1956

Milk serum equilibria as shown by dialysis, ultrafiltration and ultracentrifugation

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MILK SERUM EQUILIBRIA AS SHOWN BY DIALYSIS, ULTRAFILTRATION AND ULTRACENTRIFUGATION. I.

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subjects: Food Technology
Dairy Chemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Heads of Major Departments

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1956

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I. INTRODUCTION

The sum of the ionic equilibria of milk, which include caseinate if not other entities among the proteins, generally have been referred to by the term "salt balance". Numerous attempts have been made to elucidate the salt components of milk and to devise means of measuring at least cationic concentrations in natural milk, that is, to study the salt balance. This is logical, for not only is there theoretical interest in unraveling one of the most complex combinations of systems among biological fluids, but the technological aspects of the systems are of importance. For instance, it is well known that when the ratio of calcium plus magnesium to citrate plus phosphate is not "correct" condensed milk will curdle when sterilized in tin cans, coffee cream will "feather" and difficulty likely will be experienced in coagulating milk with rennin. The addition of small amounts of citrate, phosphate or bicarbonate ions, if the ratio is high, or of calcium ion, if the ratio is low, usually will correct the difficulties.

This study initially had a two-fold objective as regards the milk equilibria. The first was to study the cationic, permaselective resin membrane electrodes of Kressman (36), to determine whether or not they were either sufficiently selective for use in determining ionic concentrations in the mixed

system, milk, or could be useful if correction could be made for ionic interference. The second was to study milk dialysis, in the hope that some information could be obtained that would confirm or refute the electrode measurements.

Data that will be presented indicate the improbability of employing the cationic resin electrodes in mixed salt systems to measure ionic concentrations. This seems particularly true when attempts are made to measure divalent cations in systems containing monovalent cations in higher concentrations than those of the divalent cations.

Consequently attention was focused upon the dialysis aspects of the problem. As a result, a new dialysis apparatus and procedure, termed "continuous pressure dialysis" was developed. The use of this method, together with a recently acquired Spinco Model L Ultracentrifuge, made possible a study of the dialysis of milk and of its casein-free skim milk. Certain aspects of the dialysis study, suggested that it would be relatively easy to develope a simple ultrafilter, which was done.

As a result of these developments, the objectives of the project were expanded. In addition to the electrode aspects, the major objective became the physical separation of milk into caseinate, fat and casein-free skim milk and the ultrafiltrates of fat-free and of casein and fat-free skim milks, and the ionic distribution of the major cations and anions among these

physically-separated fractions, and between the ultrafiltrates and the dialysates of the milks.

II. REVIEW OF LITERATURE

A. Electrochemical Theory of Membrane Electrodes

Membrane electrodes consist of materials which are permeable to cations and/or anions. An example of this would be the glass electrode which is thought to be permeable to hydrogen ions and largely impermeable to all other cations and anions. Most membrane electrodes are either of the semi-permeable membrane type, e.g. collodion, the clay membrane type, e.g. Putnam clays, or the resin membrane type, e.g. polystyrene. These membrane types can be used for determining, in pure solutions, the activities of various anions or cations. Each membrane type has its own electrochemical characteristics and will behave accordingly. Marshall (50) considers there are three distinct types of membranes each of which has its own electrochemical properties.

The first type is unchanged by the ions on either side and merely assists in forming a normal liquid-liquid junction by preventing mixing by convection.

The second type acts as an ionic sieve and prevents either cations or anions from passing through. Such membranes were of interest in preventing passage of anions by mechanical constriction, since, with the exception of hydroxyl and fluorine, cations are smaller than anions. The equation for the behavior of this type of membrane is the Nernst equation. If the membrane

will permit passage of cations, but is impermeable to anions it is actually a hydrated membrane through which water molecules are likely to penetrate. It should follow that the $H^+ + OH^- \rightleftharpoons H_2O$ equilibrium, should allow some mobile ions within the membrane. If OH^- ions can transport electricity, other anions should behave similarly at the membrane surface. Such behavior should be negligible in acid solution.

The third type, which Teorell (99) and Meyer and Sievers (58) have proposed, restrains ionic mobility only by virtue of the electrical charge on the membrane. These authors consider that this action may be combined with "sieve action". They proposed that the relative mobilities of cations and anions within the membrane were assumed to be different than those in the pure solvent; the mobility ratio through the membrane of a pair of ions having equal mobilities in water, was a measure of the restrictive or sieve action. When no sieve action occurred the potential across the membrane was determined by: a. The charge on the membrane expressed as ionic activity; b. The relative mobilities of the anion and cation in water; and c. The ionic concentrations on the two sides of the membrane. The mathematical model for this type of effect is shown below.

If the concentrations of the cations and anions are C_{1c} and C_{1a}, the concentrations of ions on the two sides of the membrane are C_1 and C_2 and the mobilities of the anions and cations were U_a and U_c, the EMF across the membrane, the charge of which is balanced by monovalent cations of the same kind as

those in solution, is

$$E = \frac{RT}{nF} \cdot U \frac{\frac{\sqrt{4c_2^2 - 1}}{A^2} + U}{\frac{\sqrt{4c_1^2 - 1}}{A^2} + U} + \frac{1}{2} \cdot \left[\frac{\sqrt{4c_1^2 - 1}}{A^2} + 1\right] \left[\frac{\frac{\sqrt{4c_2^2 - 1}}{A_2} - 1}{\frac{\sqrt{4c_2^2 - 1}}{A^2} + 1}\right] \left[\frac{\sqrt{4c_1^2 - 1}}{A^2} - 1\right] \left[\frac{\sqrt{4c_2^2 - 1}}{A^2} + 1\right]$$

in which $U = \frac{U_c - U_a}{U_c + U_a}$ and A = ionic activity of the membrane.

If a ratio of concentration, $C_1 : C_2$, of 1 : 10 were chosen, E becomes a function of U and of C_1/A. If values were assigned to U_c/U_a, E can be expressed as a function of A/C_1 or as log A/C_1 and a family of curves can be plotted. The shape of the curve which is identical with the experimental curve defines U_c/U_a. The experimental curve can be used to measure the effective ionic charge, A, on the membrane and also the value for U_c/U_a which for corresponding water solutions of the ions gives a measure of the sieve action.

Kressman (36) using cationic resin membrane electrodes of the sulfonated polystyrene type found that, when measuring ionic activities of various solutions they obeyed the Nernst equation, $E = \frac{RT}{nF} \log \frac{A_2}{A_1}$, where A_2 and A_1 are the activities of the cations on the two sides of the membrane.

All work done in this thesis was done with the type which

Kressman (36) used. Thus, all measurements were in agreement with the Nernst equation.

B. Determination of Ionic Calcium and Magnesium in Milk by Resin Contact Methods

Several authors have attempted to study the amount of ionizable calcium and magnesium in milk by bringing milk into contact with a cationic resin. Contact time and conditioning of the resin in buffer solutions was adjusted, presumably to prevent disturbing the equilibrium between the ionizable and other forms of calcium and magnesium in milk. The ions bound to the resin then were eluted and determined.

Gehrke and Almy (28) studied the effect of mineral ion exchange resins on milk constituents. They studied the exchange characteristics of pure solution, effect of one ion on exchange of another, adsorption of lactose at various pH levels, effect of non-protein nitrogen on ion exchange and the exchange of ions from cheddar cheese whey with Zeo-Karb-H, DeAcidite and Amberlite IR-4B resins. They found that calcium and magnesium affinity was greater than that of sodium and potassium with the Amberlite resin. Zeo-Karb-H exchanged non-protein nitrogenous substances but DeAcidite did not. When the three resins were tested for lactose adsorption between pH levels of 6.0 to 8.5 none were found to have an affinity for lactose.

Baker et al. (6) used Amberlite IRC-50 and IR-4B cationic

and anionic resins, respectively, to study the exchange of ionizable calcium and magnesium in skim milk. The resin, placed in the potassium form with a potassium buffer, was allowed to contact the milk for various lengths of time. The cations were eluted from the exposed resins. Their findings indicated that 36 per cent of the total calcium in milk were removed in 10 sec. The rate of removal of calcium from 10 to 180 sec. was constant and was presumed to be the removal of dissolved salts or the conversion of dissolved salts to the ionic state. From 180 to 300 sec. the removal rate was constant and was attributed to an exchange of colloidal calcium phosphates to the dissolved calcium phosphates. When phosphorous was exchanged, 44 per cent was removed in 10 sec., from 44 per cent to 65-68 per cent removal the rate of exchange was slower than that during the first 10 sec., and beyond 65-68 per cent the rate became constant. This indicated that one third of the phosphorous in milk was bound organically and not available as ions.

Christianson et al. (17) studied the ionizable calcium and magnesium in relatively the same manner as Baker et al. (6) except that these workers used constant levels of sodium and potassium to condition their resin and varied the calcium content. The solutions of varying calcium concentrations then were mixed with the resin and allowed to equilibrate. From these standard calcium solutions ionic concentrations of exchangeable calcium were determined after elution of calcium

from the resin; the exchanged calcium was plotted against calcium concentration of the solutions employed in preparing a standard curve. Samples of skim milk treated in the same manner gave levels of exchanged calcium from 4.0-4.5 mg. per 100 ml. of milk. Christiansen et al. (18), using the same method as above, studied the ionizable calcium and magnesium and found them to be 2.0-2.3 and 0.82-0.85 millimol., respectively, per liter. They also studied the effect of pH, of addition of citrate and heating of milk. As the pH decreased the amount of calcium exchange increased. Addition of sodium citrate reduced the exchange of calcium more than did sodium chloride, suggesting that sodium ions lower exchange of calcium to a lesser degree than complexing it by citrate. Heating milk, prior to exposure to the resin, decreased the availability of calcium and, slightly, that of magnesium. Storage subsequent to heating, caused a reversal in that more calcium was free to react with the resin.

van Kreveld and van Minnen (103) studied the amount of ionizable calcium and magnesium in milk with a Duolite C-20 resin. They treated the resin with a salt solution iso-ionic with milk. Their data indicated that from 4.0-4.6 millinormal calcium and 1.4 to 1.7 millinormal magnesium were in the ionic state. These data agreed well with those of Smeets (85) and Seekles and Smeets (84). Seekles and Smeets (84) did not use a resin contact method as did the other workers but complexed the calcium and magnesium from milk ultrafiltrate with

ammonium purpurate or murexide.

Results obtained for calcium and magnesium activity, when milks were heated, indicated that heating caused a reduction in activity of both calcium and magnesium ions and that as the period of heating increased the activity decreased (18, 84, 103). Condensing milk on the other hand was found to increase the activity of calcium and magnesium. Sterilization, subsequent to condensing, caused a decrease in ionic activity (103).

C. Studies of Membranes of Different Types

Considerable data have been reported relative to various types of membranes and their physical properties. Types which have been widely studied include collodion, mineral, resin-collodion and artificial resin membranes. Little work has been done on the application of these types to measuring ionic concentrations in biological fluids. However, if this could be done easily and simply it would be of great value both clinically and for manufacturing purposes in the Dairy Industry.

1. Collodion membranes

a. Diffusion of ions through collodion membranes. Loeb (42) first studied the osmotic and diffusion effects of electrolytes and non-electrolytes through pure collodion membranes. He concluded that the rate of diffusion of non-electrolytes was directly proportional to the concentration. Electrolytes were found to behave similarly except that the

diffusion rate was greater at the higher concentrations and diffusion continued to a lower concentration than with non-electrolytes. Loeb (43) considered that the rate of diffusion depended upon the sign, valency and radius of the ions in addition to their concentration gradient. When membranes were treated with various agents, such as gelatin, casein and egg albumin, water diffused more rapidly into the salt solution than when untreated collodion membranes were used. This difference in diffusion rates for water when mono-, di- and trivalent ions were present, resulted not from differences in permeability but to the negative charge which the water displayed in presence of the high positive ionic charges. When a collodion membrane separated water and sodium gelatinate solution, the water diffused into the gelatinate solution at a definite rate but when alkalis or salts of varying valencies were added to the gelatinate solution the rate of water migration diminished (44).

Loeb (45, 46) also established that the rate of diffusion of electrolytes through a membrane was dependent upon the valency of the ion, because as the valency of the ion increased the water boundary layer became more negative. The rate of water diffusion through the membrane was accelerated by ions having large positive charges but the rate was dependent upon the concentration of the ions. When Loeb (47, 48) studied the effect of reversal of sign of the collodion membrane, he employed hydrogen and trivalent cations. Membranes were treated

with protein as before. Reversal of sign of the membrane by varying the hydrogen ion concentration varied with the isoelectric point of the protein with which the collodion was treated. Ordinarily, the pH at which the reversal of the sign of the membrane occurred was slightly higher than that of the isoelectric point of the protein. Trivalent ions caused a protein treated membrane to be charged positively as did the hydrogen ion. However, low pH caused the protein to be converted to a protein salt of the acid employed, which was capable of ionizing into a positive protein ion and the anion of the acid; reversal of sign of the protein by trivalent cations on the alkaline side of the isoelectric point of the protein caused the formation of free carboxyl groups. The reversal of sign was due to the formation of non-ionizable metal proteinate salts.

Studies of the permeability of membranes with pure solutions of sodium, potassium, lithium and hydrogen chlorides yielded theoretical potentials, calculated from Nernst equation, when measured across the collodion membranes in the ratio of 0.1/0.01 M. (60). Monovalent anions were found to diffuse at a slower rate than monovalent cations.

Transference numbers, $t = 0.5 \frac{E}{116 \log \frac{C_2}{C_1}}$, a measure of diffusion, were calculated for various monovalent ion solutions; these showed that, when the solutions were dilute, the transference numbers were small but increased as the concentration

gradient increased (59). Such measurements were found to correlate with the mobilities of the cations and anions within the membrane (61, 62). By use of this technique a correlation was established between the membrane pore size and the diameter of various molecules. These workers postulated, on the basis of their data, that 98 per cent of the pores were large enough to pass acetone while only 0.3 per cent would pass glucose (107).

b. Electrical behavior of collodion membranes. Data discussed this far, with regard to collodion membranes, concerned the diffusion of ions but did not consider their electrical characterization under varied conditions. This section concerns the electrical behavior of collodion membranes.

Sollner et al. (86) measured concentration potentials across various commercial collodion membranes with 0.1 and 0.01 M. potassium chloride solutions. They indicated that the electrochemical activity of the membranes depended directly upon the amount of acidic impurities in the collodion. When the collodion was subjected to oxidation by 1.0 M. sodium hydroxide, concentration potentials were uniform and approached the theoretical values calculated by the Nernst equation (87). Base exchange studies were conducted with collodion. The membrane was saturated with hydrogen ion and was placed in contact with a neutral salt solution, the cations of which replaced the hydrogen ion, which then was titrated. There was no correlation evidence between replacement of hydrogen ion by

other cations and the electrochemical activity of the membrane (93). Electrochemical activity was found to be dependent upon the number of dissociable groups on the membrane (88).

In order to determine whether collodion membranes were a homogenous phase or a micellar-structure phase Sollner and Carr (90) oxidized, redissolved and recast the collodion. It was indicated that concentration potentials of recast collodion membranes were lower than those of oxidized membranes. These results suggested that the membranes were of micellar- rather than homogenous-structure. Studies of the effects of the thickness of membranes indicated that concentration potentials were dependent upon the thickness of the membrane which indicated a micellar-structure as the true state of the membrane (91).

Studies of water uptake and swelling of collodion in water solutions of organic and inorganic electrolytes and non-electrolytes, indicated that in inorganic electrolyte solutions, 60-70 per cent of the water was taken up in swelling; the remainder entered intramicellar and intermicellar cavities. In water solutions of organic electrolytes and non-electrolytes, collodion swelling depended upon the solute, e.g. glycerine, glucose, citric acid (13, 89).

Special membranes prepared for study were those having high ionic selectivity, high permeability, low resistance and good mechanical properties (1, 12, 14). Some membranes were prepared which contained protamine sulfate (12, 95). Those membranes

containing the protamine yielded low resistance, low ionic selectivity and high permeability. Ionic selectivity and permeability of non-protamine-containing membranes was dependent entirely upon the time of oxidation by 1 M. sodium hydroxide. The highest selectivity, permeability and mechanical strength of the membranes was obtained when an oxidation time of 6 to 7 min. was used. Longer oxidation periods resulted in weaker membranes and shorter oxidation period yielded membranes with low selectivity and permeability. Membranes prepared as indicated above did not obey the Teorell and Meyer-Sievers theory of membrane behavior (92). However, both membrane types rapidly established their concentration potentials across 0.1 and 0.01 M. potassium chloride solutions, showing that they have permeability (96, 97).

c. Studies of ion binding by proteins using collodion membranes. Studies using protamine collodion membranes were made concerning the binding of calcium, magnesium, sodium, and potassium by various proteins (8, 9, 10, 11, 15, 16, 97). Measurements were made by immersing the membrane containing the protein plus ion into a known volume of water and titrating the water with a standard pure ion solution, usually 10 times greater than that within the membrane. The titrations were followed electrometrically until the voltage across the membrane was zero. At this point the concentration inside the membrane was considered equal to that outside and the

concentration and activities of the ions within the membrane were calculated.

Carr and Topol (15) studied the activities of sodium and chloride ions in protein solutions using a protamine collodion membrane. Their findings indicated that negative membranes were useful from pH 3.0 to 9.5. During the course of these investigations it was found that chloride ion activity was not affected by either gelatin or casein in the pH range employed. However, sodium was bound to casein at pH values above 7.0. At pH 7.5 it was found that 0.18 to 0.20 meq. of sodium was bound per gram of casein but that sodium was not bound by gelatin. Further findings indicated that the amount of sodium bound was directly proportional to the amount of casein present and that this binding was lowered when the pH was alkaline.

Carr (8) found that there was no difference in binding of chloride ion by bovine or human serum albumin below pH 7.0; above pH 7.0 there was difference. Using other proteins, Carr (10) found that proteins, with isoelectric points between pH 1.0 and 3.0, would not bind chloride ions in the pH range of 3.0 to 7.0, while those with high isoelectric points (8.5 to 11.0) showed affinity for chloride between pH values of 3.0 and 7.0. There was no direct correlation between anion binding and isoelectric point of the protein, in the pH range 4.0 to 10.0.

Binding of calcium by various proteins was studied using protamine collodion membranes (9, 11). When the binding of

bovine serum albumin was studied at physiological pH (7.4) over concentration ranges of 1 to 50 millimol. of calcium per liter, with solutions containing 1 to 3.3 per cent protein, it was found that 8 calcium ions were bound per protein molecule at the higher calcium concentrations. Binding of calcium ions per molecule was determined by assuming an average molecular weight and calculating the amount of calcium bound per molecule. It was found that as the pH increased above the isoelectric point of serum albumin there was a large increase in calcium binding by this protein. Within the pH range of 6.7 to 7.6 there was a 75 per cent increase in calcium binding. Carr (11) found that calcium binding by 0.8 per cent casein solutions increased from a low of 0.01 millimol. per liter at pH 5.6 to a high of 1.49 millimol. per liter at 10.6. With other proteins, e.g. lysozyme, gamma globulin and hemoglobin, there was no correlation between calcium binding and isoelectric points. For instance, lysozyme (I.P. 10.6) bound more calcium than gamma globulin (I.P. 6.5) but hemoglobin (I.P. 6.8) bound less calcium than gamma globulin. However, it generally was found that calcium binding among proteins was greater for those proteins which had low isoelectric points (1.0 to 3.0); among those with high isoelectric points (8.5 to 11.0) there was little difference in calcium binding.

Carr and Woods (16) found that at pH 7.4 the amount of magnesium bound by various proteins was the same as the amount of calcium bound. It was further found that binding of magnesium

increased as the pH decreased and increased as the concentration of magnesium increased until the ion concentration (depending upon the protein) was 6 to 10 millimols. of magnesium per liter.

2. Mineral membranes

Mineral membranes were prepared by grinding and casting various clays in the form of disks from 0.1 to 0.5 mm. thick (52, 53, 54, 55, 56, 57). These membranes were heated, mounted and tested for mechanical leaks.

Activities of solutions of sodium, potassium, ammonium, calcium and magnesium chlorides were measured by use of saturated calomel half cells, salt agar bridges and a Leeds-Northrop potentiometer. Potassium activities could not be measured with any degree of reproducibility with Montmorillonite membranes when the concentration was below 0.1 M. (53, 54). However, Bentonite membranes could be used to measure ammonium ion activities when the concentrations were below 0.1 M. (55). Marshall and Krinbill (57) also determined the activities of sodium ion solutions using Beidellite membranes. These membranes were sensitive and theoretical potentials were observed when the concentration of sodium was as low as 0.03 M.

Calcium and magnesium activities were measured with Putnam membranes; the results did not agree with those calculated by the Nernst equation (51, 52, 56, 64). These membranes were found non-sensitive to hydrogen ions and divalent ions but were sensitive to monovalent ions other than hydrogen.

Mineral membranes, in general, were found to be sensitive to systems involving monovalent ions but were quite erratic in a mixture of mono- and di-valent ions. However, mixtures of divalent ions were found to behave like systems of monovalent ions (51).

3. Resin membranes

Wyllie and Patnode (110) developed membranes from artificial cationic-exchange resins by molding resins at 2500 p.s.i.g. and 150° C. and used them to measure sodium ion activities. These workers found that a membrane thickness from 0.5 to 5.0 mm. did not affect the potentials measured across them by saturated calomel half cells. When 60 per cent Amberlite IR-100 was incorporated into the membrane together with methylmethacrylate and polystyrene, immediate potential readins were obtained. However, when 20 and 50 per cent resin concentrations were used, constant potentials were obtained after 30 days and 20 min., respectively. The structure of these membranes were found to be similar to the porous or sieve structure of collodion membranes.

Affsprung et al. (2) prepared membranes using Amberlite IR-120 and Dowex 50 by the molding process. When these membranes were studied in solutions of mono- and di-valent ions it was found that theoretical Nernst potentials were obtained.

Kressman (35, 36) studied permaselective resin rod membranes of the polysulfonated styrene type. Membranes of this

type were put into a cationic form, e.g. sodium, potassium, ammonium, were mounted and the potentials were measured by means of saturated calomel half cells and a Leeds-Northrop potentiometer. Kressman (36) found that ionic activities of monovalent ions could be determined when the concentration gradient across the membrane was 0.1/0.01 M. for all salts studied. Theoretical Nernst potentials were obtained for both mono- and di-valent ions in the pH range of 5.0 to 9.0. However, when ion mixtures were used, corrections had to be made for the interferring ion effect.

D. Dialysis and Ultrafiltration of Milk

Dialysis and ultrafiltration have been used for years as a tool in the purification and concentration of protein solutions. There are, however, but few studies of the dialyzable and ultrafilterable constituents of the various biological fluids. The following summary applies only to milk.

Saito (80) dialyzed milk using Pergament Paper; the paper membrane was placed in a beaker and the milk placed within it. The space between the membrane and the beaker was filled with distilled water and the beaker rotated constantly. A stirrer was placed in the milk which was agitated for 5 hrs. at 50 r.p.m. Saito was primarily interested in the dialyzable nitrogen fractions of milk. He found 2.24 per cent of the total nitrogen in milk dialyzable; this dialyzed component yielded a strong ninhydrin reaction but negative Biuret test,

indicating it was not a polypeptide but an amino acid. When milk samples were dialyzed after heating at 100° C. for various lengths of time, the amount of total and ammonia nitrogen which were dialyzable increased as the time of heating increased. Saito (81) found that when milk was treated with ultra violet light, prior to dialysis, no increase was noted in total dialyzable nitrogen. However, when milk was treated with rennin, the amount of total dialyzable nitrogen decreased. This was attributed to partial adsorption of dialyzable nitrogen by the coagulum.

When Saito (81) dialyzed milks which had been innoculated with various acid producing organisms, the amount of dialyzable amino and ammonia nitrogen increased rapidly.

Lampitt and Bushill (37) studied the distribution of phosphorous in milk by static and continuous dialysis and by ultrafiltration. Static dialysis consisted of immersing 25 ml. of distilled water, in a collodion membrane, in 900 ml. of milk at 0-5° C. for 2 days. Ultrafiltration was carried out at 5° C. by pulling a 70 cm. Hg vacuum on a collodion membrane; the yield was 2.5 ml. of filtrate per hour. Continuous dialysis of milk was accomplished by placing 20 ml. of milk in a collodion tube, which was inserted into a tube of water surrounded by a brine (5° C.) jacket. Distilled water passed between the jacketed tube and the membrane and was conducted to a still in which it was evaporated under 70 cm. Hg vacuum. The vapors from the still passed to a condenser and

the condensate returned to the dialysis water. Milk inside the membrane was stirred by a mechanical stirrer. Water was added to the milk to maintain a constant level inside the membrane.

