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COMPOSITION OF THE CASEIN-CONTAINING PARTICLES IN MILK[1]

T. F. FORD, G. A. RAMSDELL, AND SHIRLEY G. LANDSMAN
Eastern Utilization Research Branch
Agricultural Research Service, USDA, Washington 25, D. C.

It is well known that casein exists in milk in large aggregates. The purpose of this paper is to provide data on the chemical composition of these aggregates in relation to their size. Separation was achieved by centrifugation. The following chemical determinations were carried out on the separated material and on the residual liquids: total nitrogen, casein nitrogen, calcium, total phosphorus, trichloracetic-acid-soluble phosphorus, lactose, fat, and total solids.

EXPERIMENTAL PROCEDURE

Centrifuges, centrifugal velocities, and centrifuging times. Three centrifuges were used: a laboratory centrifuge having the cups provided with metal inserts to immobilize the liquid (Figure 1); various air-driven bowl rotors of 3.7 cm. diameter and 2 ml. to 2.7 ml. capacity, and a 12.4-cm. diameter, 50-ml. capacity, air-driven bowl rotor. One of the small bowl rotors and the 50-ml. bowl rotor are shown in Figure 2. The immobilizing inserts used with the laboratory centrifuge are an adaptation of a device introduced by Elford *(4)*. The bowl rotors are of the Henriot and Huguenard type *(8)* and were used without immobilizing devices.

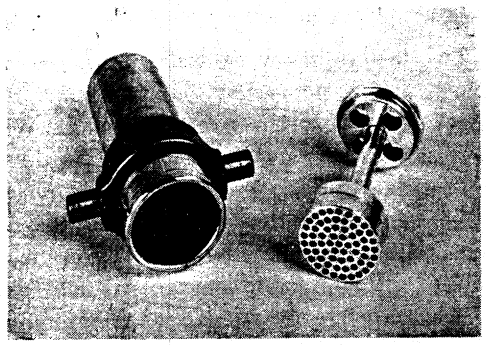

FIG. 1. One type of immobilizing insert used with the laboratory centrifuge. Length of holes, 1.24 cm.; diameter, 0.231 cm.

The laboratory centrifuge was run at about 3,000 r.p.m., giving a centrifugal force at the midpoints of the immobilized columns of about 2,300 times gravity. The 2 ml. to 2.7 ml. capacity rotors were run at 510 and 800 r.p.s., giving effective centrifugal forces of about 13,000 and 33,000 times gravity. The 50-ml. capacity rotor was run at 416 r.p.s., giving an effective centrifugal force of about 28,500 times gravity.

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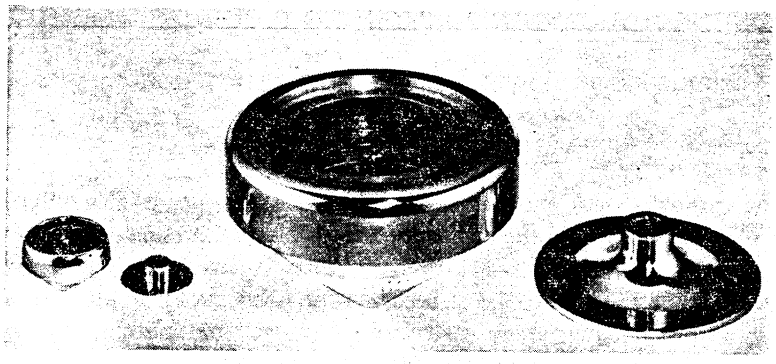

FIG. 2. Air-driven bowl rotors. Small rotor: 3.7 cm. diameter; 2.7 ml. capacity; inside and outside radii of annulus, 0.969 cm. and 1.454 cm. Large rotor: 12.4 cm. diameter, 50 ml. capacity, inside and outside radii of annulus, 3.49 cm. and 4.76 cm.

As a rule, with the small bowl rotors at 800 r.p.s., for example, roughly 14 minutes was required to remove 90-95% of the casein colloids; and with the 50-ml. rotor at 416 r.p.s. about 38 minutes was required. Although much longer times were used, up to 92 hours with the laboratory centrifuge and up to 16 hours with the bowl rotors, the final 5-10% of casein colloids was never completely removed. Some acid-precipitable protein always remained in even the clearest liquid samples.

