average, 17.0 mg. per 100 g. milk as compared with the herd bulk milk average of only 11.4 mg. per 100 g. milk and this together with the higher concentration of soluble magnesium and the lower pH also probably contributed to the instability. There

were other marked differences in composition between the colostrum and herd bulk samples e.g. in ester phosphorus, non-protein nitrogen, soluble unionized calcium and lactose contents but it seemed unlikely that these differences would be responsible for the different stability to heat of the two groups of milks.

Although the post-colostrum milks differed in composition from the herd bulk samples in the same respects as the colostrum samples, the differences were of a lesser degree and did not appreciably lower the average heat stability of the post-colostrum samples below that of the bulk milks. The average concentrations of lactalbumin plus lactoglobulin nitrogen and soluble ionized calcium in the post-colostrum milks were respectively 87.9 and 15.6 mg. per 100 g. milk compared with the corresponding values of 65.9 and 11.4 mg. per 100 g. milk for the herd bulk samples. It appeared therefore that the values for these two constituents in post-colostrum milk were below the critical level required for rapid coagulation. As in the colostrum samples, the average concentrations of soluble magnesium, ester phosphorus, non-protein nitrogen and soluble unionized calcium were higher in post-colostrum milk than in the herd bulk milk. However, since the stability to heat of the post-colostrum samples was much the same as that of the herd bulk milks it appeared that the concentration of these constituents was not related to stability. The fact that the more stable post-colostrum milks contained even more casein than the

Table 14 The average composition of groups of mid-lactation milks (fat-free) arranged in order of increasing coagulation times at 130°C

Range of coagulation time at 130°C	10-15 min.	15-20 min.	20-25 min.	25-30 min.	30-35 min.	35-40 min.	over 40 min.
No. of samples	7	11	8	7	7	9	6
Mean coagulation time(min.)	12.0	18.0	23.3	27.9	32.8	36.5	53.8
pH	6.78	6.76	6.68	6.71	6.70	6.72	6.77
Titratable acidity (ml. 0.1N NaOH/100g.milk)	16.4	15.8	17.7	16.4	17.7	16.0	13.8
				(g./100g. milk)			
Total solids	9.17	9.02	9.24	9.21	9.16	8.91	8.84
Protein (total N x 6.38)	3.38	3.22	3.40	3.39	3.33	3.12	3.30
Lactose (anhydride)	4.72	4.74	4.77	4.76	4.80	4.77	4.61
Ash	0.77	0.76	0.78	0.75	0.74	0.76	0.70
				(mg./100g.milk)			
Total calcium	116.2	118.3	121.3	118.5	112.1	113.3	103.3
Total magnesium	11.5	11.8	12.3	12.0	12.0	11.8	10.8
Total citric acid	146	159	186	184	165	165	161
Total phosphorus	101.3	97.0	100.3	93.5	98.0	93.8	78.9
Sodium	59	62	49	46	56	57	60
Potassium	156	154	158	158	152	158	140
Chloride	101.4	106.0	96.4	95.5	96.6	107.2	120.1
Nitrogen fractions							
Casein N	409.9	391.1	427.3	425.5	415.9	378.4	389.9
Lactalbumin + lactoglobulin N	72.9	66.8	63.4	63.0	58.0	63.1	63.9
Proteose-peptone N	24.0	24.4	19.5	20.3	24.1	23.8	29.5
Non-protein N	23.9	22.7	23.0	22.0	24.4	23.9	29.3
Calcium fractions							
Colloidal inorganic Ca	55.6	51.4	50.3	41.6	46.4	46.9	35.4
Caseinate Ca	31.2	31.3	30.9	32.6	29.7	27.7	29.4
Soluble unionized Ca	21.9	24.1	28.4	30.0	25.3	25.1	24.6
Soluble ionized Ca	10.9	11.3	11.7	12.6	11.7	11.9	11.5
Magnesium fractions							
Colloidal Mg	4.3	4.3	4.2	4.1	3.9	4.0	3.6
Soluble Mg	7.2	7.5	8.0	8.0	8.1	7.8	7.2
Citric acid fractions							
Colloidal citrate	18	19	22	18	14	16	12
Soluble citrate	128	140	164	167	151	148	150
Phosphorus fractions							
Colloidal inorganic P	30.9	30.0	29.5	25.7	26.6	28.2	22.2
Casein P	21.6	20.9	22.7	22.2	22.6	19.4	20.7
Soluble inorganic P	37.5	35.2	38.7	32.7	37.9	36.5	28.4
Ester P	11.3	10.9	9.4	12.9	11.0	9.7	7.6

colostrum samples, indicated that a casein content above normal, with the other prevailing conditions, did not cause instability to heat.