Static dialysis and ultrafiltration methods were compared on samples of milk held for 8 days at 4° C. Results of the comparison indicated that the concentration of phosphorous (both total and inorganic) were the same in the evaporated dialysate and the ultrafiltrate. However, when static and continuous dialysis procedures were studied, results indicated that more inorganic phosphorous was dialyzed by the continuous method than by the static method. This was attributed to disturbing the salt equilibrium by the dilution which occurred in the continuous method, causing increased solubility of inorganic phosphorous (presumably calcium-phosphorous complex). However, no difference was noted in the amount of organic phosphorous left in the dialysate residue by either method.

Results from the static dialysis procedure indicated that 46 per cent of the total phosphorous were dialyzable; while 60 per cent were dialyzable by the continuous method.

Comparison of results from static dialysis and ultrafiltration showed that 46 and 40 per cent, respectively, of the total phosphorous was found in the dialysates and ultrafiltrates. Results obtained by Norbö (66) substantiated the above data concerning the ultrafilterable phosphorous.

Lampitt and Bushill (38) further studied the distribution of calcium and phosphorous in milk by static dialysis. Twenty-

five to thirty-three per cent of the calcium was dialyzable; 33-41 per cent of the inorganic phosphorous was dialyzable.

The effects of dilution, pH and heating on the dialyzable calcium and phosphorous were studied by the static dialysis technique (38). Dilution of milk (1:2) caused an increase of 12 per cent dialyzable calcium and 5-7 per cent dialyzable inorganic phosphorous. When pH was lowered from 6.55 to 5.75 an increase of 11 and 17 per cent in dialyzable inorganic phosphorous and calcium, respectively, was noted. However, this change in pH (6.55 to 5.75) did not cause the organic phosphorous to dialyze. Heating caused a decrease in the dialyzable calcium and the inorganic phosphorous of about 4 per cent.

Lampitt et al. (39) studied the dialyzable calcium, magnesium and phosphorous in normal milk and found that 62-83 per cent of the total magnesium in milk was dialyzable. These authors also studied the effect of agitation on the dialysis of milk. Their results showed that agitation reduced the dialyzable magnesium and calcium and increased the dialyzable inorganic phosphorous.

These authors (39) indicated that as the acidity of milk increased, the dialyzable organic phosphorous increased and became maximal at 0.25 per cent acid; dialyzable calcium, magnesium and inorganic phosphorous increased steadily as the per cent acid increased (limits not given). Ling (40) reported that the entire quantity of tricalcium phosphate of milk

disappears when the milk reaches 0.68 per cent acidity and that the total increase in phosphorous in the whey is equivalent to the decrease in tricalcium phosphate.

Some workers dialyzed milk against solvents other than water in order to determine the amounts of dialyzable and soluble inorganic constituents. Rona and Michaelis (78) dialyzed 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.

Pyne (69) dialyzed milk against rennet whey and estimated that 56 per cent of the inorganic phosphorous and 34 per cent of the total calcium in fresh milk (pH 6.6-6.65) was soluble. However, at a pH value of 5.28 the solubilities of calcium and phosphorous were 88.3 and 99.3 per cent, respectively. Verma and Sommer (106), dialyzing milk against rennet wheys, found 35 per cent of the total calcium, 8 per cent of the total magnesium, 37 per cent of the total phosphorous and 85 per cent of the citric acid to exist in the soluble form.

György (31) dialyzed milk against rennet whey and found that the undialyzable phosphorous amounted to 50-60 per cent of the total and that an increase in acidity caused an increase in the dialyzable calcium and phosphorous. When casein was at the isoelectric point all the calcium and phosphorous was dissolved in the whey. He concluded that the casein and

the undialyzable dicalcium phosphate showed chemical affinity for one another.

Bell (7) obtained ultrafiltrates from heated milks and found a decrease in soluble or filterable calcium and phosphorous. He attributed this decrease to the fact that calcium and phosphorous go from the soluble to the insoluble state by forming an insoluble salt-protein compound. Centrifugal studies at 38,000 to 40,000 r.p.m. on heated milks showed that there was an increase in the amounts of calcium and phosphorous found in the portions thrown out. He also found that the amount of calcium and phosphorous centrifuged from skim milk was greater the higher the heat treatment.

Magee and Harvey (49) dialyzed fresh, pasteurized and boiled milks in collodion membranes against cold running water for various lengths of time and then analyzed the contents of the membrane. There were 26.4, 20.4 and 15.7 per cent of the calcium diffusible from fresh, pasteurized and boiled milks, respectively. They attributed the decrease in diffusible calcium to the formation of colloidal tricalcium phosphate from soluble dicalcium phosphate.

E. The State of Calcium, Phosphorous and Casein in Milk

Investigations concerning the state of calcium, phosphorous and casein in milk are of importance in manufacturing problems. The principle regions of interest are the distribution of calcium and phosphorous in the soluble and colloidal

state and the association between calcium, phosphate and casein.

The distribution of calcium and phosphorous in the soluble state were considered in the preceding section.

Pyne (68, 70, 72) considered that the colloidal calcium in milk existed as a tricalcium phosphate which in turn formed a chemical bond with casein. His premis was based upon the difference in formol titration values of milk with and without added potassium oxalate. He also investigated the electrometric titration curves of tricalcium phosphate and tricalcium phosphate plus casein over the pH range of 7.0 to 10.0. In later work Pyne and Ryan (73) using a modified Ling (40) titration with potassium oxalate and excessive phenolphthalein concluded that 88 per cent of the calcium existed as tricalcium phosphate. Porcher and Chevallier (67) had postulated that both di- and tri-calcium phosphate existed in milk.

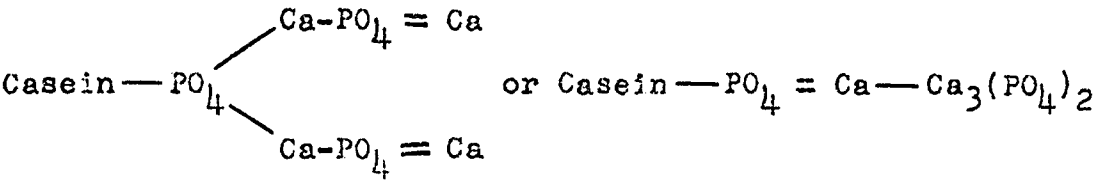
Van Slyke and Bosworth (105) considered the natural acidity of milk resulted from colloidal dicalcium phosphate. They considered that it was maintained in the colloidal state by a protective colloid action of casein. When dicalcium phosphate was titrated with calcium hydroxide these workers postulated that the dicalcium phosphate was converted to tricalcium phosphate.

Ling (40, 41) titrated oxalated and non-oxalated milk and rennet whey in order to estimate the amount of tricalcium phosphate in milk. His findings indicated that nearly one half of

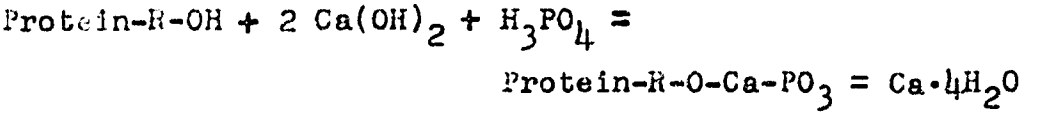
the total inorganic phosphate in milk existed as colloidal tricalcium phosphate. As the acidity in milk increased the casein "acidity" increased proportionally to the calcium removed. Furthermore, as the total calcium content of milk changed with season the amount of calcium-caseinate complex varied while the colloidal tricalcium phosphate remained constant. He interpreted this to indicate that the formation of tricalcium phosphate took precedence over that of calcium-caseinate. On the basis of this, he concluded that there was no chemical union between tricalcium phosphate and casein. ter Horst (100) criticized Ling's (41) work and agreed with Pyne and Ryan (73) that the error in the original Ling titration of milk and rennet whey resulted from titrating to phenolphthalein end points which corresponded to different pH values. Such an error indicated the presence of more tricalcium phosphate than was actually present as a result of the titration of some dicalcium phosphate in the Ling (40) method.

de Kadt and van Minnen (20) separated casein from milk by ultracentrifugation. They concluded that only calcium and phosphate were bound to casein on the basis of their analyses of casein, whey and of the original milk. Furthermore, they proposed that phosphate and calcium were bound to casein through an ester type linkage. Analyses showed that the calcium to phosphorous ionic ratio was 3 : 2, after subtracting the amount of ester linked calcium; this is the same as for tricalcium

phosphate. On the basis of their studies these workers proposed the following complex between tricalcium phosphate and casein.



Eilers et al. (23) titrated phosphoric acid with calcium hydroxide in the presence of casein between pH values of 4.0 and 9.0; they postulated the following form for the calcium-caseinate complex.



Ramsdell and Whittier (75) supercentrifuged the calcium-caseinate-phosphate complex from milk. They found that after titration of the complex with potassium oxalate an increase in acidity occurred. From this reaction it was concluded that tricalcium phosphate rather than dicalcium phosphate was the molecule attached to casein. The isolated complex contained 95.2 per cent calcium-caseinate and 4.8 per cent tricalcium phosphate which was distributed as 0.742 per cent organic and 0.985 per cent inorganic phosphorous. The casein contained 0.789 per cent organic phosphorous. Ford and Ramsdell (26) and Ford et al. (27) centrifuged skim milk at various speeds up to 48,000 r.p.m. and collected the serum and

casein colloids removed. Upon analysis of the original milk and casein free serums it was found that the calcium to phosphate ratios were 1.40 or close to that of tricalcium phosphate. Deposits obtained from centrifuging at various speeds, when analyzed, showed calcium to phosphorous ratios of 1.54, again close to that of tricalcium phosphate. They concluded that 70-90 per cent of the casein appeared as a single phosphoprotein which was considered to be a mixture of α-, β- and γ-casein. Over the range of speeds studied these authors considered the phosphoprotein to be combined with calcium and tricalcium phosphate. Larger protein particles removed at low speeds were found to have less organic phosphorous and some nonprotein substances while those obtained at higher speeds had a higher organic phosphorous content.

ter Horst (101), using an ultracentrifuge, fractionated the calcium-caseinate complex and found that the various fractions had different calcium combining capacities and isoelectric points. She also studied the ion adsorption of casein and found that casein could combine with considerable quantities of sodium and chloride even in presence of large excesses of calcium.

Van der Burg (102), employing yeast suspensions, adsorbed the calcium phosphate on the cell surfaces during the heating of milk. He showed that heating of yeast suspension in milk caused a 21 per cent decrease in the colloidal tricalcium phosphate associated with milk. There was only a very slight change

in the amount of calcium appearing as the ester phosphate of casein. He concluded that the calcium in casein was attached to the phosphate ester groups and that the remaining free amino and carboxyl groups tied up tricalcium phosphate in varying amounts. ter Horst (100) believes that this hypotheses approaches more nearly the actual state of the calcium-caseinate complex than any of the other postulations. Pyne (71) studied the behavior of barium analogs of phosphates, polarographically, in casein solutions and substantiated Van der Burg's (102) hypothesis on the heat sensitivity of, and the type of union between caseinate and phosphate complex. Evenhuis and de Vries (24) disagree with Van der Burg's (102) premis that tricalcium phosphate is adsorbed to the yeast cell upon heating. They postulate that it is not an adsorption but a crystallization process. They demonstrated this by using cotton wool instead of yeast cells. As much tricalcium phosphate was crystallized on the cotton wool as was adsorbed on the yeast cells. Thus, they propose that the influence in the crystallization of tricalcium phosphate, when milk is heated, resulted from a difference in the rate of tricalcium phosphate (present as colloidal phosphate) solublization and the rate of precipitation of this tricalcium phosphate which crystallized on the yeast cell surface.

Edmondson and Tarrassuk (21, 22) studied the effect of heat and addition of disodium hydrogen phosphate on the distribution of calcium, phosphorous and nitrogen in the fractions

obtained after centrifuging at 20,000 r.p.m. for various lengths of time. Heating of skim milk caused an increase in total sedimented casein during 40 min.; beyond 40 min. a reversal occurred. This reversal was attributed to a shift in size of the casein complex and to some settling of denatured serum proteins. Addition of disodium hydrogen phosphate caused a decrease in caseinate complex sedimentation. Heating and addition of disodium hydrogen phosphate to milk samples, followed by centrifugation gave increases in Ca:N and P:N ratios for the sediment. From the sedimentation data, a molar ratio of Ca:P of 1.5 (the same as that of tricalcium phosphate) was calculated for raw skim milk. For milks with added disodium hydrogen phosphate but without heat the same molar ratio was found to be 1.25. However, calcium in the serum phase was decreased to about 1/3 of its original value by adding 0.15 per cent disodium hydrogen phosphate.

F. Mineral Constituents in Milk

Large numbers of analyses have been made of the mineral content of milk. There is considerable variation among the values reported. The main causes of variation are probably the difference in location, breed and feeds used. Probably considerable variation results from the methods of analysis employed. More recent analyses may be more accurate because of improvement in methods and techniques.

Table 1 presents some of the values reported for the

Table 1. Reported values for the mineral constituents of normal fluid milk.

Worker	mg. per 100 ml. of milk					
	Sodium	Potassium	Chloride	Calcium	Magnesium	Total Phosphorous
Van Slyke and Bosworth (105)	57.0	124.0	78.0	136.0	12.5	65.2[a]
Hess et al. (32)	39.7	127.5	76.0	103.3	4.24	76.1[b]
Whittier (108)	57.0	124.0	-	127.0	8.0	62.0[a]
Roadhouse and Koestler (76)	46.8	161.2	85.3	113.0	6.64	87.2[b]
Robinson et al. (77)	-	-	-	123.0	-	-
Keirs and Speck (34)	46.6	138.7	-	118.9	-	-
Davis and MacDonald (19)	41.5	143.5	106.0	136.0	12.1	96.2[b]
Sommer (98)	58.4	151.8	110.0	149.0	-	93.0[b]

[a]Inorganic Phosphorous.
[b]Total Phosphorous.

mineral constituents of normal fluid milk.

The values reported by these authors are average values which were determined in their particular locality. Considerable variation is evident for most of the important constituents of milk.

III. MATERIALS AND METHODS

A. Materials

1. Milk

The raw whole milk used was bulk mixed milk including various breeds, but was from predominately Holstein, Guernsey and Jersey cattle. The majority of the samples obtained was from milk held in raw milk storage tanks, in the College Market Milk Department.

2. Water

Distilled water was used in all experiments. For the electrode measurements, for all standard salt solutions and for the ionic analyses, the water was distilled a second time in a Pyrex apparatus, equipped with a condenser having a clear quartz inner tube. Prior to the second distillation the water was treated with a sulfuric acid potassium dichromate mixture.

3. Reagents

All chemicals used were of reagent grade or of special high grades.

Reference phosphorous solutions were made from a sample of potassium dihydrogen phosphate obtained from the National

Bureau of Standards (Sample No. 186-I-a).

All standard salt solutions were made from reagent grade chemicals which had been recrystallized three times from redistilled water.

4. Membranes

Cellulose Visking tubing used was purchased from the Visking Corporation, Chicago, Illinois.

Cation membrane electrode measurements were made with Zerolit 315 cation exchange membrane rod furnished by United Water Softeners Co., Gunnersbury Ave., London W4, England.

B. Methods

1. Calcium and magnesium determinations

Calcium and magnesium were determined by the method of Jenness (33) as follows: weigh 10 g. of milk into a 100 ml. volumetric flask, dilute with 20 ml. of distilled water, add 2 ml. of 1 N. hydrochloric acid (to dissolve calcium salts and disperse casein), agitate by gently swirling the solution, and after 10 min. add 2.5 ml. of 0.5 N. sodium hydroxide (to bring pH to 4.0 to 4.1 and precipitate isoelectric casein). Make the contents of the flask to 100 ml., mix thoroughly and filter discarding the first 5-10 ml. Pass

10 ml. aliquots of filtrate through a Duolite A-4[1] column (for removal of phosphorous) and follow each aliquot by two 10 ml. portions of distilled water to rinse the column. Collect the aliquot (10 ml.) plus the 20 ml. of rinse water (chromatographed aliquot) in a 125 ml. erlenmeyer flask.

Calcium determination. Add sufficient 1.5 N. sodium hydroxide (usually 2 ml.) to bring the chromatographed aliquot to a pH above 10, add 0.2 g. murexide (ammonium purpurate) indicator (0.2 g. ammonium purpurate plus 100 g. sodium chloride, intimately mixed) and titrate to a purple color from pink with standard EDTA[2].

Calcium and magnesium determination. Add 0.5 ml. of borate buffer (4.0 g. sodium tetraborate in 80 ml. distilled water) to a chromatographed aliquot plus washings, bring the pH to 8-10 with 1.5 N. sodium hydroxide (usually 1-2 drops),

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[1]Duolite A-4 (Chemical Process Co., Redwood City, California) resin column. Place 3 g. of resin on a glass wool plug in a 7 x 250 mm. column with a 15 x 100 mm. reservoir on top and a 1 x 25 mm. capillary tip on the bottom to which polyethylene tubing and a screw clamp were attached. Back wash with distilled water to stratify the resin and to remove air, and convert the resin to its exchange cycle by passing, two 50 ml. portions of 1 N. sodium acetate through the column. Rinse with ten 10 ml. portions or distilled water. Regenerate after four aliquots (10 ml.) of de-proteinized milk have been treated (33).

[2]Make 4 g. disodium dihydrogen ethylenediamine tetraacetate dihydrate and 1 g. sodium hydroxide to 1 L. with water. Standardize against standard calcium carbonate, 1 mg. per ml. (1.0 g. triple precipitated and dried calcium carbonate plus 2 ml. concentrated hydrochloric acid, made to 1 L.) (33).

add 5 drops Eriochrome Black T (1 g. indicator plus 30 ml. distilled water containing 1 ml. 1 N. sodium carbonate made to 100 ml. with isopropyl alcohol) and titrate from pink to blue with standard EDTA.

2. Phosphorous determination

Total inorganic phosphorous was determined by the method suggested by Fontaine (25) and Graham and Kay (30) for milk.

Weigh 0.15 to 0.25 g. of milk into a 10 ml. glass stoppered volumetric flask, add 1.5 ml. of 15 per cent trichloroacetic acid, make to volume, mix and filter from precipitated proteins. Discard the first 4 ml. of filtrate and transfer 1 ml. aliquots of the remaining filtrate to 25 ml. amber g.s. flasks, add sufficient 10 N. sulfuric acid to make the total volume 5 ml. and add 2.5 ml. of 7.5 per cent sodium molybdate and enough distilled water to make to 20-22 ml. Add 2.5 ml. of dilute stannous chloride[1], mix and place the stoppered flasks in a boiling water bath for 20 min. to develop the blue color of reduced phosphomolybdate. Cool to room temperature, make to volume and read at 820 mμ in a Beckman DU spectrophotometer (corex cuvettes). Run a reagent

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[1]Dilute stannous chloride solution was prepared by diluting 1 ml. of a solution of 10 g. stannous chloride in 25 ml. of concentrated hydrochloric acid, to 200 ml. The dilute solution was not stable and should be prepared fresh every 8-10 days (25).

blank with each group of determinations. Phosphorous was calculated by a regression equation established with samples of known phosphorous concentration.

3. Titratable acidity

Acidity of milk samples was determined by titrating 18 g. of milk (containing 10 drops of 1 per cent phenolphthalein) with 0.1 N. sodium hydroxide (3). Results were recorded as per cent lactic acid.

4. Chloride determination

Heat approximately 50 g. raw whole milk in a boiling water bath for 3 min., remove, cool to room temperature and mix well. Weigh 10 g. samples of this milk into 27 x 113 mm. plastic round bottom centrifuge tubes, precipitate the casein with 5.5 ml. of 15 per cent trichloroacetic acid with constant agitation (to yield very small casein particles), remove casein from the sides of the plastic tubes with a stirring rod, and centrifuge in an International Clinical centrifuge for 3 min. at 2,500 r.p.m. Decant the centrifugate into a second clean plastic centrifuge tube. Wash the casein by resuspending in two successive 3 ml. portions of water (pH 4.7) acidified with 1 drop of 15 per cent trichloroacetic acid, and centrifuge again for 3 min., decant all washings into the tube containing the centrifugate of the original milk. Titrate the combined centrifugates to the phenolphthalein end point (5 drops

of 2 per cent phenolphthalein) with 2 N., followed by 0.1 N. sodium hydroxide (the latter used when approaching the end point), centrifuge the precipitated phosphates at 2,500 r.p.m. for 3 min. Decant the clear centrifugate into a 125 ml. Erlenmeyer flask, bring to pH 6.6 by adding 1.5 ml. of 0.1 N. acetic acid, titrate with constant stirring with a standard silver nitrate solution (1 ml. equivalent to 1 mg. chloride) to the potassium chromate (12 drops, 10 per cent solution) end point. Results were calculated and recorded as per cent chloride.

5. Potassium determination

Potassium determinations were made by the method of Haff and Brotz (74) and Schober and Fricker (82). Weigh a 10 g. sample of raw whole milk into a platinum evaporating dish, dry on a steam plate, ash at 390-410° C. for 8 hrs., cool and dissolve the ash in 10 ml. of dilute (10 per cent) hydrochloric acid. Filter through a Whatman No. 42 paper, wash the paper and contents with distilled water and collect filtrate and washings in a 250 ml. beaker. Re-ash residue and filter paper for 1 hr. at 600° C. (after charring with a Bunsen burner), cool and dissolve with 10 ml. of 10 per cent hydrochloric acid. Combine the filtrate and the second ash solution, make

alkaline* with potassium-free sodium carbonate (usually 2 g.) to precipitate phosphorous, filter to remove the precipitated phosphates and acidify* with 40 per cent acetic acid. Evaporate the solutions to 20-25 ml. on a steam plate, adjust the pH to 4.0 with dilute (5 per cent) acetic acid, warm to 70° C. and add a 50 per cent excess of sodium tetraphenyl boron[1], filter, after 5 min., through fine porosity fritted glass crucibles that have been brought to constant weight. Wash the precipitate 7 times with 5 per cent acetic acid, dry at 100° C. for 0.5 hr., cool and weigh. Run a blank with each group of samples (usual blank 0.0 to 0.0004 g.). Potassium tetraphenylboron is stable to 120° C.; it contains 3.787 per cent potassium (29).

6. Sodium determination

Weigh 20 g. raw whole milk into a platinum evaporating dish, dry on a steam plate, place in a cold muffle furnace,

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*Note: Keep beakers covered at all times with watch glasses during addition of sodium carbonate and hydrochloric acid to prevent loss by spattering. Rinse watch glasses into the beakers before proceeding.

[1]Dissolve 0.2 g. sodium tetraphenylboron (Hach Chem. Co., Ames, Ia.) in 10 ml. distilled water. Prepare and filter at least 1 hr. before using; if cloudiness appears in filtrate, refilter (29). Use within 24 hrs. because of instability; if not used immediately store in absence of light. Fifteen ml. of this solution yields a 50 per cent excess for 10 g. of normal whole milk.

heat to 400° C. and hold for 8 hrs. Cool the dishes, dissolve the ash with 10 ml. of 10 per cent hydrochloric acid, filter through a Whatman No. 42 paper and wash paper and contents with distilled water. Collect filtrates and washings in 250 ml. beakers. Return the ashless paper to the platinum dish, charr and ignite at 600° C. for 1 hr. Cool the dishes, dissolve the residue in 10 ml. of 10 per cent hydrochloric acid, and combine with filtrate from the first ashing. Sodium was determined by the magnesium uranyl acetate method (109). Reduce the combined filtrates to 50-75 ml. on a steam plate, add 3 g. of solid zinc carbonate and digest on the steam plate for at least 0.5 hr. Zinc carbonate raises the pH and precipitates the phosphates. Filter, wash the filter paper and precipitate thoroughly with 5 ml. portions of distilled water (usually 8-10 times) and collect the filtrate and washings in a 400 ml. beaker. Acidify with 0.1 N. hydrochloric acid to the methyl orange end point and evaporate to a volume of 5-7 ml. If the solution is not acid to methyl orange, adjust at this point; adjustment cannot be made after the reagent is added.

Adjust the solution to 20 ± 1° C., add 100 ml. of the magnesium uranyl acetate reagent[1] and stir (500-1,000 r.p.m.)

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[1]Magnesium uranyl acetate solution: Dissolve 45 g. uranyl acetate, 300 g. magnesium acetate and 60 ml. of glacial acetic acid in 800 ml. of distilled water, heat to dissolve all salts, cool and dilute to one liter. Store at 20° C. for a minimum of 2 hrs. with frequent stirring or agitation. Filter through a Buchner funnel, and place in a clean storage bottle and store at 20° C. (109).

for 45 min. at 20° C. Filter through a previously double washed and weighed Gooch crucible[1], wash the precipitate with two 10 ml. portions of the reagent at 20° C. and four 5 ml. portions of the sodium wash solution[2]. A blank was run on all reagents used. The crucibles were dried at 100° C. for 0.5 hr., cooled and weighed. The sodium complex, $NaMg(UO_2)_3(C_2H_3O_2)_9 \cdot 6.5\ H_2O$, contains 1.528 per cent sodium.

