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PROPERTIES AND DISTRIBUTION OF CASEIN AND NON-CASEIN PROTEINS, CALCIUM AND PHOSPHORUS IN SKIMMILK, BUTTERMILK AND BUTTERSERUM FROM THE SAME WHOLE MILK

by

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I. INTRODUCTION

The acidity of dairy products is a function chiefly of the salts and proteins in these products, provided no acid production by micro-organisms has occured. When acidity, in addition to that contributed by the natural salts and proteins, develops, it usually results from the conversion of lactose to lactic acid.

The natural acidity of dairy products (i.e. that resulting from milk salts and milk proteins) varies with the fat-free solids content of the product so that the greater the concentration of fat-free solids the higher the acidity.

The degree of acidity or the reaction of dairy products may be measured as titratable acidity (commonly expressed as per cent lactic acid by weight) or as pH. Either of these may be employed to follow changes in degree of acidity in dairy products.

In certain products, chiefly, farm-separated cream for buttermaking, it is customary to reduce the acidity which may have developed, with alkaline salts or alkalies. The aqueous phase may then exhibit the normal acidity of sweet cream; however, in addition to the natural salts it will contain the end products of the neutralization reactions, chiefly sodium, calcium or magnesium lactates depending on the neutralizing medium employed.

Initially, it was desired to determine both the acidities of the fat and the water soluble acidities of the serum (the aqueous phase) of butter, in an attempt to evaluate the "quality" of the cream from which the butter was made. A number of extraction procedures for determining the acidity of butterserum were ineffective.

Ling (1936) had presented a series of titrations of oxalated and non-oxalated milk and of the corresponding oxalated and non-oxalated rennet wheys as a means of estimating the "colloidal tricalcium phosphate" in the product. These titrations may also be employed to evaluate the "true serum acidity" of milk (that is, the acidity resulting from non-casein components).

It was proposed to employ the Ling titration procedure in butterserum to this latter end. However, it became apparent during the early stages of the investigation that butterserum, even from unsalted butter, would not yield a normal coagulum with rennet. Only in a few samples did it form a weak clot from which it was impossible to expel any whey for titration. In addition the coagulum from buttermilk frequently was weak and expelled its whey with difficulty.

These findings led to the present study the objectives of which were (1) to determine the reason for the inability of casein in some of these products to coagulate with

rennet, (2) to determine, if possible, whether a study of the protein fractions in skimmilk, buttermilk and butterserum obtained from a single milk source would throw further light on the adsorbed materials in the fat globule-serum interphase, (3) to determine whether differences could be shown among the casein and the non-casein protein preparations from skimmilk, buttermilk and butterserum obtained from a single milk source, (4) to determine the distribution of calcium, phosphorus and the common protein fractions among skimmilk, buttermilk and butterserum obtained from a single milk source and (5) to obtain, if possible, further information with regard to the mechanism of the reaction between rennet and casein.

II. REVIEW OF LITERATURE

A. Constituents Which Influence the Acidity of Milk

Investigations of the acidity factors in milk (i.e. milk salts and proteins) have been concerned primarily with (a) the distribution of calcium and phosphate between the soluble and colloidal states, (b) the type of colloidal phosphate, (c) the association between calcium phosphate and casein and (d) changes among the constituents with increasing acidity.

Rona and Michaelis (1909) estimated the proportions of soluble and insoluble or colloidal calcium by dialyzing milk against rennet whey from the original milk and whey resulting from casein precipitation with ferrous hydroxide. Calcium decreased in the iron whey and increased in the rennet whey. By this method they estimated that 40 - 50 per cent of the total calcium was soluble.

György (1923) found that the undialyzable phosphorus amounted to 50 - 60 per cent of the total and that an increase in acidity caused an increase in the amount of dialyzable calcium and phosphorus. At the isoelectric point of casein all calcium and phosphorus was dissolved in the whey. He concluded that the casein and the undialyzable dicalcium phosphate showed chemical affinity for one another.

Van Slyke and Bosworth (1914) separated soluble and insoluble calcium and phosphates by filtering milk through a porcelain filter under pressure. In this way they obtained 35 per cent of the total calcium as soluble in the filtrate. Similar results were reported by Porcher and Chevallier (1923). Van Slyke and Bosworth (1914) also found the ratio of insoluble inorganic to organic phosphorus to vary considerably and interpreted this to indicate that there was no chemical union between phosphate and casein. Some of their filtration experiments indicated that varying portions of the albumin were adsorbed on the casein.

Pyne (1940) in comparing different sera for dialysis of milk found rennet whey the most satisfactory and estimated 56 per cent of the inorganic phosphorus and 34 per cent of the total calcium in fresh milk (pH 6.56 - 6.65) to be soluble. At pH 5.28 the corresponding percentages were 96.3 and 88.3.

From ionic strength considerations and from the analyses by Van Slyke and Bosworth (1914) Whittier (1929) calculated that only 2.5 per cent of the soluble calcium in milk was ionized. He concluded that the remainder existed as a calcium citrate complex. Verma and Sommer (1950), from analyses of rennet wheys, found 35 per cent of the calcium, 8 per cent of the magnesium, 37 per cent of the phosphorus and 85 per cent of the total citric acid in milk to exist

in soluble form.

Van Slyke and Bosworth (1914a) contended that the acidity of fresh milk was due to dicalcium phosphate kept in the colloidal state by casein acting as a protective colloid. According to them the neutral dicalcium phosphate would hydrolyze during a titration to form tricalcium phosphate.

$$CaPHO_4 + 2H_2O = Ca(OH)_2 + H_3PO_4$$
$$2\ CaHPO_4 + Ca(OH)_2 = Ca_3(PO_4)_2 + 2H_2O \quad .$$
$$3\ CaHPO_4 = Ca_3(PO_4)_2 + H_3PO_4$$

They proposed to determine the "true" acidity in milk by titrating a sample from which the tricalcium phosphate had been removed with a saturated potassium oxalate solution.

Porcher and Chevallier (1923) considered both di- and tricalcium phosphate to be present in milk. Pyne (1934), on the other hand, contended that the colloidal phosphate exists as tricalcium phosphate which forms a chemical bond with the casein. He based his contention on (a) the difference in formol titration values of milk with and without added potassium oxalate and (b) potentiometric titration curves between pH 7 and 10 of solutions of tricalcium phosphate and of tricalcium phosphate plus calcium caseinate. In a later work dealing with the influence of tricalcium phosphate on rennet coagulation Pyne (1945) reiterated this view.

Pedersen (1936) separated milk proteins from the serum by ultra-centrifugal methods. He isolated casein and three other proteins with lower molecular weight which he called α, β and γ. He considered that the protein commonly called lactalbumin was a mixture of the α and β fractions and that globulin corresponded to the γ fraction. His experiments showed that casein, when dissolved in sodium and potassium phosphate buffer, forms a polydisperse complex.

De Kadt and Van Minnen (1943) separated casein from milk by supercentrifugation. On basis of analyses of the casein, the remaining whey and the original milk they concluded that only calcium and phosphate were bound to the casein. Some phosphate and an equimolecular amount of calcium were bound to the casein by ester linkages. If these values were subtracted from the total value of calcium and phosphate removed with the casein, the remaining calcium : phosphate ratio was 3:2 which is the same as that in $Ca_3(PO_4)_2$. This was interpreted as evidence for a complex between $Ca_3(PO_4)_2$ and casein and formulated as follows

$$\text{Casein} - PO_4 = Ca \cdot \cdot \cdot \cdot \cdot \cdot Ca_3(PO_4)_2 \quad ,$$

or a compound

$$\text{Casein} - PO_4 \begin{cases} Ca - PO_4 = Ca \\ Ca - PO_4 = Ca \end{cases} .$$

Ramsdell and Whittier (1944) isolated the calcium caseinate - phosphate complex from milk by supercentrifugation. By titrating a solution of this complex with potassium oxalate and observing an increase in alkalinity, they concluded that $Ca_3(PO_4)_2$ rather than $CaHPO_4$ was involved in the complex. The complex was found to contain 4.80 per cent $Ca_3(PO_4)_2$ and 95.20 per cent calcium caseinate, the latter containing 1.18 per cent calcium. The complex contained 0.742 per cent organic and 0.958 per cent inorganic phosphorus. The casein contained 0.789 per cent organic phosphorus.

Eilers et al. (1947) proposed the following formulation for a calcium caseinate-phosphate complex.

$$\text{Peptide chain - R - OH} + 2\ Ca(OH)_2 + H_3PO_4 =$$

$$\text{Peptide chain - R - O - Ca - O - } PO_3 = Ca + 4H_2O\ .$$

This hypothesis was based on limiting values obtained by titrating phosphoric acid with calcium hydroxide in the presence of casein between pH 4 and 9. The scheme was criticized by ter Horst (1947) who pointed out that the titration values reported were not necessarily representing the above equation, partly due to the fact that at pH 4.0 one third of the phosphoric acid was already neutralized.

In studying this problem, Van der Burg (1947) employed a suspension of yeasts with the ability to adsorb calcium phosphate. By adding such a suspension to milk and heating the yeast-milk suspension he was able to show a 21 per cent decrease in colloidal tricalcium phosphate associated with casein compared with a considerable increase in the milk heated without yeast. In both cases there was only a slight decrease in calcium equivalent to the ester phosphate of casein. His conclusion was that the calcium in casein is attached to the ester phosphate groups and that the remaining free carboxyl and amino groups take up the colloidal tricalcium phosphate in varying amounts.

Ling (1936) used titrations of oxalated and non-oxalated milk and rennet whey as a means of estimating the colloidal tricalcium phosphate in milk; he used this scheme in later studies (1937) of the partition of calcium and phosphorus. He found that the tricalcium phosphate accounted for nearly half the total inorganic phosphate in milk and that, during acidity development, the increase in casein acidity was proportional to the removal of calcium from the casein. He observed that when the total calcium content of milk fell sharply with season the effect was felt more by the casein calcium than by the colloidal tricalcium phosphate and interpreted this to indicate that the formation of tricalcium phosphate takes precedence

over saturation of casein with calcium. This, he claimed, could not be reconciled with a chemical union between tricalcium phosphate and casein.

Ling (1936) also observed a linear relationship between the disappearance of colloidal tricalcium phosphate and the increase in titratable acidity of the milk.

Recently Pyne and Ryan (1950) pointed out an error in the original Ling titrations which was caused by titrating milk and whey to the same phenolphthalein endpoints corresponding actually to different pH values. This resulted in overestimation of the tricalcium phosphate. They proposed a modification in which they employed 12 times as much phenolphthalein in the milk titration than they did in the whey titration and in both cases employed the same depth of color of the endpoint. Applying this modification they estimated that 88 per cent of the phosphate in milk was tricalcium phosphate and concluded that the remainder was dicalcium phosphate. This is in agreement with ter Horst's (1947) contention that some dicalcium phosphate is also titrated in this reaction.

Whittier (1929a) studied the buffer intensities of milk and milk constituents and found the buffer capacities of skimmilk and acid whey to be alike at pH 4 and pH 7, while at pH 5 the buffer capacity of skimmilk was high and that of whey was low. The difference he attributed to the casein.

Wiley (1934, 1934a) studied the buffer effects of casein, phosphates, calcium and citrates in synthetic preparations analogous to milk and whey and in these products themselves. He found that the buffer capacities of the individual constituents were not additive and postulated that the increased buffering of milk at low pH was due to the solution of the colloidal phosphate, believed to be dicalcium phosphate, and that the increased buffering in acid whey at pH 6.2 was due to the precipitation of this salt. Part of the buffering of milk was also due to casein.

B. Determination of the Milk Proteins

Casein, the largest protein fraction in milk, is colloidally dispersed; the whey proteins, albumin and globulin, are considered soluble although evidence was presented by Van Slyke and Bosworth (1914a) indicating that albumin is partly adsorbed on casein. In fresh milk the low molecular weight proteose-peptone fraction is small; upon proteolysis it will increase at the expense of casein, albumin and globulin.

A modification by Van Slyke (1893) of older methods of determining casein in milk was adopted as an "official" method (Van Slyke, 1898). It involved precipitation of casein from diluted milk at 40°C by the addition of a few milliliters 10 per cent acetic acid, filtration, washing

and determination of nitrogen in the precipitate by the Kjeldahl method. Van Slyke (1909) later presented a rapid volumetric procedure involving titration with standard alkali of the casein precipitated from a known volume of milk. A simple centrifugal method based on measuring the volume of the casein precipitate was proposed by Hart (1907), and Robertson (1909) described a method in which the casein, after precipitation with acetic acid, was dissolved in 0.1N NaOH and determined refractometrically by comparison with a standard solution of casein.

The difficulties with these early methods seem to have been the attainment of a pH in the solution corresponding to the isoelectric point of casein. Waterman (1927) proposed a buffer mixture of acetic acid and sodium acetate as precipitating reagent and determined casein nitrogen as the difference between total nitrogen and nitrogen in the non-casein filtrate. With this procedure he obtained better replication than with the official method of Van Slyke (1893). The casein percentages which he obtained with two samples were 2.65 and 2.91 respectively; maximum variations among replicates were 0.06 and 0.07 per cent compared with 0.29 and 0.31 per cent by the official method.

Moir (1931) thought that the pH (4.7 - 4.8) obtained by Waterman was too high to insure complete precipitation. He modified the procedure by adding the buffer solution

in two parts, first the 10 per cent acetic acid and then the sodium acetate (0.28N); the mixture was allowed to stand for an hour before filtering. The pH values of his precipitation mixtures were quite variable, a fact which he ascribed to varying buffer capacities of different milks. For this reason he recommended that a preliminary experiment be carried out with each milk sample to determine the correct amount of sodium acetate required to produce a pH 4.6. He reported casein nitrogen percentages between 0.3602 and 0.4046 for a number of milk samples.

Moir (1931a) pointed out that earlier methods for combined albumin and globulin gave high results when these proteins were precipitated together by tannic acid, and low results when they were precipitated by heat coagulation. He proposed a method for precipitating them with trichloroacetic acid from the hot non-casein filtrate and determining nitrogen in the precipitate by the Kjeldahl method. The determination of globulin alone was accomplished (Moir, 1931b) by saturating the neutralized (to phenolphthalein) non-casein filtrate with magnesium- or sodium sulfate, filtering and determining nitrogen in the precipitated globulin.

Rowland (1938, 1938a) suggested further refinements of Moir's procedure for isoelectric casein precipitation and presented a scheme for complete fractionation and analysis of the several protein and non-protein nitrogen

fractions in milk, including the proteose-peptone fraction ignored by Moir. His improvements included (a) separation of albumin from proteose-peptone by denaturing albumin and globulin by heat coagulation and precipitating them with the casein, (b) determination of non-protein nitrogen free of proteose-peptone nitrogen in strong trichloroacetic acid solution (12 per cent in the final mixture) at room temperature, (c) adjusting the non-casein filtrate to pH 6.8 - 7.0 before precipitating globulin with magnesium sulfate and (d) using selenium oxychloride as catalyst in the digestion. Rowland (1938b) reported the average protein and non-protein nitrogen distribution in normal milk as follows:

	Per cent of total N
Casein N	78.3
Albumin N	9.1
Globulin N	3.5
Proteose-peptone N	4.1
Non-protein N	5.0

These results were slightly different from those previously reported by Davies (1933). Rowland proposed that the differences were caused by the high globulin values obtained by Davies using Moir's method.

Memefee and Overman (1940) used Rowland's separation scheme but modified the digestion by using HgO as catalyst.

They also distilled the ammonia into a boric acid solution thus permitting direct titration with standard HCl.

Pillemer and Hutchinson (1945) separated albumin from globulin in blood serum by precipitating the former with methanol in acetate buffer. Albumin was determined on aliquots of the methanol filtrate.

Shahani and Sommer (1951, 1951a) studied the distribution of protein and non-protein nitrogen fractions in fresh and aged raw milk of different breeds. Their method was essentially that of Rowland (1938, 1938a) with the modifications of Menefee and Overman(1941). They precipitated globulin from the non-casein filtrate with methanol according to the method of Pillemer and Hutchinson (1940).

Johnson and Swanson (1952) determined whey proteins by a spectrophotometric adaptation of the biuret color reaction. The method was complicated by interference by lactose and calcium phosphate; these were removed by dialyzing the whey. NaCl, Na_2SO_4 and ethanol used in fractionating the proteins also interfered but were compensated for by including known concentrations of them in the standard protein solutions. The method could not be used with casein which gives an abnormal color response in the biuret reaction.

C. Determination of Calcium and Phosphorus in Milk

The problems in determining calcium and phosphorus in milk may be divided in two categories, namely, those involving the separation of these minerals from the milk and those involving the actual determination. Since milk and blood are biological fluids with similar properties, the same methods of separation and analyses have often been used with both.

Calcium may be separated from milk by ashing and dissolving the ash in an acid. However, Sanders (1931) following the suggestion of Bell and Doisy (1920), Roe and Kahn (1926) and others, separated the calcium in a 10 per cent trichloroacetic acid filtrate and found the results of this method to agree with those of the ashing procedure.

In the methods described, the calcium once separated from milk (or blood) was precipitated as oxalate, centrifuged, washed and titrated with standard $KMnO_4$ solution as done by Kramer and Tisdall (1921), Sanders (1931), Miethke and Levecke (1932) and Derx and Jansen (1947).

Biering (1943) offered a micromethod which was a modification of that of Rappaport and Rappaport (cited by Biering) using ceric sulfate for oxidizing the oxalate and back titrating with sodium thiosulfate.

Rothlin and von Bidder (1945) examined critically the Kramer - Tisdall method for calcium in blood serum, discussed its sources of error and prescribed a modification which gave results of high accuracy. Their modifications consisted in (1) careful ashing of the sample, (2) complete precipitation of the calcium oxalate in buffered, boiling solution and overnight digestion at room temperature, (3) filtration rather than centrifugation of the precipitate and (4) oxidation-reduction titrations with ceric sulfate and ferrous ammonium sulfate.

Salomon et al. (1946) described a precision method for calcium in blood plasma, which involved ashing with perchloric acid, precipitation as calcium oxalate and titration of the oxalate ion with ammonium hexanitrato cerate in perchloric acid.

Colorimetric determination of calcium in blood serum was described by Roe and Kahn (1926), who precipitated calcium as phosphate, converted it to molybdate, reduced with hydroquinone bisulfite and compared against a standard in a colorimeter. Elliot and Pearson (1946) determined serum calcium spectrophotometrically by reading transmittances of the potassium permanganate solution in which the calcium oxalate had been dissolved. Tsao (1952) reported a colorimetric determination based on the formation of a colored complex between calcium and 2, 3, 4 - trihydroxy benzoic acid. Keirs and Speck (1950) determined

calcium in milk by flame photometry.

Phosphorus in blood and milk may be determined as total, inorganic, acid-soluble and lipid phosphate. Greenwald (1915) apparently developed the first method for separating the different fractions in blood serum. He obtained inorganic phosphorus in the picric-acetic acid filtrate after precipitation of the proteins and lipids. Lipids were "precipitated" with chloroform and dil. HCl from acetone extracts of the serum. The residue from the acetone extract was treated with boiling ethanol and then with ether and picric acid in 4 per cent HCl. Acid soluble phosphorus was determined in this extract and was found to be equal to the inorganic phosphorus. Greenwald ultimately determined the phosphorus colorimetrically as reduced phosphomolybdate.

Lenstrup (1926) applied this scheme to human and cow's milk and found average values of 95.4 mg. total, 78.3 mg. acid-soluble, 67.1 mg. inorganic and 11.1 mg. lipid phosphorus per 100 ml. milk.

Sanders (1931) determined acid soluble phosphorus in milk directly in the trichloroacetic acid filtrate and total phosphorus in ashed samples. Phosphorus was precipitated from the trichloroacetic filtrate as molybdate; it was claimed that the precipitate thus formed was coarser and filtered better than that from sodium tungstate and H_2SO_4.

Youngburg and Youngburg (1930) determined total phosphorus in blood serum from a sample digested with H_2SO_4 using hydrogen peroxide as catalyst; inorganic phosphate was determined directly in the trichloroacetic acid filtrate, acid-soluble phosphorus in a digested aliquot of this filtrate and lipid phosphorus in a digested aliquot of an alcohol-ether (3:1) extract of the serum. Phosphorus was determined colorimetrically by the method of Kuttner and Cohen (1927).

Graham and Kay (1933) separated the phosphorus fractions in milk by a scheme similar to that which Youngburg and Youngburg used with blood serum. For digestion they used both H_2SO_4 and HNO_3. Youngburg and Youngburg had rejected the latter because of the difficulties in removing it from the solution. Traces of this oxidizing acid interfered seriously with color production in the reduced phosphomolybdate solution. Graham and Kay pointed out that colorimetric determination from the various filtrates gave more accurate results than the official gravimetric procedure of A.O.A.C. (1925).

Recently Van Slyke and Sacks (1953) demonstrated that the lipid extracts obtained with Bloor's (1914) alcohol-ether (3:1) mixture did not contain appreciable amounts of inorganic phosphorus.

The colorimetric determination of blood serum- or milk phosphorus as reduced phosphomolybdate has been

modified a number of times since the first presentation by Bell and Doisy (1920). Briggs (1922) proposed dilution of the serum and the use of hydroquinone as reducing agent. Fiske and Subbarrow (1925) reduced with 1, 2, 6-amino-naptholsulfonic acid and claimed to get maximum color production even in the presence of interfering substances. Kuttner and Cohen (1927) proposed a microtechnique using a sodium molybdatesulfuric acid reagent and dilute $SnCl_2$ for reduction. Kuttner and Lichtenstein (1930) discussed the advantages of $SnCl_2$ as reducing agent. Bodansky et al. (1932) noticed a considerable variation from Beer's law when the concentrations in sample and standard differed by more than 20 per cent and presented a table of corrections. Obermer and Milton (1932) demonstrated the use of the Vernes-Brisq-Yvon photometer for phosphorus determination.

Dyer and Wrenshall (1938) determined phosphorus with the Evelyn photoelectric colorimeter. They pointed out that the amount of stannous chloride used would influence the color and its stability. In a later work from the same laboratory Smith et al. (1939) called attention to the importance of using fresh stannous chloride free of stannic tin. Fontaine (1942) boiled the reduced phosphomolybdate solution for 20 min. before reading the transmittance. The color produced by this technique exhibited maximum absorption at 820 mμ, was very stable and obeyed

Beer's law over a wide concentration.

Simonsen et al. (1946) determined blood serum phosphorus colorimetrically as phosphovanadomolybdic acid in the Klett-Summerson photoelectric colorimeter and in the Beckman quartz spectrophotometer.

D. Rennet Coagulation of Milk

Many divergent theories have been advanced to explain rennet coagulation of milk. Loevenhart (1904) proposed that polymerization of casein particles took place resulting in formation of the less dispersed paracasein. Mellanby (1912) suggested that casein and rennet form an adsorption complex which is conditioned by the amount of calcium present. Bang (1911) believed that rennet merely changed the affinity of casein for calcium and that casein and paracasein were the first and last members respectively of a family of compounds with increasing affinities for calcium.

Some of the rennet action theories are based on the concept of a protective colloid in milk which holds the casein in suspension; rennet acts by way of destroying the protective colloid. On basis of ultra microscopic studies, Alexander (1912) concluded that in milk lactalbumin was the protective colloid. This hypothesis was criticized by Palmer and Richardson (1925) who questioned that casein in milk was an unstable suspensoid requiring a protective

colloid for its stabilization. They showed that the addition of gelatin or albumin did not prevent coagulation of calcium caseinate with rennet.

The subject was reviewed recently by Berridge (1951) who pointed out that an understanding of the coagulation mechanism rests upon a knowledge of the normal condition of casein and phosphates. In milk these substances exist as a disperse phase consisting of a double compound of tricalcium phosphate and casein molecules in which the carboxyl groups and phosphoric acid groups have formed salts with lysine and arginine and with calcium ions. He distinguished clearly between two separate stages of the coagulation process namely, (a) the action of rennet on the casein molecules and (b) formation of the coagulum, the last stage being solely a function of the casein. The coagulation stage may proceed as soon as a few molecules of the casein have been acted upon by rennet.

The first stage was studied by Hankinson and Palmer (1943) who blocked coagulation by dialysis of the milk to remove soluble calcium. They also employed synthetic sodium and calcium caseinate sols and observed changes in viscosity and zeta potential when purified rennet was added. They interpreted their results by means of the Krasny-Ergen equation relating hydration and electrokinetic potential and concluded that the primary effect of rennet on calcium caseinate was a true dehydration with a secondary

decrease in zeta potential both tending to destabilize the system.

The electrophoretic differentiations of acid and rennet casein reported by Mitschmann and Lehmann (1947a) revealed the α-fraction of the rennet casein to have two distinct peaks on the descending boundary while this was not the case with acid casein. This was interpreted to mean that rennet action was confined to the α-fraction or that rennet acted directly on some smaller fraction which again influenced the interrelationship of the low γ-fractions. Recently Gonashvili (1949) published similar findings and suggested that rennet cleaved casein by peptide link scission into two fractions which had different isoelectric points.