No relationships between coagulation time and chemical composition were found within the colostrum group of milks, and this was not surprising in view of the similarity of the coagulation times of the colostrum samples. The greater variation in the coagulation times of the post-colostrum milks offered some possibility of relating stability to composition within this group. However, apart from a direct relationship between coagulation time and pH, as found in the herd bulk milks, and a tendency for low stability to be associated with high concentration of calcium ions, there were no relationships between the concentrations of the constituents listed in Table 5 and the coagulation times of the post-colostrum milks.

Mid-lactation milk Milks in this group showed considerable variation in both stability to heat and chemical composition. The samples were therefore divided into seven sub-groups, each containing milks of similar heat stability, and the average chemical composition of the samples in each sub-group calculated to enable a first comparison to be made between composition and stability (Table 14). This table shows that no milk constituent exhibited a regular change in concentration as heat stability increased. There was, however, a tendency for the concentration of colloidal inorganic calcium and colloidal inorganic phosphorus to decrease as heat

Table 15 The average values for components of the casein complex (mg./1 g. casein) in groups of mid-lactation milks (fat-free) arranged in order of increasing coagulation time at 130°C

Range of coagulation time at 130°C	10-15 min.	15-20 min.	20-25 min.	25-30 min.	30-35 min.	35-40 min.	over 40 min.
No. of samples	7	11	8	7	7	9	6
Mean coagulation time (min.)	12.0	18.0	23.3	27.9	32.8	36.5	53.8
				mg./1 g. casein			
Colloidal inorganic Ca	21.0	20.7	18.4	15.2	17.5	20.2	14.4
Colloidal inorganic P	11.9	12.0	10.8	9.6	9.9	11.8	8.7
Tricalcium phosphate	42.0	43.5	38.1	30.3	36.0	41.5	28.4
Dicalcium phosphate	17.6	14.8	14.0	13.0	13.6	15.7	12.6
Total colloidal calcium phosphate	59.7	58.3	52.1	43.3	49.6	57.2	41.0
Colloidal magnesium	1.6	1.7	1.6	1.5	1.5	1.6	1.4
Caseinate calcium	11.7	12.6	11.3	12.1	11.3	11.7	11.9
Caseinate phosphorus	8.3	8.4	8.3	8.2	8.5	8.1	8.2
Colloidal citrate	5.7	7.7	8.6	6.9	5.2	6.7	4.7