7. Freezing point determination

Freezing point determinations were made according to the method of Association of Official Agricultural Chemists (4).

8. Continuous pressure dialysis

The apparatus employed is shown in detail in Figure 1.

Weigh a 50 g. sample of unheated whole milk into a 100 ml. beaker and add 0.1 g. of finely ground thymol. Pour the milk through a funnel into a 10 inch piece of wetted (outside)

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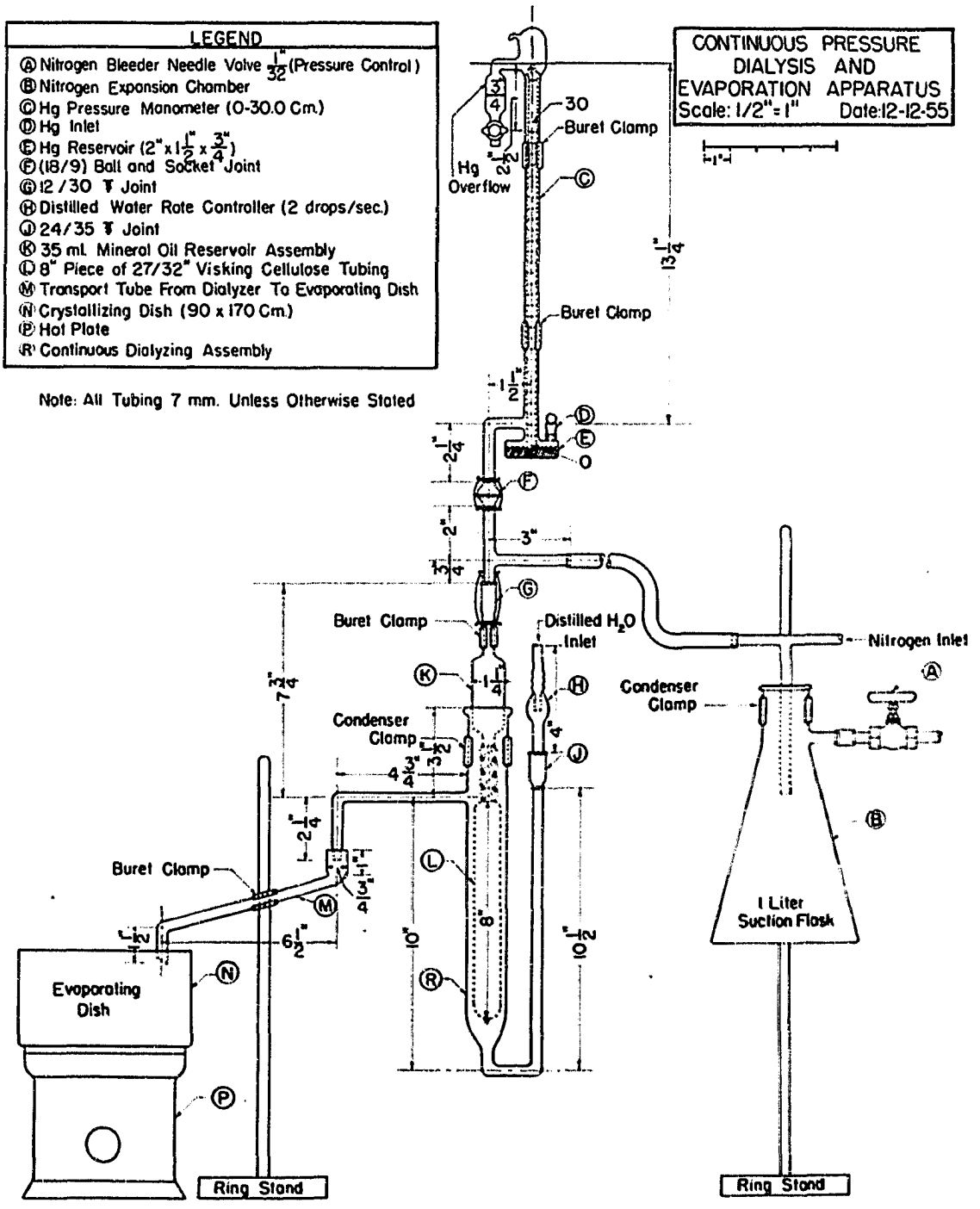
[1]Digest asbestos fibers (5) in 1 : 3 hydrochloric acid for 2-3 days, wash free of acid, digest 2-3 days with 10 per cent sodium hydroxide, wash free of alkali, digest in hot alkaline-tartrate solution several hours, wash free of alkali, digest several hours in 1 : 3 nitric acid, wash free of acid, shake into a fine pulp in distilled water and store. Prepare alkaline-tartrate solution as follows: Dissolve 173 g. sodium potassium tartrate and 50 g. sodium hydroxide in and make to 500 ml. with distilled water; after 2 days filter through prepared asbestos.

[2]The sodium wash solution: 35 ml. of glacial acetic acid, 405 ml. anhydrous ethyl acetate and 460 ml. of absolute alcohol; maintain and use at 20° C. (109).

Figure 1. Detailed drawing of continuous pressure dialysis and dialysate evaporation apparatus.


LEGEND
Ⓐ Nitrogen Bleeder Needle Valve 1/32" (Pressure Control)
Ⓑ Nitrogen Expansion Chamber
Ⓒ Hg Pressure Manometer (0-30.0 Cm.)
Ⓓ Hg Inlet
Ⓔ Hg Reservoir (2" x 1 1/2" x 3/4")
Ⓕ (18/9) Ball and Socket Joint
Ⓖ 12/30 ₸ Joint
Ⓗ Distilled Water Rate Controller (2 drops/sec.)
Ⓙ 24/35 ₸ Joint
Ⓚ 35 mL Mineral Oil Reservoir Assembly
Ⓛ 8" Piece of 27/32" Visking Cellulose Tubing
Ⓜ Transport Tube From Dialyzer To Evaporating Dish
Ⓝ Crystallizing Dish (90 x 170 Cm.)
Ⓟ Hot Plate
Ⓡ Continuous Dialyzing Assembly
Note: All Tubing 7 mm. Unless Otherwise Stated
CONTINUOUS PRESSURE DIALYSIS AND EVAPORATION APPARATUS
Scale: 1/2"=1" Date:12-12-55
30
Buret Clamp
Hg Overflow
Buret Clamp
Distilled H2O Inlet
Nitrogen Inlet
Condenser Clamp
Condenser Clamp
Buret Clamp
Evaporating Dish
1 Liter Suction Flask
Ring Stand
Ring Stand

27/32 in. (flat width) Visking Cellulose tubing, L, tied tightly, with string, at one end. Slip the untied end over the end of the mineral oil reservoir, K, and tie securely with string in three places. Float mineral oil on the surface of the milk to fill the reservoir. Remove free air from the assembly by massaging and twisting the membrane. Dry the outer surface of the membrane with facial tissue and weigh the entire membrane-reservoir assembly (G + K + L). Insert the reservoir assembly into the dialyzing apparatus, R, attach and secure to the pressure manometer, C. Apply pressure by means of compressed nitrogen; adjust pressure to 115 mm. Hg. Fill the dialyzing chamber, R, with distilled water, flow distilled water through the chamber at 2 drops per second, collect dialysate in a 90 x 170 mm. crystallizing dish, N, resting on a hot plate, P, the heat of which was turned on when the dialysis was started and the temperature of which was such that the inflow rate equaled the evaporation rate. Control pressure within ± 1 mm. Hg by an 1/8 in. needle valve, A, mounted on the one liter expansion chamber, B. Continue the dialysis and evaporation for 15 hrs., remove the reservoir-membrane assembly, dry and reweigh. Remove the milk residue from the membrane as follows: Tie a string around the membrane just above the oil-milk interface, to separate the milk residue from the oil, drain the oil from the membrane and cut the lower portion from the rest of the membrane with a scissors. Store the milk residue at 4.4° C. until analyzed.

Evaporate the dialysate to approximately 150-175 ml. in the crystallizing dish, transfer to a 250 ml. beaker, evaporate to 25-30 ml. on a steam plate, make to a volume (50 ml.) equal to that of the original milk sample and store at 4.4° C. until analyzed. Unused portions of dialysates were stored at -28.8° C. for reanalysis in case of doubtful results.

9. Ultrafiltration

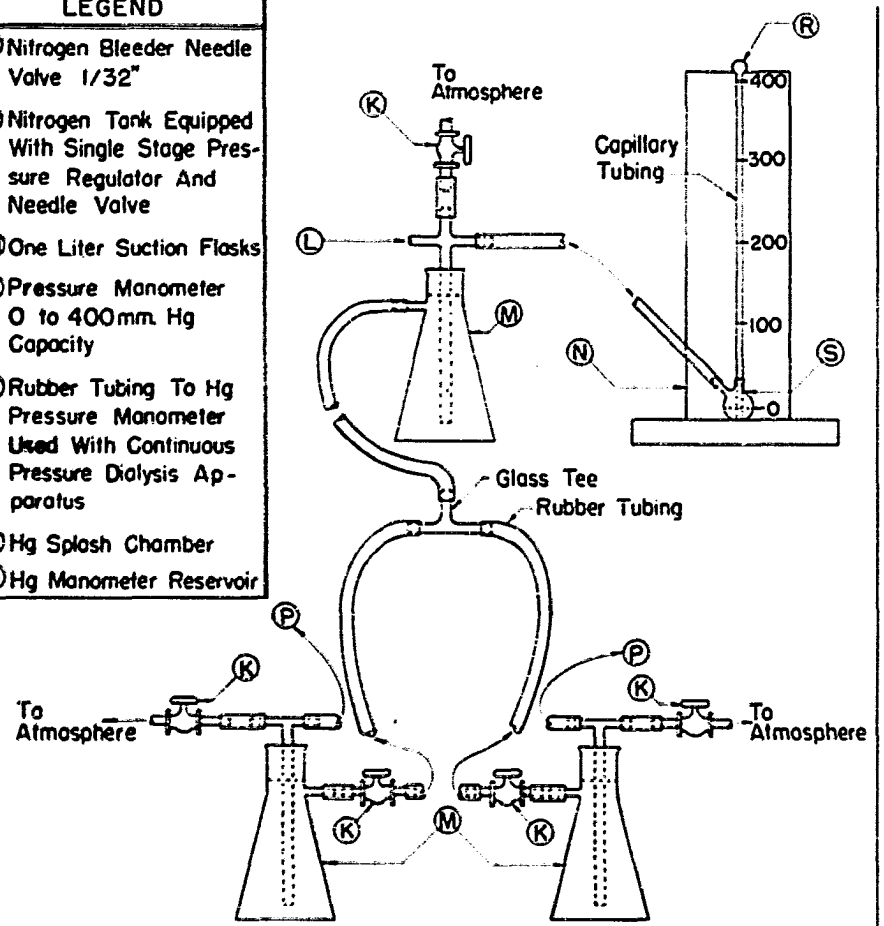
The apparatus employed was a modification of that used for pressure dialysis (Figure 1). The indicating pressure manometer (Figure 1, C) was not changed. The pressure regulation system and the ultrafiltration unit are described in detail in Figure 2.

Place 50-70 ml. of product into a 10-12 in. length of 27/32 in. (flat width) Visking tubing, G, tied tightly with string at one end, attach the untied end to the mineral oil reservoir, B, float 200 ml. mineral oil on the milk sample, dry the outside of the membrane with facial tissue, place the oil reservoir-membrane assembly in the ultrafiltration chamber, J, close the screw clamp on the Tygon tubing, H, and attach the complete assembly to the pressure-indicating manometer (Figure 1, C). Add 5 ml. water to the water trap, E, to prevent evaporation of the filtrate.

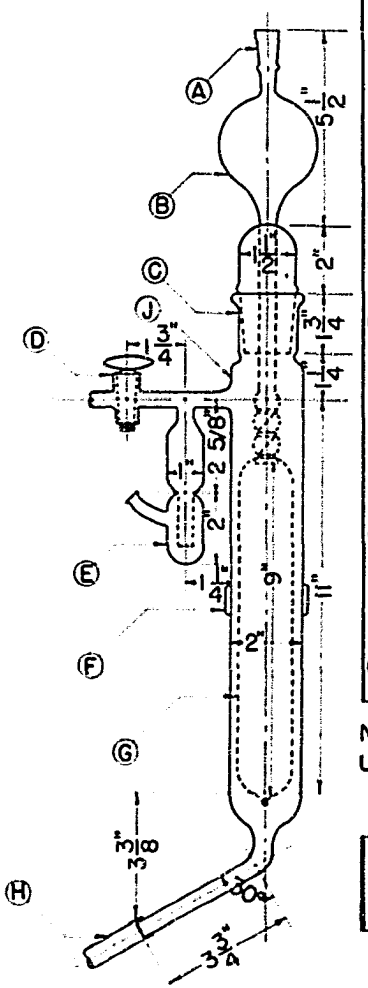
To adjust the pressure close the needle valves, K, to atmosphere, open those to the terminal suction flasks, M, open the needle valve on the nitrogen tank in such a manner that

Figure 2. Detailed drawing of ultrafiltration apparatus and pressure control system for dual ultrafiltration.

LEGEND
(K) Nitrogen Bleeder Needle Valve 1/32"
(L) Nitrogen Tank Equipped With Single Stage Pressure Regulator And Needle Valve
(M) One Liter Suction Flasks
(N) Pressure Manometer 0 to 400mm. Hg Capacity
(P) Rubber Tubing To Hg Pressure Manometer Used With Continuous Pressure Dialysis Apparatus
(R) Hg Splash Chamber
(S) Hg Manometer Reservoir



PRESSURE REGULATING SYSTEM FOR DUAL ULTRAFILTRATION



LEGEND
(A) 12/30 ⅀ Connected To Hg Pressure Manometer Used With Continuous Pressure Dialysis Apparatus
(B) 200 ml. Mineral Oil Reservoir Assembly
(C) 40/50 ⅀
(D) Stopcock Opened When Removing Ultrafiltrate
(E) Water Trap To Prevent Evaporation Of Ultrafiltrate
(F) Supporting Clamp
(G) 27/32" Cellulose Visking Tubing
(H) Tygon Tubing. Clamped During Ultrafiltration, Unclamped For Filtrate Removal
(J) Ultrafiltration Chamber

Note: All Tubing 7mm. Unless Otherwise Specified

PRESSURE ULTRAFILTRATION APPARATUS

1" Scale 1/2"=1"

the pressure rises slowly to 225 mm. in the regulating manometer, N, and then close the needle valves leading into the suction flasks until the pressure on the indicating manometers (Figure 1, C) is 200 mm. Re-regulate the needle valve on the nitrogen tank until the regulating manometer, N, is at about 225 mm.

Filtration can be continued until the protein-rich residue (PRR) is 5-10 ml. The length of time the filtration is continued will depend on the purpose of the filtration. PRR can be recovered by tieing off the membrane just above the oil-sample interface, puncturing the membrane and collecting PRR in a beaker. PRR was transferred by a 25 ml. hypodermic syringe to a 50 ml. volumetric flask. Both membrane and beaker in which PRR was caught were washed 3 times with distilled water and the washings were transferred to the volumetric flask and the contents made to 50 ml.

10. Physical separation of milk

The physical separations of milk are shown in Figure 3, which is self explanatory.

Fraction II C, labeled lipoprotein, was not studied. There is a probability that it may not be lipoprotein; it appears lipoid in nature.

11. Analyses of the milk fractions

Sodium, potassium, calcium, magnesium, chloride, inorganic

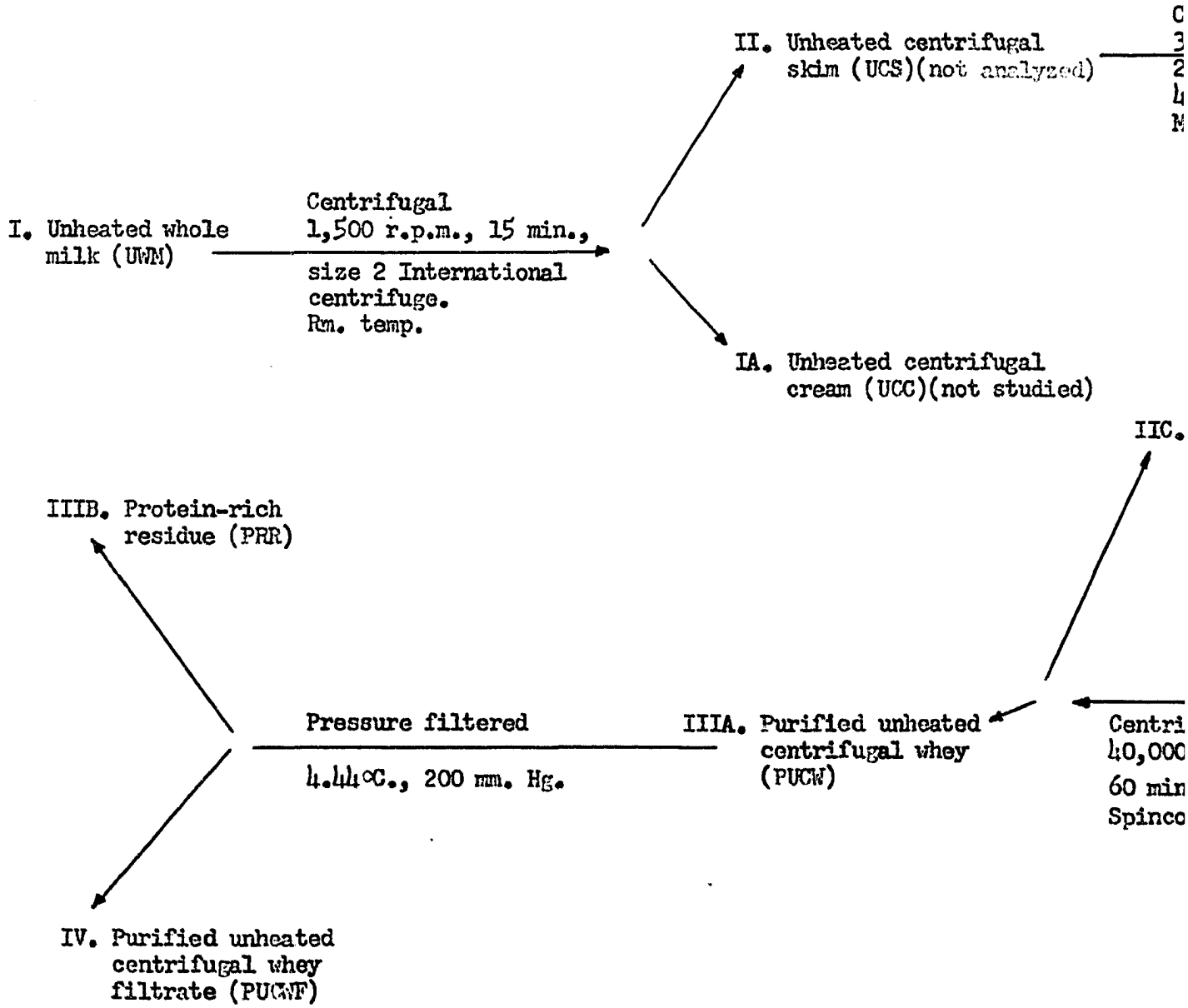
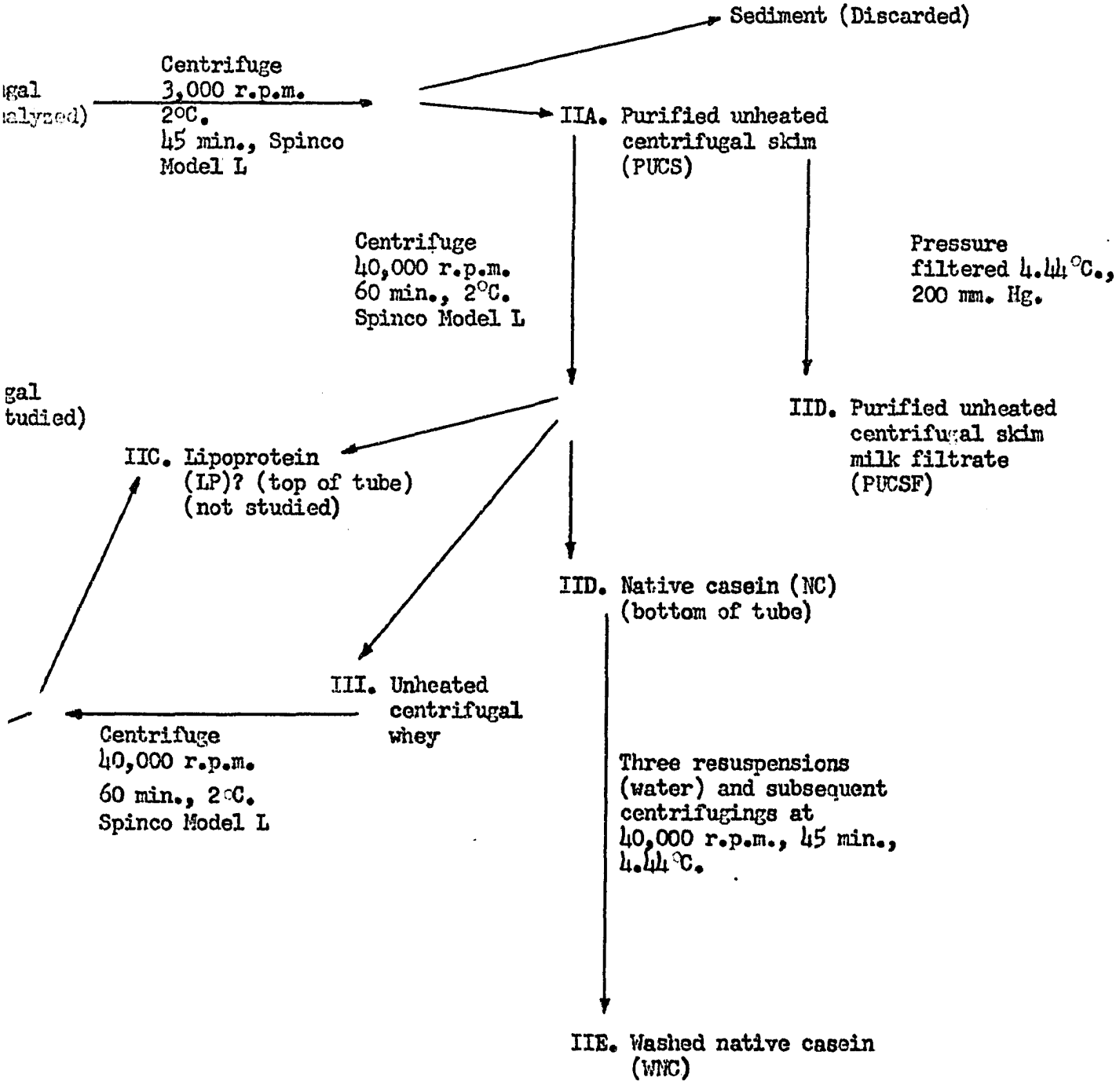


Figure 3. Flow sheet of the physical separation



al separation of milk into several components.

phosphorous and freezing point were determined on unheated whole milk (UWM), its dialysate (UWMD) and dialysis residue (UWMDR), on purified unheated centrifugal skim milk (PUCS), its dialysate (PUCSD), dialysis residue (PUCSDR) and ultrafiltrate (PUCSF), on purified unheated centrifugal whey (PUCW), its dialysate (PUCWD), dialysis residue (PUCWDR) and ultrafiltrate (PUCWF) and on the redispersed, washed, native casein (RWNC), its dialysate (RWNCD) and dialysis residue (RWNCDR).

In addition per cent nitrogen, fat and total solids were run on UWM and PUCS. Percentage nitrogen was determined on PUCW. Biuret and ninhydrin tests were made on PUCSF and PUCWF. Total phosphorous was determined on washed native casein (WNC) and nuclear phosphorous (hydroxy amino acid-esterified phosphoric acid) was determined on isoelectric casein obtained from WNC suspensions and from PUCS.

Modifications made in the sodium, potassium, calcium, magnesium, chloride and inorganic phosphate analyses of dialysates and filtrates are: a. Sodium. Transfer 5 ml. aliquots of dialysates and filtrates to 50 ml. beakers, dilute to 20-25 ml., add 3 g. solid zinc carbonate, digest on the steam plate for one hour, filter, evaporate to 30-40 ml., adjust to the methyl orange end point with 1 N. hydrochloric acid, evaporate to 3-5 ml. volume and precipitate as previously described. b. Potassium. Transfer 5 ml. aliquots of dialysates and filtrates to 50 ml. beakers, dilute to

20-25 ml. and add 2 g. sodium carbonate. Proceed from this point as previously described. c. Chloride, phosphorous, calcium and magnesium. Transfer 5 ml. of dialysates and filtrates to 100 ml. volumetric flasks and make to volume at 25° C. Use 1 ml. aliquots for total inorganic phosphorous determinations as described before. Use 10 ml. aliquots for calcium and magnesium. Pass each aliquot directly through the Duolite A-4 resin column and titrate as described previously. Use 5 ml. aliquots of dialysates and filtrates for the chloride determination. Remove phosphate by adjusting the pH to the phenolphthalein end point with 0.1 N. sodium hydroxide and centrifuge at 2,500 r.p.m. for 3 min. decanting and washing with alkaline water at pH 8.3 (sodium hydroxide). Adjust the clear centrifugate to pH 6.6 and titrate with standard silver nitrate.