Centrifugal techniques. Two techniques were used. With the laboratory centrifuge and with the small bowl rotors, many portions of each milk sample were centrifuged, each for a longer time than the preceding one. With the 50-ml. capacity rotor, in some experiments, the partially depleted liquid from one centrifugate was used to prepare the liquid and deposit in the next. The first of these techniques gives a Sven Odén type of depletion, i.e., for any single particle size the plot of concentration against centrifuging time follows a definite curve, which is almost a straight line. Sedimentation constants can be calculated from such plots *(5)*. The second technique gives results which are more difficult to interpret in terms of particle size but permits a sharper separation of fractions.

With the laboratory centrifuge only liquid samples were obtained. With the bowl rotors both liquid samples and deposits could be recovered. With the laboratory centrifuge the liquid samples were thrown out of the inserts into clean cups; with the bowl rotors the liquids were removed with hypodermic syringes or with pipettes, and the deposits were collected on celluloid liners fitted against the wall of the annulus.

Two of the laboratory centrifuge experiments were run at 17° C.; most of the other separations were at 19.5° C., although some were at temperatures as high as 25° C.

Milk samples. Effects of residual fat. Whole milk was obtained from the U. S. Department of Agriculture farm at Beltsville, Md. Both Jersey and mixed herd milks were used. No preservatives were added. All samples were stored at 4° C.

Skimmilk was prepared usually by gravity separation. It was siphoned from 3-l. flasks about two-thirds filled with whole milk, after 24 hours standing at 4° C. Twenty-four-hour gravity skimmilk obtained in this way contained roughly 0.3 to 0.7% fat. Gravity skimmilk was used primarily to insure the inclusion of large casein colloid particles but in part also to avoid even the slight heating and additional handling involved in mechanical separation. For some experiments the 24-hour gravity skimmilk was submitted to further separation by centrifuging for short times in the 50-ml. bowl rotor, at 416 r.p.s., especially for the purpose of removing the large particles and lowering the fat content. The initial fat content could be thus lowered to 0.07 to 0.08%. After even the most prolonged centrifugation, skimmilks always contain some fat.

As the data are presented here, any inaccuracies due to included fat are largely eliminated since the data are employed as ratios, as of calcium to nitrogen, or phosphorus to nitrogen, and in each case the analyses for these various elements were all on the same samples.

METHODS OF ANALYSIS

Nitrogen was determined by a semi-micro Kjeldahl method with mercuric oxide and sodium sulfate as catalysts. With the liquid samples casein was precipitated with 1 to 9 acetic acid and, after holding the samples for 15 minutes, sodium acetate buffer was added, giving a final pH of 4.6. For the determination of casein nitrogen on air-dried deposits, samples were weighed into 15-ml. centrifuge tubes, water was added, and the samples were allowed to swell. The resulting gelatinous material was then made into a suspension by adding dilute sodium hydroxide, keeping the pH below 8.0. Casein was precipitated from these redispersed samples, as from original liquid samples, with acetic acid, sodium acetate buffer was added to bring the pH to 4.6, and nitrogen was determined on the washed precipitate in the usual way.

Calcium was determined on the ash according to the micro-titration method of Tisdall and Kramer *(11)*. The Clark and Collip washing technique was used *(2)*. Total phosphorus and trichloracetic-acid-soluble phosphorus were determined by the volumetric method described in the official methods of the A.O.A.C. *(1)*. Lactose was determined by the A.O.A.C. gravimetric method. Fat was determined by the Röse-Gottlieb method.[2] The Mojonnier tester was used in place of the Röhrig tube. Total solids were obtained by transferring the samples to weighing bottles partly filled with sand and heating them in a vacuum at 98-100° C. to constant weight.