By means of paper chromatographic technique Kerns (1951) was able to follow progressive changes in casein when acted upon by rennet. His chromatograms showed considerable unfolding of the casein molecule with the appearance of several new fractions. He suggested that rennet acted specifically on the α-casein to produce several large polypeptides.

The concept of rennet action on some specific casein fraction is not new, however. By elaborate fractionation in acidified alcohol and acetone solutions Linderstrøm-Lang (1929) separated casein into three fractions; he proposed that rennet acted on only one of these to destabilize

and coagulate the entire system. His theory was substantiated by later experiments of Holder (1932) and Nitschmann and Lehmann (1947).

The importance of unionized calcium and phosphate to coagulation of casein in milk was recognized by van Dam (1908) who suggested that colloidal calcium bound to casein rather than soluble calcium salts was essential. This view was shared by Beau (1941) who postulated that rennet acts on the caseinate-phosphate micelle to depolymerize it so that carboxyl-, amino- and phosphoryl groups are set free. The casein molecules then recombine by a linkage of their carboxyl groups through calcium atoms and their amino groups through phosphate radicals to form a continually increasing network of larger, heavier molecules. This concept is in agreement with the findings of Pyne (1945) who demonstrated by several methods that the gradual increase in coagulation time of gently heated milk was proportional to an increase in ionizable calcium and phosphate and to a decrease in the calcium phosphate content of the caseinate.

In keeping with this concept are the recent observations by Chevallier et al. (1950, 1950a) who found that milk with high serum calcium content coagulated slowly in contrast to the easy coagulation of milk in which a high proportion of the calcium was attached to the casein.

Berridge (1942) observed that below 15°C milk is not coagulated although rennet has acted on the casein. He

used this fact as a means of separating the two stages of the coagulation process. By holding milk with rennet added at 0°C for several hours and then warming to various temperatures to permit coagulation he was able to derive a mathematical relationship between the rate of coagulation and temperature. He found that the temperature coefficient of coagulation was of the same order as that of protein denaturation and suggested on this evidence that the second phase of rennet coagulation is a complete or partial denaturation of the casein which has been rendered heat sensitive by the rennet enzyme. The action of rennet on the calcium caseinate-phosphate complex hydrolyzes chemical bonds which normally keep the molecules in their native configuration. Unstable molecules are formed, and at sufficiently high temperature they denature through combination of highly reactive polypeptide chains. A molecular network or gel is formed.

Calcium is essential to this process since the new linkages between the polypeptides are believed to involve "bridging" by way of calcium. Berridge noticed that the reaction for which he determined the temperature coefficient was also sensitive to calcium ions. Addition of calcium equivalent to 0.002N and 0.02N decreased the coagulation time by 18 and 61 per cent respectively. The temperature coefficient was not affected by added calcium.

In support of this theory Berridge (1951) cited Ballowitz (1933) who claimed to have measured proteolytic hydrolysis of casein during rennet action. He also suggested that the experiments by Nitschmann and Lehmann (1947) could be interpreted to that end. These investigators prepared differently colored acid and rennet caseins and subjected mixtures of these to the action of calcium chloride. From their data Berridge pointed out that precipitation occurred at calcium concentrations lower than the calculated mean of the concentrations required by the separate caseins. This suggested to him that rennet casein molecules undergoing denaturation combined with acid casein molecules and were partly kept in solution by them and partly caused denaturation of them.

He stated that this theory had much in common with the depolymerization theory of Beau (1941) and that future investigations might reconcile the two.

E. Electrophoresis of Milk Proteins

1. Casein

The discovery by Osborne and Wakeman (1918) of an alcohol-soluble protein in milk gave the first indication that casein was not a homogeneous substance as previously believed. Later Linderstrøm-Lang and Kodama (1925) and Linderstrøm-Lang (1929) in an elaborate separation scheme based on differential solubilities in acidified alcohol

obtained casein fractions with varying properties and compositions. By fractional precipitation from ammonium chloride solutions Cherbuliez and Mayer (1933) produced four casein fractions varying in composition and rennet coagulability.

The electrophoretic technique developed by Tiselius (1930) offered a new tool for physical separation of protein compounds. Mellander (1939) was first to demonstrate that casein could be separated electrophoretically in the fractions α, β and γ with mobilities and phosphorus contents decreasing in that order. He used HCl-borate (pH 8.6) and maleic acid-maleate (pH 6.14) buffers.

Krejci et al. (1941) made observations with regard to the asymmetry of the patterns on the ascending and descending boundaries and presented evidence to show that the β-component of casein forms unstable complexes with the α-component. Warner (1944) separated the α- and β-fractions by solubility methods and demonstrated electrophoretically the purity of his preparations. He discussed the existence of a complex between α- and β-casein and demonstrated it by producing electrophoretic patterns of mixtures of the purified components. He pointed out that the γ-fraction reported by Mellander (1939) was probably not a casein component since it showed no mobility; he took it to represent the well-known S and ϛ boundary effects and substantiated this by showing that the solution

above the β-boundary after electrophoresis contained only insignificant amounts of nitrogen.

By fractionation in 50 per cent alcohol at pH 5.8 Hipp et al. (1950) separated a γ-fraction which had properties similar to the alcohol-soluble protein described by Osborne and Wakeman (1918). It had lower mobility and contained less phosphorus than α- and β-casein. Later Hipp, Groves, Custer and McMeekin (1952) demonstrated a method of separating the α-, β- and γ-components by fractional precipitation in aqueous urea. Hipp, Groves and McMeekin (1952) titrated suspensions of the α-, β- and γ-fractions thus obtained and found them to have different acid- and base-binding capacities, the γ-fraction binding the most acid and the α-fraction binding the most base.

Slatter and Van Winkle (1950) subjected dialyzed skimmilk to electrophoresis and observed changes in milk proteins on heating milk to 65, 75 and 85°C. Tobias et al. (1952, 1952a) dialyzed relatively fat-free (less than 0.09 per cent) skimmilk for 15-19 hr. and obtained electrophoretic patterns directly from these preparations. The use of several fresh batches of buffer during dialysis would give slightly higher mobilities. They identified α-, β- and γ-caseins and β-lactoglobulin and noted several changes in mobilities and some interactions among the components as a result of pasteurizing the milk at 300°C in the Mallorizer.

In studies with commercial casein and iodinated casein veronal buffer at pH 7.5 Kamel and Turner (1951) observed that iodination of casein at 39°C caused the β-fraction to diminish. When casein had been incubated at 70°C, with or without iodine, only one electrophoretic component was evident.

2. Whey proteins

Palmer (1934) isolated a crystalline globulin from frozen cow's milk whey by precipitation with sodium sulphate at pH 5.8. This protein was examined electrophoretically by Pedersen (1936a). When it was dissolved in dilute HCl (pH 1.8) and dialyzed first against distilled water and then against phosphate buffer at pH 6.15 the electrophoretic pattern showed only one component. When it was dissolved at pH 10.0 and then dialyzed as above the electrophoretic pattern indicated several components.

Li (1946) demonstrated three components of this protein at pH 4.8 and 6.5 compared to one at pH 5.3 and 5.6. McMeekin *et al.* (1948) found it to have two components in acetate buffer at pH 4.8.

Cow's milk colostrum contains a large portion of proteins which give the suckling calf a certain protection against infectious diseases. They are the so-called "immune" globulins. Smith (1946) examined the whey proteins in normal cow's milk electrophoretically and reported

that 10 per cent of these could be identified as the immune globulins normally found in colostrum.

Deutsch (1947) produced electrophoretic patterns of whey proteins from the milk of several mammalian species, including the cow, in veronal-citrate buffer at pH 8.6. The patterns varied among the different species but were characteristic for any given species. Cow's milk proteins from rennet and acid whey produced identical patterns. Those from goat's milk did not.

Bain and Deutsch (1948) using low temperature ethanol fractionation of bovine whey proteins obtained a globulin which showed only one electrophoretic component at high pH but several at pH 4.2 and below.

Stanley *et al.* (1951) prepared whey proteins from cow's milk after precipitating the casein with NaCl, HCl or rennet; the proteins were obtained from these wheys by lyophilization and likewise by acid precipitation from the NaCl whey. Preparations by the four methods had the same electrophoretic components but there were differences in isoelectric points and mobilities of the largest component.

Weinstein *et al.* (1951) isolated a minor protein fraction from skimmilk which could be sensitized to produce the typical solar-activated flavor of homogenized milk. This fraction was shown to have two electrophoretic components in veronal-citrate buffer at pH 7.6.

F. Milk Fat-Protein Interphase Phenomena

Babcock (1885) studied extensively the state of fat in milk and described it as an emulsion in which the dispersed fat globules were stabilized "by a thin film of of liquid analogous to that which separates the bubbles of air in foam or soap suds". He had rejected earlier theories visualizing a membrane surrounding the fat globule, but modified this point of view as a result of studies in which he found traces of fibrin in milk.

Storch (1897) was first to isolate natural emulsifying agents in milk by repeated dilution and separation of cream and found that they had the characteristics of protein. His isolation technique has been used extensively by several investigators of this problem.

Völtz (1904) obtained the membrane material by letting fat globules rise through long columns of water and skimming them off. He considered the material to be casein and described it as labile. Palmer and Samuelson (1924) reported the isolation from buttermilk of creams washed by Storch's technique of a single globulin-like, phosphorus-free protein and suggested that it was the agent stabilizing the fat globules in milk. Titus *et al.* (1928) using Völtz's technique obtained and analyzed fat membrane material. On basis of its nitrogen, sulfur, phosphorus and tryptophane contents and its specific rotation they concluded that the

substance was related to or identical with casein.

Hattori (1925) treated milk with chloroform-saturated water and obtained a white powder which he assumed was the membrane of the fat globules. He examined a number of its physical and chemical properties (including amino acid distribution) and concluded that it was unlike any other milk protein and called haptein.

Schwarz and Fischer (1936) repeatedly diluted (with NaCl solution) and separated creams. The resulting washed creams were churned and the aqueous phase of the butter yielded a substance containing 11.25 per cent nitrogen and 0.63 per cent phosphorus. They identified it as the stabilizer of the fat globules and considered it to be a previously unknown protein.

Jack and Dahle (1936) studied electrophoretically the effect of various substances on the electrokinetic potential of milk fat globules. They found that in creams the electrophoretic mobility of the fat globules increased proportionately as the fat percentage increased from 60 to 81 per cent. Up to 60 per cent there was no change in mobility. The lipid phosphorus content per unit of fat decreased as the fat percentage increased from 60 to 81 per cent fat.

When increasing amounts of phospholipids were added to butter oil emulsified in distilled water the fat globule mobility increased until an amount equivalent to

16 mg. lipid phosphorus per 100 g. fat had been added. Addition of casein to this system caused an increase in fat globule mobility until the equivalent of 0.4 g. casein nitrogen per 100 g. fat had been added. These values corresponded to those of a 60 per cent cream.

The results indicated that the inner layer of the fat globule membrane is phospholipid and the outer layer is chiefly casein. Cream containing 60 per cent fat is composed almost entirely of fat globules plus their entire membrane.

Wiese and Palmer (1932) summarized the various identifications by earlier investigators of the fat membrane material and attempted to determine whether any one of the substances reported constituted the sole stabilizer of fat in cream. To this end they prepared artificial fat emulsions with calcium caseinate, lactalbumin, globulin and phospholipid and observed their behavior on separation (in a milk separator) and churning. The phospholipid gave the best separation and the lactalbumin the best churning, but all were slightly abnormal. The conclusion was drawn that no single one of these substances constituted the sole stabilizer of fat in milk. It was shown that some of the adsorbed material on the fat surface was so closely held that it could not be removed by repeated dilution and separation. Later Palmer and Wiese (1933) identified

tightly adsorbed material as a mixture of protein and phospholipid which had both hydrophobic and hydrophilic properties but was unlike other milk proteins. Calcium was not intimately associated with the material of which a large part was removed during churning. In another study Wiese and Palmer (1934) isolated the fat globule membrane material from the aqueous phase of butter from creams washed by the Storch technique by precipitation at pH 3.9 - 4.0 and extraction with alcohol, chloroform and ether. Two such preparations contained 11.84 and 12.64 per cent nitrogen respectively. On the basis of this and other analyses (including amino-acid assays) it was identified as the haptein previously reported by Hattori (1925).

Rumpila and Palmer (1935) extended the study and found that the percentage of protein and phospholipid in the fat globule membrane varied among different samples of cream but that it was essentially constant for any one sample after the fourth washing with water. When milk fat globules were emulsified in rennet whey, skimmilk or buttermilk the membrane produced did not have the same composition as the natural one. They postulated that, in the synthesis of milk, the natural fat globule membrane is not derived from the milk plasma but is formed before the fat globules become part of the milk.

Bird et al. (1937) questioned the concept of a membrane surrounding the fat globule and argued that the

semipermeability of a membrane would tend to exclude attack on the fat by lipolytic and other enzymes. They depicted a fat-serum interphase of serum constituents which were held at force centers on the fat globule. These were (a) a non-labile phospholipid-protein complex oriented at the fat side of the interphase and (b) a labile complex on the water side consisting of all the surface tension-lowering materials in the serum. Of these, casein was considered the most important. The importance of casein as a protective material was shown from churning data indicating a sharp reduction in per cent total fat lost when the creams were churned below pH 4.7, the isoelectric point of casein.

Palmer (1944) was essentially in agreement with this concept of fat globule stabilization when he distinguished between (a) materials which were closely associated with the globule as a result of capillary action and (b) outer layers of materials which are readily removed by repeated dilution and separation of cream.

Jenness and Palmer (1945) found that creams washed by the Storch technique had protein contents ranging from 0.46 to 0.86 g. per 100 g. fat and lipid phosphorus contents from 8.9 to 16.3 mg. per 100 g. fat. They speculated that churning might involve an "erosion" of materials from the fat globule surface so that the original lipo-protein complex of the membrane would become disrupted and release

a protein-rich portion to the buttermilk. Later Jenness and Palmer (1945a) discussed the hypothesis and suggested that during churning some of the phospholipid-protein linkages as well as some of the phospholipid-fat linkages were broken so that buttermilk would contain a phospholipid-protein complex richer in protein than the original membrane. The butterserum would contain a complex richer in phospholipid than the membrane because, upon melting the butter, the phospholipids would cling to certain high melting fat molecules and pull them into the serum. Therefore the butterserum would contain a higher melting point fraction than the melted fat.

Brunner, Duncan and Trout (1952) washed creams by the Storch technique and churned the creams. The butterserum was dialyzed and the lipids extracted with ethanol and ether. The lyophilized protein was found to contain 13.5 per cent nitrogen on dry basis and its amino-acid composition differed markedly from that of recognized milk proteins. Brunner, Lillevik *et al.* (1952) examined this fat membrane material electrophoretically and found two or three major components in the membrane proteins from non-homogenized milk and three or four components in those from homogenized milk. Temperature of extraction and protein concentration influenced the mobility pattern.

Some recent researches have indicated that the fat-protein interphase phenomena are also related to milk

coagulation. Palmer and Tarassuk (1936) investigated the hypothesis proposed by Lundstedt (1934) that the low curd tension of buttermilk was caused by adsorption on casein of the lecithin removed from the fat during churning. They isolated the membrane complex from cream by the dilution and separation technique and prepared artificial buttermilk by direct addition to milk of a sol of this complex and found the curd tension to be lowered. This was also the case with artificial buttermilk prepared by more indirect methods involving addition of the membrane complex, whereas synthetic creams involving only butterfat and skimmilk produced buttermilk with normal curd tension. They considered that the fat globule membrane complex rather than lecithin was responsible for the lower curd tension. Tarassuk and Palmer (1939) made synthetic creams by emulsifying pure butterfat in aqueous sols of dried whey, skimmilk powder, calcium caseinate, gelatin and tissue fibrin (lecithoprotein). The buttermilks from the creams made with dried whey, gelatin and calcium caseinate exhibited sharply reduced curd tensions or produced no coagulum at all with rennet. Addition of appreciable quantities of $CaCl_2$ before adding rennet restored only partly their coagulability. The buttermilks from creams made with skimmilk powder and tissue fibrinogen showed normal coagulation.

The cause of the non-coagulation phenomenon was considered to be more profound than lack of calcium since it was shown that papain was able to coagulate these buttermilks and that the whey from the buttermilks were higher in calcium than the whey from the corresponding skimmilk. They proposed the hypothesis that non-coagulation was due to the liberation of free fatty acids which were adsorbed on the calcium caseinate.

In later experiments Tarassuk and Richardson (1941) demonstrated that replacement of the natural adsorption membrane in raw cream by other surface active materials produced extensive lipolysis and that the corresponding buttermilks did not coagulate with rennet. The inhibition was presumed due to interference by high melting point fatty acids. The same effect could be achieved by adding lauric, myristic and palmitic acids to milk. Coagulability was restored if the fatty acids were liquified.

III. EXPERIMENTAL

A. Materials

1. Milk and cream

The whole milk used in preparing skimmilk, buttermilk and butterserum from the same source was obtained in the College milk plant when it had been pasteurized at 61.6°C for 30 minutes or at 71.6°C for 15 seconds. It represented the mixed milk from some 30 - 40 herds, including such breeds as Holstein, Guernsey, Jersey and Brown Swiss. The experiments were spaced so that milks were examined during all seasons.

Skimmilk used in the preliminary Ling titrations was also obtained in the College milk plant where it had been pasteurized at 61.6°C for 30 minutes.

Buttermilk for a few preliminary titrations was prepared by pasteurizing at 69-72°C for 30 minutes mixed lots of farm-separated sweet cream from the College creamery. After cooling overnight, the cream was churned in quart glass jars to yield the buttermilk.

Farm-separated cream from 20 - 30 herds was used in a few experiments involving only buttermilk and butterserum. The cream was neutralized, pasteurized and churned in commercial lots in the College creamery. Buttermilk was collected as it drained from the churn; samples of the

partly worked unsalted butter were removed from the churn and melted to yield the butterserum.

2. Water

Distilled water was used in all experiments. For the calcium and phosphorus determinations and for washing casein preparations water was used which had been distilled a second time in a Pyrex glass still equipped with a condenser having a clear quarts inner tube. Before the second distillation this water was treated with a small amount of sulfuric acid and potassium dichromate.

3. Reagents

All chemicals used were reagent grade.

The reference solutions for the phosphorus analyses were prepared from potassium dihydrogen phosphate (Standard Sample 186-I-a) supplied by the National Bureau of Standards.

Acetone was purified by refluxing over ferric chloride (0.5 g. per 1.) for 2 - 3 hours, distilling, refluxing over calcium chloride (60 g. per 1.) for several hours and distilling. The first and last 200 ml. fractions of each 5 l. batch were discarded. This method was suggested by Chapman and McFarlane (1943).

Ethyl ether was prepared by refluxing the commercial reagent grade over potassium hydroxide (10 g. per 1.) and

potassium permanganate (2 g. per 1.) for one hour and distilling from these reagents.

Absolute ethanol was prepared by refluxing commercial absolute ethanol over potassium hydroxide (10 g. per 1.) and aluminum (10 g. per. 1.) for 2 hours and distilling from these reagents.

The rennet extract used was a commercial preparation manufactured by Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin.

B. Apparatus

1. Milk separation

The whole milk was separated in a small, power driven farm separator or in a De Laval airtight power separator, No. 192 (The De Laval Separator Co., New York, N. Y.) equipped with a cold milk bowl.

2. Churning

The cream was churned in quart glass jars placed horizontally in a home-made, motor-driven, reciprocal action shaker with 75 - 80 oscillations per minute.

3. Pasteurization

Some of the creams were pasteurized in a home-made laboratory pasteurizer consisting of 6 silver plated rectangular quart containers (2-1/2 in. x 4 in. x 6 in.) submerged in an insulated, constant temperature water

bath. An agitating device with 6 stainless steel paddles, each suspended in one of the containers, was used for stirring.

4. Centrifugation

An International centrifuge, size 2, was used for centrifuging butter samples when removing the butterserum, for separating whey from curd and for removing acetone and ether extracts.

An International clinical centrifuge was employed in washing the casein preparations.

An International centrifuge, size 1, type SB with high speed (10,000 rpm.) gear attachment and head was used during one experiment where a special attempt was made to remove the fat from buttermilk and butterserum.

All centrifuges were manufactured by International Equipment Co., Boston, Massachusetts.

5. Fat and total solids determinations

Fat and total solids determinations were made with standard Mojonnier equipment (Mojonnier Bros. Co., Chicago, Illinois) consisting of hot plate, vacuum oven, vacuum desiccator, centrifuge, fat extraction flasks and aluminum weighing dishes. An Ainsworth, type D.L.B., analytical balance was used for weighing the samples.

6. Curd tension measurements

Curd tension measurements were made with a curd tension meter manufactured by the Submarine Signal Co., Boston, Massachusetts.

7. Viscosity determination

Viscosity determinations were made with an Ostwald viscosity pipette submerged in a 2 liter beaker of water held at 30°C.

8. Nitrogen determinations

The condensers used in distilling the ammonia from the digested mixture in the Kjeldahl flasks were fitted with standard tapered (24/40) joints at both ends. The male fitting at the top was connected to the trap with a bent glass tube and a rubber hose; the female part of the bottom joint was sealed to a glass tube long enough to reach into the receiving flask. This construction made it possible to remove the trap above and the glass tube below the condenser and permitted easy flushing of the system.

9. Colorimetry

A few of the phosphorus determinations were made with a Duboscq colorimeter (Bausch and Lomb Optical Co., Rochester, New York).

10. Spectrophotometry

The transmittance readings in the phosphorus determinations were made in a Beckman spectrophotometer, model DU (Beckman Instruments, Inc., Pasadena, California). In some comparative experiments a Coleman spectrophotometer, model 11, (Coleman Electric Co., Inc., Maywood, Illinois) equipped with a PC-5 filter was also employed.

11. Lyophilization

The whey proteins were prepared by vacuum sublimation in a lyophilizer made available by the poultry department. The essential part of this apparatus is a cylindrical steel condenser 10 in. high and 6 in. in diameter closed at the bottom. To the top of the condenser is welded and fitted a steel pipe with 6 horizontal branches, each with an outlet ground to serve as the male part of a standard tapered (29/42) glass joint. Round bottom flasks with standard tapered openings could be fitted to these pipes for evacuation. The system was evacuated with a vacuum pump (1/2 H.P. motor) to a pressure corresponding to 5 - 10 mm. of mercury. The vapors were condensed by placing the condenser in a chilled (-50°C) insulated methanol bath (ca. 4 gal.) which was refrigerated by a half ton freon compressor.

12. Electrophoresis

Electrophoresis of casein and whey proteins was carried out in a Tiselius cell (Klett Mfg. Co., New York, N. Y.). The optical equipment used was the Philpot-Swensson system described by Swensson (1946) but modified by replacing the usual plate holder with a Leica 35 mm. camera equipped with focal plane shutter but without the lens. Constant direct current voltage was maintained by means of a voltage regulator (Technical Apparatus Co., Boston, Massachusetts).

The electrophoresis cell was submerged in a water bath held at 2°C±0.5° by a sealed, mercury expansion type thermoregulator operating through a mercury switch relay assembly.

The electrophoresis equipment was made available through the courtesy of the biochemistry group in the chemistry department and was operated by a technician working with this group.

13. pH measurements

pH Measurements were made with Beckman glass electrode pH meters, models G and H2 (Beckman Instruments, Inc., Pasadena, California).

C. Methods

1. Preparation of skimmilk, buttermilk and butterserum from the same whole milk

Skimmilk and cream (35-40 per cent fat) were removed from their respective reservoirs when separation of the entire lot of whole milk had been completed. The cream was placed in quart glass jars (1.5 pints in each), chilled overnight (2-4°C) and churned in the shaking apparatus described above until the butter granules were the size of hazel nuts. The buttermilk was strained through several layers of cheese cloth into a flask. The butter granules were washed twice with tap water (2-4°C) allowing them to remain in contact with the last water for 10 min. After draining the water, the butter was worked with a ladle in a stainless steel tray until it appeared dry; it was stored in 4 oz. sample jars with screw-caps in a refrigerator at 2°C until needed.

Butterserum was prepared according to the technique described by Parmelee et al. (1943). While still warm the entire volume of butterserum prepared was placed in a large separatory funnel for 10-15 min. in order to allow as much as possible of the remaining fat to rise to the surface. The serum was then drained slowly from the funnel leaving the fatty layer behind. The samples were preserved by refrigeration only, except in a few runs where

they were also saturated with toluene.