UWMDR were analyzed in the same manner as was unheated whole milk. PRR were analyzed as were unheated whole milk after diluting to 50 ml. volume.

12. Preparation of caseins for nuclear phosphorous determinations

Add 1 N. hydrochloric acid slowly to approximately 200 g. UCS to precipitate isoelectric casein (pH 4.7). Place the precipitated casein into 27 x 113 mm. plastic round bottom centrifuge tubes, centrifuge for 5 min. at 2,500 r.p.m., decant supernatant, resuspend and wash with water acidified with hydrochloric acid (pH 4.7), three times. Add sufficient

1 N. sodium hydroxide to the casein precipitated from the 200 g. of UCS to cause complete peptization, following this with precipitation at pH 4.7 with 1 N. hydrochloric acid and wash with hydrochloric acid solution (pH 4.7). Repeptization, precipitation and washing were repeated five times. Suspend as much as possible of the final casein obtained in water and make to 100 ml. volume. Determine per cent total nitrogen on this suspension by Rowland's (79) method. To determine organic phosphorous ash 10 ml. aliquots of the suspension at 550° C. for 3 hrs., cool, dissolve ash in 10 ml. of 10 per cent hydrochloric acid, dilute to 100 ml. volume and determine phosphorous by Fontaine's (25) method as previously described for UCS.

Casein obtained by centrifuging PUCS at 40,000 r.p.m. in a Spinco Model L ultracentrifuge was treated in the manner described above to obtain isoelectric casein. The procedure was the same as above except that the casein was first peptized with 1 N. sodium hydroxide then precipitated with 1 N. hydrochloric acid at pH 4.7. From this point on the procedure was the same as for casein obtained from normal skim milk.

13. Preparation of cationic resin membrane electrodes

Cut cationic resin rod (2 mm. diameter) membrane (sodium form), of the sulfonated polystyrene type, (obtained from Kressman (36)) into 8 mm. lengths and place in a 10 per cent

hydrochloric acid solution to convert to the hydrogen form. Decant the acid solution after 24 hrs., wash repeatedly with redistilled water until the wash water is neutral, place the sections in test tubes containing 10 per cent solutions of either sodium, potassium, calcium or magnesium chlorides and allow to stand for 24 hrs. Decant the salt solutions and remove excess salt by repeated washing with redistilled water. Test complete salt removal by adding 5 per cent silver nitrate to the wash water. Place the 8 mm. resin rod sections in 5 mm. sections of 1/16 x 1/32 in. rubber tubing, fit this combination snugly into the tapered end of the glass electrode cell. Test for mechanical leaks by filling the electrode with a 0.01 per cent solution of Niagara Sky Blue F F and hanging it in an enclosed space to prevent evaporation. Appearance of dye on the under side of the electrode indicates mechanical leakage because the ionic diameter of the dye is too great to pass the pores of the resin membrane. Wash mechanically perfect electrodes free of dye with redistilled water and characterize against a solution of pure salt, the cation of which corresponds to the membrane form.

14. Measurement of potentials with cationic resin membrane electrodes

Voltages were measured with a Leeds and Northrop hydrogen-ion potentiometer. The circuit was standardized against an Epley cadmium standard cell. A lamp and scale galvanometer

(Leeds and Northrop) was employed (Figure 4). Saturated Schollenberger (83) type calomel half cells were used. The salt solution under consideration was placed in its respective resin membrane electrode and the electrode immersed to a depth of approximately one inch in 100 ml. of redistilled water in a 150 ml. beaker. The beaker containing the water was placed in a constant temperature (25 ± 0.01 °C.) bath. One calomel half cell was immersed to a depth of one inch in the solution contained in the resin rod membrane electrode; the other was immersed to the same depth in the water contained in the 150 ml. beaker. A concentration potential existed and in order to determine the concentration of cation inside the electrode a standard salt solution (usually 10 times greater than in the sample) was added to the water in the beaker until the potential across the membrane was zero. The null point was determined by adding the standard salt solution in 1 ml. quantities, from a 25 ml. burette, agitating the solution with nitrogen* for 1 min., then reading the voltage. Repeated 1 ml. additions of standard salt solution, agitation and voltage measurements were continued until the data ranged from a large positive to a large negative potential. The calomel half cells were removed after each

*Before the nitrogen was bubbled through the solution it was conveyed through a Kendall Tube to remove any oxygen, bubbled through a sodium stannite solution and finally through redistilled water (Figure 4).

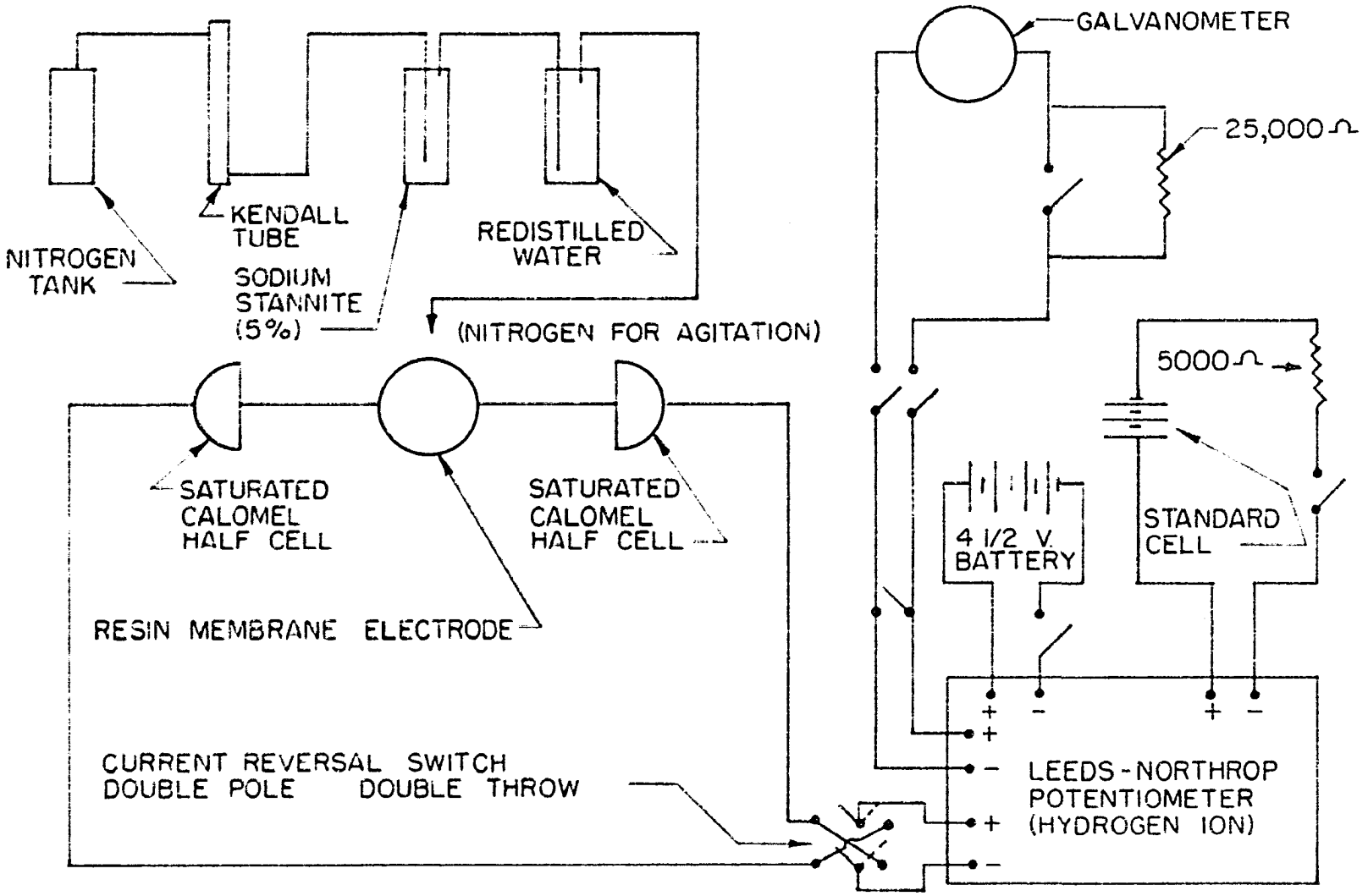


Figure 4. Schematic diagram of voltage measurement apparatus and nitrogen purification train.

measurement to prevent electrode polarization. The voltages were plotted against ml. of standard salt solution and the point of zero voltage was read from the plot. The concentration of cation within the electrode was calculated on the assumption that the concentration within the electrode and without the electrode are the same at zero voltage; the concentration outside the membrane was known.

IV. RESULTS

A. Evaluation of the Accuracy of the Analytical Methods

1. Chloride (Cl) determination

The procedure described (III, B-4) was developed because the Milk Industry Foundation method (63) was not reproducible in this laboratory, apparently because of protein interference. The method developed, denatured the whey proteins by heating the milk sample in a boiling water bath and precipitating these proteins together with casein at the isoelectric point of the latter (79); trichloroacetic acid was employed as the precipitant. The proteins were centrifuged from the sample, were washed three times with trichloroacetic acid solution (pH 4.7), the original centrifugate and washings were combined, were adjusted to the phenolphthalein end point to precipitate phosphates, the phosphates were removed by centrifugation, the supernatant was decanted quantitatively and was titrated with standard silver nitrate after adjusting the pH to 6.8 with acetic acid, to the potassium chromate end point.

Certain data obtained in the above development will be reported below.

The quantity of 0.1 N. acetic acid needed to bring the clear centrifugate to pH 6.8 was found to be 1.5 ml. as shown

in Table 2.

It was found that the number of ml. of silver nitrate required to reach the potassium dichromate end point was a function of the indicator quantity. Table 3 indicates that from 10 to 14 drops yielded the minimum titration; this was considered the correct titration because below 10 drops, additional silver ion was needed to satisfy the solubility product of silver chromate and above 14 drops, the concentration of chromate ion is apparently sufficiently large to react at an end point with some of the silver ions. Based on the data presented, 12 drops of 10 per cent potassium chromate were adopted in the determination.

Data relative to the recovery of added chloride are shown in Table 4. These data indicate reasonably satisfactory recoveries; the average was 97.8 per cent, while with one exception (94.5) the range was 95.5 to 100.0 per cent.

2. Sodium determination

The determination of sodium by the gravimetric, magnesium uranyl acetate (109) method is one of the most accurate for this element. The accuracy of the method for sodium determination in milk was tested by analyzing milk samples to which varying amounts of a standard sodium chloride solution were added. Table 5 contains the recovered amounts and percentages. The average per cent recovery for all milk samples was 96.5; the range, barring one sample (92.0) was 95.5 to 99.1 per cent.

Table 2. Quantity (ml.) of 0.1 N. acetic acid added to clear centrifugate at phenolphthalein end point to obtain a pH of 6.8.

ml. 0.1 N. acetic acid	pH
0.3	7.75
0.5	7.40
0.75	7.30
1.0	7.20
1.5	6.80[a]
2.0	6.60

[a]Correct pH.

Table 3. Effect of indicator concentration on quantity of silver nitrate required in the titration.

Drops of 10 per cent potassium chromate	ml. of silver nitrate at end point
4	20.3
6	19.6
8	19.4
10	19.2
12	19.2[a]
14	19.2
16	19.6
18	19.7

[a]Indicator concentration used.

Table 4. Recovery of chloride added to milk.

Run	Chloride added mg./10 ml.	Chloride recovered mg./10 ml.	Percentage recovery
I	16.7	15.7	94.5
	31.2	30.2	96.8
	45.7	46.0	100.5
	60.4	57.6	95.5
II	24.2	23.6	97.5
	36.5	35.8	98.2
	60.0	60.5	100.5
III	12.7	12.4	97.5
	24.8	23.8	96.0
	36.4	36.4	100.0
	61.1	59.5	97.4

Table 5. Recovery of sodium added to milk.

Run	Sodium added mg./20 ml.	Sodium recovered mg./20 ml.	Percentage recovery
I	5.00	4.90	98.0
	10.00	9.20	92.0
	15.01	14.55	97.0
II	4.99	4.95	99.1
	10.00	9.55	95.5
	15.03	15.00	99.7

This was considered satisfactory.

3. Potassium determination

Recovery runs were based on a series of milk samples to which various concentrations of potassium were added and on the milks without added potassium. Table 6 indicates that the potassium tetraphenylboron (74, 82) precipitation method yields excellent results. The average recovery of potassium was 98.0, the range 96.4 to 99.2 per cent.

4. Inorganic phosphorous determination

A regression, relating log % Transmittancy (820 $m\mu$) and mg. phosphorous was determined for the Fontaine (25) method; potassium dihydrogen phosphate (N.B.S. Sample No. 186-1-a) solutions were employed containing from 0.0 to 0.035 mg. of phosphorous per 25 ml. The regression determined is:

$$\text{Log \% Transmittancy} = 1.99817 - (30.5874)(\text{mg. P/25 ml.}),$$

which is shown, together with the data from which it was calculated, in graphical form in Figure 5. This equation was employed in calculating all phosphorous values reported. The accuracy of the phosphorous determination in this laboratory was evaluated by a series of recovery runs. Known amounts of phosphorous were added to milk; phosphorous determinations were run on milk plus phosphorous and the original milk. The data (Table 7) obtained indicate that the recoveries were

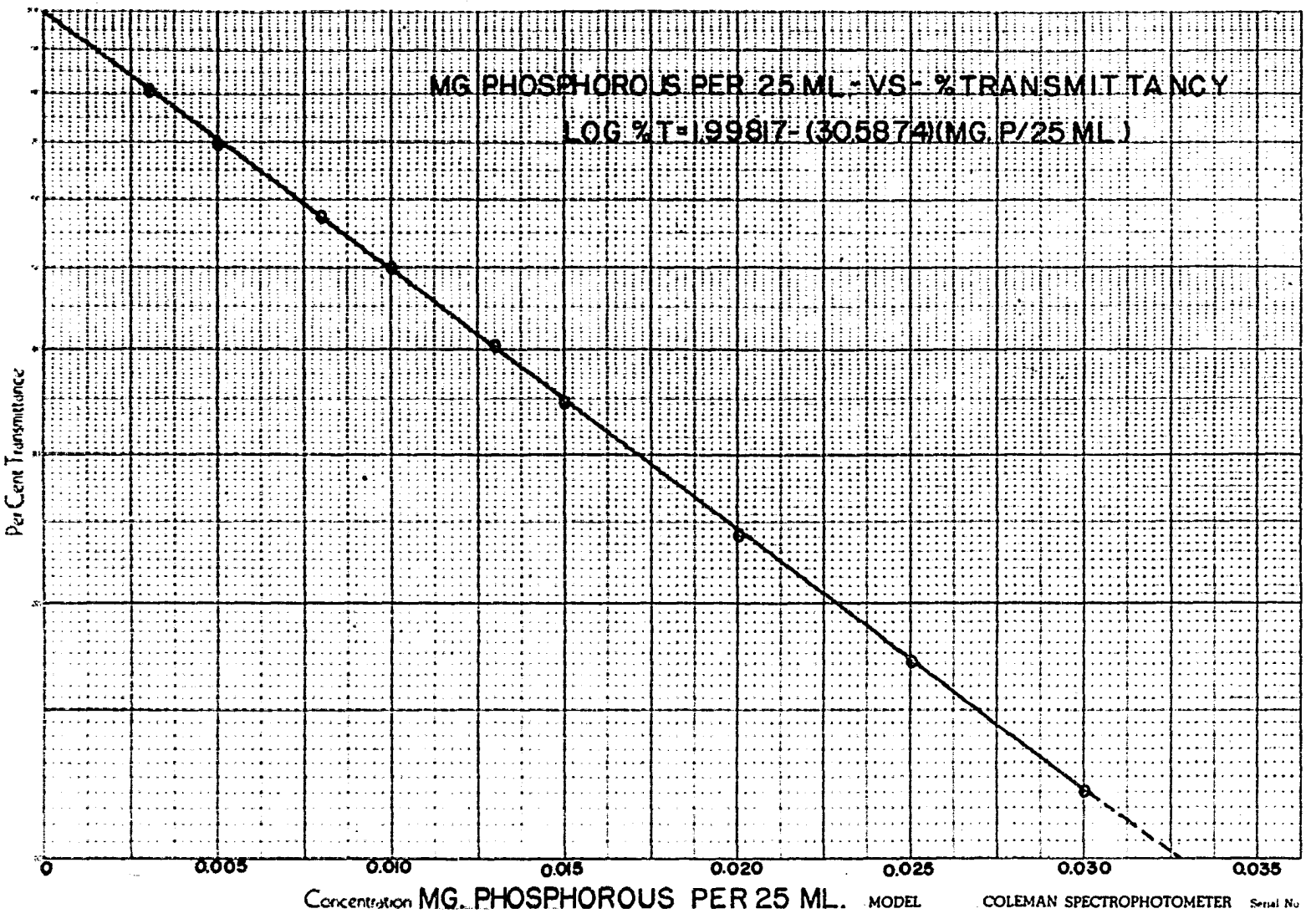
Table 6. Recovery of potassium added to milk.

Run	Potassium added mg./10 ml.	Potassium recovered mg./10 ml.	Percentage recovery
I	4.99	4.91	98.5
	10.00	9.81	98.1
	15.00	14.98	99.2
	20.05	19.69	98.1
II	4.96	4.88	98.4
	9.98	9.75	97.8
	14.93	14.62	98.0
	19.91	19.20	96.4

Table 7. Recovery of inorganic phosphorous added to milk.

Run	Phosphorous added mg./10 ml.	Phosphorous recovered mg./10 ml.	Percentage recovery
I	0.113	0.111	98.2
	0.156	0.154	98.7
	0.206	0.199	96.6
	0.252	0.249	98.8
II	0.112	0.109	97.3
	0.157	0.154	98.1
	0.203	0.200	98.5
	0.250	0.248	99.2

Figure 5. Standard regression curve for phosphorous determination.


MG. PHOSPHOROUS PER 25 ML. - VS - % TRANSMITTANCY
LOG %T = 1.99817 - (.305874)(MG. P/25 ML.)
Per Cent Transmittance
0
0.005
0.010
0.015
0.020
0.025
0.030
0.035
Concentration MG. PHOSPHOROUS PER 25 ML.
MODEL
COLEMAN SPECTROPHOTOMETER
Serial No

satisfactory. The average recovery was 98.1 per cent, the range of values was 96.6 to 99.2.

5. Calcium and magnesium determination

The accuracy of the methods for calcium and magnesium was determined in a manner similar to that used for the other methods by recovery of calcium and magnesium, added to milk. The method of Jenness (33) was employed. The data in Tables 8 and 9, indicate the method was satisfactory as used. The average recoveries and recovery ranges were; magnesium, 96.4 and 95.6 to 97.0 per cent, respectively, and calcium, 100.2 and 99.3 to 100.8 per cent, respectively. The magnesium recoveries were not as good as would have been hoped but are still within ranges frequently encoutered in biological materials.

B. Continuous Pressure Dialysis

The first dialysis attempts followed the usual pattern: milk was placed in Visking Cellulose tubing which was immersed in a stream of distilled water for 15 hrs. Considerable water was taken up by the milk residue. It was considered that this water uptake would cause a shift in milk salt equilibria and yield erroneous results. It was considered that it might be possible to maintain constant volume of the material dialyzed, if pressure were exerted on the fluid in the membrane. In the first attempts at use of

Table 8. Recovery of calcium added to milk.

Run	Calcium added mg./10 ml.	Calcium recovered mg./10 ml.	Percentage recovery
I	3.63	3.64	100.3
	7.45	7.40	99.3
	10.91	10.89	99.8
	15.46	15.51	100.3
II	3.65	3.68	100.8
	7.48	7.53	100.7
	10.90	10.95	100.5
	15.50	15.45	99.7

Table 9. Recovery of magnesium added to milk.

Run	Magnesium added mg./100 ml.	Magnesium recovered mg./100 ml.	Percentage recovery
I	1.56	1.50	96.2
	3.60	3.49	96.9
	5.43	5.27	97.0
	9.86	9.58	96.3
II	1.59	1.52	95.6
	3.64	3.52	96.7
	5.40	5.20	96.3
	9.85	9.54	96.8

pressure, the pressure was exerted upon the milk by means of a layer of mineral oil injected into the membrane cavity from a hypodermic syringe to which the membrane was attached. The pressure could be varied by the depth to which the plunger was inserted into the syringe barrel. The pressure exerted by this method decreased the amount of water uptake by the milk during dialysis, in fact the maximum pressure that could be exerted caused the final volume in the membrane to be less than that of the original milk. Because pressures could not be duplicated, this method was discontinued. In order to duplicate pressures, the apparatus in Figure 1 was designed and built. The pressure was exerted on the mineral oil and not the milk, because when milk was dialyzed alone it took up water and displaced any air present in the membrane. Mineral oil floated over the milk surface excluded all air and reduced the possibility of water uptake in the manner described.

1. Development of method

Before the continuous pressure dialysis apparatus could be used as a research tool, it was necessary to determine the optimum dialyzing pressure. This was done by dialyzing a series of samples for 11 hrs. at pressures from 70 to 170 mm. Hg. Figure 6 indicates the gain or loss of water for a 50 g. sample of milk at the end of the dialysis period for various pressures. These data indicate that there was no loss or gain

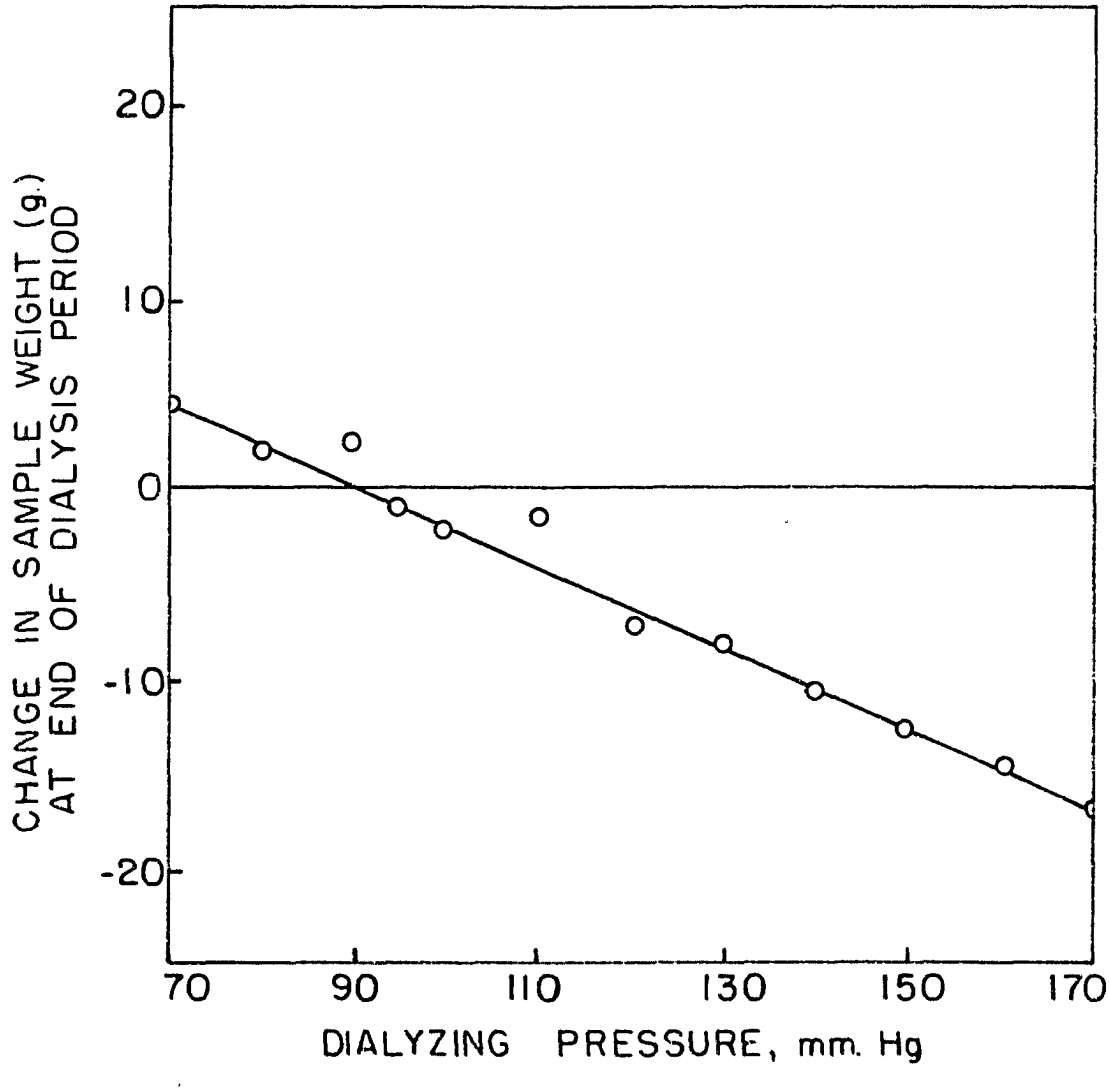


Figure 6. Sample weight change (g.) at end of 11 hour dialysis at different pressures.

of water at the end of a 11 hr. dialysis when 92 mm. Hg pressure was employed. The total weight change, at the end of the dialysis period, is indicated as a linear function of the applied pressure. It was considered that the final weight might not indicate volume changes during the dialysis period. To determine whether or not there was a fluctuation in weight during the dialysis period, several runs were made at different pressures (applied pressure 0, 115, 150 and 170 mm. Hg). Fifty g. samples of milk were used in all cases and one aliquot was dialyzed for each of the following periods: 2, 4, 6, 8, 10, 11 and 14 hrs. The weight before and after each dialysis was recorded and plotted as shown in Figure 7. These data indicate that when no pressure was applied water was taken up rapidly during 4 hrs. after which the weight remained constant. A pressure of 115 mm. Hg on the other hand allowed water uptake (4 g.) during 4 hrs., followed by a decrease to the original weight at 7 hrs.; beyond 7 hrs. the milk lost weight until at 11 hrs. the loss (4 g.) equalled the maximum gain at 4 hrs. It has been determined that this loss (4 g.) is constant through 15 hrs. With pressures of 150 and 170 mm. Hg weight loss began at 3 and 2 hrs., respectively; total losses were 14.5 and 17.0 g., respectively, at the end of 11 hrs.