RESULTS AND CALCULATIONS

Relationship between casein nitrogen and total nitrogen. Figure 3 shows plots of per cent casein nitrogen against per cent total nitrogen in the liquids as the casein-containing colloids are progressively removed by centrifuging for-

[2] Described on p. 272, 5th ed. of Official Methods of A.O.A.C.

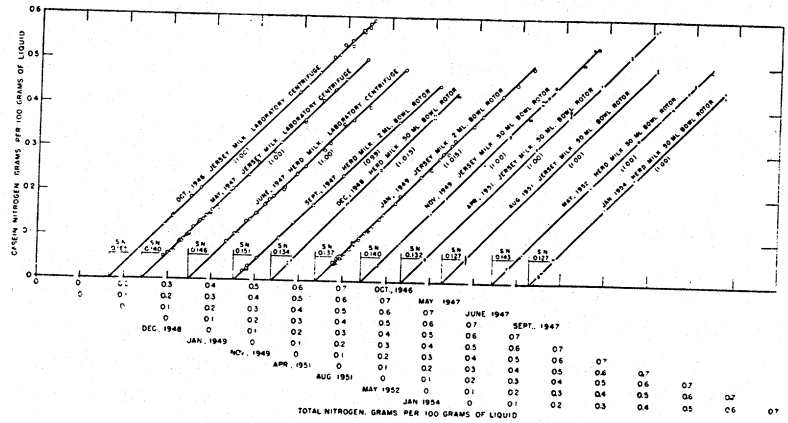

FIG. 3. Relationship of casein nitrogen to total nitrogen for skimmilk samples centrifuged for different lengths of time. Cirles represent single determinations or averages of duplicate determinations; crossed lines show limits of duplicate determinations. S.N. = serum nitrogen. Eleven plots are shown here, and each consecutive plot has its own abscissa values.

eleven milks. Since the data indicate essentially straight line relationships, there is here no evidence for differences in absorbed or otherwise associated noncasein proteins based on particle size, as might have been expected.

The large scale plot from which Figure 3 is reduced gives the following ratios between the decrease in casein nitrogen content and the decrease in total nitrogen content as the colloids are removed: 1.00, 1.00, 1.00, 0.99, 1.015, 1.015, 1.00, 1.00, 1.00, 1.00, and 1.00. These ratios are simply the slopes of the lines. It is seen that as a practical matter the casein nitrogen removed is essentially equal to the observed decrease in total nitrogen.

If casein nitrogen were the only nitrogen removed, the slopes of these lines should actually be greater than 1.00. This is because the original total nitrogen content represents grams per 100 g. of skimmilk, and the final total nitrogen content on the depleted samples represents grams per 100 g. of casein-free serum. For a typical milk containing 0.500% casein nitrogen and 0.642% total nitrogen, calculations give 1.016 for the theoretical slope of the line, assuming casein nitrogen to be the only nitrogen removed. The calculations involve a factor, 6.89, based on analyses, for conversion of casein nitrogen to dry lactose-free colloid; and a value for hydration of 0.52 g. of bound water per gram of dry colloid, or 0.55 g. per gram of casein, due to de Kadt and van Minnen (cf. ref. 3). That the actual slopes of the lines of Figure 3 are in many cases significantly less than 1.016 means that noncasein or serum proteins as well as casein are removed by the centrifugation. The amount, although small, is several times as great as the amount of albumin and globulin which would theoretically be independently sedimented in the centrifuging times used.

In general, total nitrogen determinations are more reliable than casein nitrogens. In 32 sets of duplicate total nitrogen determinations representing five complete centrifugal fractionations over a 3-year period, the average deviation of the duplicates from the mean values was ±0.29%; for the corresponding

duplicate casein nitrogens the average deviation was ±0.88%. Therefore, casein nitrogen is referred to total nitrogen in Figure 3, and for the same reason calcium, total phosphorus, and acid-soluble phosphorus are referred to total nitrogen in subsequent plots.