2. Ling titrations

Titrations of skimmilk, buttermilk and butterserum were, in the main, carried out according to Ling (1936) except that 10 g. rather than 10 ml. samples of milk and its rennet whey were employed. To one 10 g. portion each of milk and its rennet whey was added 0.4 ml. of saturated potassium oxalate solution (made neutral to phenolphthalein) and the mixture was allowed to stand for 10 minutes before titration with 0.1 N NaOH. Ten drops of phenolphthalein (saturated in 50 per cent alcohol) was added and the titration was carried to a definite pink color. The p-rosaniline hydrochloride color standard described by Ling was not employed. Initially the rennet wheys were prepared by coagulating 100 ml. of milk in a beaker at 30°C with 0.7 ml. rennet, cutting the coagulum with a stainless steel spatula and filtering through filter paper (Whatman No. 42) in long stem funnels. Later the following rapid modification was developed: Place 75 ml. of milk in a large test tube (1-1/2 in. wide, 5 in. deep), add 0.53 ml. rennet and let stand for 5 min. Cut the coagulum with a stainless steel spatula and agitate slightly. Centrifuge for 10 min. at 1500 rpm. and decant the whey without filtration.

Acidity was developed in the milk products by adding 2 per cent of cheese culture and incubating at 21°C. Samples were withdrawn at various acidity levels and titrated immediately or they were placed in ice water and titrated later.

In some experiments titrations were made also on milk samples from which the proteins had been precipitated with an alcohol-acetone mixture as follows: To a 5 g. sample of milk in a large test tube add 45 ml. of a mixture of 7 vol. ethanol and 3 vol. acetone. Allow to digest for 5 min. and centrifuge for 10 min. at 1500 rpm. Transfer all the supernatant liquid to an Erlenmeyer flask and titrate with 0.1 N NaOH until the pink phenolphthalein color remains for 1 min. Titrate a blank of 45 ml. alcohol- acetone mixture and subtract this value from that of the sample. Multiply the result by 2 to obtain the titration value equivalent to a 10 g. sample.

3. Dialysis and concentration of rennet extracts

Commercial rennet extract was placed in Visking cellophane bags (1 in. diameter), the bags were immersed in running distilled water at room temperature during 12 - 14 hrs. For concentration the cellophane bags were placed at room temperature for 3 - 4 hrs. in an air current produced by an office fan.

4. Curd tension measurements

Milk samples were coagulated in 250 ml. beakers at 30°C using the required amount of rennet (0.7 or 1.4 ml. per 100 ml. milk) and allowing 5 or 10 min. for coagulation. The knife of the curd tension meter was then forced to cut through the coagulum and the maximum resistance observed.

5. Viscosity measurements

Comparative viscosities of butterserum samples were measured in an Ostwald pipette submerged in a water bath at 30°C. The time required to empty the bulb of the pipette through the capillary was recorded.

6. Fat and total solids determinations

Fat and total solids were determined by the Mojonnier modifications of the A.O.A.C. (1945) methods recommended by the Milk Industry Foundation (1949).

7. Protein determinations

Protein determinations on skimmilk, buttermilk and butterserum were made according to the method presented by Rowland (1938, 1938a). The procedure for globulin precipitation was modified as follows: Transfer 20 ml. of the non-casein filtrate to a 300 ml. Kjeldahl flask, add 4 drops of brom thymol blue indicator and adjust to pH 6.8 - 7.0 with 0.1 N NaOH. Add powdered, crystalline magnesium

sulfate (9 g. per 10 ml.) and agitate until the solution is saturated. Warm to 25-30°C to facilitate this. Allow to stand at room temperature overnight, filter and wash the precipitate with saturated magnesium sulfate solution. Return the filter paper to the Kjeldahl flask for digestion.

Nitrogen in casein preparations was determined by weighing 0.02 to 0.04 g. of the dried preparation into a small (0.5 ml.) glass vial. The vial was placed in the neck of a 300 ml. Kjeldahl flask and was carefully pushed to the bottom of the flask. Digestion and distillation were carried out as described above.

8. Calcium determinations

Calcium was determined by a modification of a procedure for blood serum presented by Rothlin and von Bidder (1945): Weigh a 1 g. sample into a platinum crucible and add 1 ml. dilute HCl (1 vol. conc. HCl and 1 vol. water). Evaporate the mixture to dryness at 105-110°C on a hot plate under the hood or under an inverted glass funnel connected to a suction pump. Heat the crucible gently over a microburner until the contents are well charred, then over a Bunsen burner until ashing is complete and finally over a Meeker burner for 30 min.

Dissolve the ash in dilute HCl, evaporate to dryness on the hot plate, dissolve in 0.5 ml. HCl, evaporate to half the volume and transfer to a 50 ml. beaker with 4

washings of 2 ml. portions of hot water. Add a few drops of brom thymol blue indicator (0.04 per cent in ethanol), make the solution alkaline (pH 7.6) with 2N NH_4OH and add 2 ml. of 50 per cent ammonium acetate. Acidify with 1 ml. N acetic acid and heat to boiling over a microburner. Precipitate the calcium with 2 ml. boiling ammonium acetate solution (4 per cent). Boil 2 min., add 0.5 ml. 2N NH_4OH, boil an additional 5 min; remove from the flame, cool to 50°C and add 2N NH_4OH dropwise until a blue-green color (pH 7.2 - 7.4) is obtained.

Allow the mixture to stand overnight and filter with suction through a micro porcelain filter crucible. Wash the precipitate 6 times with 2 ml. portion of warm (50°C) 0.5N NH_4OH and place the crucible in a 50 ml. beaker. Add sufficient 10N H_2SO_4 (10-15 ml.) to submerge the crucible, warm the solution for 10 min. over a boiling water bath and cool to room temperature. Add a known volume (15-20 ml.) of 0.005 N ceric sulfate and 3 drops of o-phenanthroline ferrous sulfate indicator.[1] Titrate the excess ceric sulfate with 0.005 N ferrous ammonium sulfate (microburette) to a faint orange-red endpoint. For each experiment determine a blank value including the

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[1] Prepared by dissolving 0.5 g. o-phenanthroline in 10 ml. 0.1 N ferrous ammonium sulfate and diluting to 500 ml.

volume of 0.005 N ceric sulfate required to oxidize the 3 drops of indicator.

$$\text{Per cent Ca} = (b - a)(N_{FeSO_4})_2SO_4 \cdot 2H_2O) \frac{100}{\text{sample weight}}$$

where

b = ml. ferrous ammonium sulfate ⟷ blank

a = ml. ferrous ammonium sulfate ⟷ sample .

Ferrous ammonium sulfate was prepared as 0.1 N solution which was standardized against 0.1 N $KMnO_4$ and diluted to 0.005 N. This was used to standardize the 0.005 N ceric sulfate solution. The latter is stable and was used for standardization of the relatively unstable ferrous ammonium sulfate solution each time an analysis was made.

9. Phosphorus determinations

a. Phosphorus fractions. The phosphorus fractions in skimmilk, buttermilk and butterserum were separated by the method for phosphorus in blood serum suggested by Youngburg and Youngburg (1930) and used for milk by Graham and Kay (1932). Slight modifications in the technique were introduced.

(1) Total phosphorus. Weigh 0.10-0.15 g. sample into a 10 ml. glass-stoppered volumetric flask, dilute to volume with 0.2 per cent NaCl solution and mix. Transfer 2 ml. aliquots to 10 ml. volumetric flasks, add 2 ml. 10 N H_2SO_4 to each and evaporate at 105-110°C until the residue turns

dark brown. Remove from the hot plate, add 3 drops of 30 per cent hydrogen peroxide, place a small glass funnel (3/4 in. diameter of cone and 1-1/4 in. stem) in the neck of each flask[1] to prevent loss by spattering and continue the digestion until the solution again turns dark. Remove from the hot plate, add 3 drops of hydrogen peroxide and digest. Repeat until the residue in the flask remains clear upon prolonged digestion at high temperature (200-250°C). The final digestion may be made over the flame of a microburner, placing the flasks on an asbestos wire gauze. Cool, remove the funnel and rinse it and the neck of the flask with 3-4 ml. water. Heat in boiling water bath for 5 min., cool and make to volume. Transfer aliquots (2 - 5 ml.) to 25 ml. amber colored, glass stoppered volumetric flasks for colorimetric analysis.

(2). Inorganic phosphorus. Weigh a 0.15 - 0.25 g. sample into a 10 ml. glass stoppered volumetric flask. Add 2 ml. 10 per cent trichloroacetic acid to precipitate the proteins, make to volume, mix and filter. Transfer 1 or 2 ml. aliquots of the filtrate to 25 ml. amber colored, glass stoppered volumetric flasks.

(3). Acid-soluble phosphorus. Transfer 1 or 2 ml. aliquots of the non-protein filtrate from (2) to a 10 ml.

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[1] These funnels were made from Pyrex glass tubing by shaping one end of a piece of tubing as the cone of a funnel.

volumetric flask, digest and proceed as for total phosphorus.

(4). Lipid phosphorus. Weigh 0.12 g. (butterserum) to 0.8 g. (skimmilk and buttermilk) of sample into a 10 ml. glass stoppered volumetric flask. Add 5 - 6 ml. ethanol-ether mixture (3 vol. 95 per cent ethanol and 1 vol. ethyl ether), mix and bring to boiling for 5 min. in a hot water bath. Cool and make to volume with alcohol-ether mixture; filter and transfer 2 ml. aliquots of the filtrate to 10 ml. volumetric flasks for digestion and treatment as for total phosphorus.

b. Spectrophotometry. Spectrophotometric determination of phosphorus was made according to the method outlined by Fontaine (1942) as follows: To the aliquots in the 25 ml. volumetric flasks add sufficient 10 N H_2SO_4 to make the total amount equal to 5 ml. after allowance for the acid in the aliquots; add 2.5 ml. 7.5 per cent sodium molybdate and enough water to make 20 - 22 ml. Add 2.5 ml. dilute stannous chloride solution[1], mix and place the glass stoppered flasks in a boiling water bath for 20 min. to develop the blue color of reduced phosphomolybdate. Cool to room temperature and make to volume. A blank

[1] Made by dissolving 10 g. fresh $SnCl_2$ in 25 ml. conc. HCl and diluting 1 ml. of this solution to 200 ml. Prepare the dilute solution fresh every 8 - 10 days.

containing all the reagents but no phosphate was prepared in the same manner.

The transmittances were read at 820 mμ in the Beckman spectrophotometer using the Corex cuvettes. Two runs were also read at 740 mμ in both the Beckman and Coleman spectrophotometers. The per cent phosphorus was calculated from regression equations established for each of these wave lengths with the respective instruments.

The cuvettes used with the Coleman spectrophotometer were standardized according to the method suggested by Van Devender, Jr. (1948) by determining the transmittances of a $CuSO_4$ solution (8 g. per liter) at 740 mμ with a number of tubes. Of the cuvettes tested 8 were selected which showed no greater variation than 0.2 per cent transmittance when measured against an arbitrarily chosen tube as standard. These cuvettes were then used without applying corrections to the readings.

c. Phosphorus determination with the Dubosca colorimeter. In one run the colorimeter determinations were made as originally suggested by Youngburg and Youngburg. An aliquot of the digest was transferred to a 10 ml. glass stoppered volumetric flask; 2 ml. of a molybdate-sulfuric acid solution (equal volumes of 7.5 per cent sodium molybdate and 10 N H_2SO_4) and 1 ml. dilute stannous chloride

solution[1] were added, mixed and the solution made to volume. A known standard was prepared simultaneously in the same manner, and the two solutions were compared in the colorimeter after 10-15 min.

d. Phosphorus determination of protein preparations. Phosphorus was determined in 0.01 - 0.02 g. samples of protein preparations weighed into 10 ml. glass-stoppered volumetric flasks, digested and analyzed with the Beckman spectrophotometer as above.

e. Phosphorus determination of ether extracts. Phosphorus was determined in appropriate aliquots of the ether extracts of the proteins, digested and treated as above.

10. Preparation of casein and whey proteins

a. Casein. Skimmilk, buttermilk or butterserum (250-400 ml.) was placed in a 1 l. beaker and 0.1 N HCl was added dropwise with mechanical agitation until pH 4.6 had been obtained. The precipitated casein was allowed to settle at 2°C for several hours whereupon the whey was removed with suction through Buchner-type funnels with coarse, fritted discs and saved for the preparation of whey proteins.

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[1] See footnote p. 54.

The casein was distributed in several conical centrifuge tubes and washed 6 times with distilled water. After each washing the tubes were centrifuged for 5 minutes at 1800 rpm., and the supernatant water discarded. The washed casein was transferred to a 1 l. beaker and suspended in 300 - 400 ml. water. Sufficient 0.1 N NaOH was added to adjust the pH to 6.8 - 7.0 and the mixture was allowed to stand in the refrigerator overnight or longer in order to permit complete suspension. If necessary the pH was readjusted from time to time by adding more NaOH. This suspension was extracted twice with an equal volume of ethyl ether, and the casein was reprecipitated at pH 4.6 with 0.1 N HCl, washed 6 times with water, once with ethanol and once with ethyl ether. It was then dried in vacuum for several hours and left in a desiccator over $CaCl_2$ at atmospheric pressure for several days to become completely dry. The granular product was ground finely with glass mortar and pestle.

Part of the washed casein was removed before the final alcohol and ether washings and stored in a water suspension (saturated with toluene) until needed.

b. Whey proteins. The whey resulting from the casein precipitation was distributed in several Visking cellophane casings (1 in. diameter) and dialyzed at 35°C against a large volume (2 l.) of distilled water which was

changed 2 - 3 times during 48 hours. Dialysis was considered complete when the last water did not give a test for chlorides. The dialyzed whey was distributed among several 1 l. round bottom flasks with standard tapered (29/42) openings. The contents were caused to freeze in a thin layer over the entire inside surface of the flasks by rotating them in the chilled methanol bath of the lyophilizer after which they were fitted to the condenser and evacuated until the proteins were dry. This could usually be achieved in 12 - 18 hrs.

11. Extraction of casein and whey proteins.

Casein and whey proteins were extracted by a procedure used by Blix (1941) for extracting lipids from blood sera. The proteins were suspended in a phosphate buffer (pH7.7) or in water, 15 ml. of the suspension was placed in each of several glass stoppered 60 ml. flasks and dry, chilled acetone was added to fill the flasks. The contents were mixed for 10 min., placed at - 23°C and centrifuged for 5 min. at 1800 rpm. The supernatant mixture of acetone and buffer was discarded and the flasks again filled with acetone, agitated in a shaking device at - 23° for 30 min. and centrifuged as above. This extraction was repeated once. A chilled mixture of acetone and ether was then added to the flasks, mixed and allowed to stand at 0°C for 5 min. and centrifuged. Two extractions

were carried out with ethyl ether alone at - 23°C for 15 min. and both of these extracts together with that of the acetone-ether mixture were collected, filtered until clear and evaporated to dryness at moderate heat on the steam plate. The lipid residue was taken up in fresh ether, transferred to a 25 or 50 ml. volumetric flask and made to volume. Aliquots of this solution were used in estimating the amount of lipid and its phosphorus content.

The extracted protein was dried in vacuum for 2 - 3 hours to remove the ether and desiccated at atmospheric pressure for several days.

12. Electrophoresis of casein and whey proteins

For electrophoresis the caseins were dissolved in a veronal-citrate buffer (0.05 M sodium barbital, 0.0075 M sodium citrate, 0.05 M NaCl; adjusted to pH 8.45 with citric acid; ionic strength = 0.15) and the whey proteins in a phosphate buffer (0.0321 M K_2HPO_4, 0.0036 M KH_2PO_4, 0.1 M NaCl; pH 7.7; ionic strength = 0.20). The solutions were dialyzed for at least 24 hrs. against 2 l. of the corresponding buffers. Electrophoresis was carried out at 2°C $\pm$ 0.5° at a constant field strength of 4.5 - 6.0 volts cm^{-1} and was continued until maximum resolution was obtained (usually 160 - 180 min.). Exposures were made of the initial boundary, of the final resolution and of one or two intermittent patterns.

Tracings of the negatives, magnified under a standard photographic enlarger to 8 times the actual cell dimensions, were made on co-ordinate paper. The initial boundary exposures were superimposed on these tracings. The component areas were separated according to the method described by McInnes and Longsworth (1944) by dropping vertical lines from the peaks to the base line. The areas were measured with a compensating polar planimeter taking the average value of 5 projections. The line bisecting the area under each gradient curve was used as a basis for mobility measurements.

D. Results

1. Ling titrations

a. Removal of whey from curd by centrifugation. A routine method of separating whey from curd by centrifugation was adopted. The following experiment demonstrated that the whey obtained in this manner was comparable to that obtained by filtration as described by Ling (1936).

Samples of a batch of skimmilk were removed at 3 acidity levels, and the wheys from each were prepared as follows: (1) Coagulate 150 ml. skimmilk in a beaker, cut with a spatula, stir and filter. (2) Coagulate 150 ml. skimmilk in a beaker, cut, stir, transfer to 4 centrifuge tubes and centrifuge for 10 min. at 1500 rpm. (3) Coagulate

75 ml. skimmilk in each of two centrifuge tubes, cut, stir and centrifuge for 10 min. at 1500 rpm. In all instances 0.7 ml. rennet was used per 100 ml. milk, and the milk was coagulated for 5 min. at 30°C.

Duplicate titrations were made of the oxalated and non-oxalated wheys prepared by each method. The results are presented in Table 1.

Maximum deviation among methods (0.08 ml.) was no greater than maximum deviation between duplicates (0.10 ml.). The sample curdled in a tube at the highest acidity level is an exception because of an accidental delay during which acidity developed before titration could be completed. The results indicate that the rapid centrifugation method yield s whey which is comparable to that obtained by the slower method of filtration.

b. Ling's concept of quantitative estimation of colloidal tricalcium phosphate. When milk is titrated with a base, dicalcium phosphate is considered to be converted as follows (Van Slyke and Bosworth, 1914a):

$$3\ CaHPO_4 = Ca_3(PO_4)_2 + H_3PO_4 \quad .$$

An"overrun" acidity (o) is thus produced which is equivalent to one hydrogen per phosphate molecule. This acidity increase may be prevented by precipitating the calcium with potassium oxalate, in which case an alkalinity

Table 1. Comparison of the titratable acidity of skimmilk whey prepared by three methods

ml. 0.1 N NaOH per 10 g. milk	Duplicates	ml. 0.1 N NaOH per 10 g. whey					
		Without potassium oxalate (W_1)			With potassium oxalate (W_2)		
		Coagulated in beaker and filtered	Coagulated in beaker and centrifuged	Coagulated in tube and centrifuged	Coagulated in beaker and filtered	Coagulated in beaker and centrifuged	Coagulated in tube and centrifuged
1.77	1	1.20	1.16	1.18	0.85	0.90	0.90
	2	1.18	1.18	1.20	0.84	0.80	0.90
2.53	1	1.62	1.68	1.72	1.20	1.20	1.22
	2	1.70	1.70	1.62	1.20	1.22	1.22
5.74	1	3.90	3.88	4.14	2.68	2.70	2.90
	2	3.90	3.90	4.18	2.68	2.70	2.95

factor (x) is introduced because of the formation of potassium phosphate, as indicated by the equation presented by Pyne and Ryan (1932):

$$Ca_3(PO_4)_2 + 3K_2C_2O_4 = 3\ Ca\ C_2O_4 + 2\ K_3PO_4\ .$$

Ling (1936) reasoned that by estimating the alkalinity (x), equivalent to the third potassium in the salt, it would be possible to determine the colloidal tricalcium phosphate in milk. If the ordinary titration value of milk (M_1) is the sum of casein acidity (c), serum acidity (s) and the "overrun" (o) we have

$$M = c + s + o\ .$$

When oxalate is added the "overrun" acidity (o) is removed and the alkalinity factor (x) is introduced. Hence the titration of oxalated milk may be represented as

$$M_2 = c + s - x\ ,$$

non-oxalated whey

$$W_1 = s + o\ ,$$

and oxalated whey

$$W_2 = s\ .$$

Assuming that (c) and (s) are not affected by the oxalate we have

$$M_1 - M_2 = o + x$$

$$W_1 - W_2 = o$$

and

$$(M_1 - M_2) - (W_1 - W_2) = x.$$

Using the relationship

$Ca_3(PO_4)_2 \rightleftharpoons 2K_3PO_4 \rightleftharpoons$ 2 l. N acid or base the (x) value may be converted to g. $Ca_3(PO_4)_2$.

c. Ling titrations of skimmilk, buttermilk and butterserum. In preliminary experiments 7 random lots of skimmilk and 3 of buttermilk were incubated after addition of a lactic acid culture, and samples were removed for titration at various acidity levels.

Figure 1 is a composite of data of these 10 samples and illustrates the linear relationship of the M_2 to the M_1 values. Similar linear functions were obtained when W_1 and W_2 were plotted against M_1. This is in agreement with Ling's observation with whole milk. It will be noted from Figure 1 that in this composite set of data the buttermilk titration values fall on the same line as the skimmilk values.

Data from the same experiments are presented in Figure 2 and illustrate the relationship between the titratable acidity (M_1) and the value (x) equivalent to colloidal tricalcium phosphate. Although there are considerable variations among samples the gradual decrease of the (x) value with increasing titratable acidity is apparent. The colloidal tricalcium phosphate has disappeared completely

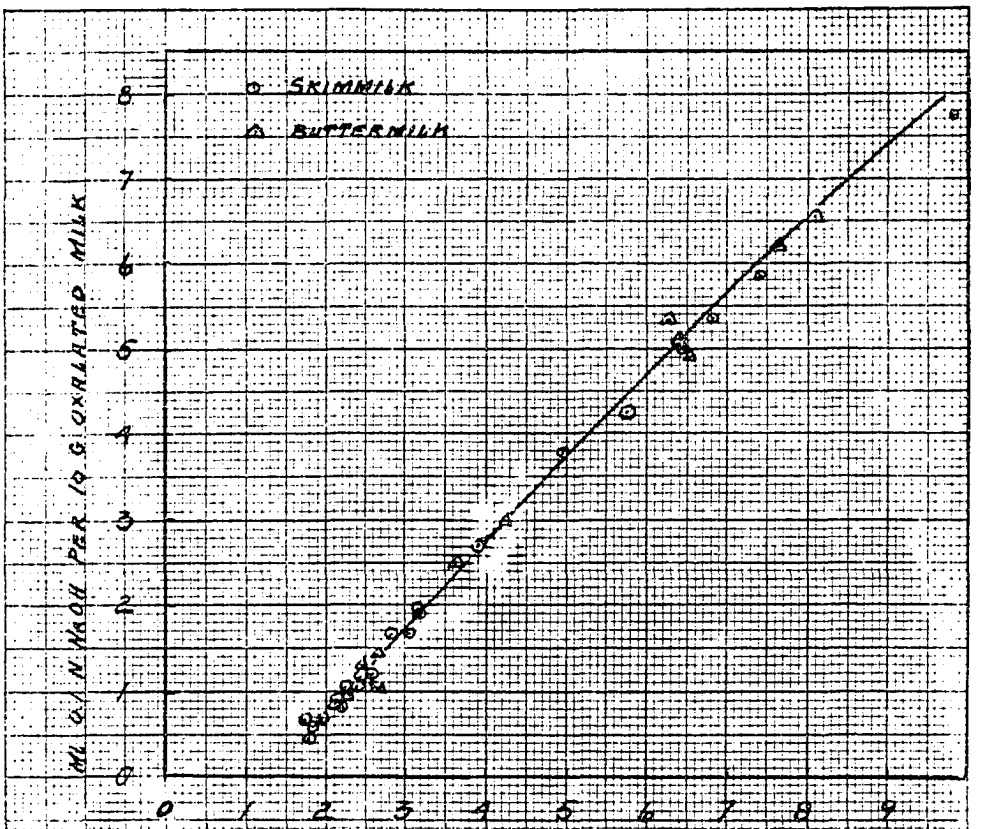


FIG. 1. TITRATION OF OXALATED SKIMMILK AND BUTTERMILK VS. NON-OXALATED SKIMMILK AND BUTTERMILK.

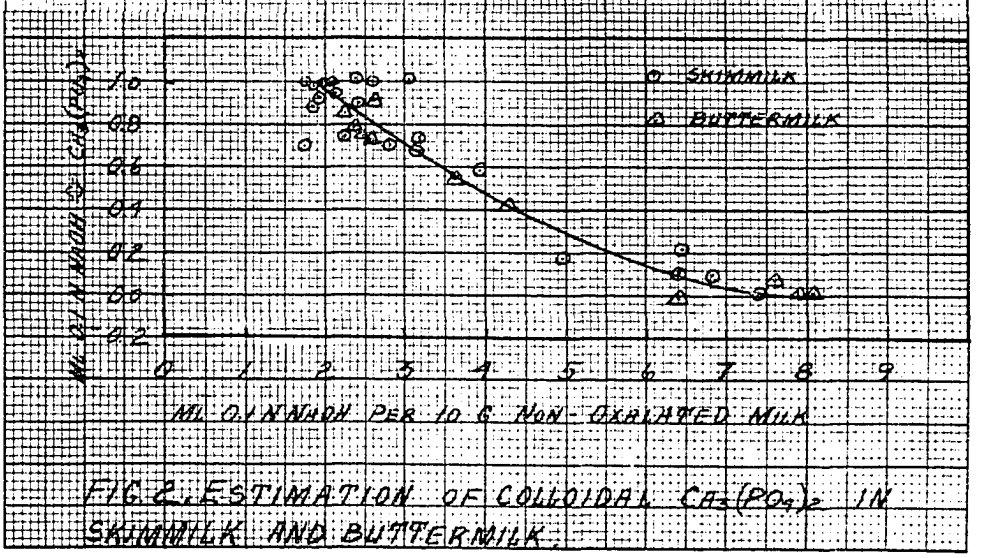


FIG. 2. ESTIMATION OF COLLOIDAL $Ca_3(PO_4)_2$ IN SKIMMILK AND BUTTERMILK.

when M_1 is 7.0 - 7.5 ml. This corresponds approximately to the acidity at which milk coagulates spontaneously.