On the basis of these results it was considered that a pressure of 115 mm. Hg gave the most nearly satisfactory results, because the maximum loss of water was equal to the

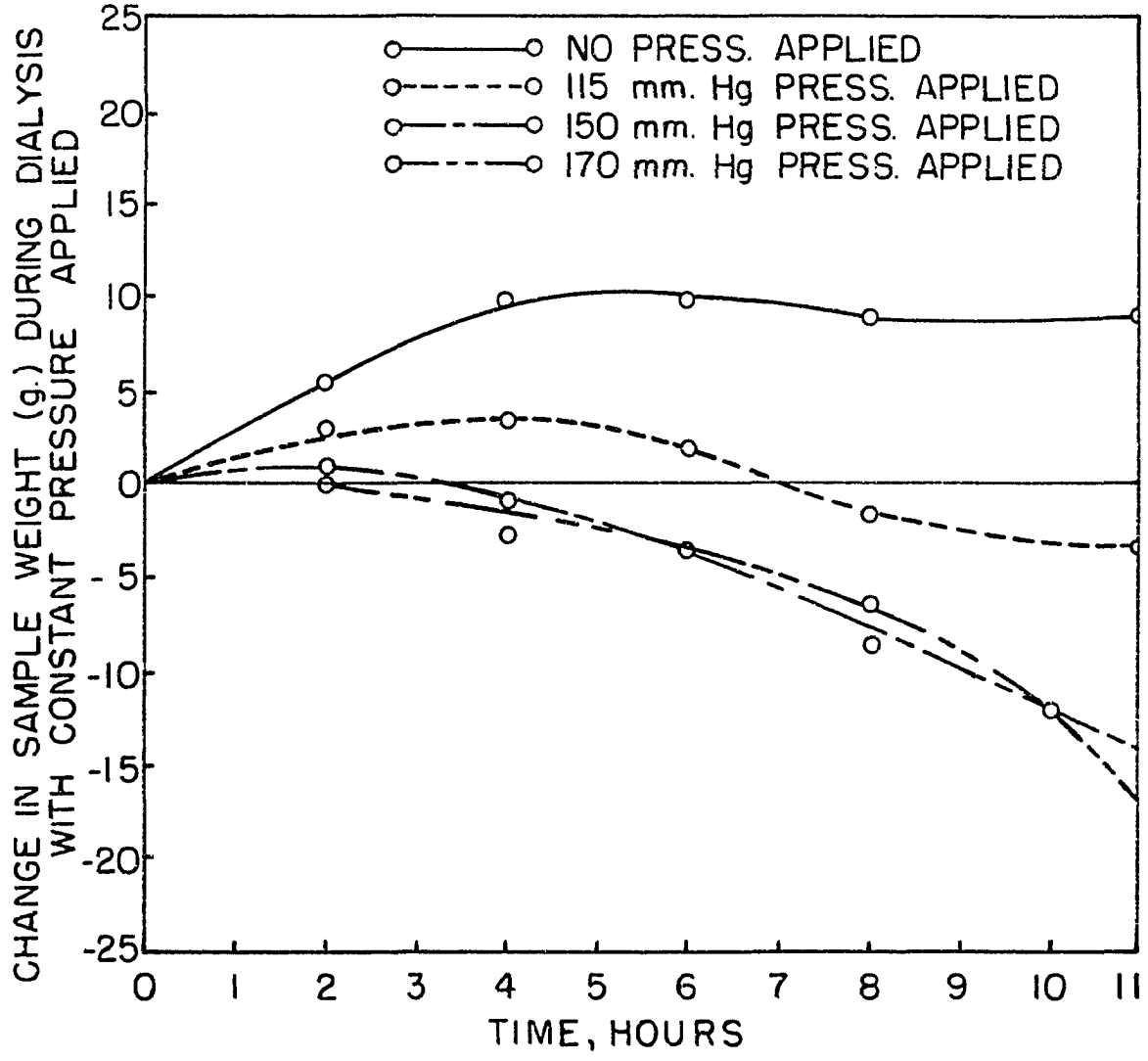


Figure 7. Sample weight changes (g.) during 11 hour dialysis at different pressures.

gain, with the initial weight the reference point. This pressure kept the milk closest to its original weight throughout the dialysis. All succeeding dialyses were made at 115 mm. Hg.

When dialysis pressure was established it was considered advisable to ascertain the distribution of dialyzable elements with respect to dialysis time. This was done by dialyzing 50 g. samples of milk at 115 mm. Hg and collecting the dialysate in 500 ml. fractions. Each fraction was collected in approximately 45 min. The fractions were evaporated to approximately 30-40 ml., transferred to 100 ml. volumetric flasks and made to volume. Each fraction was analyzed for calcium, magnesium and phosphorous. Ten ml. and 1 ml. aliquots of each fraction were used for calcium and magnesium, and for phosphorous, respectively. All results were calculated as milliequivalents per fraction. The data are presented in Figure 8. The removal of calcium, magnesium and phosphorous proceeded in logarithmic manner during the first 7 hrs., during which the weight within the membrane was equal to or greater than the original weight. Beyond 7 hrs. the weight within the membrane became less than the original weight (Figure 7) and although the removal of ions was still approximately logarithmic, the rate was less than during the first 7 hrs. and a discontinuity occurs in the curve at the point at which the weight within the membrane equalled the original weight. This suggests that during

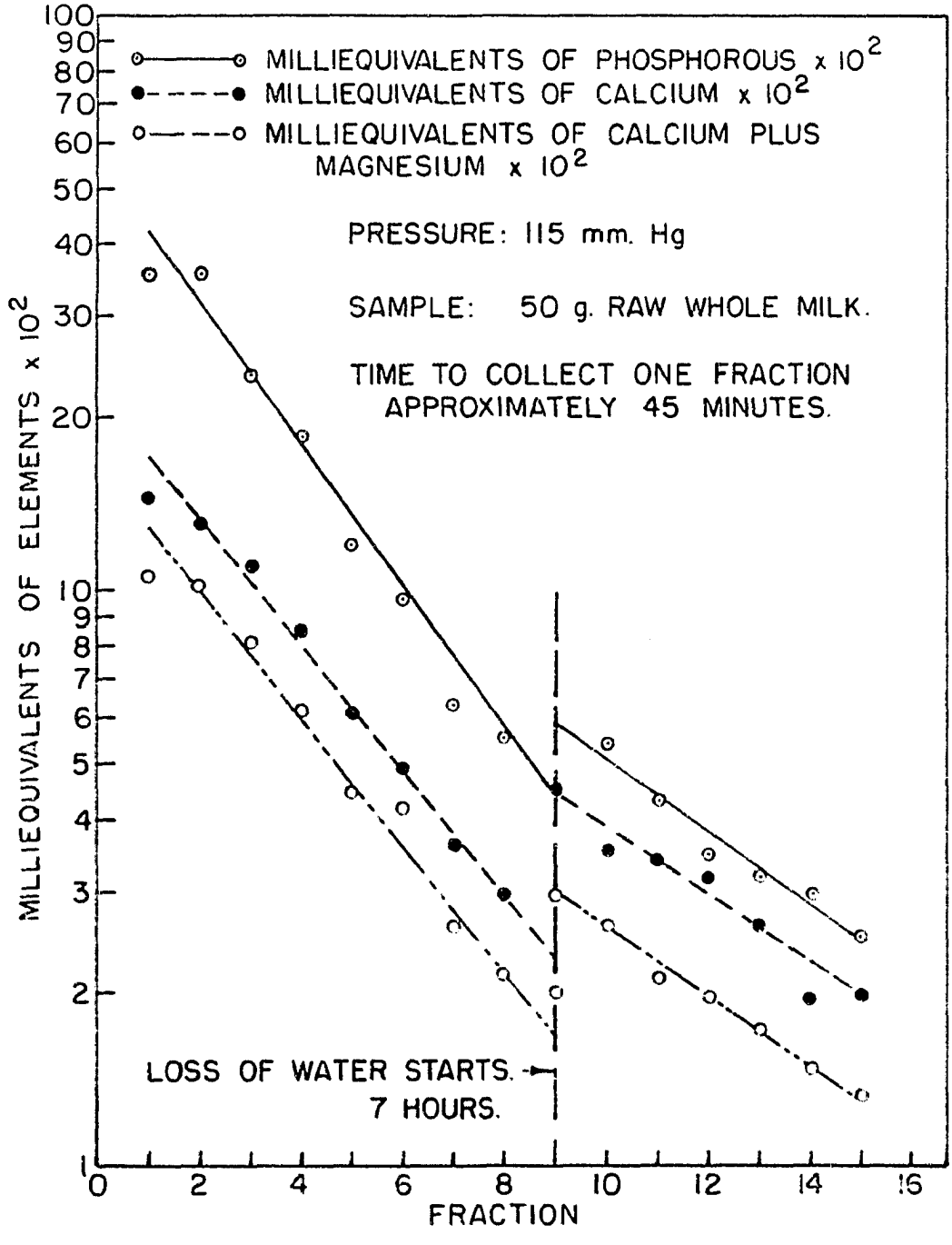


Figure 8. Distribution of phosphorous, calcium and calcium plus magnesium during a 15 hour dialysis at 115 mm. Hg pressure.

the first 7 hrs. the action probably is a true dialysis and that from 7 to 15 hrs. it may be a combination of dialysis and ultrafiltration. Another explanation may be that during the first 7 hrs. the majority of the readily dialyzable constituents are removed and that from 7 to 15 hrs. the constituents removed are those released by a slow hydrolysis of colloidal salts and/or by desorption of ions from ion-protein complexes, allowing the ions thus released to pass through the membrane. Evidence for the rapidity of dialysis during the first 7 hrs. is shown by the fact that of the total calcium, magnesium and phosphorous dialyzable in 15 hrs. an average of 83.7, 85.8 and 86.4 per cent, respectively, was dialyzed in the first 7 hrs.

To further study the efficiency of dialysis freezing points were determined on the dialysis residues after various dialyzing periods. Fifty g. samples of the same parent milk (held in ice water) were dialyzed for various lengths of time ranging from 1 to 25 hrs. The residues were removed from the membrane and freezing points were determined with a Hortvet Cryoscope by the A.O.A.C. (4) method. The addition of thymol used as a preservative was found to have no effect upon the freezing point; therefore, its use was continued.

Figure 9 shows changes in freezing points of dialysis residues with increased time of dialysis. These data indicate that the majority of the ions or molecules which have

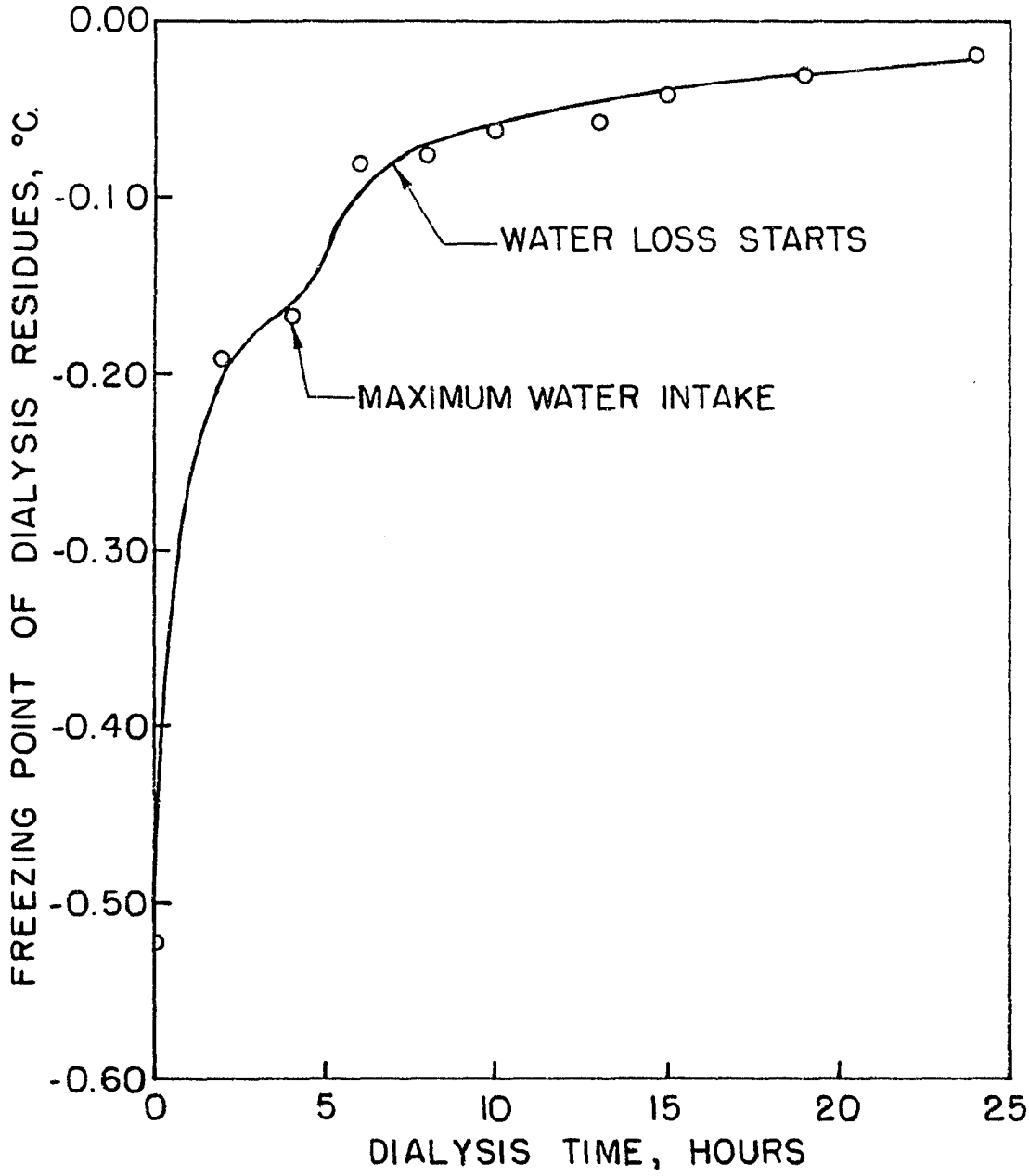


Figure 9. Freezing points of unheated whole milk samples dialyzed at 115 mm. Hg pressure for different times.

the greatest effect on the freezing point are removed during the first 7 hrs., for during this time the freezing point of the residue rises approximately 0.51° C., whereas the rise from the 7th to the 25th hr. is approximately 0.05° C. Except for the change in curvature caused by water intake which appears to occur and diminish rapidly, the curve indicates a logarithmic change in curvature and suggests that several hours additional dialysis might be needed before the freezing point was dependent only on non-dialyzable constituents. It is possible therefore, that the increase in ionic concentration of the dialysates after 7 hrs. results from some ultrafiltration in addition to dialysis.

The inflection occurring at that point of greatest water uptake (4 hrs.) by the membrane contents, very likely results from a dilution effect of the contents of the dialysis membrane, because the rate of change in freezing point decreases from a freezing point of about -0.2° C. and increases shortly after the maximum water intake is reached. The curve apparently assumes a natural curvature again at about -0.08° C. If the above interpretation is correct it corroborates the hypothesis that the increase in ionic removal after 7 hrs. may result from inclusion of some ultrafiltration. Despite this fact it is considered that this type of dialysis will yield a more nearly representative picture of the ionic species of milk than will static dialysis.

2. Data obtained with continuous pressure dialysis

a. Comparison of 15 and 20 hr. dialysis times. The data of Figure 9 indicate that at 15, 19 and 24 hrs., 93.9, 94.4 and 96.4 per cent, respectively, of the osmotically active materials had been dialyzed from the milk samples, if it is assumed that some materials, that were not osmotically active in the original milk, did not become active as equilibria were changed during dialysis. These data together with those in Figure 8 suggested that possibly little would be gained by dialysis times longer than 15 hrs.

As a check on the above the same milk was dialyzed for 15 and 20 hrs. The original milk and the dialysates were analyzed; the data are shown in Table 10. The data indicate that there would be no advantage in 20 hrs. as compared to 15 hr. periods for sodium, potassium or chloride. There is some advantage in using the longer time as regards calcium and inorganic phosphorous and considerable advantage as regards magnesium. At the time these data were obtained, a refrigerator of sufficient size was not available for the dialysis apparatus and an excessive amount of thymol was considered undesirable. Thymol dialyzed from the sample, together with other constituents and it was considered that 15 hrs. might be as long a time as it was feasible to use.

It was considered that 15 hrs. might give a better estimate of the dialyzable constituents than longer times if

Table 10. Amounts and percentages of elements dialyzed from 50 g. unheated whole milk

Element	Analyses of				
	Original milk	Dialysate 0 to 15 hrs.	Dialysate 15 to 20 hrs.	Dialysate residue	Dial 20 h
	mg./50 g. (1)	mg./50 ml. (2)	mg./50 ml. (3)	mg./50 g. (4)	(sum 3)
Sodium	21.08	20.58	0.0	0.0	20
Potassium	87.24	87.15	0.0	0.0	87
Calcium	57.95	17.39	1.79	39.28	19
Magnesium	5.23	3.19	0.61	1.92	3
Chloride	49.90	49.00	0.2	0.08	49
Inorganic Phosphorous	29.58	18.85	0.86	11.85	19

50 g. unheated whole milk in 15 and 20 hours

s.	Dialysate residue mg./50 g. (4)	Dialysate 20 hrs. (sum 2 and 3)	Percentage Elements in original milk dialyzed in 15 hrs.	20 hrs.	Elements dialyzed in 20 hrs. obtained in 1st 15 hrs.	15 to 20 hrs.
	0.0	20.58	97.6	97.6	100.0	0.0
	0.0	87.15	99.9	99.9	100.0	0.0
	39.28	19.18	30.0	33.1	90.7	9.3
	1.92	3.80	60.9	70.7	83.9	16.1
	0.08	49.20	98.2	98.6	99.6	0.4
	11.85	19.71	63.7	66.6	95.6	4.4

some ultrafiltration occurred after 7 hrs. Some justification for selecting 15 hrs. exists in the curve for 115 mm. Hg pressure in Figure 7. These data suggest that the balance of gain and loss should not be far out of line at 15 hrs.

Lampitt and Bushill (38) reported dialyzable calcium and phosphorous (by continuous dialysis) as 25-33 and 60 per cent of their totals in milk. The percentages of the total calcium and phosphorous that were found to dialyze in 15 hrs. were 30.0 and 63.7, respectively. These data are in good agreement.

Ca/P and (Ca + Mg)/P atomic ratios* were calculated for each of the fractions shown in Figure 8, to determine whether or not these would throw any light on the length time of dialysis to adopt. These ratios (Figure 10) indicate that the (Ca + Mg)/P ratio gradually increases from 0.8 in fraction 1 to 1.33 in fraction 15, with inflections at about fraction 6 (about 4.5 hrs.) and fraction 10 (7.5 hrs.). Ca/P ratios behave peculiarly in that the curve passes through two maxima (fractions 6 and 12). The data indicate that the ions appear to dialyze in a manner independent of each other and do not aid in choosing a dialysis time. It is interesting that both ratios most closely approach that of mono-phosphate during the initial stages of the dialysis and that of tri-phosphate

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*Ca/P atomic ratios for $Ca_3(PO_4)_2$ = 1.5, $CaHPO_4$ = 1.0 and $CaH_4(PO_4)_2$ = 0.5.

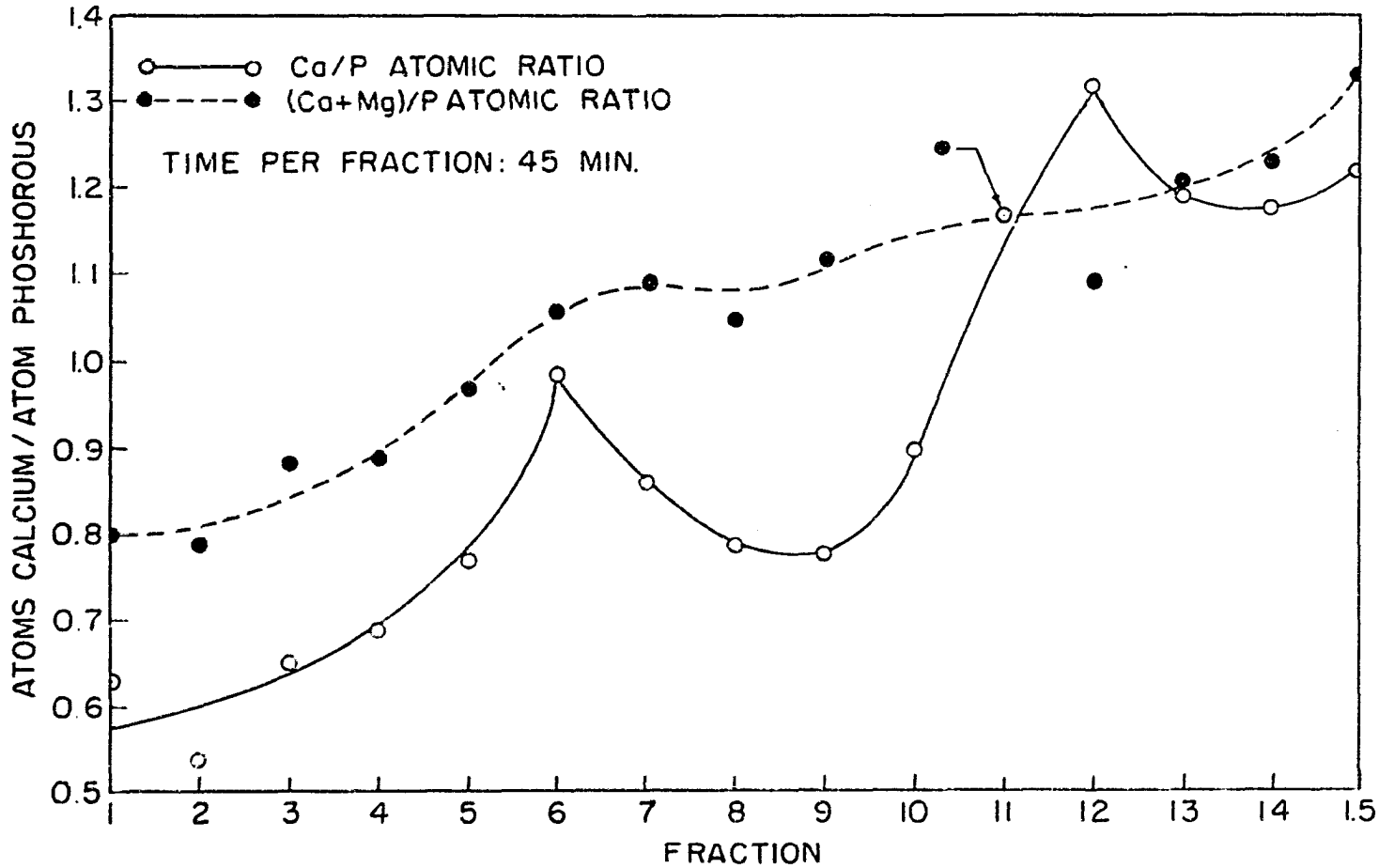

Figure 10. Ca/P and (Ca + Mg)/P ratios of fractions taken during dialysis at 115 mm. Hg for 15 hours.

toward the end of the dialysis. The (Ca + Mg)/P ratios in the plateau region (fractions 6 through 12) are close to that for the di-phosphate. These ratios are not particularly helpful.

b. Elements dialyzable from raw whole milks obtained from August 1955 through March 1956. During the period August 1955 through March 1956, a series of unheated whole milk samples were dialyzed. All dialysates, parent milks and dialysate residues were analyzed for sodium, potassium, calcium, magnesium, chloride and inorganic phosphorous. The quantities of elements dialyzed during 15 hrs., were calculated as per cent of these elements found in their respective original unheated whole milk and are plotted in Figure 11. These data indicate that chloride, sodium and potassium were almost completely dialyzed (96-100 per cent) from the milks. Average percentages of the total calcium, magnesium and inorganic phosphorous which dialyzed, were 24.9, 36.6 and 61.3, respectively. Reported values (31, 39, 49) for dialyzable calcium and inorganic phosphorous agree with those obtained in this study. However, Lampitt et al. (39) reported that 62-83 per cent of the magnesium was dialyzable; the data obtained in this study indicated 27-60 per cent to be dialyzable. The data of Lampitt et al. (39) were obtained by static dialysis procedures; no data were reported for magnesium by their continuous method.