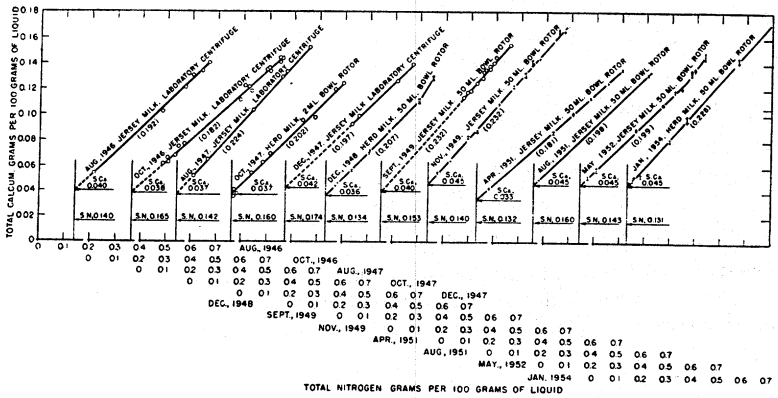

FIG. 4. Relationship of total calcium to total nitrogen for skimmilk samples centrifuged for different lengths of time. Circles represent single determinations or averages of duplicate determinations; crossed lines show limits of duplicate determinations. S.N. = serum nitrogen. S.Ca = serum calcium. Twelve plots are shown here, and each consecutive plot has its own abscissa values.

Calcium: casein-nitrogen ratios. Figure 4 shows plots, for 12 milks, of total calcium against total nitrogen as the colloids are progressively removed. Single straight lines are drawn through the sets of points except for December, 1948, and May, 1952, where a break in the region of large particles seems apparent.

For these 12 milks the extreme ratios of calcium removed to casein nitrogen removed are 0.181 and 0.232. The average is 0.206. In calculating these ratios, the serum calcium in the original skimmilks was found by proportioning the extrapolated value for the calcium in 100 g. of casein-free serum to the calculated weight of such serum in 100 g. of the skimmilk. Here, as before, the numerical constants 6.89 and 0.52 were used. For these plots, therefore, unlike the casein nitrogen : total nitrogen plots, the ratios given are not exactly the slopes of the lines.

Total phosphorus: casein-nitrogen ratios. Here again (Figure 5) single straight lines are drawn, with one exception. In this case, May, 1952, a deviation is indicated in the same region in which a deviation in calcium is shown in Figure 4. The five total phosphorus : casein-nitrogen ratios given by the lines as drawn are 0.125, 0.145, 0.119, 0.122, and 0.128. These ratios were calculated in the same way as the calcium : casein-nitrogen ratios.

Acid-soluble phosphorus: casein-nitrogen ratios. Since the calcium and total phosphorus plots are linear with only two exceptions, they indicate at least approximately constant composition for the casein colloids removed over the whole range or almost the whole range of sizes. The acid-soluble phosphorus plots of

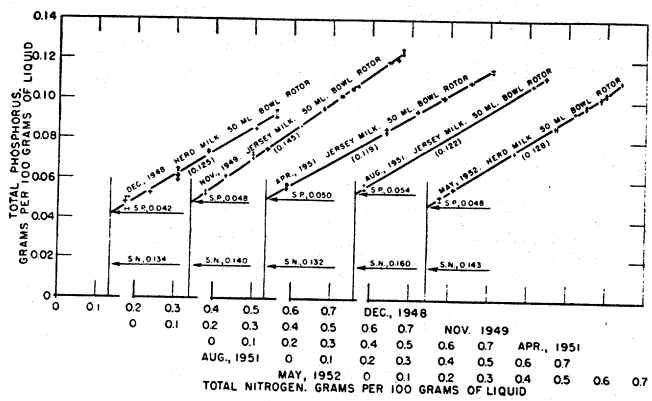

FIG. 5. Relationship of total phosphorus to total nitrogen for skimmilk samples centrifuged for different lengths of time. Crossed lines show limits of duplicate determinations. S.N. = serum nitrogen. S.P. = serum phosphorus. Five plots are shown here, and each consecutive plot has its own abscissa values.

Figure 6, however, seem to show a break in homogeneity in the region of the largest sizes.