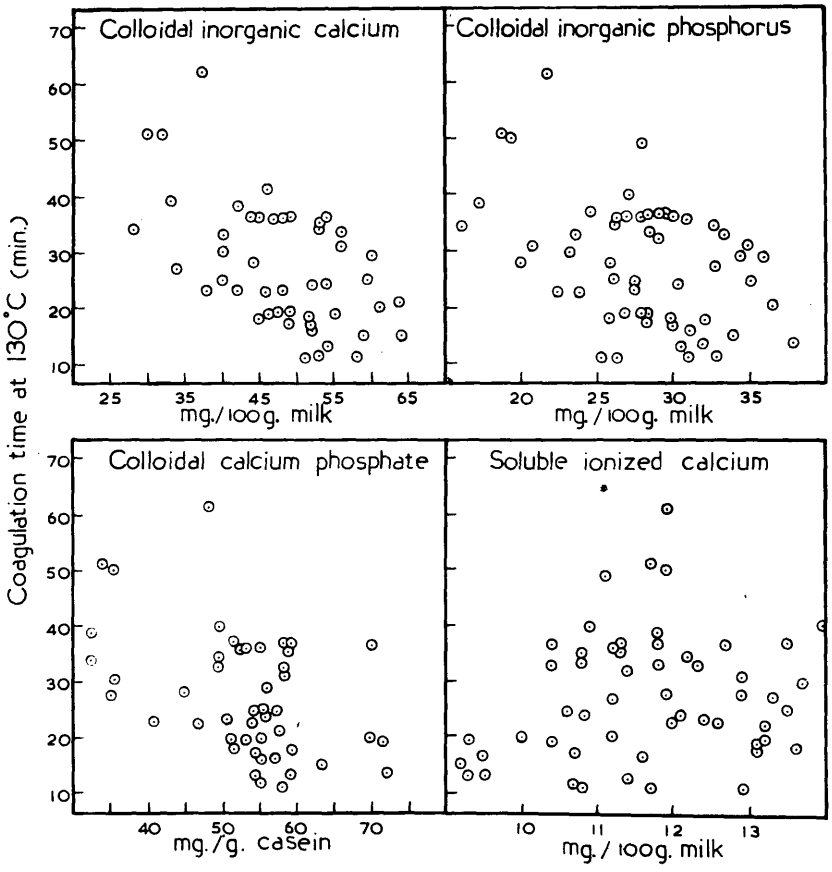

Fig. 30 The relationship of concentrations of colloidal inorganic calcium, colloidal inorganic phosphorus, colloidal calcium phosphate (per g. casein) and soluble ionized calcium to coagulation time at 130°C in mid-lactation milks.

stability increased and these changes were reflected in similar trends with the concentrations of total calcium and total phosphorus. There was also a tendency for the concentration of chloride to increase and that of lactalbumin plus lactoglobulin to decrease as coagulation time increased.

It seemed from Table 14 that the amounts of colloidal inorganic calcium and phosphorus in the casein complex might be related to the heat stability of the complex. This possibility was examined by calculating, for each of the seven sub-groups, the average amounts of colloidal inorganic calcium and phosphorus, and of the other components of the casein complex, associated with 1 g. of casein (Table 15). The table revealed, however, no consistent variation in amount of colloidal calcium phosphate, or of any other component of the casein complex, with increasing stability to heat.

That there was little relationship between the chemical composition and heat stability of the mid-lactation milks, was confirmed when the concentrations of all constituents listed in the above tables, in individual samples, were plotted against the corresponding coagulation times. The only relationships found were very poor (Fig. 30); the most that could be said was that the concentrations of colloidal calcium phosphate or of its components, whether expressed per 100 g. milk or per 1 g. casein, tended to be low in the more stable milks and vice versa. Fig. 30 also shows the complete lack of relationship

between concentration of soluble ionized calcium and coagulation time. pH which had been found to be related to coagulation time in the herd bulk and post-colostrum milks, was unrelated to heat stability in the mid-lactation samples.

Late lactation milk A larger proportion of the samples in the late lactation group of milks had coagulation times exceeding 40 min. at 130°C than in any of the other groups (Fig. 26d). This tendency to a greater heat stability was associated with considerable differences in chemical composition compared with the herd bulk milks (Table 7). Of these compositional differences the higher pH values, and the lower values for the soluble fractions of the mineral constituents, especially of calcium, may have been responsible for the late lactation milks having a tendency to be more heat stable. The lower lactose values may have resulted in a less rapid development of acidity during heating and this also may have been partly responsible for the increased coagulation times. The higher values for sodium and chloride and the lower values for potassium were unlikely to have been the cause of the enhanced stability. As for the post-colostrum milks, values for lactalbumin plus lactoglobulin above normal did not affect stability adversely.

Although there was a wide variation in the coagulation times of the late lactation milks there was no evidence of any relationship between chemical composition and heat stability when the concentrations

of constituents listed in Table 7, in the individual samples, were plotted against the corresponding coagulation times.