This preliminary experiment demonstrated that Ling titrations could be applied to skimmilk and buttermilk and give results comparable to those for whole milk reported by Ling (1936).

Figure 3 summarizes titration of skimmilk, buttermilk and butterserum derived from the same whole milk source. In all of these products the M_2 value increased with the M_1 value at the same rate, but the buttermilk M_2 titrations were slightly higher and the butterserum M_2 titrations significantly higher than those of skimmilk at the same M_1 values. If the W_2 to W_1 relationships were relatively the same in all three products this would suggest that the alkalinity factor (x) resulting from colloidal tricalcium phosphate is slightly less in buttermilk than in skimmilk and appreciably less in butterserum.

Figure 3 might also be interpreted by taking the M_2 titration as the independent variable and consider that the difference between M_1 and M_2 represents the titration of the caseinate-phosphate complex. It may then be concluded that in skimmilk and buttermilk this complex is of greater magnitude than in butterserum.

The decrease in colloidal tricalcium phosphate with increasing acidity in skimmilk and buttermilk is shown in Figure 7. The rate of decrease is about the same in the

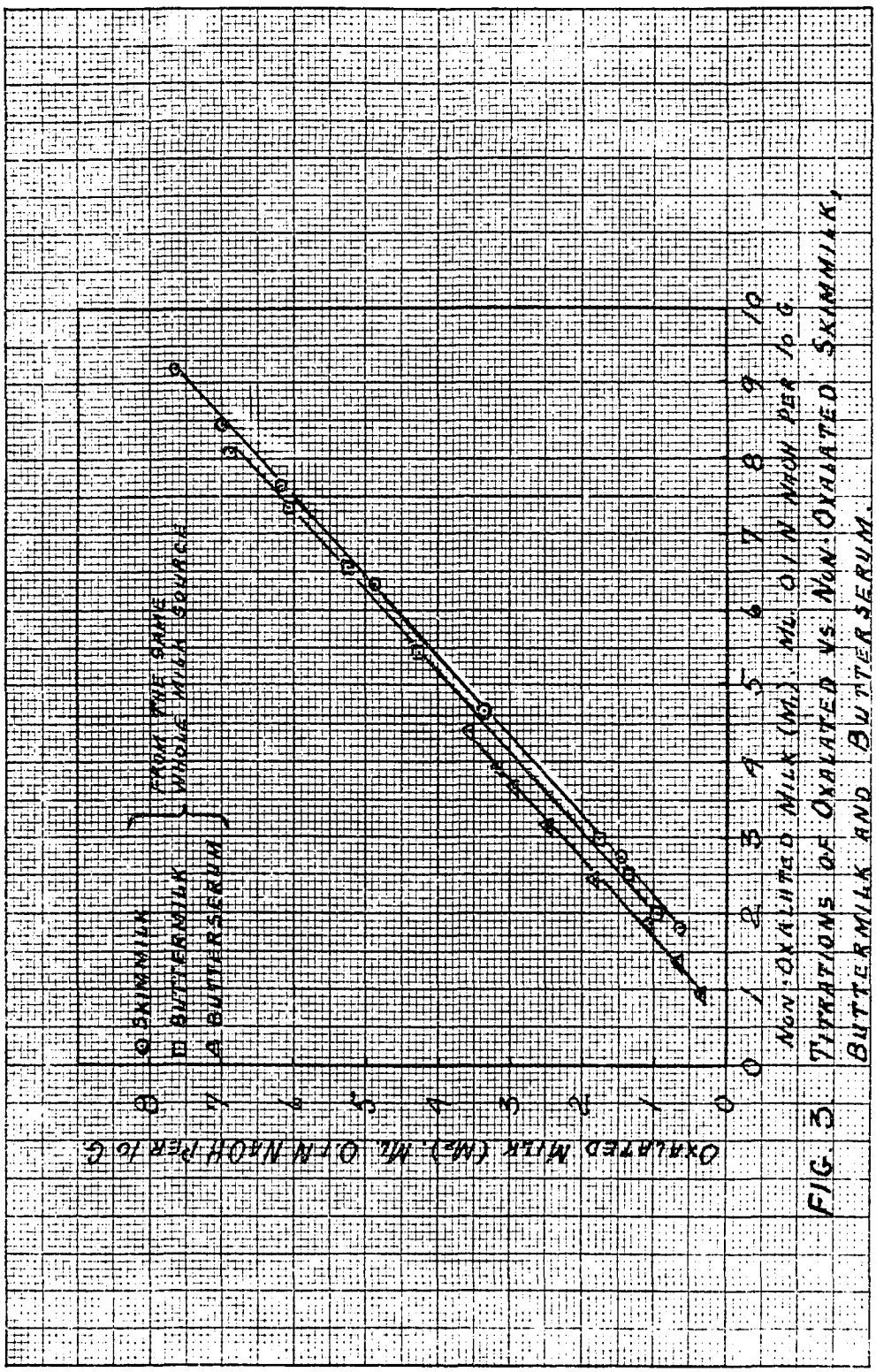


FIG. 3 Titrations of Oxalated vs. Non-Oxalated Skimmilk, Buttermilk and Buttersérum.

two products, but the data indicate that the colloidal tricalcium phosphate phase in buttermilk is smaller than in skimmilk.

The linear relationships between the W_1, W_2 and M_1 values for skimmilk and buttermilk respectively are shown in Figures 4 and 5. They also illustrate the more rapid rate of increase in the W_1 value compared with the W_2 value. This difference has been interpreted as resulting from the gradual solution of tricalcium phosphate as the acidity increases.

Titrations of oxalated and non-oxalated whey from butterserum could not be obtained due to the failure of butterserum to coagulate with rennet.

d. Alcohol-acetone titrations. An attempt was made to measure the serum acidity (W_2) by titrating the filtrate from a sample of milk after precipitating the proteins with an ethanol-acetone mixture (7:3). The curves representing these titrations are also drawn into Figures 4 and 5 and illustrate that the alcohol-acetone titration is not identical with the oxalated whey titration (W_2). In general the alcohol-acetone values are lower than the W_2 values until M_1 reaches 7.0 - 7.5 ml. Then they increase rapidly and become greater than the W_2 values. This is approximately the acidity at which milk coagulates spontaneously, and the curves indicate that the alcohol-acetone titration does not include the colloidal tricalcium

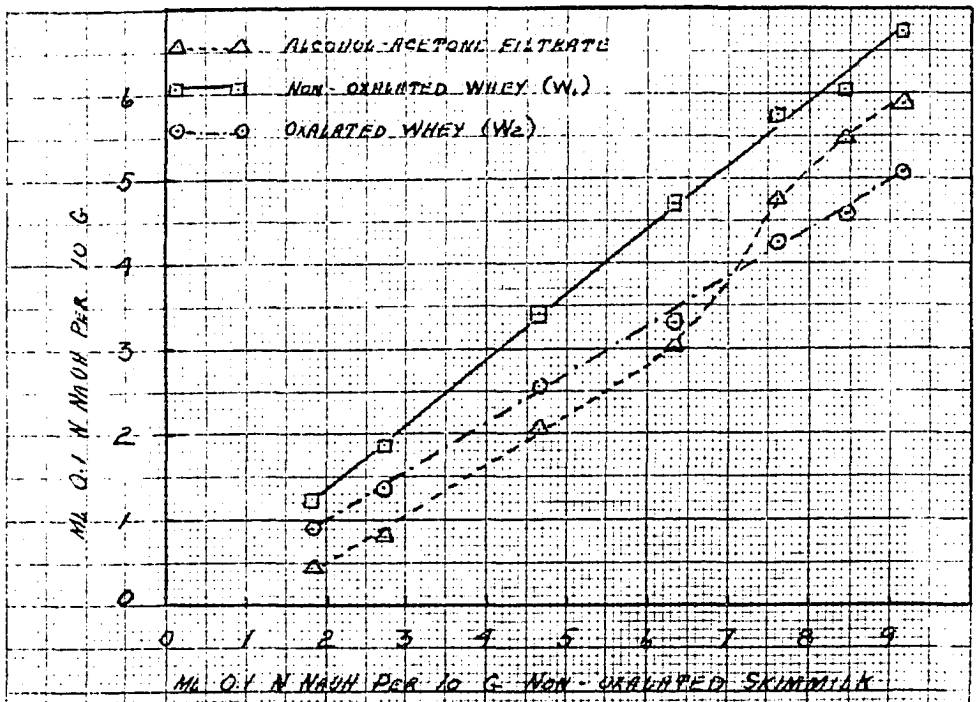


FIG. 4. TITRATION OF OXALATED AND NON-OXALATED WHEY AND ALCOHOL-ACETONE FILTRATE VS. NON-OXALATED SKIMMILK

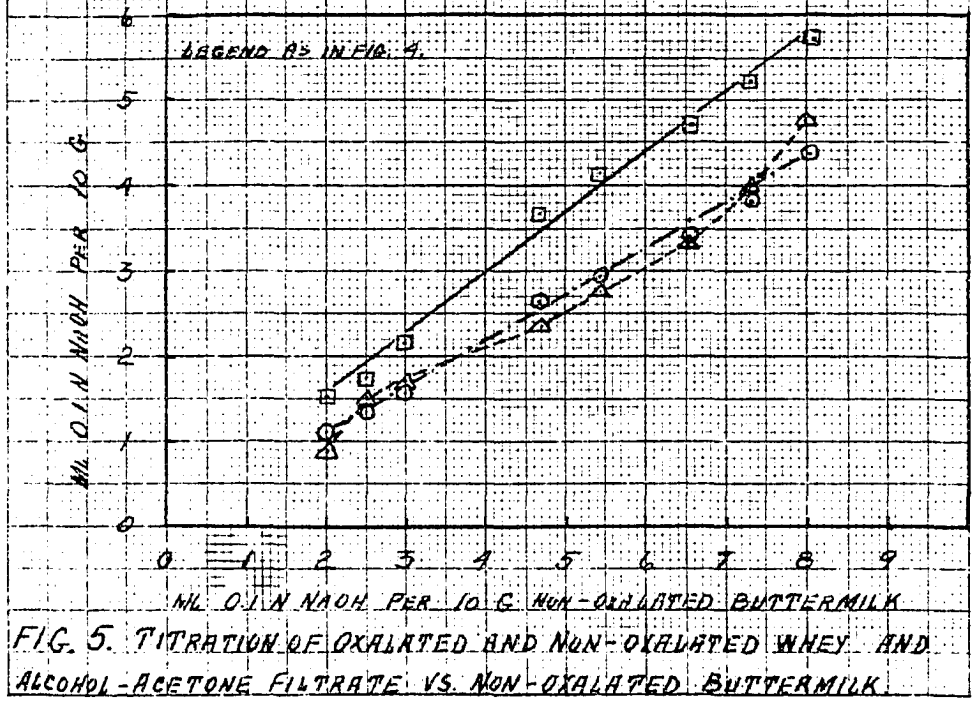


FIG. 5. TITRATION OF OXALATED AND NON-OXALATED WHEY AND ALCOHOL-ACETONE FILTRATE VS. NON-OXALATED BUTTERMILK

phosphate until the acidity in the milk is sufficiently high to precipitate the casein. With buttermilk the difference between alcohol-acetone and W_2 values are smaller than with skimmilk, suggesting a less completely formed caseinate-phosphate complex in buttermilk.

Within the acidity range in which the butterserum was examined the alcohol-acetone titrations yielded an almost linear relationship to M_1, but the values were much higher than those obtained with skimmilk or buttermilk. This is illustrated in Figure 6 and may be interpreted to indicate an extremely incomplete caseinate-phosphate complex in butterserum or the absence of one. The evidence is not adequate, however, since titrations of the butterserum were not made at acidities high enough to include the coagulation point of casein.

The absence of a rennet coagulum in butterserum, the soft coagulum often obtained in buttermilk and the failure to substitute alcohol-acetone serum titrations for the oxalated whey titrations made it impossible to apply Ling's titration scheme in a study of the acidity factors in skimmilk, buttermilk and butterserum.

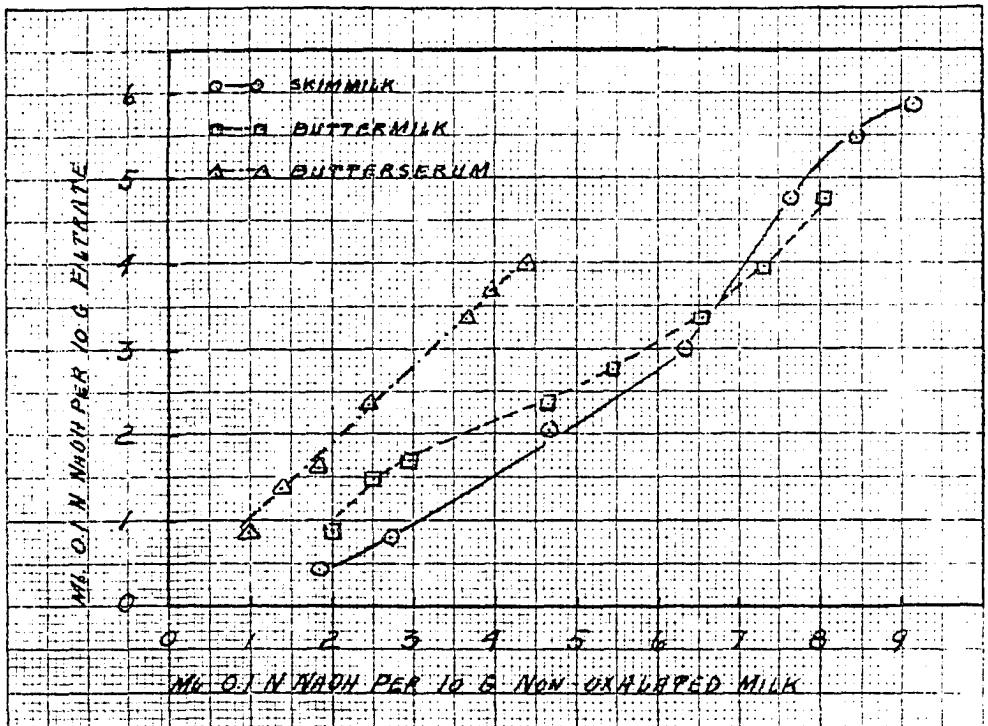


FIG. 6. TITRATION OF ALCOHOL ACETONE FILTRATES VS. NON-OXALATED SKIMMILK, BUTTERMILK AND BUTTERSERUM.

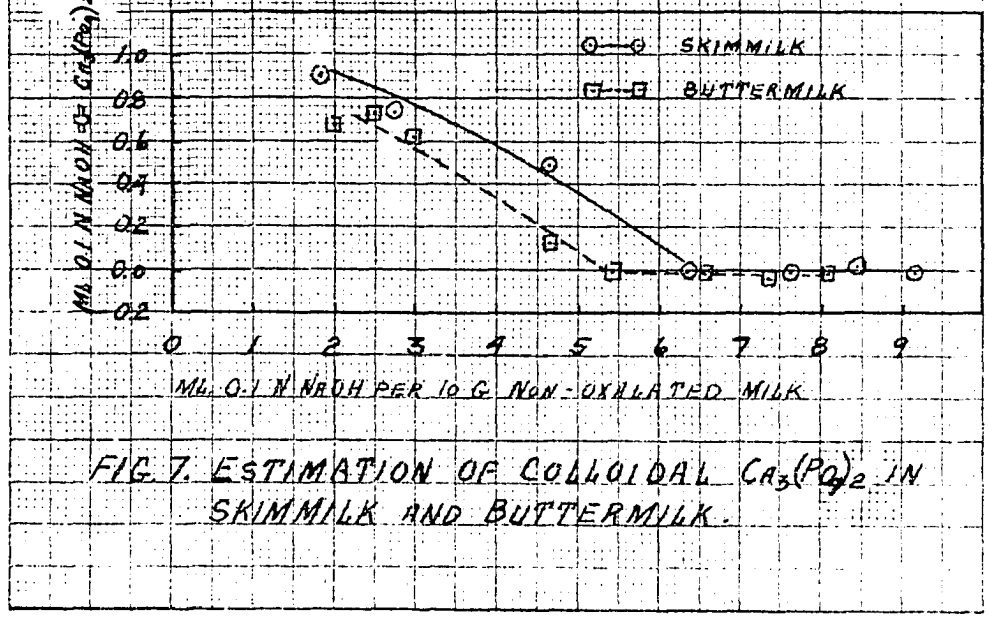


FIG. 7. ESTIMATION OF COLLOIDAL $Ca_3(PO_4)_2$ IN SKIMMILK AND BUTTERMILK.

2. Coagulation of buttermilk and butterserum using different amounts of rennet

A batch of sweet cream was ripened with a cheese culture. At various acidity levels samples were removed, neutralized with calcium lime[1] to approximately the original acidity, pasteurized and churned. One sample was removed and processed before any acidity had developed. Buttermilk and butterserum from these creams were used for coagulation experiments in which varying amounts of rennet (0.7, 1.4, 3.5 and 7.0 ml. per 100 ml. milk) were added. Table 2 summarizes the results of the buttermilk coagulations. With all rennet additions satisfactory coagulation was accomplished in 10 min. although the smallest rennet addition produced a slightly soft coagulum and a slightly turbid whey. The recovery of whey (by centrifugation) was practically the same with all rennet additions.

The neutralized buttermilk without rennet yielded a small casein precipitate and milky whey when centrifuged; this was not the case with the sweet non-neutralized buttermilk and must be ascribed to the formation of a calcium caseinate binding all the calcium it can at a

[1] Use of a sodium-containing neutralizer would have inhibited coagulation further, while the use of a calcium-containing neutralizer should have aided coagulation.

Table 2. Coagulability of buttermilks to which various volumes of rennet were added

Source of buttermilk	ml. Rennet per 100 ml. milk	ml. Buttermilk used	ml. Whey recovered	Appearance of coagulum before centrifuging coagulation time: 10 min.	Appearance of coagulum after centrifuging at 1500 rpm for 10 min.
Run 4	0	50	none	No coagulation	No coagulation
Sweet cream; not	0.7	50	38	Soft	Soft curd; milky whey
neutralized	3.5	50	38	Firm	Firm curd; dark whey
	7.0	50	38	Firm	Firm curd; dark whey
Run 4					
Sour cream; neutral-	0	50	None	Soft	Small volume of soft curd
ized with lime from	0.7	50	35	Firm	Firm curd; clear whey
0.29 to 0.14 per	3.5	50	35	Firm	Firm curd; clear whey
cent acid	7.0	50	28	Firm	Firm curd; clear whey
Run 4					
Sour cream; neutral-	0	50	38	Soft	Soft curd; milky whey
ized with lime from	0.7	50	36	Firm	Firm curd; clear whey
0.44 to 0.12 per	3.5	50	38	Firm	Firm curd; clear whey
cent acid	7.0	50	39	Firm	Firm curd; clear whey
Run 4					
Sour cream; neutral-	0	50	38	Soft	Soft curd; milky whey
ized with lime from	0.7	50	32	Soft	Soft curd; clear whey
0.54 to 0.06 per	3.5	50	36	Soft	Soft curd; clear whey
cent acid	7.0	50	38	Soft	Soft curd; clear whey

lower pH than under normal conditions. The lime effect did not interfere with coagulation in the tubes of the same buttermilk to which rennet had been added.

The results of the corresponding buttersera are summarized in Table 3. Coagula were formed in non-neutralized sweet cream sera and in one butterserum from neutralized cream. In no case did the buttersera "set up" and yield firm coagula as does skimmilk. Increased amounts of rennet did not improve the type of curd.

3. Experiments with dialyzed, concentrated and dialyzed-concentrated rennet

With large rennet additions some dilution of the milk takes place, and difficulties are likely to arise because the rennet extract itself contains 17 - 20 per cent sodium chloride. Dilution of the milk and addition of sodium chloride both tend to retard coagulation.

In order to be able to add rennet enzyme without these effects it was decided to prepare salt-free and concentrated rennet extracts and use these in comparison with the normal extract.

A quantity of commercial rennet was divided in 3 parts; one was dialyzed until it was free of salt, one was concentrated to half its volume, and one was first dialyzed then concentrated to one third its original volume. The dialyzed sample increased in volume by about 40 per cent.

Table 3. Coagulability of buttersera to which various volumes of rennet were added.

Source of butterserum	ml. Rennet per 100 ml. serum	ml. Butter serum used	ml. Whey re-covered	Appearance of coagulum before centrifuging coagulation time: 10 min.	Appearance of coagulum after centrifuging at 1500 rpm. for 10 min.
Run 4					
Sweet cream; not neutralized	0.7	50	None	Very soft	Very soft; no separation of whey
	1.4	50	None	Very soft	
Run 4					
Sweet cream; neutralized with lime from 0.29 to 0.14 per cent acid	0.7	40	25	No coagulation	Small volume of soft curd; milky whey
	3.5	40	29	No coagulation	
Run 4					
Sour cream; neutralized with lime from 0.44 to 0.12 per cent acid	0.7	50	None	Very soft	Small volume of soft curd; milky whey
	1.4	50	None	Very soft	
Run 4					
Sour cream; neutralized with lime from 0.53 to 0.06 per cent acid	0.7	40	7	No coagulation	Scant separation of curd and whey
	3.5	40	9	No coagulation	
	7.0	40	10	No coagulation	
Run 5					
Sweet cream; not neutralized	0.7	40	20	Very soft	Soft; milky whey
	3.5	40	20	Very soft	Soft; milky whey
	7.0	40	20	Very soft	Soft; milky whey

Chloride titration (with silver nitrate) of the dialyzed whey showed that all the salt had been removed. The salt percentage in the concentrated whey did not increase in proportion to the reduction in volume because some of the salt passed through the membrane to the outside of the casing while still in solution. Evaporation left large crystals, presumably sodium chloride, on the cellophane bag. The salt analyses are shown in Table 4.

These rennet preparations were added to normal milk samples on basis of the original volume of the normal extract, making proper allowances for increase or decrease in volume during dialysis and concentration. Their coagulating powers were determined by adding 1.4 ml. of each preparation to 100 ml. of milk; duplicates were run on several milks; these were allowed to coagulate for 5 and 10 min. respectively. The curd tension of each coagulum was measured immediately after each coagulation period. Results of the measurements are shown in Table 5. They indicate that the different modified rennets when applied in this manner have practically the same coagulating power as the original rennet.

A series of 4 different buttersera was obtained from commercial churnings before salt was added to the butter. Each normal or modified rennet extract was added to separate samples of each butterserum at the rate of 1.4 ml. per 100 ml. milk (on basis of the original extract) and

Table 4. Per cent sodium chloride in normal, dialyzed and concentrated rennet extracts.

Rennet preparation	Normal	Dialyzed	Concentrated
I	17.1	0	23.8
II	20.5	0	27.0

Table 5. Curd tension of milk samples coagulated with normal, dialyzed, concentrated and dialyzed-concentrated rennet extracts.

	Normal		Dialyzed		Concentrated		Dialyzed-concentrated	
	5 min.	10 min.	5 min.	10 min.	5 min.	10 min.	5 min.	10 min.
	Curd tension in grams							
Rennet prep. I with buttermilk. (Past. at 91°C)	0	0	0	0	0	0	-	-
Rennet prep. I with raw whole milk	4	11	5	11	4	12	-	-
Rennet prep. II with whole milk. (Past. at 63°C for 30 min.	5	5	5	5	2	2	2	2
Rennet prep II with raw whole milk	4	7	4	7	3	7	2	8

allowed to stand for 10 min. at 30°C. Coagulation did not take place in any of these samples even after 12 - 14 hrs; control samples of buttermilks from the same churnings coagulated immediately.

At the end of the 10 min. coagulation period 6 ml. of each non-coagulated butterserum sample was transferred to an Ostwald pipette and the time required for this volume to flow through the capillary was recorded. The results are presented in Table 6. One butterserum shows an increase in viscosity in all the samples to which rennet was added but there is no significant change in viscosity as a result of the various modifications of rennet.

It was concluded that coagulation of butterserum could not be accomplished either by increasing the amount of rennet added or by modifying the rennet by dialysis, concentration or both.