The data in Figure 11 indicate that dialyzable calcium

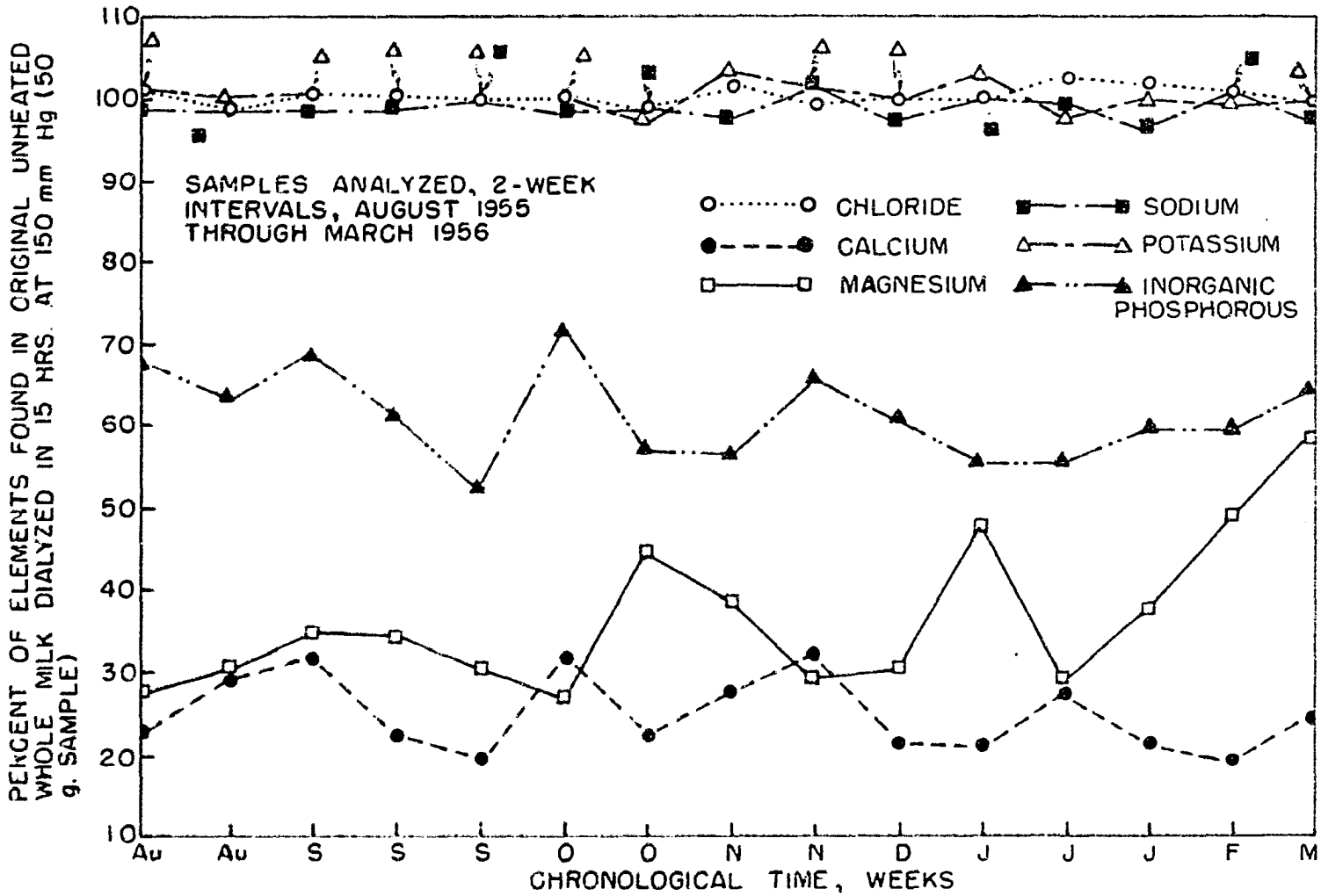

Figure 11. Percentages of inorganic constituents in original unheated whole milk dialyzed in 15 hours at 115 mm. Hg pressure from August 1955 through March 1956.

and inorganic phosphorous vary in the same directions with chronological time. Magnesium seems to vary inversely with calcium. The similarity between variation of calcium and inorganic phosphorous suggests, when first noted, the possibility that they may be dialyzing in definite atomic ratios. The calculated ratios presented in Table 11 indicate considerably variation in the Ca/P ratios, and those in Figure 10 suggest that in as much as the atomic ratios change with time, it should not be expected that definite atomic ratios would exist in the total dialysate.

As was pointed out above dialyzable magnesium tends to vary inversely with calcium and inorganic phosphorous. Such a variation could mean that as the calcium and inorganic phosphorous decrease the magnesium increases. This may indicate that milks which have low calcium content might be expected to have high magnesium contents.

Data in Figure 11 also indicate a tendency for the dialyzable inorganic phosphorous to be low during the late fall months, (October, November and December) and rise in the early winter months, (January, February and March). Dialyzable calcium tended to follow this same pattern except for one analyses during January. The trend in dialyzable magnesium was generally upward from August 1955 through March 1956, despite considerable fluctuation.

Average values for dialyzable elements from unheated whole milk were calculated from the data plotted in Figure 11;

Table 11. Calcium, magnesium and inorganic phosphorous in unheated whole milk dialysates.[a]

Date	Calcium mg.	Magnesium mg.	Phosphorous mg.	Atomic ratios Ca/P	(Ca+Mg)/P
8-6-55	295.59	87.70	458.44	0.50	0.74
8-19-55	398.38	71.53	429.55	0.72	0.93
9-1-55	428.85	122.96	404.15	0.82	1.20
9-16-55	301.04	50.57	387.40	0.60	0.77
9-29-55	273.08	37.10	378.51	0.55	0.68
10-13-55	438.44	28.67	488.44	0.69	0.77
10-27-55	309.42	67.98	363.45	0.66	0.90
11-10-55	359.81	57.14	367.29	0.76	0.93
11-29-55	427.18	28.24	508.91	0.65	0.72
12-22-55	292.79	98.97	437.67	0.52	0.81
1-4-56	290.50	62.85	452.28	0.49	0.68
1-16-56	342.25	57.09	393.19	0.67	0.88
1-30-56	290.84	57.09	478.40	0.81	0.62
2-15-56	281.29	70.87	458.76	0.47	0.67
3-14-56	342.52	85.65	492.24	0.53	0.76
Average	331.47	65.63	433.25	0.59	0.78

[a]Calculated as mg. per 100 g. solids-not-fat in the original milk.

they were: sodium 98.7, potassium 99.9, calcium 24.88, magnesium 36.6, chloride 100.1 and inorganic phosphorous 61.3. The per cent dialyzable inorganic phosphorous, 61.3 agrees well with the amount of phosphorous found in purified unheated centrifugal whey, 64.1 (Table 14). The percentages dialyzable calcium (24.9) and magnesium (36.6) are considerably lower than the percentages of the total calcium (42.0) and magnesium (89.2) found in the centrifugal whey. These

differences may be considered to result from liberation of weakly bound calcium and magnesium possibly not phosphate combined, which subsequently appears in the whey. If this is correct it would suggest that the bonding between salts and proteins is very weak and is easily broken by the centrifugal forces applied. It is also possible that during dialysis, proteins (especially casein) may buffer the system by serving as a sequestering agent which would cause slower liberation of calcium and magnesium than of phosphorous. Such a situation might change equilibria as ionic concentrations lower by having caseinates function as sequestering agents or as cationic exchangers.

c. Distribution of calcium, magnesium and phosphorous in whole milk dialysates. The data for calcium, magnesium and inorganic phosphorous (Figure 11) were calculated as mg. per 100 g. solids-not-fat (Table 11).

The average Ca/P and (Ca + Mg)/P atomic ratios calculated from the data (Table 11) were 0.59 and 0.78, respectively. If it is assumed that the salts in milk appear as simple calcium or mixed calcium-magnesium phosphate salts, e.g. tri-phosphate or di-phosphate, the ratios presented would suggest that the phosphates were mixtures of mono- (Ca/P = 0.5) and di- (Ca/P = 1.0) phosphates. It should be kept in mind, however, that Ca/P ratios change as dialysis proceeds (Figure 10), although the data do suggest mixtures of mono- and di-phosphates during the first 12 hrs.

The Ca/P ratios (Table 11) show a tendency to decrease in the late fall and increase in the early winter, which may indicate that dry feeds increase the amount of calcium to phosphate in milk.

d. Effect of heat on dialyzable elements from milk. Heating milk is considered to precipitate tricalcium phosphate (7, 49).

Magee and Harvey (49) and Lampitt and Bushill (38) have shown that less calcium dialyzed from heated than from unheated milk. In these studies a milk sample was divided, one half was dialyzed as unheated whole milk, the other half was pasteurized at 62.8° C. for 30 min., adjusted to the original weight with distilled water and dialyzed, at 115 mm. Hg for 15 hrs. The dialysates were evaporated and made to 50 ml. (approximately the original volume dialyzed). Both dialysates and the original milk were analyzed for sodium, potassium, calcium, magnesium, chloride and inorganic phosphorous. The results obtained were calculated as per cent of the total for each element in the original whole milk (Table 12).

Laboratory pasteurization of unheated whole milk caused a decrease in the amount of dialyzable inorganic phosphorous and dialyzable calcium to the extent of 2.7 and 3.2 per cent, respectively. However, pasteurization increased the amount of dialyzable magnesium from 58.4 to 64.5 per cent. One would suspect that dialyzable magnesium would have behaved in a manner similar to calcium; this was not the case, suggesting

Table 12. Per cent of total elements in original milk that were dialyzed from unheated and pasteurized[a] samples of the original milk.

Element	Percentage of elements in original milk found in	
	Unheated milk dialysate	Pasteurized milk dialysate
Sodium	99.5	100.2
Potassium	100.1	100.2
Calcium	24.1	20.9
Magnesium	58.4	64.5
Chloride	99.7	99.8
Inorganic phosphorous	63.9	61.2

[a]Pasteurized at 62.8° C. for 30 min.

that magnesium is not involved with phosphate in the precipitation. This anomolous behavior of magnesium is similar to its behavior on centrifugation of milk, as a result of which magnesium appears to be freed from some combination in the original milk and appears in the purified unheated centrifugal whey (Tables 13 and 14).

Pasteurization at 62.8° C. for 30 min. apparently does not reduce the amount of dialyzable sodium, potassium or chloride.

C. Ultracentrifugation and Continuous Pressure Dialysis

It was proposed to conduct a dialysis study of the distribution of inorganic constituents of the various milk

fractions obtainable with a Spinco Model L Ultracentrifuge. The ultracentrifuge afforded an opportunity to separate native casein from milk so that a clear whey and the native caseinate could be studied. Fractionation procedures used in these studies are shown in Figure 3.

1. Distribution of elements in various milk fractions

To determine more completely the distribution of sodium, potassium, calcium, magnesium, chloride and inorganic phosphorous in milk, a study involving continuous pressure dialysis, ultracentrifugation and freezing points of the fractions was undertaken. All milk samples were mixed herd milks and were taken from raw milk storage tanks after thorough agitation. Samples collected were held at 4.44° C. (40° F.) for not longer than 3 hrs. before analyses and treatments were started.

All samples studied were fractionated or otherwise treated according to scheme shown in Figure 3. The data were calculated as mg. of element per 50 g. original milk (Table 13).

Freezing points were determined for all fractions (Table 13). The freezing points of the original unheated whole milks were -0.550 and -0.523° C. those of the corresponding purified unheated centrifugal wheys, were -0.553 and -0.523° C. These freezing points indicate that the contribution of casein to the total freezing point or milk is

Table 13. Analysis of elements in the various milk fractions.

Fraction		Freezing point °C.	mg. elements, calculated as per 50 g. whole milk				C
			Potassium	Sodium	Calcium	Magnesium	
Unheated whole milk (UWM)	S_1	-0.558	79.65	23.43	58.70	4.50	
	S_2	-0.523	71.55	24.94	61.85	5.41	
Unheated whole milk dialysate (UWMD)	S_1	-0.515	78.80	24.20	18.59	1.22	
	S_2	-0.504	71.30	25.20	12.07	1.64	
Unheated whole milk dialysate residue (UWMDR)	S_1	-0.028	0.0	0.0	40.25	3.18	
	S_2	-0.034	0.0	0.0	49.22	3.55	
Purified unheated centrifugal whey (PUCW)	S_1	-0.553	80.50	23.24	23.30	4.46	
	S_2	-0.523	69.80	24.41	20.54	5.45	
Purified unheated centrifugal whey dialysate (PUCWD)	S_1	-0.509	78.95	23.80	18.18	3.64	
	S_2	-0.498	69.60	24.31	12.58	4.26	
Purified unheated centrifugal whey residue (PUCWR)	S_1	-0.031	0.0	0.0	5.59	0.73	
	S_2	-0.030	0.0	0.0	4.69	1.02	
Washed native casein (WNC)	S_1	-0.021	0.0	0.0	4.12[a]	0.14[b]	
	S_2	-0.022	0.0	0.0	3.26[a]	0.18[b]	
Washed native casein dialysate (WNCD)	S_1	+0.021	0.0	0.0	0.79	0.0	
	S_2	+0.021	0.0	0.0	0.90	0.0	
Washed native casein residue (WNCR)	S_1	-0.012	0.0	0.0	3.19[a]	0.12[b]	
	S_2	-0.008	0.0	0.0	2.24[a]	0.16[b]	

[a]Total calcium
[b]Total magnesium
[c]Total phosphorous
S=Sample

lements in the various milk fractions.

Freezing point °C.	mg. elements, calculated as per 50 g. whole milk, for each fraction					
	Potassium	Sodium	Calcium	Magnesium	Chloride	Inorganic Phosphorous
-0.558	79.65	23.43	58.70	4.50	51.60	28.63
-0.523	71.55	24.94	61.85	5.41	49.76	32.04
-0.515	78.80	24.20	18.59	1.22	51.60	20.71
-0.504	71.30	25.20	12.07	1.64	51.55	16.73
-0.028	0.0	0.0	40.25	3.18	0.0	10.61
-0.034	0.0	0.0	49.22	3.55	0.0	15.59
-0.553	80.50	23.24	23.30	4.46	50.10	20.10
-0.523	69.80	24.41	20.54	5.45	52.55	16.39
-0.509	78.95	23.80	18.18	3.64	51.60	20.10
-0.498	69.60	24.31	12.58	4.26	51.55	16.44
-0.031	0.0	0.0	5.59	0.73	0.0	0.19
-0.030	0.0	0.0	4.69	1.02	0.0	0.14
-0.021	0.0	0.0	4.12[a]	0.14[b]	0.0	8.90[c]
-0.022	0.0	0.0	3.26[a]	0.18[b]	0.0	7.60[c]
+0.021	0.0	0.0	0.79	0.0	0.0	2.80
+0.021	0.0	0.0	0.90	0.0	0.0	3.12
-0.012	0.0	0.0	3.19[a]	0.12[b]	0.0	5.85[c]
-0.008	0.0	0.0	2.24[a]	0.16[b]	0.0	4.31[c]

negligible. Data of this nature have been found to be reproducible during many trials. As regards washed native caseinates, the freezing points of their suspensions (-0.021 and -0.022° C.) made to concentrations present in the original milk together with the freezing points of washed caseinate dialysates (-0.021 and -0.022° C.) further show that caseinates exert essentially no osmotic activity.

Percentages of the total sodium, potassium and chloride in the original whole milk that were found in the milk and whey dialysates were calculated and found to be similar to the values in Table 10 (97.6 to 99.9). Per cent dialyzable calcium, magnesium and phosphorous from the whole milk and centrifugal whey were also found to be essentially the same as those in Table 10 except for the dialyzable magnesium in the centrifugal whey. The amount of dialyzable magnesium (4.46 and 5.45 per 50 g. milk) from the centrifugal whey was much greater than that dialyzable from the original milk (1.22 and 1.64 mg. per 50 g. whole milk) indicating that centrifugation may have caused a desorption of magnesium from protein or that magnesium may occur in a different form than calcium.

The amounts of inorganic phosphorous dialyzable from whole milk and centrifugal whey were the same; however, all the inorganic phosphorous in the centrifugal whey was dialyzable, while approximately 61.3 per cent of the inorganic phosphorous was dialyzable from the whole milk. It is presumed

that the remaining inorganic phosphorous in milk is present as non-dialyzable phosphates, or is bound in a calcium-phosphate-calcium caseinate complex.

The analysis of the whole milk dialysate residues and centrifugal whey dialysate residues confirm the previous findings that almost all the potassium, sodium and chloride are dialyzed in 15 hrs. at 115 mm. Hg.

Most of the sodium, potassium and chloride present in the milk was found in the centrifugal whey. There are slight differences between the quantities of these elements in the milk and in the whey; these differences may be indicative of binding or adsorption by the caseinates. Amounts of calcium and inorganic phosphorous, found in the centrifugal whey are approximately one-third and three-fourths, respectively, of those in the original whole milk. Furthermore, almost all of the magnesium in the whole milk was found in the centrifugal whey. Since there are smaller amounts of calcium and inorganic phosphorous found in the whey than in the whole milk this difference may offer some suggestion as to the amount of these elements bound by the caseinates. Thus upon centrifugation there is an average of 62.8 and 39.0 per cent of the calcium and inorganic phosphorous, respectively, in the whole milk, remaining in the caseinates. Since most of the magnesium in the whole milk was found in the centrifugal whey this tends to further substantiate the premis that centrifugation causes a desorption of magnesium from protein or that

magnesium exists in a different form in milk than does calcium.

It was considered that the amount of inorganic phosphorous remaining in the caseinates upon centrifugation was chemically bound with the caseinate. It was assumed, for purposes of calculation, that the difference in inorganic phosphorous contents of the whole milk and centrifugal whey (UWM-PUCW) represents the amount of non-dialyzable tricalcium phosphate in the milk. If an amount of calcium is calculated which is equivalent to this inorganic phosphorous (UWM-PUCW) and this quantity is compared to the quantity of calcium obtained when the calcium of washed native casein (WNC) is subtracted from the difference in calcium between the whole milk and whey, (UWM-PUCW)-WNC, and found to be similar, then it would suggest that the phosphorous remaining with the caseinate upon centrifugation occurred as tricalcium phosphate. These calculations were made but the values did not corroborate such a hypothesis. However, Ca/P ratios were calculated for the experimental values of calcium and inorganic phosphorous in the caseinates and were found to be 2.53 and 1.95. These values are not indicative of any known calcium and phosphorous compound but they may be indicative of the ratio of calcium and inorganic phosphorous bound to the caseinate.

The caseinate obtained by ultracentrifugation was washed three times with redistilled water, made to the concentration occurring in the parent milk and dialyzed for 15 hrs. at 115 mm.

Hg. The average Ca/P and (Ca + Mg)/P ratios calculated for the washed native casein residues reported in Table 13 were 0.41 and 0.43, respectively. The average Ca/P ratio for the washed native casein dialysate was found to be 0.22. Essentially no magnesium was detected in the washed native caseinate and none was present in its dialysate. This suggests that the caseinate essentially was not a mixed calcium and magnesium "salt".

The Ca/P and (Ca + Mg)/P ratios for the washed native caseinate residues, 0.41 and 0.43, respectively, may be indicative of the amount of calcium and phosphorous bound to the caseinate aggregate. The ratio of Ca/P (4.56) in the washed native caseinate dialysate would be the dialyzable calcium and phosphorous associated with the caseinate. These values may indicate the proportion in which the calcium, and phosphorous are bound to the caseinate or they may be residual adsorbed calcium, and phosphorous.

If the amount of nuclear phosphorous (0.514 per cent) present in the casein, obtained by centrifugation, is subtracted from the total phosphorous in the washed native caseinate this difference (inorganic phosphorous) may be indicative of the phosphorous bound to the caseinate. Calculations were made and the Ca/P ratios for the bound calcium and phosphorous were found to average 0.245.

a. Calcium, magnesium and phosphorous content of purified unheated centrifugal whey obtained from unheated whole milk.

The data of Table 14 were obtained as confirmatory evidence regarding the distribution of calcium, magnesium and inorganic phosphorous between purified unheated centrifugal wheys and the parent milks. These data show that 42.0 per cent of the calcium, 87.8 per cent of the magnesium and that 64.1 per cent of the inorganic phosphorous in the unheated whole milk were found in the purified unheated centrifugal whey. The average percentages of calcium, magnesium and inorganic phosphorous that had been found to dialyze (Figure 11) were 24.8, 36.6 and 61.3 per cent, respectively, of the amounts in the milks. The percentage of inorganic phosphorous that dialyzed from the milk was the same as that found in the whey. The percentages of the totals of calcium and magnesium in the centrifugal whey were greater than those in the milk dialysates. Whether or not, this difference in calcium and magnesium between the centrifugal whey and the milk dialysate results from a possible cation exchange or a sequestering activity of the caseinates, as equilibria shift during dialysis, is not clear. It is offered as a possible explanation because it seems unlikely that the cations would be combined with the proteins in a manner that could be changed to this degree by centrifugal force.

The average Ca/P ratio, 0.92, in the centrifugal whey varies considerably from the theoretical Ca/P ratio in the triphosphate (1.5). It agreed closely with the Ca/P ratio, in the di-phosphate (1.00). The Ca/P ratio, 1.39, in the unheated

Table 14. Calcium, magnesium and inorganic phosphorous in unheated whole milk and in purified unheated centrifugal whey.[a]

mg. per 50 g. sample			Ratios	
Calcium	Magnesium	Inorganic phosphorous	Ca/P	(Ca + Mg)/P
Unheated Whole Milk (Fat and Protein Free)				
55.33	8.49	33.85	1.21	1.58
54.63	4.19	26.65	1.59	1.78
57.76	5.03	29.82	1.49	1.69
53.17	8.16	32.53	1.27	1.59
59.18	5.18	31.91	1.45	1.64
Av. 56.01	6.21	30.95	1.39	1.66
Purified Unheated Centrifugal Whey[a] (Fat and Protein Free)				
24.21	6.83	21.33	0.88	1.28
23.06	4.41	19.89	0.89	1.18
20.32	5.09	16.22	0.97	1.39
24.32	5.88	21.42	0.88	1.22
25.53	4.21	20.04	0.98	1.25
Av. 23.48	5.96	19.78	0.92	1.27
Percentage Elements in Milk Found in Purified Unheated Centrifugal Whey				
43.8	80.4	63.0		
42.2	105.3	74.6		
35.3	100.1	54.4		
45.7	72.1	65.9		
43.1	81.3	62.8		
Av. 42.0	87.8	64.1		

[a]Calculated to an equivalent basis of the parent milk.

whole milk approaches the theoretical for the tri-phosphate (1.50). However, in unheated whole milk, caseinates are considered to bind considerable calcium. (Ca + Mg)/P ratios for both whole milk (1.66) and centrifugal wheys (1.27), suggest the possibility of mixed phosphates.

2. Nuclear phosphorous determinations

The nuclear hydroxyaminoacid-esterified phosphorous contents of various isoelectric caseins were determined as an adjunct to the studies of salt distribution. It was thought that there might be a difference in the nuclear phosphorous contents of isoelectric caseins obtained by centrifugation followed by isoelectric precipitation and those obtained directly from unheated skim milk by isoelectric precipitation. The casein obtained from milk by ultracentrifugation was washed, reconstituted and acid precipitated in the same manner as used when casein was precipitated from unheated skim milk at its isoelectric point. After repeptization and precipitation (5 times) the phosphorous contents of the two isoelectric caseins were determined. The phosphorous found was considered esterified with the hydroxyl of hydroxy amino acids in the casein (nuclear phosphorous). Nuclear phosphorous in casein centrifuged from the milk and then precipitated was found to be 0.523, 0.519 and 0.502 per cent; that for casein acid precipitated directly from unheated centrifugal skim milk contained 0.569, 0.566 and 0.534 per cent.