Mastitis milk Although the mastitis milks differed considerably from the herd bulk milks in chemical composition (Table 8), they showed no bias towards a lower or higher stability to heat (Fig. 26). This implied that the differences in composition between these two types of milk, e.g. in pH, lactose, sodium, chloride, casein, serum proteins and soluble phosphorus, were unimportant in relation to heat stability, and that the constituents present in similar concentration, e.g. soluble calcium and soluble magnesium, might be important. Further evidence of the probable unimportance of some of the above constituents is provided by the fact that the mastitis milks tended to be less stable than the late lactation milks despite many similarities in composition (Fig. 26, Table 7 & 8). For example, both were high in pH, sodium, chloride and serum proteins, and both were low in lactose, soluble citrate and soluble phosphorus. One major difference between mastitis and late lactation milk was in the casein content, but the mastitis milks did not show the increased stability that might be expected from a low casein content.

When the composition and heat stability of the individual mastitis milks were compared, little relationship was found. Where relationships did exist they were poor and the opposite of what might have been expected. For example, low heat stability tended to be associated with high pH and low

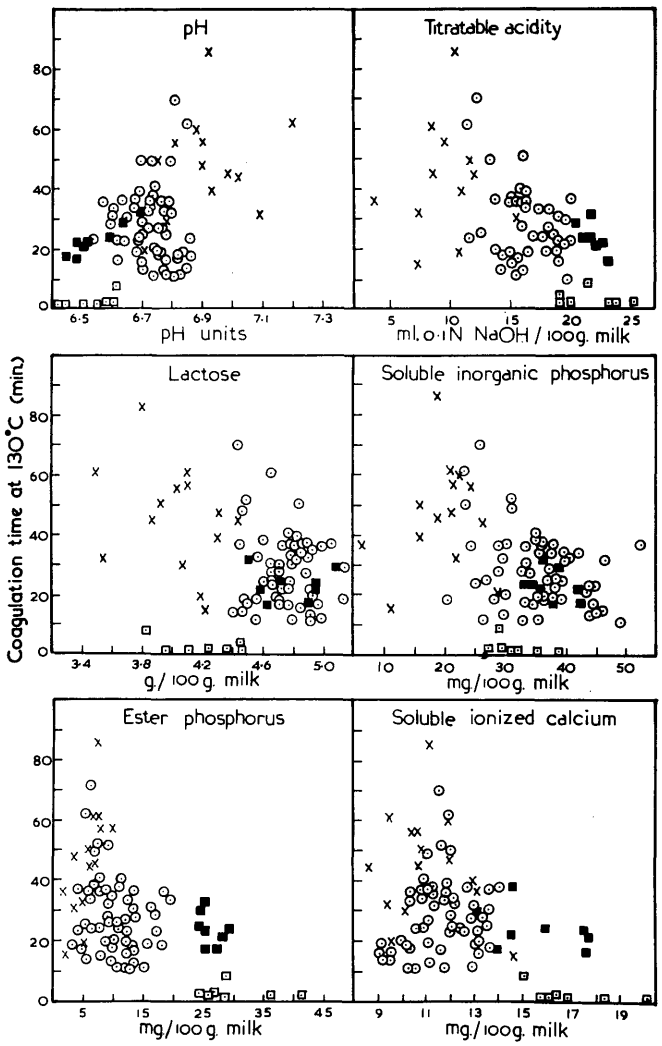

Fig. 31 The relationship of pH, titratable acidity and concentrations of lactose, soluble inorganic phosphorus, ester phosphorus and soluble ionized calcium to coagulation time at 130°C in milks from individual cows in different stages of lactation (⊡ colostrum, ■ post-colostrum milk, ⊙ mid-lactation milk, X late lactation milk).

concentrations of soluble magnesium and soluble ionized and unionized calcium.