4. Distribution of protein fractions in skimmilk, buttermilk and butterserum

a. Purpose of the experiment. The non-coagulation of butterserum and the variations in Ling titration curves of skimmilk, buttermilk and butterserum suggested the possibility of important differences in the distribution of salts and proteins among these products. The distribution of total proteins, casein, albumin, globulin, proteose-peptone and non-protein nitrogen in skimmilk, buttermilk

Table 6. Viscosities of four buttersera coagulated with normal, dialyzed, concentrated, dialyzed-concentrated rennet extracts.

Source of butterserum	No. seconds required to empty Ostwald pipette (30° C)				
	Normal	Dialyzed	Concentrated	Dialyzed-concentrated	Without rennet
Sweet cream; not neutralized. Past. 71° C for 30 min.	331	359	367		
Cream neutralized with NaOH from 0.24 to 0.12 per cent acid. Past. 71° C - 30 min.	401	605	578	440	
Cream neutralized with NaOH from 0.24 to 0.12 per cent acid. Past. 71° C for 30 min.	1041	1092	869	1045	542
Sweet cream; not neutralized. Past. 73° C for 30 min.	348	395	354	441	468

and butterserum from the same whole milk source was explored in the following series of experiments. The series included 9 runs which were spaced so that milks from all seasons were included.

b. Fat and total solids contents. Fat contents of the products were determined in all runs, and total solids were determined in runs 4 to 9. Results of these analyses are shown in Table 7. The fat contents of the skimmilk were slightly higher than might be expected in the commercial product. In some runs this resulted from the use of a small farm separator; in the others it was due to separation at low temperature (4 - 6°C) in a cold bowl separator. The fat contents of the buttermilks were also slightly higher than normal for commercial churning. Overnight chilling (2 - 4°C) of the cream samples before churning in the glass jars was helpful in keeping this at a minimum. The fat content of the buttermilk in run 9 was exceptionally low because a special attempt was made to remove the fat from it by centrifugation in plastic tubes at 10,000 rpm. After centrifugation the buttermilk was pipetted from below the fat layer.

The fat contents of the buttersera were high because of the difficulties of removing it completely. Although the sample in run 9 was centrifuged at 10,000 rpm. as was the corresponding buttermilk it still contained 3.17 per cent fat. The high fat contents of the buttersera are

Table 7. Fat, solids and fat-free solids contents of skimmilks, buttermilks and buttersera from the same whole milks.[1]

Run	Skimmilk	Buttermilk	Butterserum
	Per cent fat		
1	0.12	1.94	3.42
2	0.13	3.01	4.20
3	0.12	2.08	4.04
4	0.13	1.88	7.24
5	0.09	0.83	3.64
6	0.12	1.20	8.64
7	0.09	1.21	3.58
8	0.11	0.76	4.65
9	0.13	0.42	3.17
	Per cent total solids		
1	-	-	-
2	-	-	-
3	-	-	-
4	8.08	9.80	13.70
5	9.14	9.90	10.30
6	8.56	9.62	13.90
7	9.14	9.78	8.52
8	9.48	10.00	11.37
9	9.12	8.90	9.28
	Per cent fat-free solids		
1	-	-	-
2	-	-	-
3	-	-	-
4	7.95	7.92	6.46
5	9.05	9.07	6.66
6	8.44	8.42	5.26
7	9.05	8.57	4.94
8	9.37	9.24	6.72
9	8.99	8.48	6.11

[1]Determined by the Mojonnier method.

partly responsible for their high total solids contents although their fat-free solids contents are lower than those in butter milk and skimmilk.

c. Use of selenium oxychloride. One sample of skimmilk was analyzed for total-, non-casein-, non-protein- and proteose-peptone nitrogen. To one set of duplicates was added 2 drops of selenium oxychloride before digestion; the other set of duplicates was digested without the catalyst. The comparative results are shown in Table 8 and indicate that selenium oxychloride does not cause any significant difference in the results. Variations between duplicates were as great as variations between the two methods.

d. Distribution of protein fractions. Distribution of the protein fractions in fat-free skimmilks, buttermilks and buttersera is shown in Tables 9 and 10. Each analysis is the average of triplicate determinations.

Total protein and casein contents were practically alike in skimmilk and buttermilk from the same source but were slightly lower in the corresponding buttersera. With a few exceptions (runs 5, 6 and 9) the albumin content of the buttermilk was slightly higher than that of the corresponding skimmilk. In all runs the albumin content of butterserum was significantly lower than in the corresponding skimmilk and buttermilk and constituted on the average

Table 8. The effect of selenium oxychloride on the values determined for the nitrogen distribution in skimmilk.

Nitrogen fraction	Replicates	No selenium oxychloride		Two drops of selenium oxychloride 1)	
		ml. 0.02 N NaOH = N	Per cent nitrogen	ml. 0.02 N NaOH = N	Per cent nitrogen
Total	1	20.65		20.85	
	2	20.45		20.40	
	Ave.		0.56		0.56
Non-casein	1	9.55		9.85	
	2	9.55		9.65	
	Ave.		0.129		0.132
Non-protein	1	5.10		5.40	
	2	5.10		5.10	
	Ave.		0.035		0.036
Proteose-peptone	1	4.85		4.52	
	2	4.50		4.40	
	Ave.		0.064		0.061

[1]Technical grade.

Table 9. Distribution of protein fractions as per cent of fat-free skimmilks, buttermilks and buttersera from the same whole milks.

Run		Total protein	Casein	Albumin	Proteose-peptone	Globulin
		As per cent of fat-free product				
	Skimmilk	2.95	2.48	0.229	0.140	0.102
1.	Buttermilk	2.93	2.48	0.274	0.108	0.070
	Butterserum	2.63	2.30	0.184	0.102	0.044
	Skimmilk	2.85	2.39	0.153	0.198	0.096
2.	Buttermilk	2.75	2.34	0.249	0.146	0.023
	Butterserum	2.56	2.29	0.077	0.115	0.083
	Skimmilk	2.78	2.34	0.255	0.133	0.051
3.	Buttermilk	2.89	2.45	0.267	0.140	0.032
	Butterserum	2.28	2.01	0.076	0.146	0.051
	Skimmilk	3.09	2.58	0.246	0.156	0.109
4.	Buttermilk	3.06	2.56	0.319	0.134	0.051
	Butterserum	2.62	2.23	0.050	0.266	0.076
	Skimmilk	3.21	2.71	0.279	0.150	0.071
5.	Buttermilk	3.18	2.69	0.242	0.164	0.091
	Butterserum	2.60	2.27	0.085	0.137	0.111
	Skimmilk	3.12	2.62	0.290	0.128	0.082
6.	Buttermilk	3.16	2.63	0.188	0.170	0.175
	Butterserum	2.37	2.03	0.029	0.158	0.152
	Skimmilk	3.19	2.69	0.247	0.167	0.089
7.	Buttermilk	3.26	2.64	0.349	0.232	0.040
	Butterserum	2.60	2.31	0.087	0.149	0.056
	Skimmilk	3.19	2.69	0.285	0.127	0.089
8.	Buttermilk	3.14	2.55	0.342	0.193	0.061
	Butterserum	2.55	2.12	0.051	0.255	0.116
	Skimmilk	3.10	2.61	0.198	0.178	0.108

6.	Buttermilk	3.16	2.63	0.188	0.170	0.175
	Butterserum	2.37	2.03	0.029	0.158	0.152
	Skimmilk	3.19	2.69	0.247	0.167	0.089
7.	Buttermilk	3.26	2.64	0.349	0.232	0.040
	Butterserum	2.60	2.31	0.087	0.149	0.056
	Skimmilk	3.19	2.69	0.285	0.127	0.089
8.	Buttermilk	3.14	2.55	0.342	0.193	0.061
	Butterserum	2.55	2.12	0.051	0.255	0.116
	Skimmilk	3.10	2.61	0.198	0.178	0.108
9.	Buttermilk	-	-	0.185	-	0.140
	Butterserum	2.21	1.94	0.006	0.185	0.082
	Skimmilk	3.05	2.56	0.242	0.153	0.089
Ave.	Buttermilk	3.05	2.54	0.268	0.161	0.076
	Butterserum	2.49	2.17	0.072	0.168	0.086

Table 10. The averages of the protein fractions in buttermilk and butterserum from Table 9 as per cent of the corresponding fractions in skimmilk.

	Total protein	Casein	Albumin	Proteose–peptone	Globulin
Buttermilk	100.00	99.2	110.7	105.2	85.4
Butterserum	81.6	84.8	29.8	109.8	96.6

only 29.8 per cent of the skimmilk albumin.

The globulin fraction was small and variable but had no significant tendency to be high or low in any product. The proteose-peptone fraction was slightly higher in buttermilk and butterserum than in the skimmilk.

In runs 4 to 9 the protein contents were also calculated as per cent of fat-free solids. These results are presented in Tables 11 and 12. Calculated in this manner total protein and casein in skimmilk and buttermilk are again alike. In butterserum, on the other hand, total protein is 110.8 per cent and casein 113.9 per cent of that in skimmilk. The albumin content of butterserum is only 28.5 per cent of that of skimmilk. The proteose-peptone values for butterserum are higher than those for skimmilk and buttermilk.

Proteoses and peptones are so-called derived proteins. In milk they result from break-down of the more complex casein. It can hardly be assumed that proteolysis has taken place in any of these products since they were adequately pasteurized and stored under refrigeration. In several runs total plate counts were made on each product immediately before it was analyzed. These plate counts were generally lower than 10,000 per ml. and were never high enough to warrant suspicion that proteolysis resulting from bacterial action might have taken place.

Table 11. Distribution of protein fractions as per cent of fat-free solids in skimmilks, buttermilks and buttersera from the same whole milks.

Run[1]		Total protein	Casein	Albumin	Proteose-peptone	Globulin
		As per cent of fat-free solids				
	Skimmilk	38.76	32.34	3.08	1.38	1.96
4.	Buttermilk	38.04	31.84	3.82	0.65	1.73
	Butterserum	37.99	32.28	0.74	1.11	3.85
	Skimmilk	35.48	29.92	3.10	0.79	1.67
5.	Buttermilk	34.79	29.41	2.59	0.99	1.80
	Butterserum	37.69	32.85	1.24	1.61	1.98
	Skimmilk	37.00	31.07	3.48	0.96	1.51
6.	Buttermilk	37.19	30.94	2.19	2.06	2.00
	Butterserum	41.08	35.15	0.53	2.63	2.75
	Skimmilk	35.34	29.79	2.73	0.98	1.85
7.	Buttermilk	37.58	30.43	4.08	0.47	2.61
	Butterserum	50.84	45.17	1.67	1.08	2.92
	Skimmilk	34.05	28.71	3.04	0.94	1.36
8.	Buttermilk	33.78	27.37	3.67	0.66	2.09
	Butterserum	36.12	30.11	0.77	1.65	3.59
	Skimmilk	34.76	29.35	2.26	1.94	1.21
9.	Buttermilk	-	-	2.19	-	1.63
	Butterserum	35.15	30.82	0.11	2.95	1.28
	Skimmilk	35.9	30.2	2.95	1.12	1.59
Ave.	Buttermilk	36.3	30.0	3.09	0.97	1.97
	Butterserum	39.8	34.4	0.84	1.84	2.73

[1]Total solids were not determined on the skimmilks, buttermilks and buttersera in runs 1, 2 and 3. For that reason they could not be included in this table.

Table 12. The averages of the protein fractions in buttermilk and butterserum from Table 11 as per cent of the corresponding fractions in skimmilk.

	Total protein	Casein	Albumin	Proteose-peptone	Globulin
Buttermilk	101.1	99.3	104.7	86.6	123.9
Butterserum	110.8	113.9	28.5	164.3	171.4

The non-protein nitrogen contents presented in Table 13 are an even better indication that proteolysis did not take place.

Table 13. Non-protein nitrogen content of skimmilks, buttermilks and buttersera from the same whole milks.

Run	Non-protein nitrogen Per cent of fat-free Skimmilk	Buttermilk	Butterserum
1	0.032	0.038	0.031
2	0.033	0.035	0.028
3	0.036	0.037	0.025
4	0.034	0.037	0.029
5	0.024	0.027	0.022
6	0.027	0.026	0.018
7	0.026	0.014	0.020
8	0.027	0.027	0.031
9	0.026	-	0.024
Ave.	0.029	0.030	0.025

Non-protein nitrogen shows few variations between products or between runs. It is only slightly lower in butterserum than in skimmilk or buttermilk.

On the basis of fat-free solids the globulin fractions in buttermilk and buttersera are 123.9 and 171.4 per cent respectively of that in skimmilk. The globulin fraction is comparatively small in all the products, and no significance can be attached to the large percentage increase

in butterserum as compared with skimmilk.

e. Effect of heating on the albumin and non-casein fractions. The most significant result of the analyses reported above is the consistently low albumin content in butterserum. It might be argued that this was the result of some treatment of the product rather than a property of it.

Butterserum is removed from butter by a melting process in which the butter is heated to 55°C for 60 - 80 min. If this treatment results in heat denaturation of the albumin one would expect the albumin content to decrease and the casein content to increase proportionally. The averages in Table 14 indicate that in butterserum albumin constitutes a smaller percentage of the total protein than in skimmilk or buttermilk, but only part of this difference is accounted for in the larger percentage of casein in butterserum.

In order to test the effect of heat-treatment on butterserum during melting of the butter a sample of skimmilk was divided in two parts; one was heated in water bath at 55°C for 90 min., the other was not heated. The various protein fractions were determined in the two samples; the results are shown in Table 15. Although there were some differences in globulin, proteose-peptone and non-protein nitrogen contents there was no evidence that the

Table 14. Protein fractions as per cent of total protein in skimmilks, buttermilks and buttersera from the same whole milks.

Run		Casein	Albumin	Proteose-peptone	Globulin
		As per cent of total protein			
	Skimmilk	84.1	7.7	4.7	3.5
1	Buttermilk	84.6	9.3	3.7	2.4
	Butterserum	87.5	7.0	3.8	1.7
	Skimmilk	83.9	5.6	6.9	3.6
2	Buttermilk	85.1	9.0	5.1	0.8
	Butterserum	89.4	3.0	4.5	3.2
	Skimmilk	84.1	9.4	4.7	1.8
3	Buttermilk	84.8	9.3	4.8	1.1
	Butterserum	88.2	3.5	6.2	2.1
	Skimmilk	83.5	8.0	5.0	3.5
4	Buttermilk	83.5	10.4	4.4	1.7
	Butterserum	85.0	1.9	10.2	2.9
	Skimmilk	84.4	8.7	4.7	2.2
5	Buttermilk	84.5	7.6	5.1	2.8
	Butterserum	87.3	3.2	5.2	4.3
	Skimmilk	84.0	9.3	4.1	2.6
6	Buttermilk	83.2	5.9	5.4	5.5
	Butterserum	85.7	1.2	6.7	6.4
	Skimmilk	84.3	7.7	5.2	2.8
7	Buttermilk	81.0	10.7	7.1	1.2
	Butterserum	88.8	3.3	5.7	2.2
	Skimmilk	84.3	8.9	4.0	2.8
8	Buttermilk	81.2	10.8	6.1	1.9
	Butterserum	83.4	2.1	9.9	4.6
	Skimmilk	84.3	6.4	3.5	5.8
9	Buttermilk	-	-	-	-
	Butterserum	87.6	0.3	3.7	8.4
	Skimmilk	84.1	8.0	4.7	3.2
Ave.	Buttermilk	83.5	9.1	5.2	2.2
	Butterserum	87.0	2.8	6.2	4.0

Table 15. Effect of heat treatment[1] on the distribution of the protein fractions in a sample of skimmilk.

	Total protein	Casein	Albumin	Globulin	Proteose-peptone	Non-protein nitrogen
	As per cent of skimmilk					
Not heated	3.21	2.63	0.258	0.115	0.206	0.032
Heated at 55° C for 90 min.	3.34	2.65	0.282	0.069	0.346	0.018

[1]The heat treatments applied to the skimmilks are comparable to the heating of the buttersera when melting the butter.

Table 16. Effect of heat treatment[1] on the non-casein nitrogen in three samples of skimmilk.

Sample		Per cent non-casein nitrogen
1	Not heated	0.108
	Heated at 55° C for 80 min.	0.108
2	Not heated	0.107
	Heated at 54° C for 120 min.	0.113
3	Not heated	0.123
	Heated at 55° C for 90 min.	0.127

[1]The heat treatments applied to the skimmilks are comparable to the heating of the buttersera when melting butter.

heat-treatment lowered the albumin or increased the casein contents.

In Rowland's scheme of milk protein analysis the albumin nitrogen is found indirectly by subtracting the sum of globulin-, proteose-peptone- and non-protein nitrogen from the non-casein nitrogen. Thus by determining the non-casein nitrogen in heated and unheated duplicates of the same milk an easy check could be made on the effect of melting the butter on heat denaturation of albumin.

The results of 3 such experiments are shown in Table 16. They indicate that the heat exposure alone during preparation of the butterserum did not cause any reduction in the non-casein nitrogen and hence in the albumin content.

f. Significance of the low albumin content in butterserum. Two hypotheses may be presented to explain the low albumin content in butterserum. One is that the washing of butter granules during the buttermaking process has caused part of the albumin to "dissolve" and to be washed away. This is not likely, however, since butterserum is relatively well dispersed in butter in the granular stage. Washing of butter granules removes only the buttermilk which is not dispersed within the butter granules. It seems logical to assume that all fat-free compounds are removed at the same rate.

The other hypothesis is that albumin has been removed by some mechanism of the churning process.

During the churning of cream a foam develops which, according to Bird et al. (1937) is stabilized primarily by the labile materials in the fat globule-serum interphase; these materials will then detach from the fat surface and migrate into the water phase. When the fat globules are packed sufficiently tightly in the foam walls the condition there will be like that in heavy cream (60 - 65 per cent fat) which is composed only of the fat globule and its adsorbed membrane as suggested by Jack and Dahle (1936).

Under these conditions the fat emulsion will be destabilized and the fat globules coalesce to form macroscopic granules. The non-labile hydrophilic proteins still adsorbed on the fat globules will also be released from the fat surface and become part of the buttermilk.

The suggestion is offered here that albumin is one of the principal components of the labile protective materials on the fat globule and that it is removed from the fat surface faster and in greater proportion than casein which is likewise one of the labile materials.

The figures in Tables 9 and 10 would substantiate such a hypothesis. They show that there is, on the average, a slightly higher albumin content in buttermilk than in skimmilk. From any given churning the weight of buttermilk obtained is a little more than 10 times the weight of butterserum which may be removed from the butter. On the

basis of these relative quantities and the differences in per cent albumin between skimmilk and buttermilk (0.026 per cent) and buttermilk and butterserum (0.196 per cent) it may be estimated that the slightly higher albumin content in buttermilk over that in skimmilk will more than account for the loss of albumin in the butterserum.

The evidence is weakened somewhat by the fact that, in runs 5, 6 and 9, the albumin content of buttermilk was slightly lower than in the corresponding skimmilk. This does not necessarily exclude the possibility that albumin from the fat-serum interphase is removed with the buttermilk.

In the light of current rennet coagulation theories it is doubtful that the low albumin content in butterserum in itself could be responsible for the non-coagulation phenomenon. It is possible, however, that the albumin carries with it into the buttermilk some of the colloidal phosphate that had been adsorbed on the casein in the manner of the yeast preparations used by Van der Burg (1949).

According to van Dam (1908), Beau (1941), Pyne (1945) and Berridge (1951) the phosphate-caseinate complex plays an important role in the formation of a normal coagulation in milk and the disturbance of such a complex might conceivably be a cause of the non-coagulability of butterserum. The calcium and phosphorus assays reported below are in agreement with this hypothesis.

5. Distribution of calcium in skimmilk, buttermilk and butterserum

Total calcium was determined in skimmilk, buttermilk and butterserum from the same whole milk source. The series included 4 runs, and the results are presented in Table 17.

Rothlin and von Bidder's (1945) modification of the Kramer - Tisdall (1921) method used in these experiments gave excellent checks with duplicate samples. The authors of the modification claimed the method to be accurate to ± 0.5 per cent.

The calcium content of buttermilk (on fat-free product basis) was approximately 80 per cent of that in skimmilk, and the calcium content of butterserum was 55 - 58 per cent of that in skimmilk. For each product there were only insignificant variations among the 4 runs (with the exception of the skimmilk sample in run 1, which was low).

When calculated as per cent of fat-free product the differences between the calcium content in buttermilk and butterserum became much smaller.

In butterserum both sets of values are so constant from one run to another that it suggests a limiting value for calcium in this product. There are too few analyses, however, to permit any conclusion in that respect.

Table 17. Calcium contents of skimmilks, buttermilks and buttersera from the same whole milks.

Run		Replicates	Fat	Total solids	Calcium		
			Product		As per cent of	Fat-free product	Fat-free solids in product
1	Skimmilk	1			0.108		
		2			0.109		
		Ave.	0.04	7.49	0.109	0.109	1.46
	Buttermilk	1			0.103		
		2			0.103		
		Ave.	0.90	8.86	0.103	0.104	1.29
	Butterserum	1			0.069		
		2			0.069		
		Ave.	7.53	13.47	0.069	0.075	1.17
2	Skimmilk	1			0.128		
		2			0.129		
		Ave.	0.11	8.72	0.129	0.129	1.47
	Buttermilk	1			0.097		
		2			0.097		
		Ave.	1.12	9.80	0.097	0.098	1.12
	Butterserum	1			0.073		
		2			0.072		
		Ave.	4.04	10.54	0.073	0.075	1.10
3	Skimmilk	1			0.122		
		2			0.125		
		Ave.	0.15	9.07	0.124	0.124	1.39
	Buttermilk	1			0.097		
		2			0.097		
		Ave.	1.44	8.75	0.097	0.098	1.33
	Butterserum	1			0.064		
		2			0.064		
		Ave.	6.11	11.93	0.064	0.068	1.17
4	Skimmilk	1			0.122		
		2			0.122		
		Ave.	0.13	9.12	0.122	0.122	1.36
	Buttermilk	1			0.103		
		2			0.102		
		Ave.	0.42	8.90	0.103	0.103	1.21
	Butterserum	1			0.069		
		2			0.068		
		Ave.	3.17	9.28	0.069	0.071	1.13

The figures lend support to the hypothesis discussed in the succeeding section that calcium phosphate has been removed from the calcium caseinate-phosphate complex in butterserum and indicate that the calcium remaining is bound more tenaciously to casein.

When milk, which will not coagulate satisfactorily with rennet, is encountered in commercial practice the addition of calcium ions (as $CaCl_2$) is the common remedy employed to effect satisfactory coagulation.

An experiment was performed to investigate the possibility of improving the coagulability of butterserum by this means. Sufficient $CaCl_2$ was added to a sample of butterserum to increase its total calcium content to that of the skimmilk from the same whole milk source. Control samples of skimmilk and buttermilk from the same whole milk, a sample of butterserum without added $CaCl_2$ and the butterserum with added $CaCl_2$ were included in the experiment. All samples were set with 0.7 ml. rennet per 100 ml. milk, in 50 ml. beakers at 30°C.

The results are presented below.

	pH	Coagulum after 30 min.
Skimmilk	7.25	firm
Buttermilk	7.20	firm
Butterserum	7.20	very soft
Butterserum + $CaCl_2$	7.40	very soft

The addition of $CaCl_2$ did not produce a firmer coagulum in the butterserum than was obtained without it. All samples were allowed to remain at room temperature overnight. At the end of this period the coagulum in the skimmilk and buttermilk had shrunk and produced a large volume of whey. The coagulum in the two butterserum samples was still soft and milky and had expelled no whey.

This experiment suggests that the significantly low calcium content in butterserum in itself is not the cause of the non-coagulability of butterserum.

6. Distribution of phosphorus in skimmilk, buttermilk and butterserum

a. Evaluation of methods.

(1). Digestion of samples. The technique described by Youngburg and Youngburg (1930) for digesting the various blood serum fractions presented several difficulties. The use of graduated test tubes did not afford proper volumetric accuracy. The introduction of silica pebbles in the digestion mixtures likewise caused errors. Finally it proved difficult to carry out digestion in test tubes without frequent spattering and loss of sample.

Digestion in 10 ml. volumetric flasks standing upright on a hot plate and equipped with small glass funnels enabled the digestion to be carried out without spattering. It also permitted preparation of accurately measured aliquots.

(2). Stability of blanks. In the method originally described the phosphomolybdate was reduced in cold solution and compared visually with a known standard in a colorimeter. It seemed desirable to make the color comparisons spectrophotometrically, in order to increase the accuracy. Likewise, it seemed advisable to investigate the stability of the color of the reduced solution.