The November, 1949, milk sample (cf. Figure 6) was 24-hour gravity skim and therefore contained all of the large particles originally present. The April, 1951, milk sample was partially depleted of large particles, and the August, 1951, and May, 1952, samples were very nearly or completely depleted of large particles, by preliminary centrifugation, before the experiments were started. The plots for the last two milks disclose no deviation from a straight line relationship.

The acid-soluble phosphorus : casein-nitrogen ratios shown in Figure 6, 0.088, 0.063, 0.067, and 0.071, are based on the major straight lines and were calculated in the same way as those for calcium and total phosphorus.

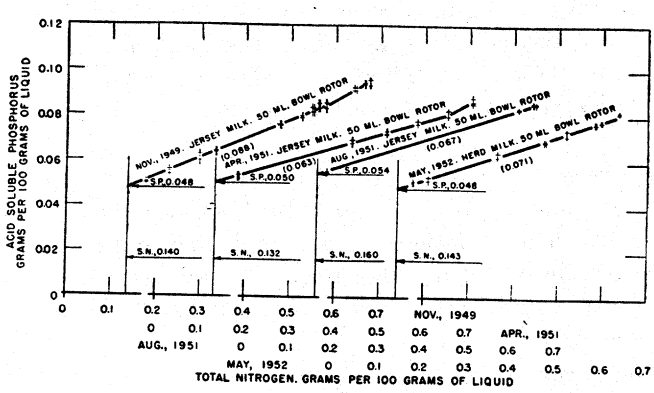

FIG. 6. Relationship of acid-soluble phosphorus to total nitrogen for skimmilk samples centrifuged for different lengths of time. Crossed lines show limits of duplicate determinations. S.N. = serum nitrogen. S.P. = serum phosphorus. Four plots are shown here, and each consecutive plot has its own abscissa values.

The plots of casein nitrogen and total nitrogen against centrifuging time for November, 1949, i.e., the depletion plots for this experiment, show that about 15% of the casein-containing colloid consisting of large particles must have differed in composition from the rest to give a break in the acid-soluble phosphorus plot at the point indicated. Other depletion experiments on gravity skimmilks in which acid-soluble phosphorus was not determined give proportions of particles in this size range not over, and usually less than, 15% of the total. Sometimes these large particles appear to be entirely absent.

Composition of casein in milk as indicated by the analysis of liquid samples. In the calculations to be outlined it is assumed that the total phosphorus content of a sample less the acid-soluble phosphorus represents organic phosphorus.

Considering only the analyses of the original skimmilks and of the casein-free serums as given by extrapolation (cf. Figures 5 and 6), the ratios of organic phosphorus to casein nitrogen found are 0.053, 0.052, 0.055, and 0.057. These ratios are for whole casein in the native state. The variation is about the same as in analyses of purified casein prepared by different methods. Linderstrøm-Lang *(7)* lists seven analyses by various authors; the phosphorus:nitrogen ratios calculated from these analyses are 0.054, 0.054, 0.056, 0.045, 0.051, 0.053, and 0.050.

TABLE 1

Summary of results of analyses of liquid samples, and calculation of the organic phosphorus:nitrogen weight ratio for the main component of native casein in milk

1	2	3	4	5
Date of milking	Ca/C.N.[a]	T.P./C.N.[a]	A.S.P./C.N.[a]	O.P./C.N.[a]
	(g/g)	*(g/g)*	*(g/g)*	*(g/g)*
Dec., 1948	0.207	0.125	—	—
Nov., 1949	0.232	0.145	0.088	0.057
Apr., 1951	0.181	0.119	0.063	0.056
Aug., 1951	0.198	0.122	0.067	0.055
May, 1952	0.199	0.128	0.071	0.057

[a] C.N. = casein nitrogen; T.P. = total phosphorus; A.S.P. = acid-soluble phosphorus; O.P. = organic phosphorus.

Table 1 lists the various weight ratios taken from Figures 4, 5, and 6. These ratios refer not to the total casein but to that part contained in the group of particles in the broad intermediate size range, which represents about 80% of the total. Here, the organic phosphorus:nitrogen ratios obtained by subtracting the acid-soluble phosphorus ratios from the total phosphorus ratios are 0.057, 0.056, 0.055, and 0.057. These ratios vary much less than those given above for the total casein. The average is 0.056.