Comparison of chemical composition and heat stability of all samples from individual cows (mastitis milks excluded) To study the effect of chemical composition on heat stability over the whole range of values obtained, the results for all samples from individual cows free from sub-clinical mastitis were collectively examined. This comparison, however, as in the different lactational groups, revealed only very poor relationships between composition and heat stability (Fig. 31). High acidity, as indicated by low pH or high titratable acidity, was usually associated with low heat stability and vice versa. With the exception of the results for the colostrum samples, heat stability tended to decrease as the concentrations of lactose and soluble inorganic phosphorus increased. There also appeared to be an inverse curvilinear relationship between the concentration of ester phosphorus and coagulation time but since the instability of the colostrum samples could be otherwise explained, it seemed very probable that the concentration of this constituent was of little importance in relation to heat stability. The concentration of soluble ionized calcium which from the literature would be expected to have an important influence on heat stability, was not closely related to coagulation time. It was true that some of the samples with a high concentration of calcium ions coagulated rapidly while all milks taking longer than 40 min. to

coagulate contained less than 12 mg. per 100 g. milk of this calcium fraction. However, the samples of intermediate stability, which constituted the majority, showed no relationship between concentration of soluble ionized calcium and coagulation time.

Neither the concentration of casein nor the concentration of the serum proteins was related to heat stability, except, as already shown, that the very unstable colostrum samples were rich in lactalbumin plus lactoglobulin. Salt-balance, calculated as described on p. 58 , was not related to coagulation time.

The concentration of colloidal calcium phosphate, whether expressed per 100 g. of milk or per 1 g. of casein, was unrelated to stability, and this was true also of the other components of the caseinate complex. However, since the caseinate complex varied considerably with respect to the proportion of calcium phosphate to casein (Tables, 5, 6, 7 & 15), it was possible that any effect which the soluble constituents of milk might have on the heat stability of the complex might vary with the calcium phosphate content of the complex. To test this hypothesis the milks, except for the colostrum samples, were divided into three groups according to the calcium phosphate content of their caseinate complexes. The groups contained milks in which 1 g. of casein was associated with respectively 31.0 - 49.9, 50.0 - 59.9 and 60.0 - 73.5 mg. of calcium phosphate. The milks in the second group had the same range of values for the ratio of

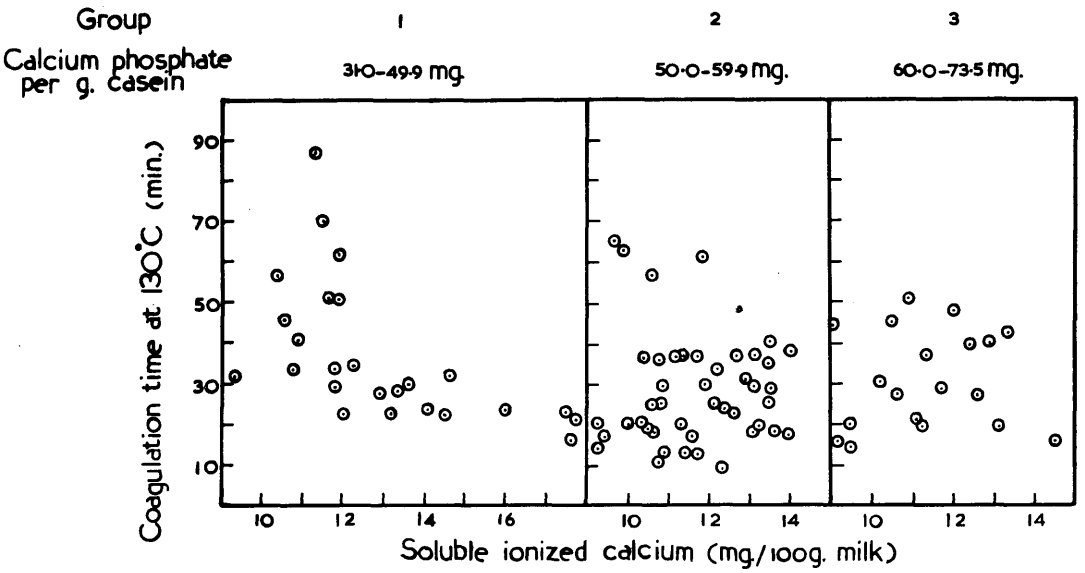

Fig. 32 The relationship of concentration of soluble ionized calcium to coagulation time at 130°C in milks grouped according to the amount of calcium phosphate associated with 1 g. of casein.

calcium phosphate to casein as the bulk milks (see Table 4). Those in the first and third groups had ratios which were therefore respectively lower and higher than average.