The latter was tested by the following experiment. To each of two 10 ml. glass stoppered volumetric flasks was added 2 ml. of standard phosphate solution (1 ml. = 0.01 mg. P), 6 ml. water and 2 ml. of a solution containing equal volumes of 7.5 per cent sodium molybdate and 10 N H_2SO_4. One ml. of dilute $SnCl_2$ solution was added and the volume made to 10 ml. Transmittances were read in the Coleman, model 11, spectrophotometer at 660 mμ (Dyer and Wrenshall, 1938) at short intervals during 20 - 25 min. These readings are presented in Figure 8. They demonstrate the instability of the color and the difficulty of producing the same color intensity with identical concentrations of phosphorus when the reaction proceeded at room temperature.

Later Smith et al. (1939) called attention to the influence of $SnCl_2$ concentration and to the importance of using fresh $SnCl_2$ free of stannic tin. Fontaine (1942)

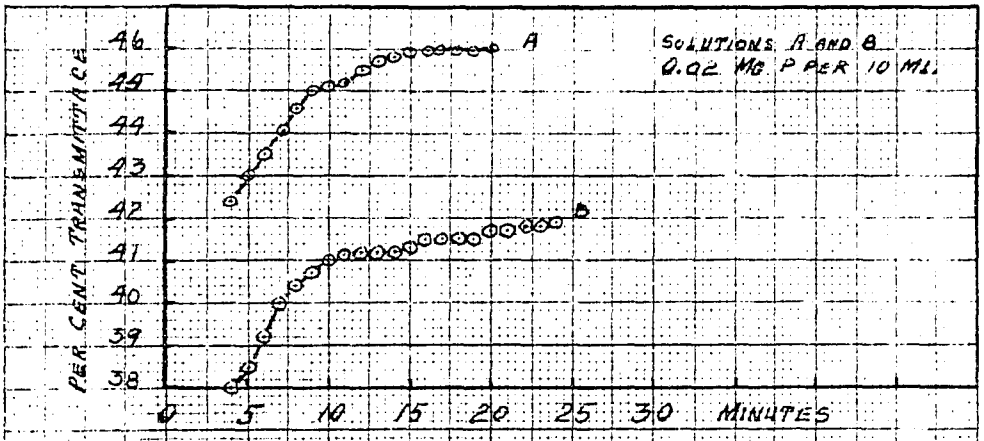


FIG. 8 STABILITY OF COLOR OF REDUCED PHOSPHOMOLYBDATE PRODUCED IN COLD SOLUTION.

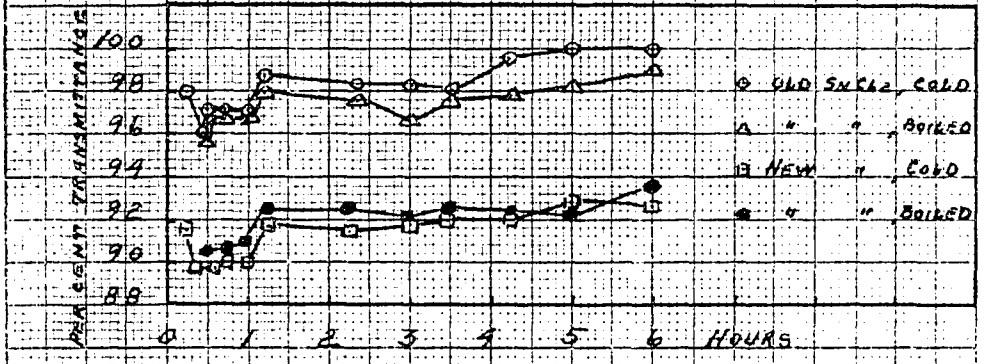


FIG. 9. STABILITY OF BLANKS. COMPARING OLD AND FRESH $SnCl_2$ AND COLD TREATMENT WITH BOILING. 1 ML $SnCl_2$ PER 25 ML SOLUTION.

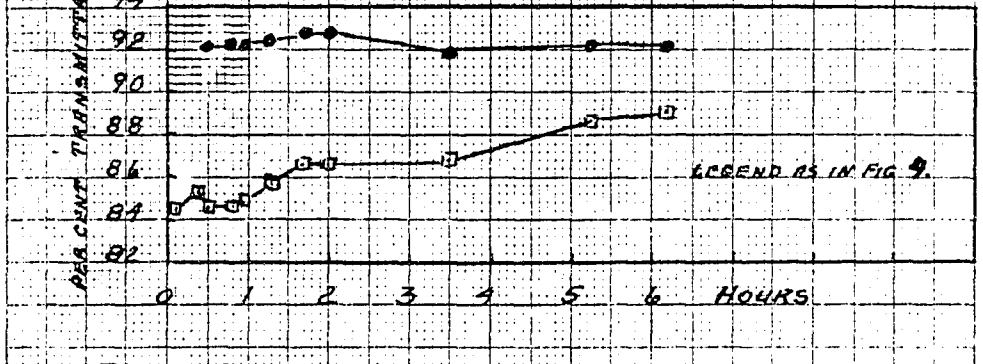


FIG. 10. STABILITY OF BLANKS USING FRESH $SnCl_2$ ONLY. COMPARING COLD TREATMENT WITH BOILING. 2.5 ML $SnCl_2$ PER 25 ML SOLUTION.

reported a method of reducing the phosphomolybdate in boiling solution which produced a stable color. These factors were examined in two experiments.

In the first experiment 4 blanks were prepared by mixing 2 ml. 10 N H_2SO_4, 2 ml. 7.5 per cent sodium molybdate and 1 ml. dilute $SnCl_2$ solution in 25 ml. amber-colored, glass-stoppered volumetric flask. Two of the blanks were made with an old $SnCl_2$ sample, the other two with $SnCl_2$ from a freshly opened bottle. One blank with old and one with fresh $SnCl_2$ were mixed at room temperature, and the other two were boiled for 20 min. after addition of $SnCl_2$. After cooling and making to volume the transmittances were read against distilled water in the Coleman spectrophotometer. Readings were made at various intervals during 6 hrs. The results are shown in Figure 9. The transmittances of the samples prepared at room temperature decreased after 30 min. After 1 hr. transmittances increased in all samples. Those prepared with old $SnCl_2$ gradually lost all color during the 6 hrs. while those prepared with fresh $SnCl_2$ stayed fairly stable during 5 hrs. The blanks prepared with fresh $SnCl_2$ had lower transmittances than those produced with old $SnCl_2$.

In another experiment 2.5 ml. of the fresh $SnCl_2$ solution was added to each blank, one was boiled, the other prepared at room temperature. Figure 10 illustrates the superior stability of the blank prepared by boiling.

Standard phosphate solutions prepared in this manner were observed to produce colors which remained stable over a period of 6 hrs.

(3). Influence of boiling time. Three samples each containing 0.02 mg. P per 25 ml. were prepared according to Fontaine's (1942) method. The flasks were removed from boiling water after 4, 9 and 20 min. respectively. After cooling and making to volume spectral transmittance curves of each were prepared in the Coleman spectrophotometer (PC-4 filter). All solutions showed maximum absorption at 760 mμ regardless of boiling time. The sample boiled for 4 min. did not develop the same color intensity as those boiled for 9 and 20 min. This demonstrated that boiling for 9 min. is sufficient to produce maximum color. The curves are presented in Figure 11.

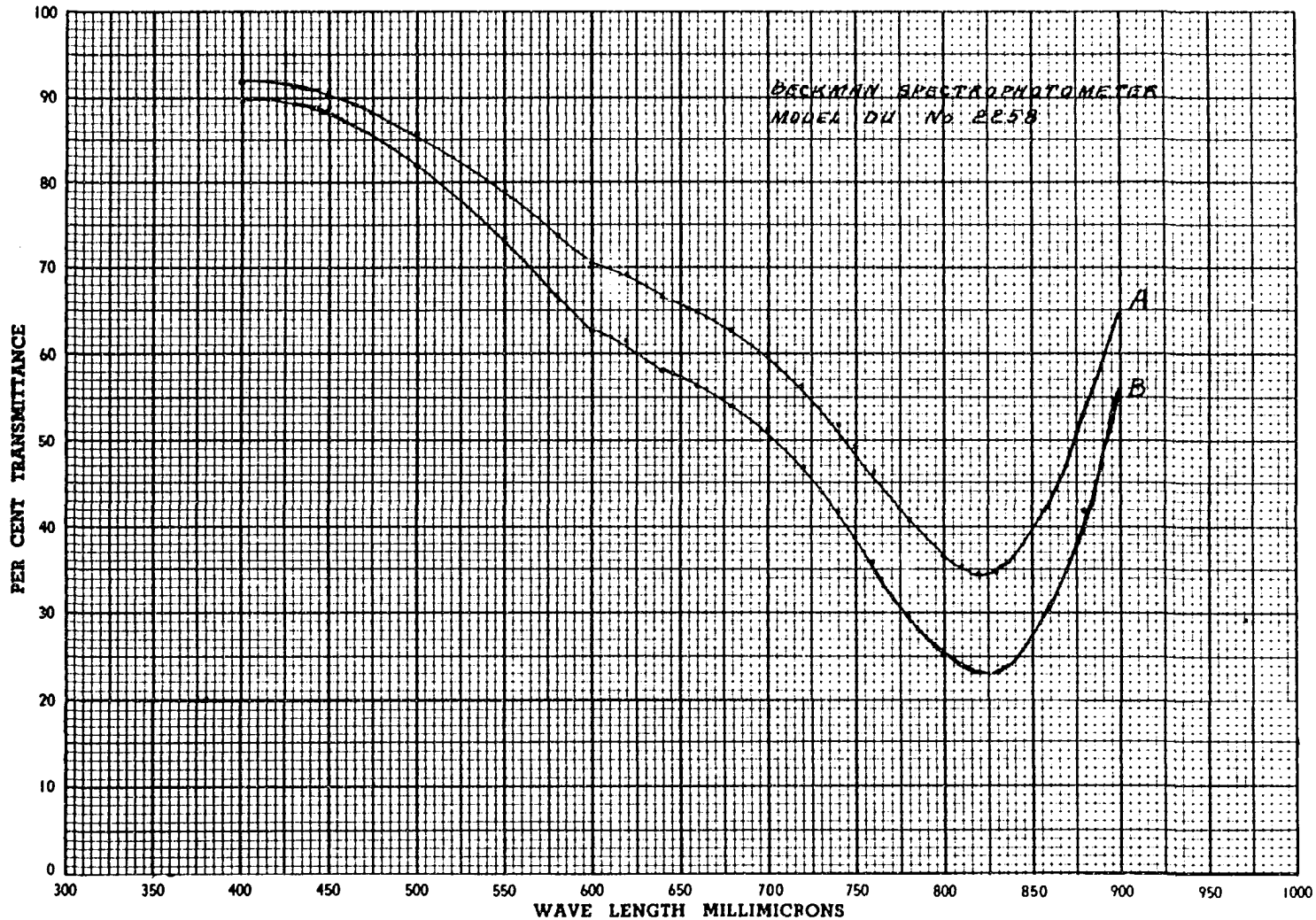
(4). Influence of filter. When readings are made above 650 mμ in the Coleman, model 11, spectrophotometer a PC-5 (red) filter must be used to prevent the effects of stray light. With this filter a reduced phosphomolybdate solution did not exhibit maximum absorption within the spectrum available with this instrument. This situation is illustrated in Figure 11 (curve 4).

The spectral transmittance curves of two phosphate solutions obtained with the Beckman, model DU spectrophotometer are presented in Figure 12. They show maximum

Fig. 11. Effect of heat treatment on transmittance of reduced phosphomolybdate solution (0.20 mg. P per 25 ml.).


1 BOILED 4 MIN. (P6-4 FILTER)
2 " 9 " (" " ")
3 " 20 " (" " ")
4 " 20 " (P6-5 ")
PER CENT TRANSMITTANCE
WAVE LENGTH MILLIMICRONS
100
90
80
70
60
50
40
30
20
10
0
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

Fig. 12. Spectral transmittance of two concentrations of reduced phosphomolybdate (A: 0.015 mg. P per 25 ml.; B: 0.020 mg. P per 25 ml.).


BECKMAN SPECTROPHOTOMETER
MODEL DU No. 22.58
A
B
PER CENT TRANSMITTANCE
100
90
80
70
60
50
40
30
20
10
0
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
WAVE LENGTH MILLIMICRONS

absorption at 820 mμ , which is in agreement with Fontaine (1942).

b. Calibration curves. Six standard solutions and a blank were prepared according to Fontaine's method. These were used in establishing the points for reference curves at 740 mμ and 820 mμ in the Beckman and at 740 mμ in the Coleman spectrophotometer (PC-4 and PC-5 filters). The wave length, 740 mμ , was chosen in order not to operate too near the end of the spectrum in the Coleman instrument. The following regression equations were calculated statistically. In these equations G represents the per cent transmittance.

Beckman (820 mμ):

Log G = 1.99322 - 30.7787 (mg. P per 25 ml.).

Beckman (740 mμ):

Log G = 2.00886 - 19.6966 (mg. P per 25 ml.).

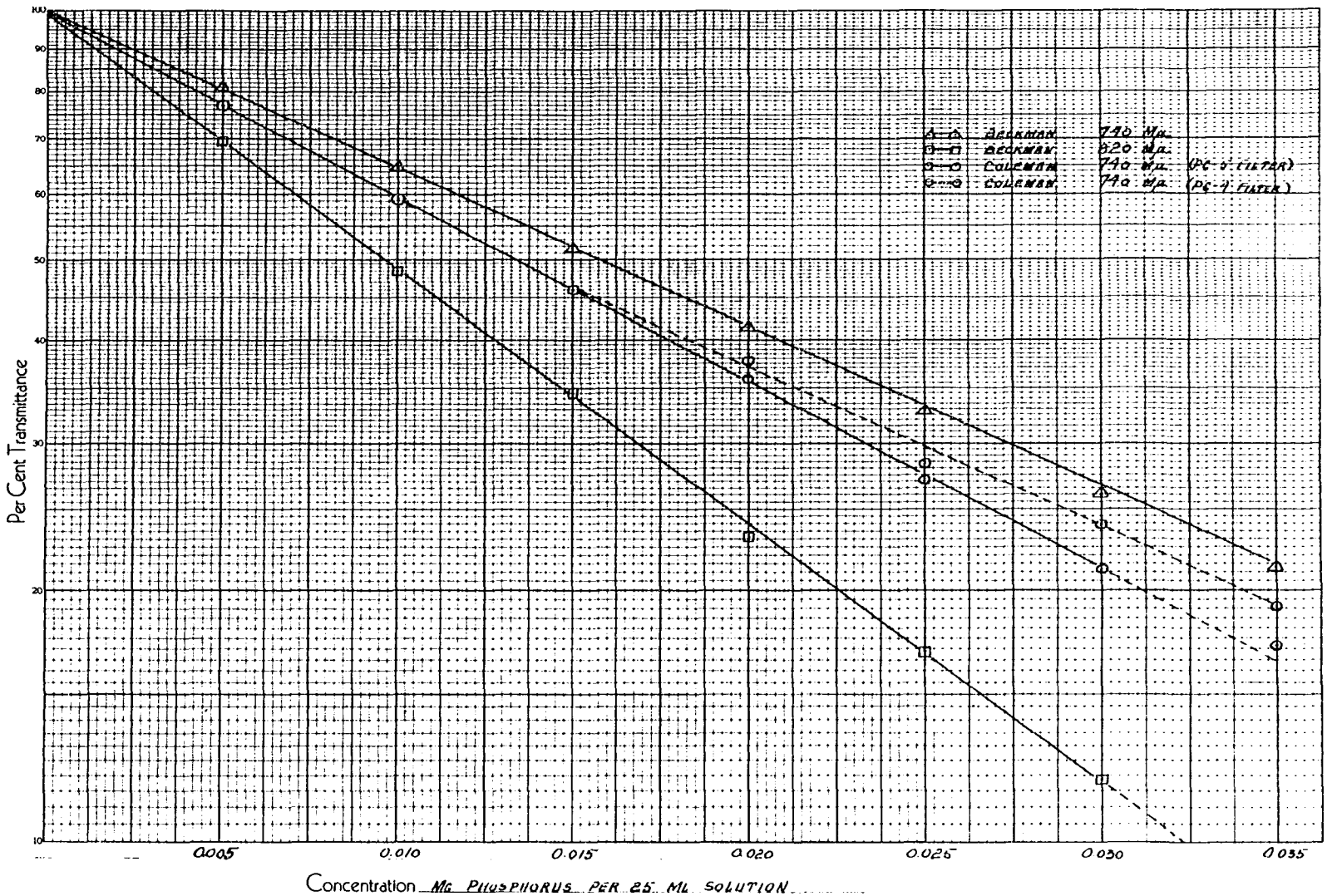
Coleman (740 mμ):

Log G = 2.00000 - 22.4165 (mg. P per 25 ml.).

The curves are presented in Figure 13. Beer's law applies for concentrations up to 0.030 mg. P per 25 ml. except when the PC-4 (purple) filter is used with the Coleman spectrophotometer.

c. Recovery experiment. A sample of skimmilk was analyzed for total, inorganic and acid soluble phosphorus. To another portion of this sample a known quantity of Na_2HPO_4 was added to increase the total phosphorus content

Fig. 13. Standard phosphorus reference curves from reduced phosphomolybdate solutions.


Per Cent Transmittance
100
90
80
70
60
50
40
30
20
10
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Concentration Mg Phosphorus per 25 ml solution
Beckman 740 mμ.
Beckman 820 mμ.
Coleman 740 mμ. (Pc-1 filter)
Coleman 740 mμ. (Pc-7 filter)

4 times. The sample to which Na_2HPO_4 was added was likewise analyzed for total, inorganic and acid-soluble phosphorus. The results are shown in Table 18.

Recovery of total and inorganic phosphorus was satisfactory; that of acid soluble phosphorus slightly higher than the calculated value.

d. Phosphorus analyses with the Beckman and Coleman spectrophotometers. Although a reduced phosphomolybdate solution prepared by boiling does not exhibit maximum absorption within the spectrum of the Coleman, model 11, spectrophotometer it is evident (Figure 13) that the calibration curve with a PC-5 filter at 740 mμ follows Beer's law. Likewise the slope of the curve indicates a reasonably large change in transmittance with phosphorus concentration. For this reason it was considered of interest to compare the results obtained with the Coleman and Beckman instruments on the same samples.

In runs 2 and 3 transmittances were measured at 740 and 820 mμ in the Beckman spectrophotometer and at 740 mμ in the Coleman spectrophotometer. The results are summarized in Table 19. They show that variations between duplicate samples are as large as variations among the three sets of readings for the same sample. It may be concluded that the Coleman, model 11, spectrophotometer equipped with a PC-5 filter may be used satisfactorily for the phosphorus analyses.

Table 18. Recovery of phosphorus added to a sample of skimmilk.

Phosphorus fraction	Replicates	Phosphorus content g. per 100 g. milk			Per cent recovery
		In original skimmilk	In skimmilk after addition of disodium phosphate		
			Calculated	Found	
Total	1	0.0833		0.3333	
	2	0.0798		0.3610	
	3	0.0805			
	Ave.	0.0812	0.3485	0.3472	99.6
	Max. dev.	0.0021		0.0138	
Inorganic	1	0.0610		0.3218	
	2	0.0595		0.3178	
	3	0.0605		0.3370	
	Ave.	0.0603	0.3278	0.3255	99.3
	Max. dev.	0.0008		0.0115	
Acid-soluble	1	0.0706		0.3519	
	2	0.0687		0.3466	
	Ave.	0.0697	0.3372	0.3493	103.6
	Deviation	0.0010		0.0026	

Table 19. Phosphorus contents of skimmilks, buttermilks and buttersera from the sam two different instruments and at two wave lengths.

Phosphorus fraction	Run no.	Replicates	Per cent phosphorus Skimmilk Coleman[1] 740 mμ	Beckman 740 mμ	Beckman 820 mμ	Max. var.	Buttermilk Coleman[1] 740 mμ	Beckman 740 mμ	Bec 820
		1	0.112	0.111	0.113	0.002	0.080	0.081	0.0
	2	2	0.118	0.119	0.123	0.005	0.083	0.084	0.0
		Ave.	0.115	0.115	0.118		0.082	0.083	0.0
Total		1	0.225	0.229	0.236	0.011	0.192	0.190	0.1
	3	2	0.230	0.231	0.233	0.003	0.174	0.170	0.1
		Ave.	0.228	0.230	0.235		0.183	0.180	0.1
		1	0.075	0.073	0.069	0.006	0.057	0.058	0.0
	2	2	0.077	0.075	0.065	0.012	0.057	0.055	0.0
		Ave.	0.076	0.074	0.067		0.058	0.057	0.0
In-		1	0.068	0.068	0.070	0.002	0.034	0.032	0.0
organic	3	2	0.076	0.077	0.076	0.001	0.037	0.036	0.0
		Ave.	0.072	0.073	0.073		0.036	0.034	0.0
		1	0.083	0.084	0.079	0.005	0.076	0.075	0.0
	2	2	0.080	0.080	0.072	0.008	0.066	0.066	0.0
Acid		Ave.	0.082	0.082	0.076		0.071	0.071	0.0
soluble		1	0.190	0.195	0.190	0.005	0.142	0.145	0.1
	3	2	0.171	0.171	0.163	0.008	0.135	0.135	0.1
		Ave.	0.181	0.183	0.174		0.139	0.140	0.1
		1	0.012	0.013	0.011	0.002	0.011	0.011	0.0
	2	2	0.012	0.012	0.011	0.001	-	-	-
Lipid		Ave.	0.012	0.013	0.011		0.011	0.011	0.0
		1	0.035	0.036	0.032	0.004	0.018	0.017	0.0
	3	2	0.035	0.036	0.033	0.003	-	-	-
		Ave.	0.035	0.036	0.033		0.018	0.017	0.0

[1]With PC-5 filter.

buttermilks and buttersera from the same whole milks. Transmittance readings made in at two wave lengths.

Per cent phosphorus in Buttermilk						Butterserum			
Beckman 820 mμ	Max. var.	Coleman[1] 740 mμ	Beckman 740 mμ	Beckman 820 mμ	Max var.	Coleman[1] 740 mμ	Beckman 740 mμ	Beckman 820 mμ	Max. var.
0.113	0.002	0.080	0.081	0.075	0.006	0.179	0.188	0.184	0.009
0.123	0.005	0.083	0.084	0.085	0.002	0.159	0.166	0.171	0.012
0.118		0.082	0.083	0.080		0.169	0.177	0.178	
0.236	0.011	0.192	0.190	0.197	0.007	0.214	0.216	0.223	0.009
0.233	0.003	0.174	0.170	0.182	0.012	0.226	0.232	0.215	0.017
0.235		0.183	0.180	0.190		0.220	0.224	0.219	
0.069	0.006	0.057	0.058	0.059	0.002	0.043	0.043	0.043	0.0
0.065	0.012	0.057	0.055	0.056	0.003	0.035	0.035	0.035	0.0
0.067		0.058	0.057	0.058		0.039	0.039	0.039	
0.070	0.002	0.034	0.032	0.031	0.003	0.036	0.037	0.038	0.002
0.076	0.001	0.037	0.036	0.030	0.007	0.036	0.036	0.036	0.0
0.073		0.036	0.034	0.031		0.036	0.037	0.037	
0.079	0.005	0.076	0.075	0.070	0.006	0.075	0.076	0.069	0.007
0.072	0.008	0.066	0.066	0.068	0.002	0.059	0.061	0.059	0.002
0.076		0.071	0.071	0.069		0.067	0.069	0.070	
0.190	0.005	0.142	0.145	0.139	0.006	0.093	0.095	0.092	0.003
0.163	0.008	0.135	0.135	0.137	0.002	0.108	0.112	0.109	0.004
0.174		0.139	0.140	0.138		0.101	0.101	0.104	
0.011	0.002	0.011	0.011	0.011	0.0	0.076	0.079	0.075	0.004
0.011	0.001	-	-	-		0.084	0.087	0.085	0.003
0.011		0.011	0.011	0.011		0.080	0.083	0.080	
0.032	0.004	0.018	0.017	0.017	0.001	0.136	0.139	0.142	0.006
0.033	0.003	-	-	-		-	-	-	
0.033		0.018	0.017	0.017		0.136	0.139	0.142	

e. Phosphorus in skimmilk, buttermilk and butterserum. Total, inorganic, acid-soluble and lipid phosphorus contents were determined in skimmilk, buttermilk and butterserum from the same whole milk source. The series included the same 4 runs used for calcium determinations. The phosphorus contents as per cent of the product are presented in Table 20 and as per cent of fat-free product and of fat-free solids in Table 21.

Total phosphorus in a given product showed wide variations between runs. It was generally lowest in the buttermilk.

Inorganic phosphorus varied considerably in the buttermilks but was rather constant in the skimmilk and butterserum although it was significantly lower in the latter than it was in skimmilk.

Acid-soluble phosphorus includes the inorganic phosphorus plus that which can be obtained in the trichloroacetic acid filtrate upon digestion. Except for the skimmilk sample in run 3 its value in all products is higher than the corresponding value for inorganic phosphorus. This increase may represent part of the phosphorus bound to casein by phosphate ester linkages, some of which might be broken during the trichloroacetic acid precipitation.