Average values for the centrifuged and acid precipitated caseins and for the caseins acid precipitated directly were 0.511 and 0.556 per cent, respectively. The difference in the two nuclear phosphorous values may result from the manner in which the caseins were obtained. One possible explanation for the lower nuclear phosphorous content of centrifuged casein may be that some of the smaller phosphorous rich casein particles (27) were lost during the washing of the casein. This would result in a concentration of the larger particles with lower phosphorous content. Another explanation might be that the casein precipitated directly from skim milk may adsorb calcium and phosphate ions despite the acidity of the medium.

Ramsdell and Whittier (75) reported a value of 0.742 per cent nuclear phosphorous in casein acid-precipitated from skim milk. Values obtained in the study reported in this thesis were 0.514 and 0.556 per cent for the centrifuged, acid-precipitated casein and for the casein acid-precipitated from normal skim milk, respectively, The latter value, when compared to that reported by Ramsdell and Whittier, tends to substantiate the hypothesis that the value for nuclear phosphorous, determined on casein acid-precipitated from skim milk may be influenced by adsorbed phosphate.

D. Ultrafiltration Studies

The method of ultrafiltration reported in these studies was an outgrowth of the work with continuous pressure dialysis. It was thought that some aspects of ultrafiltration were concerned in later stages of the pressure dialysis. For this reason it was considered advisable to determine whether or not so simple an apparatus could be employed for ultrafiltration.

1. Development of method

Most reported methods of ultrafiltration (7, 37, 84) were operated by pulling a high vacuum on a vessel wherein the sample, contained in a semipermeable membrane, was placed. The vacuum caused the filtrate to pass through the membrane and collect at the bottom of the container. It is considered that this method is not reliable because evaporation of water from the filtrate would cause the analytical data to be high.

The method reported in this study does not employ a vacuum but a positive pressure which is exerted on the sample contained in a semipermeable membrane. The pressure was exerted by nitrogen gas on a layer of mineral oil covering the sample surface. The filtrate passing through the membrane was collected at the bottom of the ultrafiltration chamber (Figure 2) and drawn off through the sampling tube.

To prevent evaporation of the filtrate a water trap was placed on the filtration chamber. This prevented loss of water to the refrigerating unit in the refrigerator. It likewise served as an expansion valve to allow excess air to escape when pressure was applied to the membrane. It was considered that if any change in concentration of the filtrate occurred, it should be one of dilution since the vapor pressure of water in the water trap should be greater than that above the filtrate. The maximum operating pressure was determined with water-filled membranes. The limiting factor was found to be the strength of the membrane. It was found that a pressure of 200 mm. Hg was satisfactory as far as rate of filtration was concerned and that generally membranes remained intact at this pressure for at least 72 hrs.

a. Filtration rate. Before using this method as a routine research tool, the filtration rate was studied. The filtration rate was determined by filtering centrifugal whey and purified centrifugal skim milk for varying lengths of time and measuring the volume of filtrate obtained. When two ultrafiltrations were carried on simultaneously, the pressure regulating device shown in Figure 2 was employed. Representative data obtained from one dual ultrafiltration are shown in Figure 12. The rate curve for centrifugal whey shows that the filtration rate was rapid during the first 20 hrs.; approximately 35 ml. were obtained from 55 ml. of skim

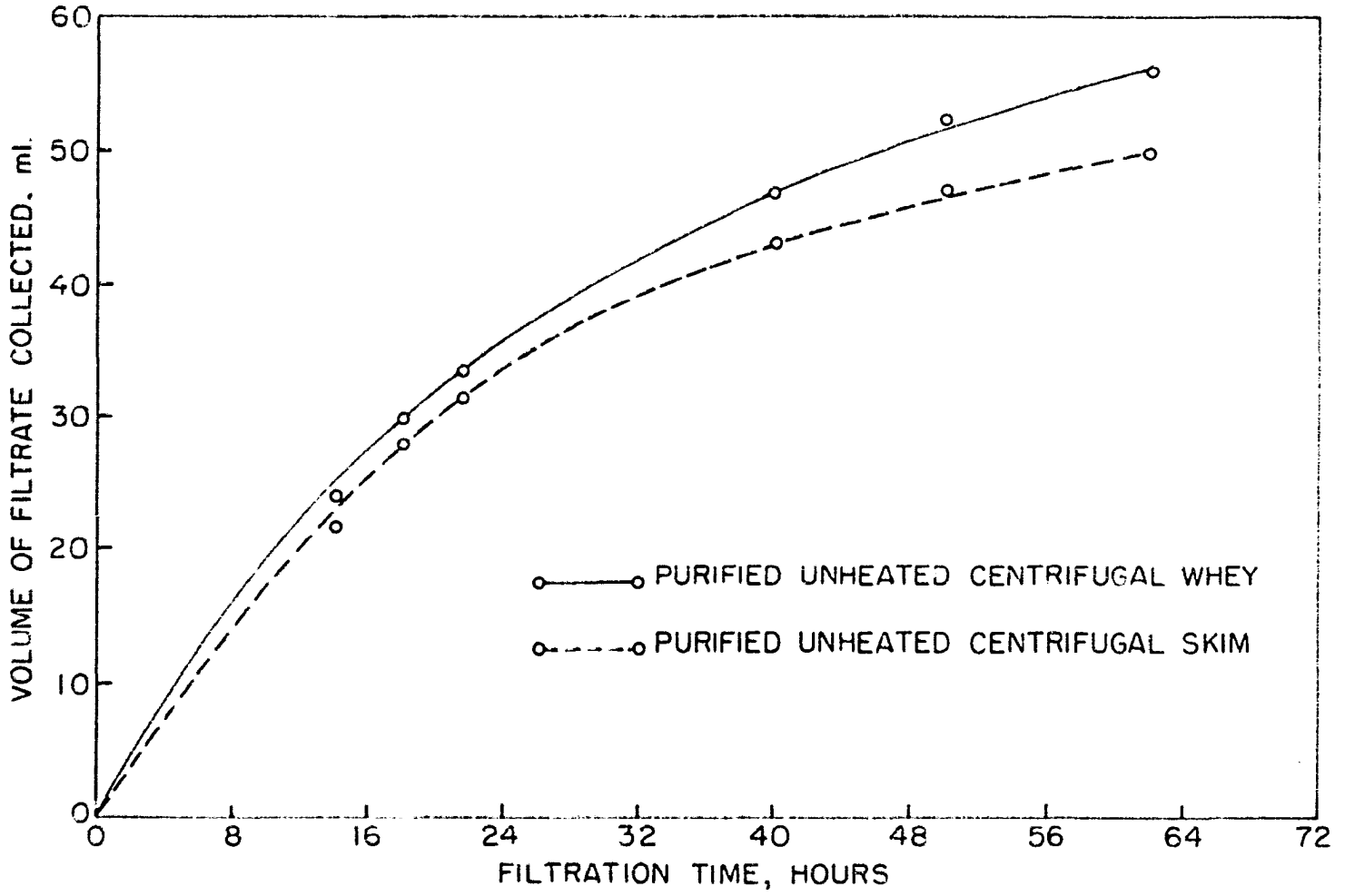

Figure 12. Ultrafiltration rates of purified unheated centrifugal whey and skim at 200 mm. Hg pressure.

milk. From 20 hrs. to 64 hrs. 20 ml. of filtrate were collected. All purified centrifugal skim milks were found to filter at a slower rate than centrifugal wheys.

b. Distribution of elements in filtrate fractions. To ascertain whether or not the filtrate collected from purified centrifugal whey at different times during a filtration were alike, composition-wise, approximately 10 ml. quantities of filtrate were collected during a 60 hr. filtration. These fractions were analyzed for sodium, potassium, calcium, magnesium, chloride and inorganic phosphorous. The data (Table 15) indicate that any variations that occurred as the filtration proceeded were within the experimental error of the analytical methods. These data indicate that it would be necessary to collect only that amount of filtrate that would be needed for the particular inorganic analyses desired.

Because, all ultrafiltrations were carried out at 4.4° C., water condensed on the inside of the ultrafiltration chamber. It was considered that this water distilled from the membrane surface and consequently it was rinsed from the surface by the filtrate collected to avoid higher concentrations of elements than should have been in the filtrates.

2. Results obtained with ultrafiltration

The ultrafiltration apparatus was employed to determine

Table 15. Distribution of elements in fractions of ultrafiltrate collected over a 60 hour period.

Fraction	Cumulative Volume ml.	Cumulative Time hrs.	mg. elements per ml. filtrate					
			Sodium	Potassium	Calcium	Magnesium	Chloride	Inorganic phosphorous
1	11.5	5	0.494	2.987	0.399	0.0973	0.119	0.454
2	22.0	12	0.475	3.033	0.387	0.0973	0.131	0.446
3	33.5	21	0.487	3.098	0.360	0.103	0.135	0.457
4	42.5	30	0.511	2.920	0.399	0.0951	0.125	0.460
5	52.5	45	0.487	2.800	0.399	0.0946	0.124	0.475
6	58.0	60	-	-	0.399	0.0973	0.121	0.487

whether or not there were differences in the concentrations of inorganic elements in the filtrates obtained from purified unheated centrifugal whey and the skim milk from which the whey was obtained. Freezing point determinations were made on all milks, wheys and filtrates and biuret and ninhydrin tests were run on all centrifugal wheys and filtrates.

a. Comparison of the inorganic constituents in the ultrafiltrates from purified unheated centrifugal whey and its parent skim milk. Samples of centrifugal whey and the parent skim milk were ultrafiltered for approximately 65 hrs. to obtain as much filtrate as possible. The filtrates were analyzed for the major inorganic elements (Table 16). Results for all products obtained were calculated on a fat- and protein-free basis.

The data (Table 16) indicate a difference between the quantities of elements contained in the ultrafiltrate from the centrifugal whey and that from its parent skim milk. Percentages of the total sodium, calcium, magnesium and inorganic phosphorous present in the parent skim milk, that were found in the whey ultrafiltrate, were greater in all cases than those in the parent skim milk ultrafiltrate. A possible reason for the lesser amounts of elements in the ultrafiltrate from skim milk may be binding or adsorption of ions by the proteins or to a sequestering or ion-exchange activity of caseinate. If it were the former, ultrafiltration might possibly be used to study the ion binding properties

Table 16. Distribution of inorganic constituents of various milk fractions.

Fraction	mg. elements per 50 g. (fat and protein free) product					
	Sodium	Potassium	Calcium	Magnesium	Chloride	Inorganic phosphorous
Purified Unheated Centrifugal Skim	29.36	74.74	55.33	8.49	55.68	33.85
Purified Unheated Centrifugal Whey	29.69 (101.0)	74.77 (100.2)	24.21 (43.8)	6.83 (80.4)	55.41 (99.5)	21.33 (63.0)
Purified Unheated Centrifugal Skim Ultrafiltrate	20.16 (68.7)	74.62 (99.8)	22.18 (40.1)	5.78 (68.1)	55.75 (100.1)	20.04 (59.2)
Purified Unheated Centrifugal Whey Ultrafiltrate	23.52 (80.1)	70.15 (93.9)	22.79 (41.1)	7.42 (87.4)	55.90 (100.4)	22.65 (66.9)

( ) Percentage elements in skim milk found in various milk fractions.

of various proteins.

Here again is evidence which may indicate centrifugal desorption of ions and/or salts by proteins. The percentages of calcium, magnesium and phosphorous in the original skim milk found in the whey ultrafiltrates were larger than those in the skim ultrafiltrates, again indicating that these become more dialyzable or filterable when caseinates are centrifuged from the system, skim milk.

b. Ca/P and (Ca + Mg)/P ratios in purified skim milk, its whey and their ultrafiltrates. Table 17 contains the Ca/P and (Ca + Mg)/P ratios for the various fractions analyzed.

Average Ca/P (0.84) and (Ca + Mg)/P (1.16) ratios for the centrifugal whey, for the skim milk ultrafiltrate, 0.81 and 1.18, respectively, and for the whey ultrafiltrate, 0.79 and 1.09, respectively, are in good agreement. However, none of the Ca/P ratios agree with those of the di- or triphosphate, 1.00 and 1.50, respectively, indicating that the elements may occur in milk as mixed salts or may be bound by or adsorbed to proteins. Ca/P ratios for skim milk approximate that of tricalcium phosphate, however, this ratio would include all of the ester- and carboxy-linked calcium which if not considered would lead one to think that all the calcium in milk was in the form of tricalcium phosphate.

Ca/P and (Ca + Mg)/P ratios for the above data as well as those for whole milk and centrifugal whey (Table 14) and

Table 17. Ca/P and (Ca + Mg)/P ratios found in various milk fractions.

Run	Fractions							
	Purified unheated centrifugal skim[a]		Purified unheated centrifugal whey[a]		Ultrafiltrates of purified unheated centrifugal skim		whey	
	Ca/P	(Ca + Mg)/P	Ca/P	(Ca + Mg)/P	Ca/P	(Ca + Mg)/P	Ca/P	(Ca + Mg)/P
1	1.45	1.64	0.89	1.14	-	-	0.78	1.02
2	1.27	1.59	0.88	1.28	0.85	1.22	0.78	1.19
3	1.22	1.59	0.76	1.08	0.78	1.15	0.79	1.05
Av.	1.32	1.60	0.84	1.16	0.81	1.18	0.79	1.09

[a]Calculated as fat and protein free.

for milk dialysates are discussed in the following section and are summarized in Table 18.

c. Summary of Ca/P and (Ca + Mg)/P ratios for various fractions. Ca/P and (Ca + Mg)/P ratios, found in this study, for all milk fractions and dialysates are summarized in Table 18.

Table 18. Average Ca/P and (Ca + Mg)/P ratios for milks, milk fractions and dialysates.

Milk Fractions	Ratios	
	Ca/P	(Ca + Mg)/P
Unheated Whole Milk (UWM)	1.39	1.67
Unheated Whole Milk Dialysate (UWMD)	0.59	0.78
Purified Unheated Centrifugal Skim (PUCS)	1.32	1.58
Purified Unheated Centrifugal Skim Ultrafiltrate (PUCSU)	0.81	1.18
Purified Unheated Centrifugal Whey (PUCW)	0.89	1.27
Purified Unheated Centrifugal Whey Dialysate (PUCWD)	0.64	0.93
Purified Unheated Centrifugal Whey Ultrafiltrate (PUCWU)	0.78	1.09

Average Ca/P ratios for unheated whole milk (1.39) and purified unheated centrifugal skim (1.32) approach the theoretical ratio for tri-phosphate (1.50). This might

suggest a mixture of di- and tri-phosphates milk. The ratios would be lower were it possible to deduct the calcium that is bound by carboxyl or nuclear phosphate groups of casein from the total calcium found and then recalculate the values.

Average Ca/P and (Ca + Mg)/P ratios for centrifugal skim milk, whey ultrafiltrates and centrifugal whey reduce from about the value of tri-phosphate in the skim, to that of an approximately equi-molar mixture of di- and tri-phosphates in unheated centrifugal whey, to approximately that of di-phosphate in unheated centrifugal whey ultrafiltrate. These data suggest that the salts in milk do not occur as single salts but as mixtures.

The Ca/P ratios for the whole milk and centrifugal whey dialysates, 0.59 and 0.64, respectively, are much lower than any of the other Ca/P ratios obtained. Such low values may indicate that during dialysis the proteins had a tendency to bind the cations and leave anions free to dialyze; this would entail preferential binding for calcium and allow other cations to dialyze to maintain cation:anion balance in the dialysates. If this were the case it would account for the small ratios.

Several workers (20, 21, 22, 27, 73) have studied the Ca/P ratios in the clear whey resulting from centrifugation of skim milk and have found values approximating that of tri-phosphate (1.50). Ratios of Ca/P in centrifugal whey, determined in these studies, were found to average 0.89 which was

considerably lower than the ratio of the tri-phosphate and the ratios for centrifugal whey reported by others. However, the Ca/P ratios obtained from whole milk (1.39) and purified centrifugal skim milk (1.32) were found to approach that of tri-phosphate. If it is considered that centrifugation causes a desorption of salt and/or ions by protein complexes, and these studies have indicated that this might be the case, it would be necessary to release more phosphate than calcium to decrease the Ca/P ratio. As was indicated previously, the Ca/P ratios in products containing native caseinates likely have out little meaning because of calcium bound by caseinate as an ion.

(Ca + Mg)/P ratios for skim milk and whey ultrafiltrates and whey dialysates all approach 1.0 (di-phosphate) indicating similarity among these salt systems. The (Ca + Mg)/P ratio for whey more nearly approximates that of an equi-molar mixture of di- and tri-phosphates.

When comparing theoretical and experimental Ca/P and (Ca + Mg)/P ratios it must be considered that mixed salts of calcium, magnesium and phosphorous may appear in milk as well as single salts. Thus, the assumption that all Ca/P ratios must compare favorably with that of the tri-phosphate may be completely in error. It must be kept in mind, however, that an amount of magnesium equivalent to more than 80 per cent of that in skim milk is present in and will filter from centrifuged whey, whereas but 68 per cent will

filter from skim milk (Table 18). A similar relationship occurs with regard to the dialyzable magnesium from whole milk and its centrifugal whey (Table 14). This raises a question as to the similarity of occurrence of magnesium and calcium and the presence of mixed phosphates.

d. Other physical and chemical characteristics of the original milk and its whey, dialysates and ultrafiltrates. Freezing points and pH values were run on all original milks, centrifugal wheys, dialysates and ultrafiltrates. Biuret and ninhydrin tests were run on the centrifugal wheys and ultrafiltrates. Data obtained from these determinations are shown in Table 19.

The average freezing point for the centrifugal wheys (-0.520° C.) was close to that of the original milk (-0.530° C.) while the average freezing point of the milk (-0.510° C.) and of the whey (-0.506° C.) dialysates was higher than that of the parent substance. The values for the milks and their centrifugal wheys indicate that the greatest portion of the smaller ions, those responsible for freezing point depression, were in the centrifugal whey indicating that the caseinates had little effect upon the freezing point.

Ultrafiltrates of centrifugal skim milk and its whey had lower average freezing points (-0.560° C.) than the parent skim milk (-0.530° C.) and the parent whey (-0.521° C.). This depression of freezing point in the skim milk and

Table 19. Some physical and chemical properties of the various milk fractions.

Fractions	Average freezing points °C.	Average pH	Biuret reaction	Ninhydrin reaction
Purified Unheated Centrifugal Skim	-0.530	6.70	+	+
Unheated Whole Milk Dialysate[a]	-0.510	6.70	NR[b]	NR[b]
Purified Unheated Centrifugal Skim Ultrafiltrate	-0.560	6.90	-	-
Purified Unheated Centrifugal Whey	-0.520	6.80	+	+
Purified Unheated Centrifugal Whey Dialysate[a]	-0.506	6.65	NR[b]	NR[b]
Purified Unheated Centrifugal Whey Ultrafiltrate	-0.560	6.85	-	-

[a]Dialysate evaporated to small volume and made to the volume of the skim milk that was dialyzed.
[b]NR = Not Run.

centrifugal whey ultrafiltrates was found to be caused by water soluble substances coming from the cellulose membrane. Distilled water was filtered through a section (10 in.) of membrane and the freezing point taken. The freezing point depression of the distilled water filtrate was found to be equivalent to the difference in freezing points of the skim

milk and whey filtrates and their parent fractions.

The pH values of the original milk and the skim milk and the whey dialysates were in good agreement with each other and were similar to that usually found in normal milks. The pH values of the centrifugal whey and the skim milk and the whey ultrafiltrates were higher than those of their parent fractions. Such an effect may result from a buffering effect of proteins in the skim milk although it would not seem likely in the centrifugal whey, in which the protein concentration is small. There is a possibility that protein bound ions may have been released during filtration thus causing this increase in pH.

Biuret and ninhydrin tests for proteins or peptides and for amino acids, respectively, were run on the centrifugal wheys and ultrafiltrates. It was surprising to find that all ultrafiltrates yielded negative ninhydrin tests when unheated milks were the starting materials. One sample of ultrafiltrate did give a positive ninhydrin reaction; the centrifugal whey from which it was obtained was 5 days old and some enzymatic decomposition may have occurred. This sample was not included in the reported results. It would be expected that if the parent whey or milk gave positive ninhydrin and biuret reactions that the ultrafiltrates might also be positive. Since they are not, this would indicate that little enzymic decomposition has occurred and that the smallest polypeptides that might have formed were

greater in diameter than the pore diameters of the membrane, and that the membrane character was such that amino acids (in the quantities present) were not released.

E. Distribution of Inorganic Elements of Milk from April 1955 to March 1956

At the time the resin membrane electrode studies were started it was decided to determine the inorganic constituents of milk for possible purposes of calculating the ionic, colloidal and bound salt concentrations. Although the resin membrane electrodes were found unusable, the gross analysis of the milk samples was continued for a one year period at intervals of approximately two weeks. The elements for which the milks were analyzed were sodium, potassium, calcium, magnesium, chloride and inorganic phosphorous. The results were calculated as mg. element per 100 g. solids-not-fat. These data are shown in Table 20 and plotted in Figure 13.

According to Figure 13 the magnesium concentration in milk stayed relatively constant throughout one year. Sodium did not vary much during the year but seemed to increase slightly during the winter months and then decrease in the early spring. Inorganic phosphorous decreased slightly during the summer months and increased in the winter. The inorganic phosphorous and calcium seemed to fluctuate more or less simultaneously indicating that the two were directly

Table 20. Inorganic constituents of milk from April 1955 through March 1956[a].

Date	Elements					
	Sodium mg.	Potassium mg.	Calcium mg.	Magnesium mg.	Chloride mg.	Ino
4-15-55	534.9	1,795.2	1,338.0	136.5	1,103.3	
4-29-55	519.3	1,694.3	1,564.4	136.7	1,151.1	
5-12-55	540.3	1,636.9	1,358.3	109.2	1,100.1	
5-26-55	532.3	1,472.6	1,326.1	127.2	1,063.9	
6-10-55	509.6	1,379.1	1,305.5	139.6	1,132.2	
6-23-55	509.3	1,489.4	1,520.5	160.2	1,166.9	
7- 8-55	493.3	1,511.6	1,352.1	120.1	1,172.5	
7-21-55	559.1	1,432.7	1,278.7	144.9	1,160.5	
8- 5-55	527.9	1,705.6	1,304.4	141.1	1,425.3	
8-18-55	483.8	1,659.9	1,327.6	119.2	1,135.1	
9- 1-55	537.7	1,749.6	1,350.2	176.9	1,254.2	
9-16-55	687.5	1,621.5	1,354.7	143.6	1,329.1	
9-29-55	558.4	1,618.3	1,399.3	122.3	1,125.3	
10-13-55	565.5	1.878.5	1,384.4	106.2	1,215.8	
10-27-55	595.4	1,590.2	1,373.7	151.1	1,171.1	
11-10-55	618.2	1,564.7	1,306.1	132.2	1,172.9	
11-29-55	571.7	1,710.9	1,336.9	96.0	1,271.7	
12-22-55	612.5	1,753.5	1,333.8	164.0	1,243.0	
1- 4-56	613.7	1,598.9	1,382.3	131.2	1,313.0	
1-16-56	645.9	1,136.2	1,294.6	198.3	1,063.4	
1-30-56	631.8	1,258.1	1,374.4	169.9	1,130.3	
2-15-56	562.2	1,453.8	1,456.2	144.3	1,117.2	
3-14-56	572.0	1,733.8	1,421.2	146.6	1,109.9	
Average	560.1	1,584.6	1,367.1	139.8	1,181.8	

[a]Calculated as mg. element per 100 g. solids-not-fat in the original milk

onstituents of milk from April 1955 through March 1956[a].