Within each group, the concentrations of constituents in the aqueous phase of the milks were plotted against the corresponding coagulation times but little relationship was found. The results obtained by this procedure for soluble ionized calcium are shown in Fig. 32. Only with milks in which the caseinate complex was relatively poor in calcium phosphate (group 1) was there any relationship between calcium ion concentration and coagulation time. Even so, all that this relationship indicated was that milks which coagulated in less than 30 min. had calcium ion contents ranging from 12-18 mg. per 100 g. and that milks taking longer than 30 min. to coagulate contained less than 12 mg., scarcely evidence of a close dependence of coagulation time on calcium ion concentration.

Milks from cows sampled at intervals during lactation

The absence of a close relationship between heat stability and chemical composition noted above, was found also when the results for milks taken from the same cow were compared.

Discussion

As in previous experiments, a great variation was found in the stability of milk samples to heat. Coagulation times at 130°C ranged from 0.6 to 86.2 min.

at 140°C from 0.6 to 35.0 min. and at 150°C from 0.5 to 7.2 min. With most samples there was a linear relationship between the logarithm of coagulation time and temperature, and this type of relationship was also found by Holm, Deysher & Evans (1923), Webb & Holm (1932) and Cole & Tarassuk (1946). However, as also noted by Cole and Tarassuk, significant deviations from the straight line relationship were found with some samples and this usually occurred when there was difficulty in detecting the exact moment of coagulation. This suggests that attention should be paid to the rate of increase in the size of the particles of coagulated protein and that a method of assessing heat stability not wholly dependent on the visual observation of coagulation might be preferable. A possible alternative method is suggested by Whitney, Paulson & Murthey (1952) in which the percentage of filterable nitrogen in the milk sample is plotted against the time of heating of the sample.

The slope of the lines relating logarithm of coagulation time and temperature was reasonably constant when coagulation times were easy to determine and indicated that the temperature coefficient ($Q_{10°C}$) of the coagulation reaction was about 2.7 between 130° and 140°C and about 3.1 between 140° and 150°C. These temperature coefficients are similar to the temperature coefficient ($Q_{10°C}$) of 3 obtained by Howat & Wright (1936) for the dephosphorylation and coagulation of neutral solutions of calcium caseinate

heated at temperatures ranging from 90° to 115°C for periods of 1 to 45 hr. Howat & Wright observed that visual coagulation of the caseinate occurred when about 45% of the casein phosphorus had been liberated as inorganic phosphate. The variation found in the stability of the caseinate complex in different milks, at a given temperature, indicates either that its coagulation is not dependent on a particular degree of dephosphorylation or, less likely, that the rate of dephosphorylation varies from milk to milk.

Despite the wide variation in the coagulation times and in the chemical composition of the samples very little relationship between heat stability and chemical composition was found. This was true when the results for all samples from individual cows, the herd bulk milks, the mastitis milks and the milks from different stages of lactation, except colostrum, were examined.

The marked instability to heat of the colostrum samples appeared to be the result of the very high levels of serum proteins. The average concentration of lactalbumin plus lactoglobulin nitrogen in the colostrum samples was 163.4 mg. per 100 g. milk, which is about 2.5 times the average for the herd bulk milks. According to Marsden (1953), the increase is mainly in lactoglobulin whose components, the two immune globulins, are more rapidly denatured by heat than any of the other serum proteins (Wegelin, 1952; Larson & Rolleri, 1955). Average values for serum proteins greater than the herd bulk milk average, but

less than the colostrum average, were also found, however, for the post-colostrum milks which were similar in stability to most of the herd bulk milks, and for the late lactation milks which tended to have longer coagulation times. It would appear therefore that there is a critical concentration of serum proteins which has to be exceeded before rapid heat coagulation results. The slight overlap in values for lactalbumin plus lactoglobulin in the colostrum and post-colostrum samples made it difficult to decide the precise level above which instability ensued, but it was probably in the region of 100-110 mg. per 100 g. milk, in terms of nitrogen, or 0.6 - 0.7% w/w lactalbumin plus lactoglobulin. This estimate of the critical concentration agrees reasonably well with the statement of Davies (1939) that in the transition from colostrum to normal milk, rapid coagulation by heat ceases when the concentration of lactalbumin plus lactoglobulin falls below 0.9%. Below the critical concentration, the amount of serum proteins was unrelated to coagulation time which is consistent with the observation of Pyne & McHenry (1955) that the removal of lactalbumin and lactoglobulin from a mid-lactation milk did not alter its stability to heat.