With the exception of run 3 the lipid phosphorus content of skimmilk and buttermilk generally is low. Run 3 shows

Table 20. Phosphorus contents of skimmilks, buttermilks and buttersera from the same whole milks.

	Duplicates	1	2	3	4
	Total phosphorus as per cent of product				
Skimmilk	1	0.095	0.113	0.236	0.081
	2	0.094	0.123	0.233	0.105
	Ave.	0.095	0.118	0.235	0.093
Buttermilk	1	0.095	0.075	0.197	0.099
	2	0.088	0.085	0.182	0.093
	Ave.	0.092	0.080	0.190	0.096
Butterserum	1	0.093	0.184	0.223	0.107
	2	0.082	0.171	0.215	0.105
	Ave.	0.088	0.178	0.219	0.106
	Inorganic phosphorus as per cent of product				
Skimmilk	1	0.063	0.069	0.070	0.066
	2	-	0.065	0.076	0.073
	Ave.	0.063	0.067	0.073	0.070
Buttermilk	1	0.060	0.059	0.031	0.076
	2	0.062	0.056	0.030	0.083
	Ave.	0.061	0.058	0.031	0.080
Butterserum	1	0.035	0.043	0.038	0.036
	2	0.034	0.035	0.036	0.033
	Ave.	0.035	0.039	0.037	0.035
	Acid soluble phosphorus as per cent of product				
Skimmilk	1	0.071	0.079	0.190	0.078
	2	-	0.072	0.163	0.072
	Ave.	0.071	0.076	0.174	0.075
	1	0.075	0.070	0.047	0.081

Inorganic phosphorus as per cent of product

Skimmilk	1	0.063	0.069	0.070	0.066
	2	-	0.065	0.076	0.073
	Ave.	0.063	0.067	0.073	0.070
Buttermilk	1	0.060	0.059	0.031	0.076
	2	0.062	0.056	0.030	0.083
	Ave.	0.061	0.058	0.031	0.080
Butterserum	1	0.035	0.043	0.038	0.036
	2	0.034	0.035	0.036	0.033
	Ave.	0.035	0.039	0.037	0.035

Acid soluble phosphorus as per cent of product

Skimmilk	1	0.071	0.079	0.190	0.078
	2	-	0.072	0.163	0.072
	Ave.	0.071	0.076	0.174	0.075
Buttermilk	1	0.075	0.070	0.047	0.081
	2	0.074	0.068	-	0.076
	Ave.	0.075	0.069	0.047	0.079
Butterserum	1	0.043	0.069	0.092	0.042
	2	0.043	0.059	0.109	0.043
	Ave.	0.043	0.064	0.101	0.043

Lipid phosphorus as per cent of product

Skimmilk	1	0.013	0.011	0.032	0.002
	2	0.011	0.011	0.033	0.002
	Ave.	0.012	0.011	0.033	0.002
Buttermilk	1	0.011	0.011	0.017	0.011
	2	0.012	-	-	0.010
	Ave.	0.012	0.011	0.017	0.011
Butterserum	1	0.068	0.075	0.142	0.055
	2	0.063	0.085	-	0.055
	Ave.	0.066	0.080	0.142	0.055

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[1]The transmittances in run 1 were read in a Duboscq colorimeter; those in runs 2, 3 and 4 were made in the Beckman spectrophotometer at 820 mμ.

Table 21. Phosphorus contents of skimmilks, buttermilks and buttersera from the same whole milks.

Run	Phosphorus As per cent of fat-free product			Phosphorus As per cent of fat-free solids in product		
	Skimmilk	Buttermilk	Butterserum	Skimmilk	Buttermilk	Butterserum
			Total			
1	0.095	0.092	0.095	1.27	1.15	1.48
2	0.118	0.081	0.185	1.37	0.92	2.74
3	0.235	0.193	0.233	2.63	2.60	3.76
4	0.093	0.096	0.109	1.03	1.13	1.73
			Inorganic			
1	0.063	0.062	0.038	0.85	0.77	0.58
2	0.067	0.059	0.041	0.78	0.67	0.60
3	0.073	0.031	0.039	0.82	0.42	0.64
4	0.070	0.080	0.036	0.78	0.94	0.57
			Acid soluble			
1	0.071	0.075	0.047	0.95	0.94	0.73
2	0.076	0.070	0.067	0.88	0.79	0.98
3	0.174	0.048	0.108	1.95	0.64	1.73
4	0.075	0.079	0.043	0.83	0.93	0.69
			Lipid			
1	0.012	0.012	0.069	0.16	0.15	1.10
2	0.011	0.011	0.084	0.13	0.13	1.23
3	0.033	0.017	0.151	0.37	0.23	2.44
4	0.002	0.011	0.057	0.02	0.13	0.90

Lipid phosphorus in butterserum was 5 to 25 times higher than in skimmilk or buttermilk. This agrees with the findings of Jenness and Palmer (1945), who observed that washed cream butterserum had a higher phospholipid : protein ratio than washed cream buttermilk.

Their explanation of this fact was based on the concept presented by Bird et al. (1937) of a non-labile phospholipid-protein complex irreversibly associated with the fat globule surface. Jenness and Palmer (1945a) postulated that, during churning, the phospholipid-protein complex is disrupted in such a manner that some of the protein-phospholipid linkages as well as some of the phospholipid-fat linkages are broken. Therefore, the buttermilk would contain a phospholipid-protein complex which was richer in protein than the original complex at the fat surface. The butterserum is left with a phospholipid-protein complex relatively richer in phospholipid than the original complex. When the butter is melted the phospholipid, still clinging to the fat, is finally released in the butterserum carrying with it a number of high-melting fat globules.

The buttersera used in these experiments were not prepared from washed-cream butter, but the high lipid phosphorus content of the butterserum compared to the corresponding buttermilks is still evidence that phospholipids are intimately associated with the fat globule

surface. Additional evidence of a phospholipid-protein complex will be presented in a succeeding section.

In Table 21 the differences between inorganic phosphorus in skimmilk and butterserum are relatively constant. In Table 17 the same may be observed with respect to total calcium. If these differences are calculated as gram equivalent weights it is found that the ratios of Ca to P, represented by these differences, in runs 1 to 4 are 1.1, 1.6, 1.3 and 1.2 respectively. Thus butterserum contains less inorganic calcium and phosphorus in amounts which suggest that they have been removed as $CaHPO_4$ or $Ca_3(PO_4)_2$. The Ca/P ratios in these compounds are 1 and 1.5 respectively.

According to ter Horst (1947), Pyne and Ryan (1950) and others, both of these phosphates may be present in the calcium caseinate-phosphate complex. If they are bound to casein by rather weak forces it is entirely possible that the variations observed above might occur.

The fate of the inorganic phosphorus in butterserum cannot be accounted for by an increase in inorganic phosphorus in buttermilk except in run 4. Otherwise the inorganic phosphorus in buttermilk is lower than in skimmilk and in run 3 actually lower than in the butterserum. The problem is also complicated by the fact that total phosphorus is higher in butterserum than in the other two

products, although much of this results from the high lipid phosphorus content of this product.

The data from these experiments may be taken in support of the evidence from the Ling titrations which indicated that the calcium caseinate-phosphate complex in butterserum is either disrupted or incompletely formed.

7. Casein and whey protein preparations from skimmilk, buttermilk and butterserum

a. General characteristics of the preparations. Two different lots of whole milk were employed. From each lot casein and whey proteins were prepared from the skimmilk, buttermilk and butterserum. Skimmilk casein produced a coarse, well-defined precipitate at the isoelectric point. The whey was clear and filtered easily. The dried casein had a clean, white color.

The casein precipitate in buttermilk was much finer than that in skimmilk, and the whey was slightly turbid. Separation of the precipitate was easily accomplished by centrifugation; the whey filtered well.

The volume of 0.1 N HCl required to reach a pH 4.6 in skimmilk and buttermilk was 125 - 150 ml. per 400 ml. milk. In butterserum it was 60 - 80 ml. per 400 ml. serum. This indicated a lower buffer capacity in butterserum than in skimmilk or buttermilk; it correlates with the observation reported in the preceding section that

the inorganic phosphate content in butterserum was lower than that in skimmilk or buttermilk.

There was no visible separation of the casein precipitate in butterserum at pH 4.6 suggesting that possibly the true isoelectric condition may not be at this pH or that, if casein is united with phospholipid, the combination does not aggregate to large clots. Prolonged centrifugation (15 - 20 min.) at 1500 rpm. caused the casein to settle out, but the supernatant whey was turbid and filtered slowly. The casein from buttermilk and butterserum had a slightly yellow color indicating possible contamination with fat or other lipid material.

No difficulty was experienced in lyophilizing the dialyzed wheys; the protein preparations were white and had a fluffy texture.

The casein and whey proteins from skimmilk dispersed readily in their respective buffers and gave clear transparent suspensions. The caseins from buttermilk and butterserum dispersed less readily and gave turbid suspensions which tended to obscure the electrophoretic boundaries. The whey proteins from buttermilk and butterserum were readily suspended in the buffer but yielded milky suspensions which were difficult to examine electrophoretically. Extraction with acetone and ether at -23°C of all the protein preparations improved their dispersibility and made their suspensions more transparent.

b. Acetone-ether extractions of casein and whey proteins. The turbid buffer suspensions of casein and whey proteins from buttermilk and butterserum suggested either that extremely small fat globules were occluded in the protein particles or that other lipid compounds were present in appreciable quantities. Another possibility is the existence of a submicroscopic emulsion of extremely small fat globules protected by the proteins.

Blix (1941) extracted human blood serum with acetone and ether at -14°C and was able to remove all the cholesterol and 75 per cent of the phospholipids by the treatment. The extracted phospholipid was chiefly lecithin while that remaining was mostly cephalin. After extraction the serum protein dispersed easily in the buffers used.

McFarlane (1942) observed that filtered human blood serum became turbid because of a lipid precipitate. He made the observation that human blood serum dried from the frozen state gave milky suspensions when reconstituted with water; serum dried from the liquid state gave clear suspensions. This suggested to him that the association of a lipid with a stabilizing substance, depends on the presence of liquid water and that this association is destroyed by freezing. When thawed and redissolved the unprotected lipid aggregates to visible particles which reassociate with the original stabilizer. He claimed that

the lipid particles may be observed to travel with the boundary of the stabilizing substance in the electrophoretic cell. These lipids are not extracted with ether unless the extraction is accompanied by freezing. By extracting with ether at low temperatures he obtained lipid-free, lyophilized serum proteins without destroying their physical properties.

Delsal (1949) extracted blood serum at -15°C with a mixture of acetone and ethanol or with ether and was able to remove cholesterol and part of the phospholipids.

In the present experiment casein and whey protein were extracted according to the method of Blix (1941) except that extraction was carried out at -23°C instead of -14°C. A room at this temperature was the only one available.

The casein preparation in the first run were suspended in a phosphate buffer temporarily made strongly alkaline to enable complete dispersion of the casein. The whey proteins in the first run were readily dispersed at the original pH 7.7 of the buffer. Both were extracted from these buffer suspensions. In the second run both caseins and whey proteins were extracted from water suspensions in order to avoid possible interference of phosphate from the buffer in subsequent phosphorus analyses.

The acetone extracts were filtered and evaporated to dryness. The extracts of skimmilk proteins contained little residue, those of buttermilk and butterserum had

residues of fatty material.

The residues from the filtered ether extracts were weighed and analyzed for phosphorus. All the protein preparations were analyzed for phosphorus before and after extraction. Nitrogen determinations were made on the caseins in the second run before and after extraction.

Data of the casein extractions are summarized in Table 22; phosphorus and nitrogen analyses of the caseins are shown in Tables 23 and 24.

Only insiginficant amounts of lipid material were extracted from skimmilk and buttermilk casein, but appreciable quantities were removed from the butterserum casein. None of the extracts contained phosphorus with the exception of that of butterserum casein in the first run, which had only a trace. The amount extracted from an acidified suspension of butterserum casein was only half of that extracted from the water suspension.

Phosphorus in buttermilk casein was slightly lower than in skimmilk or butterserum casein. The phosphorus contents of the extracted skimmilk and buttermilk caseins were insignificantly lower than in the non-extracted preparations. Extraction caused an appreciable increase in phosphorus content of butterserum casein. This may be explained partly by dehydration with acetone and partly by the fact that fat and other lipid materials have been

Table 22. Acetone-ether extracts of casein preparations from skimmilks, buttermilks and buttersera from the same whole milks.

Product from which casein was prepared	Medium in which casein was suspended	Lipid extracted by ether[1] as per cent of protein preparation	Phosphorus as per cent of extracted lipid
Skimmilk (1)	Phosphate buffer pH 7.7	0.3	none
Buttermilk (1)	" [2]	0.3	none
Butterserum (1)	" [2]	6.7	0.1
Skimmilk (2)	Water	0.15	none
Buttermilk (2)	"	none	none
Butterserum (2)	"	9.8	none
Butterserum (2)	Water acidified with HCl to pH 2.0	4.3	none

[1]Lipids extracted after dehydration of proteins with acetone. First extraction was made with a mixture of two volumes of acetone to one of ethyl ether, second and third extractions with ethyl ether.

[2]The phosphate buffer in which the caseins from buttermilk and butterserum were suspended were made alkaline to pH 11.0 - 12.0 with 2 N Na_2OH in order to bring about complete suspension of the protein. When this had been accomplished the system was again adjusted to pH 7.7 with N HCl.

Table 23. Phosphorus contents of casein preparations from skimmilks, buttermilks and buttersera from the same whole milks.

	Phosphorus as per cent of casein in		
	Skimmilk	Buttermilk	Butterserum
Casein 1 (not extracted)	0.80	0.62	0.99
Number of replicates	4	4	4
Maximum deviation from mean	0.01	0.02	0.02
Casein 2 (not extracted)	0.73	0.69	0.73
Number of replicates	4	2	4
Maximum deviation from mean	0.03	0.01	0.01
Casein 2 (extracted from water)	0.70	0.66	0.92
Number of replicates	3	4	4
Maximum deviation from mean	0.04	0.02	0.03
Casein 2 (extracted at pH 2.0)	-	-	0.55
Number of replicates	-	-	2
Maximum deviation from mean	-	-	0.03

Table 24. Nitrogen contents of casein preparations from skimmilk, buttermilk and butterserum from the same whole milk.

		Nitrogen as per cent of casein		P/N ratio	
		Not extracted	Extracted	Not extracted	Extracted
Skimmilk	1	13.38	14.15		
	2	13.50	14.38		
	Ave.	13.44	14.27	0.054	0.049
Buttermilk	1	13.95	14.30		
	2	13.93	14.32		
	Ave.	13.94	14.31	0.049	0.046
Butterserum	1	10.86	11.52		
	2	10.82	11.90		
	Ave.	10.84	11.71	0.067	0.079

removed in the acetone and ether extracts. Fat and cholesterol would have been extracted with the acetone, phospholipids with the ether. Thus the total phosphorus in the extracted casein was really calculated on a "fat-and-moisture-free" basis.

Little or no phosphorus was found in the lipid material extracted with ether. This suggests that dehydration with acetone is not sufficiently strong to permit subsequent extraction of phospholipids. Dehydration with alcohol, followed by ether extraction or extraction with an alcohol-ether mixture permitted extraction of phospholipids from the butterserum itself, as reported in connection with the phosphorus analyses. It is possible that the acetone dehydration destabilized the sub-microscopic fat globule emulsions just enough to permit extraction of triglycerides leaving the phospholipids behind.

It may also be suggested that a chemical union rather than a physical orientation of phospholipids is the cause of non-extraction of phospholipids. This correlates with the concept of Jenness and Palmer (1945a) who suggested that the phospholipid at the fat globule surface "clings" to certain high-melting triglycerides which are removed with the phospholipid-protein complex upon melting the butter. These investigators were able to make alcohol extractions of such high-melting glycerides from butterserum of washed cream butter.

From Table 24 it is apparent that the nitrogen contents of extracted skimmilk and buttermilk caseins are practically alike. The nitrogen content is slightly higher in the non-extracted buttermilk casein than in the non-extracted skimmilk casein. No explanation can be offered for this difference. Extraction with acetone and ether caused the nitrogen content of both to increase and the P/N ratios to decrease slightly. This is probably due to removal of lipid material other than phospholipids.

Both the extracted and non-extracted butterserum casein had a much lower nitrogen content than the skimmilk and buttermilk caseins. The P/N ratio was higher in the butterserum casein than in the others and was increased materially by acetone-ether extraction. Again this increase may have been caused by extraction of lipids from submicroscopic fat globule emulsions.

With respect to nitrogen and phosphorus contents, the butterserum casein prepared in run 2 resembles the fat membrane lipid-protein complex (11.84 and 12.64 per cent N respectively) in 2 preparations reported by Wiese and Palmer (1934) or that isolated by Schwarz and Fischer (1936) and called a new protein (11.25 per cent N and 0.63 per cent P).

These investigators isolated the protein material from butter made from creams which were washed by the dilution and separation technique. They did not separate it as

casein by isoelectric precipitation but identified it merely as a new protein or a lipid-protein complex. The electrophoretic experiments described subsequently will demonstrate that the protein prepared here resembles casein.

The data presented in Tables 25 and 26 indicate that extraction of skimmilk whey protein removed only traces of lipid material which contained no phosphorus. Appreciable quantities of lipid were extracted from the buttermilk whey protein, and large quantities (20-25 per cent of the original weight) were extracted from the butterserum whey protein. The high, but variable phosphorus contents of the lipids extracted from phosphate buffer suggest contamination with phosphorus from this buffer. However, this is not in agreement with the recent report of Van Slyke and Sacks (1953) that ethanol-ether extractions of lipids are free of inorganic phosphorus.

The phosphorus contents of the two non-extracted skimmilk whey proteins were practically alike, and there was no change resulting from the extraction of the skimmilk whey protein in run 2. The phosphorus contents in the buttermilk whey proteins were higher than those from skimmilk, and there was a slight increase caused by extraction with acetone and ether.

The non-extracted butterserum whey protein contained almost three times as much phosphorus as the skimmilk whey

Table 25. Acetone-ether extracts of whey protein preparations from skimmilks, buttermilks and buttersera from the same whole milks.

Product from which whey protein was prepared	Medium in which whey protein was suspended	Lipid extracted by ether[1] as per cent of protein preparation	Phosphorus as per cent of extracted lipid
Buttermilk (1)	Phosphate buffer pH 7.7	3.7	0.39
Butterserum (1)	"	14.0	4.00
Butterserum (1)	"	19.3	1.74
Butterserum (1)	Water	21.9	0.96
Skimmilk (2)	"	0.4	none
Buttermilk (2)	"	2.0	trace
Butterserum (2)	"	25.0	0.12
Butterserum (2)	Water acidified with HCl to pH 2.0	11.3	0.20

[1]Lipids extracted after dehydration of proteins with acetone. First extraction was made with a mixture of two volumes of acetone to one of ethyl ether, second and third extractions with ethyl ether.

Table 26. Phosphorus contents of whey protein preparations from skimmilks, buttermilks and buttersera from the same whole milks.

	Phosphorus as per cent of whey protein in		
	Skimmilk	Buttermilk	Butterserum
Whey protein 1 (not extracted)	0.39	0.45	1.76
Number of replicates	3	4	4
Maximum deviation from mean	0.01	0.02	0.03
Whey protein 2 (not extracted)	0.40	0.58	1.15
Number of replicates	3	4	3
Maximum deviation from mean	0.01	0	0.03
Whey protein 2 (extracted from water)	0.39	0.65	1.75
Number of replicates	4	4	4
Maximum deviation from mean	0.03	0.03	0.16
Whey protein 2 (extracted at pH 2.0)			1.35
Number of replicates			4
Maximum deviation from mean			0.07

protein, and the extracted preparation contained over four times as much. This again is due to removal of lipid material and to determination of the remaining phosphorus on "fat-free" basis.

Only half as much lipid material was extracted from the acidified suspension as from the water suspension. The phosphorus content of the acid extracted protein was lower than in that extracted from water but slightly higher than in the non-extracted preparation. The same relationship was observed with the extraction of casein from acid solution. The lower phosphorus content of the proteins extracted from acid suspension may be due to removal of inorganic phosphate in acid solution. This inorganic phosphorus remained in the aqueous solution and was removed with the first acetone extraction. The lipid residue from the acid extraction of butterserum whey protein contained 0.20 per cent phosphorus.

In contrast to casein, which is classified as a conjugated phospho-protein, the whey proteins are considered as simple proteins containing no significant amount of phosphorus. The small phosphorus content of the two skimmilk and buttermilk whey proteins may be due to incomplete removal of phosphates during dialysis or to a reaction, during dialysis, between phosphates and the proteins.

The high phosphorus content of the butterserum casein and whey protein is indication of the presence of a phospholipid-protein complex in butterserum. Since the acetone-ether extractions failed to remove appreciable quantities of phosphorus-containing material they did not verify the presence of phospholipids as did the lipid phosphorus extraction with ethanol and ether of the butterserum itself. They indicate that acetone dehydration is not drastic enough to cause subsequent separation of phospholipids from the protein with ether.

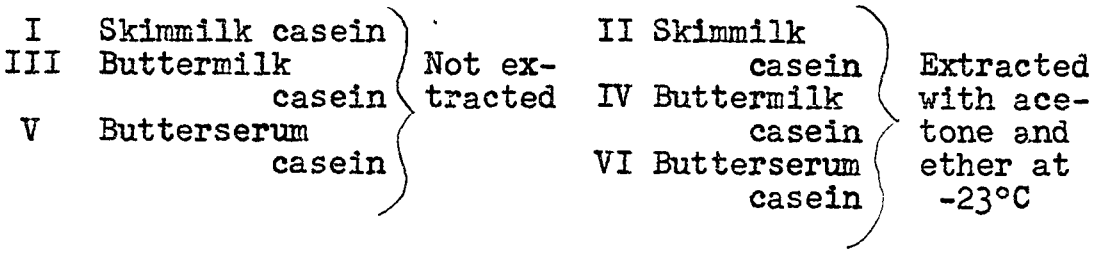
8. Electrophoresis of casein and whey proteins from skimmilk, buttermilk and butterserum

a. Casein. For electrophoresis the caseins were dissolved in veronal-citrate buffer at pH 8.45 and ionic strength 0.15. The whey proteins were dissolved in phosphate buffer at pH 7.7 and ionic strength 0.20.

Electrophoretic patterns of caseins and whey proteins from skimmilk, buttermilk and butterserum from the same whole milk are presented in Figures 14 and 15 respectively. Areas of peaks and mobilities of components are listed in Tables 27 and 28.

The patterns of skimmilk casein are similar to those previously shown by Krejci et al. (1941), Warner (1944) and Hipp et al. (1952). The extracted preparations gave better resolution of components and slightly increased

Figure 14. Electrophoretic patterns of caseins from skimmilk, buttermilk and butterserum from the same whole milk. Veronal-citrate-NaCl buffer; pH 8.45; ionic strength 0.15; protein concentration 1 per cent.