Elements				
Potassium mg.	Calcium mg.	Magnesium mg.	Chloride mg.	Inorganic Phosphorous mg.
1,795.2	1,338.0	136.5	1,103.3	700.1
1,694.3	1,564.4	136.7	1,151.1	775.3
1,636.9	1,358.3	109.2	1,100.1	706.1
1,472.6	1,326.1	127.2	1,063.9	710.2
1,379.1	1,305.5	139.6	1,132.2	756.8
1,489.4	1,520.5	160.2	1,166.9	668.7
1,511.6	1,352.1	120.1	1,172.5	694.2
1,432.7	1,278.7	144.9	1,160.5	575.4
1,705.6	1,304.4	141.1	1,425.3	672.3
1,659.9	1,327.6	119.2	1,135.1	677.3
1,749.6	1,350.2	176.9	1,254.2	681.6
1,621.5	1,354.7	143.6	1,329.1	636.9
1,618.3	1,399.3	122.3	1,125.3	724.6
1.878.5	1,384.4	106.2	1,215.8	675.2
1,590.2	1,373.7	151.1	1,171.1	790.1
1,564.7	1,306.1	132.2	1,172.9	791.1
1,710.9	1,336.9	96.0	1,271.7	784.1
1,753.5	1,333.8	164.0	1,243.0	711.9
1,598.9	1,382.3	131.2	1,313.0	819.6
1,136.2	1,294.6	198.3	1,063.4	703.9
1,258.1	1,374.4	169.9	1,130.3	816.3
1,453.8	1,456.2	144.3	1,117.2	785.9
1,733.8	1,421.2	146.6	1,109.9	770.7
1,584.6	1,367.1	139.8	1,181.8	722.9

. element per 100 g. solids-not-fat in the original milk

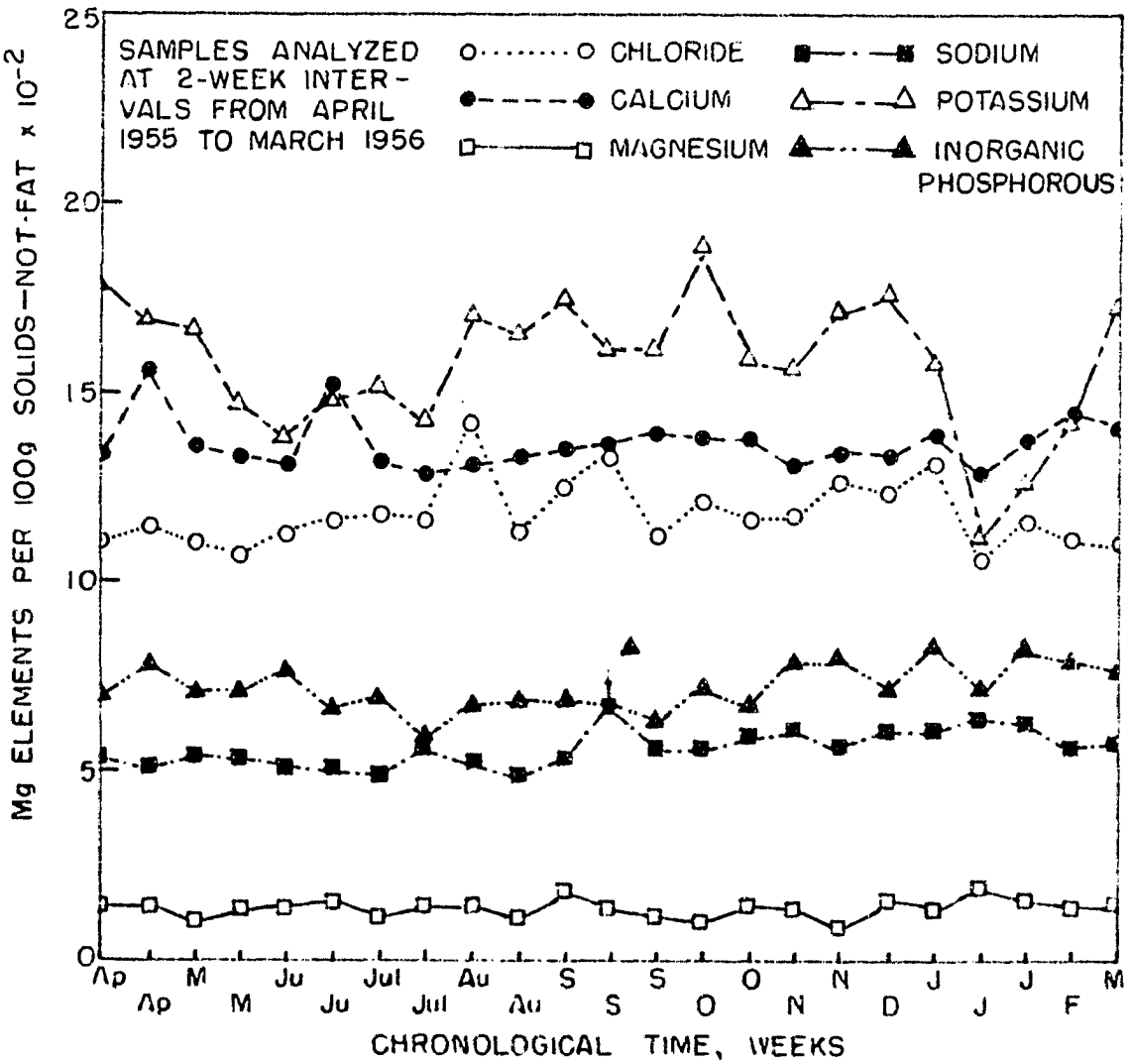

Figure 13. Distribution of the inorganic constituents in normal whole milk from April 1955 through March 1956.

related, however, calcium did not vary greatly over the period studied.

Chloride content of milk had a tendency to increase during the summer reaching its peak in the late summer and fall then decreasing during the winter.

Potassium varied considerably over the entire period studied. The tendency for potassium variation was to decrease during the early spring to a low in June and July then increase in August and September when the pasture conditions improved. Another low appeared in December and January followed by an increase to the early spring level. Potassium did not seem to correlate with any particular ion over the entire year of study. High values occurred in March to April, August to October and during November.

The average values for the main inorganic constituents in milk were sodium 560.1, potassium 1,584.6, calcium 1367.1, magnesium 139.8, chloride 1,181.8 and inorganic phosphorous 722.9 mg. per 100 g. solids-not-fat. Table 21 compares the values reported by other workers (II-F) to those found in these studies. Other workers reported their values on a mg. per 100 ml. of milk basis while values reported in these studies are on a mg. per 100 g. solids-not-fat basis. The values from the literature were calculated to the 100 g. solids-not-fat basis using an average value of 8.54 per cent solids-not-fat found in these studies.

The average sodium and calcium values (Table 21) found

Table 21. Comparison of reported and experimental amounts of constituents in milk as mg. per 100 g. solids-not-fat.

Worker	Elements					
	Sodium	Potassium	Calcium	Magnesium	Chloride	Phosphorous
Van Slyke and Bosworth (105)	667.4	1,451.9	1,593.0	146.4	913.3	731.9[a]
Hess et al. (32)	464.9	1,492.9	1,206.0	49.6	889.9	891.1[b]
Whittier (108)	667.4	1,451.9	1,487.1	93.7	-	725.9[a]
Roadhouse and Koestler (76)	548.0	1,887.6	1,373.2	77.8	1,000.0	1,021.1[b]
Robinson et al. (77)	-	-	1,440.3	-	-	-
Keirs and Speck (34)	545.6	1,624.1	1,392.3	-	-	-
Davis and MacDonald (19)	485.9	1,680.3	1,593.0	141.7	1,241.1	1,126.2[b]
Sommer (98)	683.8	1,777.5	1,745.0	-	1,288.0	1,088.9[b]
Av.	580.4	1,623.7	1,478.2	101.2	1,066.4	728.9[a]
Av. of data reported in Table 20	560.1	1,584.6	1,367.1	139.8	1,181.1	722.9[a]

[a]Inorganic phosphorous.
[b]Total phosphorous.

in these studies agree well with those of Roadhouse and Koestler (76) and Keirs and Speck (34) and with the average of the data from the literature. Potassium values did not agree well with any of the reported values except those of Keirs and Speck (34). The experimental magnesium value substantiates the values reported by Van Slyke and Bosworth (105) and Davis and MacDonald (19). The average chloride value approaches the value reported by Davis and MacDonald (19). Values reported by Van Slyke and Bosworth (105) and Whittier (108) agree with the inorganic phosphorous value determined in this study. The other workers (19, 32, 76, 98) reported their values as total phosphorous which could not be compared to the inorganic phosphorous values obtained in this study.

Values of this nature do not ordinarily have to agree exactly with one another for factors such as location, environment, feed, herd type and duration of the experiment will affect the quantity of milk constituents. It is gratifying, however, that in essentially all cases the values obtained in this study are in good agreement with averages of the values reported in the literature.

A further use for these data, reported on mg. per 100 g. solids-not-fat, may be in calculating diabetic and dietetic diets. This is of special importance for many dairy products are often used in such diets.

F. Resin Membrane Electrode Studies

Before Kressman's (36) cationic resin membrane electrodes could be applied to the study of ions in milk, the membranes had to be electrically characterized. Membrane electrodes were characterized for asymmetry potentials, stability and effect of pH on titration end points.

1. Asymmetry potentials

The asymmetry potential of each membrane was measured at 25° C. (Figure 4) by placing the same solution (0.1 M.) on either side of the membrane. Potentials obtained ranged up to ± 3 mv. among membranes but was consistent for any one membrane. The asymmetry potential of an electrode was added algebraically to any readings made with it.

2. Stability of resin membrane electrodes

The stability of each membrane electrode type, sodium, potassium, calcium or magnesium, was determined by placing a 0.01 M. solution of its cation inside the electrode and immersing the electrode (1 in.) in a 0.1 M. solution of the same salt. Potentials were read at one minute intervals for 20 min. the potentials were constant but beyond 20 min. the voltages dropped. However, if the electrode, containing the 0.01 M. salt solution, was inverted once and the potential reread; the original potential was attained.

At the same time the stability of each electrode type was being determined, the agreement between theoretical and actual voltage drops was noted. Theoretical voltage drops for a 0.01/0.1 M. system were calculated by the Nernst equation, $E = \frac{RT}{nF} \ln \frac{A_2}{A_1}$, for each electrode type. The theoretical EMF values for sodium, potassium, calcium and magnesium were calculated to be 55.28, 55.00, 50.54 and 50.61 mv., respectively. The potentials read by electrodes of all types agreed with the calculated values; all electrodes showed good stability under these conditions.

3. Effect of pH on titration endpoints

Solutions of sodium, potassium, calcium and magnesium chlorides were made up in 1 L. quantities at various pH levels, e.g. 4.0, 4.5, 5.0, 5.7, 6.0, 6.5, 7.0 and 8.0; they were adjusted to pH values greater than those of the salt solutions with 0.01 M. solutions of their respective bases, and for pH values below that of the pure salt solutions, with 0.1 M. hydrochloric acid. Although the amount of base added, in the adjustment of the pH, was known each finished solution was analyzed to verify the concentration. The null point for each solution then was determined as previously described (III-14). During the course of study it was found that a different resin membrane had to be used at each pH value for any one membrane type. The membranes apparently were sensitive to hydrogen ions and exhibited a hysteresis effect

probably because they were partially converted to a mixed hydrogen-metal form. If a new membrane was not used for each pH value the concentration measured at zero voltage varied considerably from its true value. Concentrations obtained at zero voltages for the sodium, potassium, calcium and magnesium membranes are shown in Table 22. Each pH value shown was not run with every electrode type.

The theoretical amount of 0.1 M. solution at zero potential was 11.11 ml. in all cases. When this amount of 0.1 M. standard salt solution was added to 100 ml. of redistilled water the concentration was 0.01 M., or the concentration of the solution within the membrane electrode. The primary purpose of this study was to determine whether the different resin membrane electrodes would function at the normal pH (6.6) of milk and secondly, to fine the pH values at which the salt concentrations were the same on either side of the membrane. The pH values at which the salt concentrations were the same on both sides of the membrane were found to be 6.3, 7.7, 7.1 and 6.5 for the sodium, potassium, calcium and magnesium membranes, respectively. These results indicated that the membranes could be used effectively in measuring ionic concentrations at or around the pH of milk.

When it was found that the membrane electrodes could be easily characterized and that they would function satisfactorily at the pH of normal milk, a study involving mixed

Table 22. Effect of pH on titration values of resin membrane electrodes.

pH	ml. 0.1 M. standard salt solution[a]			
	Sodium	Potassium	Calcium	Magnesium
4.0	11.40	-	-	-
4.3	-	-	11.40	-
4.5	11.35	11.30	-	11.40
4.7	-	-	11.35	-
5.0	11.20	11.23	11.20	11.30
5.6	-	-	11.15	11.20
5.7	-	11.25	-	-
6.0	11.10	-	11.10	11.15
6.4	-	-	11.20	-
6.5	11.10	-	-	-
6.6	-	11.15	-	11.10
7.0	11.00	-	-	-
7.1	-	-	10.00	-
7.2	-	-	-	10.90
7.8	-	11.10	-	-
8.5	-	11.10	-	-

[a]Theoretical quantity for all electrodes: 11.11 ml.

salt solutions was initiated. Mixed solutions of two cations were made and standardized. One cation concentration was held constant while the second was varied to yield a range of concentrations above and below that occurring in milk. Concentrations used were based upon Whittier's (108) data. The primary purpose was to determine the interference of one cation on the behavior of a membrane of another cationic type. When potential measurements were made, the data suggested that the interfering cation caused a partial conversion of the membrane from a single cationic type to a mixed cationic type, e.g., converted a sodium form membrane to a sodium-potassium form. As a result of this interference, the data obtained were unreliable. The interference of sodium ion in a calcium membrane measurement was more pronounced than that of sodium on potassium. When a calcium form membrane was titrated in presence of sodium ion the sodium apparently replaced the calcium so rapidly that no reliable estimate of the calcium concentration could be obtained.

After finding that the interference of one cation on another was so great it was decided to try an equilibration of the membrane with a salt solution similar to that of the milk dialysate. Stock solutions were prepared leaving out the calcium ion, then calcium was added at concentrations above, below and at the concentration occurring in the milk dialysates. The solution containing the concentration of

calcium found in milk dialysates was placed on the inside of a calcium form membrane electrode and the electrode was immersed (1 in.) in stock solutions containing from no calcium ion to calcium concentrations twice that occurring in the milk dialysate. Potentials were recorded at 10 min. intervals for 3.5 hrs.; the systems did not attain equilibrium.

On the basis of these data it was decided that it was improbable that these resin membrane electrodes could be applied to the measurement of ionic concentrations or of equilibrium shifts in mixed salt solutions because of the ease with which base exchange occurred in the membrane.

V. SUMMARY AND CONCLUSIONS

1. An evaluation of the accuracy of the analytical methods employed for the chief inorganic constituents of milk was made and the methods were considered to be satisfactory. Average percentage recovered found were: sodium 96.5, ptassium 98.0, calcium 100.2, magnesium 96.4, chloride 97.8 and inorganic phosphorous 98.1.

2. A new method of dialysis, "continuous pressure dialysis", was developed. Briefly the method consisted of placing a sample (50 g.) of milk into a semipermeable membrane (cellulose Visking tubing tied off at one end), which then was tied to a mineral oil reservoir. Mineral oil was floated over the sample surface, the membrane inserted into the dialyzing chamber and the membrane-reservoir attached to a Hg pressure manometer (0 to 300 mm. Hg capacity) (Figure 1). Pressure was exerted by nitrogen gas on the mineral oil and at the same time the flow of the dialysis water (distilled) was started. Pressure was adjusted to the desired level (115 mm. Hg) by means of a needle valve on an expansion flask and the water rate was adjusted to 2 drops per second. Water which passed the membrane was conducted to a crystallizing dish, in which it was continuously evaporated at a rate equivalent to that of the water intake (2 drops per sec.). The dialysis period was 15 hrs. in most

of these studies; in some instances longer periods were employed.

3. The development of the method of continuous pressure dialysis was considered necessary because of the fact that usual dialysis methods appeared to have definite shortcomings when applied to milk. Various dialysis pressures were studied. It was found that 92 mm. Hg pressure resulted in a residue weight, equal to that of the original milk, after dialysis for 11 hrs. However, this pressure (92 mm. Hg) did not maintain a constant weight of milk (50 g.) in the membrane during the dialysis period. A pressure of 115 mm. Hg was found to maintain a nearly constant weight; the maximum gain above and loss below the original weight was 4.0 g. during dialysis. To study the completeness of dialysis, a series of samples of the same milk were dialyzed for various lengths of time and the freezing points were run on the dialysis residues. At 15, 19 and 24 hrs. dialysis 93.9, 94.4 and 96.4 per cent, respectively of the osmotically active materials were dialyzed. The data likewise suggested that beyond 7 hrs. dialysis there may be a combination of dialysis and ultrafiltration. A 15 hr. dialysis period was selected because at the time these data were obtained a refrigerator large enough to hold the apparatus was not available and excessive use of thymol as preservative was thought undesirable.

Some confirmation of a 15 hr. dialysis was found by

dialyzing several milk samples for 15 and 20 hrs.; approximately 98 per cent of the sodium, potassium and chloride were dialyzed in 15 hrs. while 30.0, 61.0 and 64.0 per cent of the calcium, magnesium and inorganic phosphorous, respectively, in the original whole milk, were dialyzed during the same period. Reported values for dialyzable calcium and inorganic phosphorous agreed closely with those above, but not with those for magnesium; the results reported by others for magnesium were by static dialysis.

It would be of interest to determine the time at which the freezing point of the dialysate residue indicated that the residual osmotic materials were non-dialyzable and non-filterable. The described method may also be applicable to the simultaneous purification and concentration of protein solutions. Likewise this method could be applied to following enzymic or bacterial proteolysis by removal of amino acids as the proteolysis occurred.

4. A study of the dialyzable elements from normal whole milk was made from August 1955 through March 1956. It was found that 96-100 per cent of the sodium, potassium and chloride in the original milk were dialyzable during 15 hrs. while only 24.9, 36.6 and 61.3 per cent of the calcium, magnesium and inorganic phosphorous, respectively, were dialyzable. Ca/P and (Ca + Mg)/P ratios for the complete dialysates from whole milk did not indicate that any known combinations of calcium, magnesium or phosphorous were

dialyzed exclusively. However, a tendency was noted for Ca/P ratios to decrease in the fall and increase in the winter, indicating that dry feeds may increase the ratio.

5. Laboratory pasteurization of milk (62.8° C. for 30 min.) was found to decrease the amount of dialyzable calcium and inorganic phosphorous but to increase the amount of dialyzable magnesium. Pasteurization did not seem to have any effect upon dialyzable sodium, potassium and chloride.

6. Unheated whole milk samples were fractionated into clear whey and native caseinate (Figure 3) using a Spinco Model L Ultracentrifuge. Fractions obtained in this manner were analyzed for the major inorganic constituents. Dialysates of these fractions and of the parent milks were also obtained and analyzed. Freezing points were determined for all fractions and dialysates.

Freezing point data showed that the native caseinate had little if any osmotic activity.

The percentages of sodium, potassium, chloride, calcium, magnesium and inorganic phosphorous, present in the original milk, that were found in the centrifugal whey and milk dialysates were the same except for dialyzable magnesium which was greater in the centrifugal whey.

All of the inorganic phosphorous in the centrifugal whey (equivalent to 64.1 per cent of that in the original milk) was dialyzable while only 61.3 per cent of the total inorganic phosphorous was dialyzable from whole milk itself.

In addition 87 per cent of the magnesium in the original milk was found in the centrifugal whey and was completely dialyzable from the whey; only 36.6 per cent was dialyzable from the original milk. These data indicated that the remaining almost 39 per cent of the inorganic phosphorous in milk, possibly was adsorbed to the caseinates and that centrifugation caused some desorption or freeing of magnesium or that magnesium appears in milk in a different form than does calcium.

Ca/P ratios in the centrifugal whey approached that of di-phosphate (1.00) whereas the same ratio for whole milk and centrifugal skim milks approached that of tri-phosphate (1.5). However, in the milk and skim milk, the amounts of calcium and phosphorous chemically bound to the caseinate are included in these ratios.

Nuclear (hydroxyaminoacid-esterified) phosphorous values for caseins obtained by isoelectric precipitation of centrifuged caseinates and by isoelectric precipitation from normal skim milk were obtained. The average nuclear phosphorous value for isoelectric casein, from precipitated centrifuged caseinate was smaller than that of casein obtained by isoelectric precipitation from normal skim milk. This difference may result from loss of some phosphorous rich casein particles during washing of the centrifuged caseinate prior to peptization and isoelectric precipitation or from adsorption of inorganic phosphorous to the casein particles

precipitated from normal skim milk.

It would be of interest to study the electrophoretic mobilities of the various casein aggregates which would be obtainable by differential centrifugation, to study the calcium and phosphorous bound to these different casein aggregates, to analyze the lipoid material (Figure 3) to see whether it contained a high content of phospholipid and to determine the effects of heat on the sizes of the casein aggregates obtained by centrifugation.

7. A new ultrafiltration apparatus was designed, built and tested. The method of ultrafiltration, in brief, consisted of tieing a section of Visking cellulose tubing (tied off at one end) onto the mineral oil reservoir (Figure 2), introducing the sample into the membrane, floating 200 ml. of mineral oil over the surface, inserting the membrane into the ultrafiltration chamber (equipped with water trap), fastening the mineral oil reservoir to the pressure manometer and adjusting the pressure of nitrogen gas to the desired level by a needle valve on an expansion chamber. As filtrate was obtained, it was removed, as desired, through the Tygon sampling tube at the bottom of the filtration chamber.

The filtration rate of centrifugal whey was found to be more rapid than that of centrifugal skim milk (Figure 12). The filtrate was found to be homogenous from one portion to another; this was shown by the fact that a series of

subsequent 10 ml. fractions of ultrafiltrate from centrifugal whey were in agreement as regards analyses for the major inorganic constituents. It was concluded from this study that the various fractions were the same, compositionwise, and that samples could be drawn off at any time during the ultrafiltration.

Filtrates of centrifugal whey and skim milk were analyzed for the major inorganic elements and it was found that the percentages, of the elements in the original milk found in the centrifugal whey filtrate, were greater in all cases than percentages found in the skim milk filtrate. This indicated that casein possibly may adsorb the ultrafilterable elements in the skim milk to a greater degree than do the proteins in the centrifugal whey (albumin and globulins), or that it may serve as an ion exchange entity or a sequestering agent.

Ca/P and (Ca + Mg)/P ratios for centrifugal whey and centrifugal skim milk filtrates were calculated and were found to be in close agreement with each other suggesting that the calcium, magnesium and inorganic phosphorous are in the same form in these two fractions.

pH and freezing points were determined on all ultrafiltrates, wheys and skim milks. Freezing points of filtrates were found to be lower than those of the parent fractions. The pH values for all filtrates were found to be higher than those of the parent fractions. The higher pH

values for filtrates suggested that ions were released from proteins upon filtration or that caseinate exerted a buffering effect toward the acidic side. The lower freezing points of the filtrates than of their parent fractions may result, on the basis of preliminary data, from water soluble substances dissolved from the cellulose membrane. All filtrates gave negative ninhydrin and biuret reactions, although their parent fractions (centrifugal whey and skim milk) were positive. Such data indicated the absence of polypeptides and α-amino acids in the filtrates and suggest that the positive ninhydrin in the parent substances resulted from polypeptides or proteins rather than amino acids.

This method of ultrafiltration possibly could be applied to the concentration of protein solutions, the study of ion binding of various proteins and possibly the separation of amino acids from proteins and peptides during proteolysis by native enzymes or those from microorganisms.

8. Samples of milk taken at two week intervals were analyzed for the major inorganic constituents, fat and total solids from April 1955 through March 1956. The results showed that magnesium stayed relatively constant, sodium increased slightly during the winter and decreased during early spring, inorganic phosphorous and calcium fluctuated simultaneously but calcium did not vary greatly during the year. Chloride increased during the summer and decreased during the winter while potassium values were high

in March to April, August to October and during November.

Average values for amounts of inorganic elements as mg. per 100 g. solids-not-fat were sodium 560.1, potassium, 1,584.6, calcium 1,367.1, magnesium 139.8, chloride 1,181.8 and inorganic phosphorous 722.9.

These experimental values reported on the above basis may be of value in calculating various diets since many dairy foods usually are included in a number of dietetic regimes.

9. Cationic resin membrane electrodes studied were found to have good electrochemical stability and would function at the pH value of milk (6.6) in pure salt solutions. However, interference of monovalent with divalent ions apparently caused a partial conversion of the resin to a mixed cationic form, giving unreliable results. It was impossible to equilibrate the resin membrane electrodes against a stock solution (made to the concentration of elements in milk dialysate) or these stock solutions containing calcium concentrations above and below that appearing in milk dialysates, within usable time limits. This lack of equilibration was attributed to the strong base exchange properties of the resin which would make it difficult to apply the electrodes to milk.

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VII. ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to the following:

Dr. Emerson W. Bird for suggesting the problem, for encouragement, patience and advice during the course of investigation and for his helpful criticism during the writing of this dissertation. Also for designing and fabricating the pressure dialysis and ultrafiltration apparatus.

Professor C. A. Iverson and the Dairy Industry Department for making available the time, funds and facilities used in this study.

Mr. Lawrence Eschelman for his aid and assistance in some of the investigations.

Dr. Earl G. Hammond for his advice and counsel.

Mr. Robert W. Baughman and/or Mrs. Annie Olson for running the fat and total solids analysis.

Mr. T. H. E. Kressman for having furnished the membrane materials.

Note: Phosphorus is spelled as phosphorous throughout this thesis.