Several workers have suggested that the stability of milk to heat is influenced by the natural variation in the levels of some of the calcium fractions but the present results showed little relationship between the content of total calcium, or of the various calcium fractions in milk, and coagulation time. The absence

of a close relationship between concentration of soluble ionized calcium and heat stability was especially significant. Pyne & McHenry (1955) found an inverse curvilinear relationship between the concentration of calcium ions and coagulation time in mid-lactation milk and they suggested that the concentration of calcium ions is one of the more important factors controlling stability. The present results for mid-lactation milk did not show this relationship (Fig. 30). Also, the sub-division of all samples from individual cows, except colostrum, into three groups according to the calcium phosphate content of the caseinate complex, so as to minimize the variation in the composition of the complex, revealed little relationship between calcium ion concentration and heat stability. Only in the group of milks in which the caseinate complex was relatively poor in calcium phosphate was there a suggested inverse curvilinear relation between concentration of soluble ionized calcium and coagulation time (Fig. 32). This relationship and that found by Pyne & McHenry (1955) would appear to be of little value in predicting the coagulation time of a milk from a knowledge of its calcium ion concentration. All that either shows is that in a selected population of samples, when the calcium ion concentration exceeds a certain level, about 12 mg. per 100 g. milk in this experiment, the milk will be relatively unstable and that below this level stability will be greater but independent of calcium ion concentration. The fact that the herd

bulk milks had similar values for soluble ionized calcium (range 10.5 - 12.8 mg. per 100 g. milk) yet had coagulation times at 130°C varying from 17.2 to 59.0 min. also shows that this calcium fraction could not be regarded as a major factor controlling heat stability.

The view of Smeets (1952,1955), Smeets & Seekles (1952), Seekles & Smeets (1954) and Boogaerdt (1954), that milk is very unstable to heat when the concentration of calcium ions exceeds about 15 mg. per 100 ml. of milk ultrafiltrate, could not be confirmed. Apart from the colostrum samples, only four milks exceeded this level of calcium ion concentration and they were not markedly unstable to heat. The present results also did not substantiate the suggestion of Sandelin (1943) that the instability of colostrum is caused by a high concentration of soluble ionized calcium. The very unstable colostrum samples, it is true, were rich in this calcium fraction having a range of values of 15.1 - 20.1 mg. per 100 g. milk, but four of the seven post-colostrum milks contained similar amounts yet were not very unstable to heat.

There was a slight tendency in the mid-lactation milks for the relatively unstable samples to have a greater proportion of calcium phosphate in the caseinate complex than stable samples. This suggested that heat stability might be inversely related to the size of the caseinate micelles since Hostettler, Rychener & Künzle (1949), Ford, Ramsdell & Landsman

(1955) and others have shown that the larger the particles of the caseinate complex in milk, the more calcium phosphate they contain. However, with the results for all samples from individual cows and for the herd bulk milks there was no relationship between the composition or concentration of the caseinate complex and heat stability.

That the composition of the colloidal phase in milk did have some effect on heat stability was demonstrated by a series of experiments in which milks with widely differing coagulation times were dialyzed against a large volume of a composite sample, prepared by mixing equal volumes of the individual milks. After dialysis the range of coagulation times for the individual samples was less than before dialysis but there were still considerable differences between the individuals. It must be assumed that after dialysis the aqueous phase in each individual milk will have the same composition and that the differences found in coagulation time were due to differences in the colloidal phase or in the casein itself.

Salt-balance, calculated as described on p. 58 and believed by Sommer & Hart (1919, 1922, 1926) to be an important factor in relation to heat stability, was not correlated with coagulation time within any of the groups of milks or when the results for all samples were collectively examined. This is in agreement with the findings of Holm, Webb & Deysher (1932).