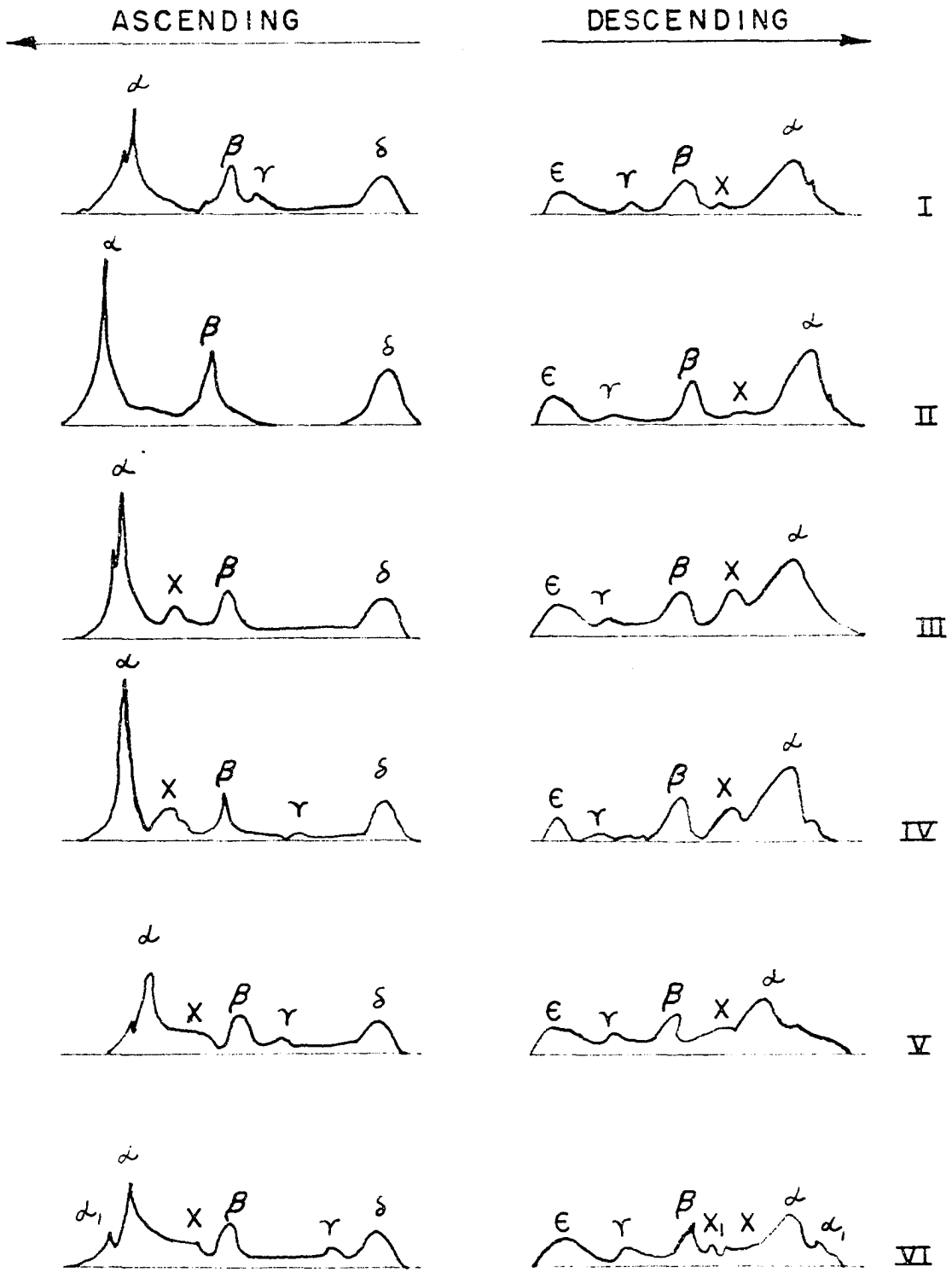

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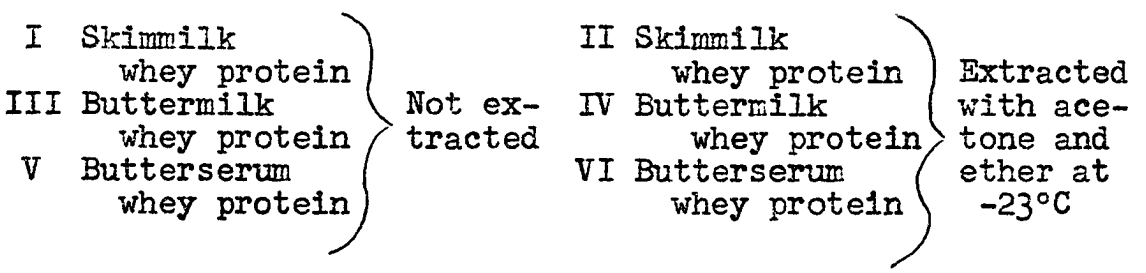
Table 27. Electrophoresis of caseins from skimmilk, buttermilk and butterserum from the same whole milk. Areas of peaks and mobilities of components in veronal-citrate - NaCl buffer; pH 8.45; ionic strength 0.15; protein concentration 1 per cent.

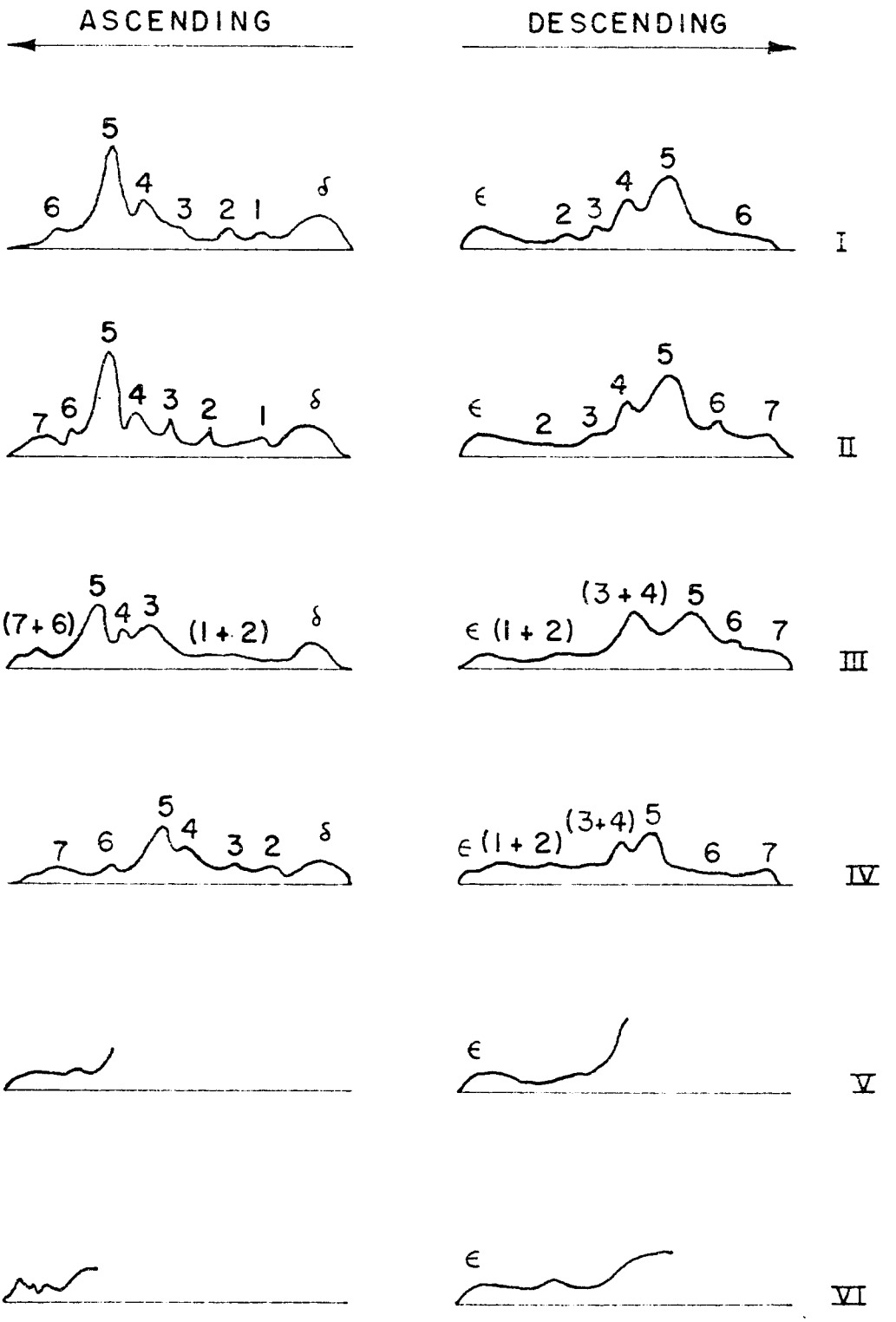
Component	Ascending		Descending	
	Per cent of total area	Mobility (-) cm^2 $volt^{-1}$ sec^{-1} $\times 10^5$	Per cent of total area	Mobility (-) cm^2 $volt^{-1}$ sec^{-1} $\times 10^5$
	Skimmilk casein; not extracted			
α	74.5	8.3	73.8	7.5
x	-	-	2.8	5.0
β	23.5	5.1	21.5	4.0
γ	2.0	4.0	1.9	2.0
	Skimmilk casein; extracted[1]			
α	75.8	8.7	71.6	7.7
x	-	-	2.6	5.5
β	24.2	5.3	23.8	4.1
γ	-	-	2.0	1.5
	Buttermilk casein; not extracted			
α	64.5	8.4	63.8	7.7
x	11.6	6.7	15.4	5.6
β	23.9	5.3	17.8	3.9
γ	-	-	3.0	1.7

β	23.5	5.1	21.5	4.0
γ	2.0	4.0	1.9	2.0
	Skimmilk casein; extracted[1]			
α	75.8	8.7	71.6	7.7
x	-	-	2.6	5.5
β	24.2	5.3	23.8	4.1
γ	-	-	2.0	1.5
	Buttermilk casein; not extracted			
α	64.5	8.4	63.8	7.7
x	11.6	6.7	15.4	5.6
β	23.9	5.3	17.8	3.9
γ	-	-	3.0	1.7
	Buttermilk casein; extracted[1]			
α	65.6	8.8	67.0	7.8
x	15.6	7.2	13.9	5.9
β	16.3	5.4	17.0	4.2
γ	2.5	2.9	2.1	1.7
	Butterserum casein; not extracted			
α	61.0	8.2	62.4	7.6
x	17.3	6.9	18.8	6.1
β	20.6	5.2	16.2	4.3
γ	1.1	3.5	2.0	2.5
	Butterserum casein; extracted[1]			
α_1	6.0	8.2	5.2	8.0
α	58.0	7.6	52.2	7.1
x	15.0	5.6	6.0	5.3
x_1	-	-	17.2	4.9
β	19.0	4.6	14.9	4.1
γ	2.0	1.4	4.5	2.2

[1]Extracted with acetone and ether at - 23° C.

Figure 15. Electrophoretic patterns of whey proteins from skimmilk, buttermilk and butterserum from the same whole milk. Phosphate buffer; pH 7.7; ionic strength 0.2; protein concentration 1 per cent.

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(1+2)
δ
ϵ
(1+2)
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6
7
III
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7
6
4
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2
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ϵ
(1+2)
(3+4)
5
6
7
IV
ϵ
V
ϵ
VI

Table 28. Electrophoresis of whey proteins from skimmilk, buttermilk and butterserum from the same whole milk. Areas of peaks and mobilities of components in phosphate buffer; pH 7.7; ionic strength 0.2; protein concentration 1 per cent.

Component	Ascending		Descending	
	Per cent of total area	Mobility (-) cm^2 $volt^{-1}$ sec^{-1} x 10^5	Per cent of total area	Mobility (-) cm^2 $volt^{-1}$ sec^{-1} x 10^5
	Skimmilk whey protein; not extracted			
1	0.9	1.1	-	-
2	1.6	2.2	0.7[2]	1.8
3	3.2	3.5	3.5	3.3
4	18.8	4.3	26.0	3.9
5	73.0	5.1	59.6	4.8
6	2.5	6.5	10.2	5.7
	Skimmilk whey protein; extracted[1]			
1	2.2	1.0	-	-
2	3.8	2.3	2.7	2.3
3	4.4	3.4	6.5	3.2
4	17.9	4.2	21.4	3.8
5	65.0	5.0	61.6	4.7
6	2.9	5.9	3.9	5.9
7	3.8	6.5	3.9	6.6
	Buttermilk whey protein; not extracted			

3	3.2	3.5	3.5	3.3
4	18.8	4.3	26.0	3.9
5	73.0	5.1	59.6	4.8
6	2.5	6.5	10.2	5.7
	Skimmilk whey protein; extracted[1]			
1	2.2	1.0	-	-
2	3.8	2.3	2.7	2.3
3	4.4	3.4	6.5	3.2
4	17.9	4.2	21.4	3.8
5	65.0	5.0	61.6	4.7
6	2.9	5.9	3.9	5.9
7	3.8	6.5	3.9	6.6
	Buttermilk whey protein; not extracted			
1	-	-	-	-
2	1.6[2]	1.7	1.7[2]	1.2
3	31.4	4.3	-	-
4	13.3	4.8	49.6[2]	3.7
5	49.6	5.6	45.3	5.1
6	-	6.8	-	-
7	4.1[2]	7.2	3.4[2]	6.0
	Buttermilk whey protein; extracted[1]			
1	-	-	-	-
2	4.5[2]	2.0	6.0[2]	1.7
3	5.5	3.0	-	-
4	32.2	4.5	32.0	4.1
5	51.2	5.2	48.0	4.9
6	3.3	6.8	8.0	6.4
7	3.3	8.4	6.0	8.2

[1]Extracted with acetone and ether at - 23° C.

[2]Assumed to include the next slower component where this could not be measured.

mobilities. The small γ-fraction and the secondary α-peak (also observed by Warner) do not appear on the ascending boundary of the extracted casein. In the descending patterns of both the extracted and non-extracted skimmilk casein there is evidence of a small component designated as x. This may be the α + β-complex previously discussed by Krejci et al. (1941) and Warner (1944).

The principal difference between skimmilk and buttermilk casein is that the x-component becomes more prominent in both the extracted and non-extracted buttermilk caseins. This is apparent in both the ascending and descending patterns. It comprises 11.6 to 15.6 per cent of the total area and has greater mobility than the corresponding rudimentary component in skimmilk casein. In the pattern of the extracted buttermilk casein the secondary α-peak is not apparent.

The patterns of the non-extracted butterserum casein shows poor resolution of components. The area of the α-component constitutes a much smaller percentage of the total than in the patterns of skimmilk casein. The secondary α-component is prominent in both extracted and non-extracted butterserum casein. It has the same mobility as the primary α-component in skimmilk and buttermilk casein while the primary α-component in butterserum has slightly lower mobility than in skimmilk and buttermilk casein. The x-

component in the non-extracted butterserum casein is poorly resolved and tends to be a part of the α-boundary. In the descending pattern of the extracted preparation resolution is better, and the x-component shows two distinct peaks.

The differences in electrophoretic patterns of butterserum casein as compared with skimmilk and buttermilk casein are indicative of denaturation or of the presence of phospholipid-protein complexes which appear as individual components or cause abnormalities in the boundaries of the original components. The greatest change occurs in the α-fraction which, according to Mellander (1939) and Warner (1944), contains more phosphorus than the β- and γ-fractions. It is reasonable to assume that with the higher phosphorus content the α-fraction would offer the greatest opportunity for phospholipid-protein linkages and for complex formation. The pronounced differences in the patterns of extracted and non-extracted preparations may be evidence of such complex formation.

In the light of these findings the hypothesis may be presented that the non-coagulation of butterserum casein with rennet results, in part, from the changes in the α-fraction. The rennet enzyme may be unable to attack certain linkages in the α-casein because these are engaged in the phospholipid-protein complex. Previous investigators such as Nitschmann and Lehmann (1947a), Gonashvili (1949)

and Kerns (1951) has presented evidence that the α-casein primarily is involved in rennet coagulation.

b. Whey proteins. The ascending patterns of the non-extracted skimmilk whey proteins show evidence of six components. The patterns of the extracted preparations are resolved more completely and suggest a seventh component with high mobility. Component five is the largest and constitutes 59.6 to 73.0 per cent of the total area. The skimmilk whey protein patterns are similar to those presented by Stanley et al. (1951).

The non-extracted buttermilk whey proteins yield patterns with relatively poor resolution of components. This is only slightly improved in the extracted preparation. In buttermilk whey protein the peak area of component five is a smaller portion of the total than in the skimmilk whey protein.

The patterns of both extracted and non-extracted butterserum whey proteins were incomplete due to the opacity of the solutions which they yielded. On the ascending boundary only the fastest moving components were visible, and on the descending boundary only the slowest components were visible. Presumably this is due to the presence of lipids or lipid-protein complexes in the buffer, and it may be taken as further evidence that the phospholipids were not extracted.

The phenomenon is similar to that observed by Blix et al. (1941) and McFarlane (1942). Both of these investigators reported that the opacity of solutions containing lipid-protein complexes would move with the boundaries of specific components in the electrophoretic cell.

In the pattern of butterserum whey protein the opacity seems to be associated with component five to a greater extent than with the others. Likewise in the pattern of the extracted buttermilk whey protein the height of the peak of component five is lower than that of the non-extracted preparation while its mobility is only slightly less. A similar observation was made by Blix and Pedersen (1947) in the electrophoresis of human serum proteins. They noted that after extraction of the serum, the greatest decrease in peak area took place in the β-globulin fraction but that it retained its electrophoretic mobility after delipidation. They stated that the change in pattern brought about by extraction of the serum was caused by incipient denaturation or to the breaking up of lipid-protein complexes.

In the electrophoretic patterns of the buttermilk and butterserum whey proteins presented here there are differences between extracted and non-extracted preparations. This indicates that lipid-protein complexes have been broken up to some degree or that some denaturation has taken place. Such differences are not apparent in the

patterns of skimmilk whey proteins, which would suggest that rupture of lipid complexes and not denaturation might be responsible for the differences between extracted and non-extracted buttermilk whey proteins.

It may be stated that the extraction data and the electrophoretic patterns together indicate the presence of phospholipid-protein complexes in buttermilk and butterserum, that the complexes formed with whey proteins have a greater lipid content than those formed with casein and that the tenacity of the complex is likely greater among the whey proteins than among the casein fractions.

For additional work on this problem it is proposed that more drastic extractions of lipid material from the proteins in buttermilk and butterserum be attempted. Ethanol, for instance, appears to be more effective as a dehydrating agent than acetone, but also more likely to cause denaturation of the proteins. By treating the relatively lipid-free skimmilk proteins with the same solvent it should be possible to learn whether possible changes in electrophoretic patterns were due to denaturation or to extraction of lipids.

Protective material from the fat globule surface might be obtained from cream washed by the Storch or Völtz techniques. Examination of this material from the standpoint of composition, lipid extraction and electrophoretic behavior would help to establish whether the protective

materials at the fat globule surface are identical to the butterserum proteins which have been studied in the present work.

IV. SUMMARY AND CONCLUSIONS

1. Skimmilks, buttermilks and buttersera from the same whole milk sources were prepared. These products were studied with respect to (a) behavior when titrated according to the method of Ling (1936), (b) coagulability with increased rennet addition and with addition of dialyzed or concentrated or dialyzed and concentrated rennet, (c) distribution of protein fractions, (d) distribution of calcium and phosphorus and (e) composition and electrophoretic properties of the casein and whey proteins prepared from them.

2. Complete Ling titrations involving titration of oxalated and non-oxalated samples of the product and its rennet whey could not be made on butterserum because of its inability to coagulate with rennet.

3. Ling titrations made on skimmilk and buttermilk, and titration curves of these products were found comparable to those for whole milk presented by Ling (1936). Complete titrations of skimmilk and buttermilk and incomplete titrations of butterserum from the same whole milk source indicated a slightly lower colloidal phosphate content in buttermilk than in skimmilk and an appreciably lower phosphate content in butterserum.

4. Titration curves of alcohol-acetone (7:3) filtrates of skimmilk and buttermilk were found not to resemble those of oxalated and non-oxalated wheys from these products. The shape of the alcohol-acetone titration curves indicated that the calcium phosphate in these products might not be titrated in the alcohol-acetone sera until the titratable acidity of the product had reached a point corresponding approximately to the acidity at which casein precipitates isoelectrically.

5. Increased addition of rennet and the use of dialyzed, of concentrated and of dialyzed and concentrated rennet did not improve the coagulability of butterserum.

6. A series of protein analyses were made on skimmilk, buttermilk and butterserum from the same whole milks. The series was comprised of 9 runs which included milks from all seasons of the year.

It was found that skimmilk and buttermilk, on a fat-free basis, contained practically the same percentages of total protein and casein; buttermilk contained slightly more albumin and proteose-peptone and slightly less globulin than skimmilk. Butterserum contained slightly more proteose-peptone and less total protein, globulin and albumin. The latter fraction in butterserum was only 29.8 per cent of its quantity in skimmilk.

On a fat-free solids basis the albumin content in butterserum was 28.5 per cent of the quantity in skimmilk,

while total protein, casein, proteose-peptone and globulin contents were 110.8, 113.9, 164.3 and 171.4 per cent of those in skimmilk. In buttermilk, total protein and casein contents were like those in skimmilk. Albumin, proteose-peptone and globulin contents were 104.7, 86.6 and 123.9 per cent of those in skimmilk.

The significance of the low albumin content in butterserum was discussed, and the hypothesis was presented that albumin as one of the labile components of the protective material on the fat globule was removed from the fat-serum interface during churning and released in the buttermilk.

6. Total calcium and total, inorganic, acid-soluble and lipid phosphorus were determined in 4 series of skimmilk, buttermilk and butterserum from the same whole milks.

Total calcium contents of buttermilk and butterserum were 80 and 55 per cent respectively of the quantities in skimmilk.

Total phosphorus in a given product varied among runs but was lowest in buttermilk. Inorganic phosphorus in butterserum was much lower than in skimmilk and buttermilk and had a nearly constant value in all runs. Acid soluble phosphorus varied among products; in each product it was generally only slightly higher than the inorganic phosphorus. Lipid phosphorus was low in skimmilk and

buttermilk; in butterserum it was 5 to 25 times higher than in skimmilk.

If the differences in calcium and inorganic phosphate radical between skimmilk and butterserum are calculated in terms of calcium phosphates the resulting figures suggest that di- or tri- calcium phosphate or a mixture of these that are present in skimmilk are not present in butterserum. Such lack of calcium and phosphate was considered as a possible factor contributing to the non-coagulability of butterserum. It was likewise considered that the high phospholipid content might seriously influence coagulation of butterserum.

Addition of calcium ions (as $CaCl_2$) to butterserum to make its calcium concentration equal to that of skimmilk did not improve its coagulability.

7. Skimmilk, buttermilk and butterserum were prepared from two different lots of whole milk. From each of these products casein was precipitated at a pH of 4.6, and the whey proteins were prepared by lyophilization of the dialyzed wheys. Part of each protein preparation (from one lot of whole milk) was extracted with acetone and ether at -23°C. Extractions were made from water or phosphate buffer suspensions.

Phosphorus was determined in the extracted and non-extracted proteins and in the lipid residue from the ether extracts.

Insignificant quantities of lipid material were found in the ether extracts of skimmilk and buttermilk caseins and of the skimmilk whey proteins. The extracts of buttermilk whey protein contained small lipid residues (2.0 and 3.7 per cent respectively of the original protein in 2 preparations). The lipid residues from 2 butterserum caseins were 6.7 and 9.3 per cent respectively of the weight of the original protein. The lipid residues recovered from the extracts of several preparations of butterserum whey protein were from 14.0 to 25.0 per cent of the original weight of protein.

Ether extracts from acidified (pH 2.0) suspensions of butterserum casein and whey protein yielded only half the amount of lipid which could be extracted from water or phosphate buffer suspensions at pH 7.0 - 7.7.

The phosphorus content in buttermilk casein was slightly lower than in skimmilk casein. The P/N ratios in extracted skimmilk and buttermilk caseins were 0.049 and 0.046 respectively. Butterserum casein contained 11.71 per cent nitrogen when extracted (compared with 14.27 and 14.31 per cent respectively in extracted skimmilk and buttermilk casein). The phosphorus content of butterserum casein increased from 0.73 to 0.92 per cent after extraction with acetone and ether, and the P/N ratio increased from 0.067 to 0.079.

The phosphorus content in two non-extracted skimmilk whey proteins was 0.39 and 0.40 per cent respectively. Extraction did not change this value. The phosphorus content in two buttermilk whey protein preparations were 0.45 and 0.58 per cent respectively; in the latter this increased to 0.65 per cent after extraction. Two unextracted butterserum whey proteins contained 1.76 and 1.15 per cent phosphorus respectively; in the latter this increased to 1.75 per cent as a result of extraction.

The high phosphorus content of butterserum casein and whey protein indicated the presence of phospholipid-protein complexes in butterserum. The acetone-ether extractions of the protein preparations failed to remove phosphorus-containing lipids and did not verify the presence of these complexes. It was assumed that the appreciable amounts of lipid extracted from butterserum casein and whey protein constituted milk fat in submicroscopic globules which were held in suspension by the hydrated protein.

8. Electrophoretic patterns were presented of extracted and non-extracted caseins and whey proteins from skimmilk, buttermilk and butterserum from the same whole milk. The patterns of buttermilk and butterserum caseins showed evidence of components which were not apparent in the patterns of skimmilk casein.

Skimmilk whey proteins appeared to contain 6 or 7 components. Buttermilk whey proteins showed poorer resolution of components than those from skimmilk. Butterserum whey proteins yielded incomplete patterns because of an opacity associated with the major component.

The possibility of the presence of phospholipid-protein complexes in butterserum and their possible interference in the electrophoretic pattern formation was discussed.

9. The method of phosphorus determination in blood serum presented by Youngburg and Youngburg (1930) and the colorimetric methods of phosphorus determination by Dyer and Wrenshall (1938) and Fontaine (1942) were studied critically.

The phosphorus fractionation scheme of Youngburg and Youngburg was modified with respect to the technique and was applied to milk. Spectrophotometric determinations were made according to Fontaine.

Calibration curves were prepared and regression equations calculated for use with the Beckman, model DU, spectrophotometer at 740 mμ and 820 mμ and the Coleman, model 11, spectrophotometer at 740 mμ. All curves obeyed Beer's law within the concentration range studied.

It was demonstrated that it was possible to use the Coleman, model 11, spectrophotometer for the determination

of phosphorus by reading transmittances of the reduced phosphomolybdate solutions of 740 mμ with a PC-5 filter even though this was not the wavelength of maximum absorption.

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