The results for herd bulk and post-colostrum milks, and also for milks from individual cows in all stages

of lactation showed a tendency for pH to be directly related to coagulation time. However, the relationships were poor, and this agrees with the generally held view that natural acidity and heat stability are not closely related.

It is apparent from the preceding discussion, that despite the detailed nature of the chemical analysis of the milks in this experiment, no adequate explanation for the variation in coagulation times, except with the colostrum samples, was found. The stability to heat of the calcium caseinate - calcium phosphate complex did not appear to be closely dependent on its concentration or chemical composition, or on the chemical composition of the surrounding aqueous phase. The results therefore suggested that the primary factors controlling the heat stability of the caseinate complex in milk could not be detected by the analytical methods used, and that the rate of heat-induced changes in the complex which lead to coagulation, possibly depended on an unmeasured physical or chemical property of the complex.

Conclusions

1. The coagulation times of most of the samples decreased by a factor of about 3 with an increase in temperature of 10°C over the range 130 - 150°C. Because of the proportionality of the coagulation times at 130, 140 and 150°C, the coagulation times at 130°C only were used as a measure of the stability of the samples to heat.
2. The coagulation times of the herd bulk milks

ranged from 17.2 to 59.0 min. at 130°C while the range for the samples from individual cows was 0.6 to 86.2 min.

3. Samples of colostrum were very unstable to heat and late lactation milks tended to have the longest coagulation times but otherwise there was little relationship between the heat stability of milk and the stage of lactation of the cow. The coagulation times of the herd bulk milks tended to increase as the average stage of lactation of the cows in the herd increased.

4. Although the colostrum samples were comparatively rich in ionized calcium, their marked instability to heat appeared to be caused solely by their high content of lactalbumin plus lactoglobulin.

5. The stability to heat of the calcium caseinate-calcium phosphate complex in all samples, other than colostrum, could not be closely related either to the composition of the complex or to the composition and salt-balance of the surrounding aqueous phase.

6. It appeared that when the calcium phosphate content of the caseinate complex was low, the heat stability of the complex tended to be inversely related to the concentration of ionized calcium in the milk.

7. In most of the samples, the concentration of ionized calcium was not related to coagulation time.

8. The absence of a close relationship between coagulation time and chemical composition suggested that variation in some unmeasured physical or chemical

property of the caseinate complex was responsible for the variation in its stability to heat.

General Summary

1. The work described in this thesis deals with the relationship between the chemical composition of milk and the stability of the milk protein, primarily the caseinate complex, to ethanol, rennet and heat.

2. It was found that stability of milk protein to ethanol was dependent principally on the concentration of soluble ionized calcium. As the concentration of this calcium fraction increased the strength of ethanol required for coagulation decreased, and approximately 60% of the variation in stability was related to variation in the concentration of soluble ionized calcium.

3. The stability of milk protein to rennet was found to be more closely related to pH than to any other property of milk. As pH increased from 6.40 to 6.80 renneting time increased from 1.5 min. to 4 min. Above pH 6.80, increase in renneting time with increasing pH was more pronounced; a pH of 6.90 corresponded to a renneting time of approximately 6 min. and a pH of 7.20 to a renneting time of 13 min.

4. Stability of milk protein to ethanol and rennet was related to the concentration of several other milk constituents. These relationships, however, could usually be attributed to the relationships that existed between these milk constituents and calcium ion concentration where stability to ethanol was concerned and with pH where stability to rennet was concerned.

5. The stability of milk protein to heat varied

greatly for different samples of milk but no adequate explanation for the variation was found. Coagulation times were largely independent of such factors as concentration of ionized calcium, ratio of colloidal calcium phosphate to casein and the ratio of soluble calcium plus magnesium to soluble phosphate plus citrate, all of which have been regarded in the past as being important in relation to heat stability. The results suggested that some undetermined physical or chemical property of the caseinate complex determined its stability to heat. Colostrum samples were particularly unstable to heat but this is attributable to the very high levels of lactalbumin plus lactoglobulin that they contained.

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