The present data are insufficient to draw any detailed conclusions as to composition in the range of large particles. It can be said at least that the over-all organic-phosphorus:nitrogen ratio is appreciably less than that given above. These large particles are complex. Deviations in lactose:nitrogen plots show that they contain lactose or are deposited with lactose in excess of that associated

TABLE 2

Various mol ratios for the colloid containing the main component of native casein, based on analyses of liquid samples

1	2	3	4	5	6
Date of milking	Mols Ca[a] / Mols C.N.	Mols T.P.[a] / Mols C.N.	Mols A.S.P.[a] / Mols C.N.	Mols O.P.[a] / Mols C.N.	Mols Ca-Mols O.P. / Mols A.S.P.
Dec., 1948	0.0714	0.0565	(0.0312)	(0.0253)	1.48
Nov., 1949	0.0811	0.0655	0.0398	0.0258	1.39
Apr., 1951	0.0633	0.0538	0.0285	0.0253	1.33
Aug., 1951	0.0692	0.0552	0.0303	0.0249	1.46
May, 1952	0.0696	0.0578	0.0321	0.0258	1.36

[a] C.N. = casein nitrogen; T.P. = total phosphorus; A.S.P. = acid-soluble phosphorus; O.P. = organic phosphorus.

with the solvate liquid. They also appear to contain citric acid (cf. Ref. *3*). They contain fat, probably vitamins, and certainly also leucocytes, fragments of cells, and other foreign matter.

In Table 2 the data of Table 1 are presented in terms of mol ratios. For the December, 1948, experiment the organic phosphorus:nitrogen weight ratio 0.056 has been assumed. In the last column of the table, column 6, the mols of calcium, per mol of casein nitrogen, have been arbitrarily diminished by amounts equivalent to the organic phosphorus, and these differences divided by the mols of acid-soluble phosphorus. The average of the resulting ratios, 1.40, is close to the mol ratio of calcium to phosphorus in tricalcium phosphate.

Composition as indicated by analyses of deposits. Results of analyses of deposits, or sediments, are given in Tables 3 and 4. The 50-ml. rotor was used for these experiments, and each of the successive deposits was made from the previously partially depleted liquid by longer centrifuging. Most of the deposits were washed, as indicated in the tables. This was done by redispersing them in distilled water and recentrifuging. By such washing the lactose content is reduced to a negligible value, but some serum protein is always retained even after repeated washing. It is assumed that the soluble serum salts are removed with the lactose.

In the August, 1951, experiment only two fractions were prepared, one composed predominantly of the largest particles present and one composed of particles of small and intermediate sizes. This was not a separation of those largest particles that might differ in composition from the rest. These had been removed in the preparation of the milk sample. It was a separation of the major, apparently homogeneous component, into two size groups.

The purpose of Table 3 is to present the analytical data and to find the ratio of organic phosphorus to nitrogen in the deposits. These ratios are given in the last column. The average of all the values is 0.0563. This is in satisfactory agreement with the ratio 0.056 for the phosphoprotein of the main component found by the analyses of liquid samples.

The purpose of the calculations indicated in Table 4 is to find the mol ratio of calcium not equivalent to organic phosphorus to acid-soluble phosphorus in the colloids removed. This calculation requires that the calcium and phosphorus

TABLE 3

Summary of results of analyses of centrifugally deposited colloids and calculations of the organic phosphorus: nitrogen ratio for the main component of native casein

1				2	3	4	5	6	7	8	9	10
Sample				H_2O[b] (In wet deposit)	T.N.	C.N.	S.N.	T.P.	A.S.P.	O.P.	Ca	O.P./C.N.
No.	Centrifuging time at 28,500 × G	Treatment[a]	Approximate per cent of total colloid									
				(g. per 100 g. vacuum dry solids)								
	(min.)				January, 1951. Jersey 24-hr. gravity skimmilk							
A-1	2	One water wash	7	—	14.45	13.67	0.78	1.661	0.886	0.775	2.81	0.0567
A-2	4	"	10	—	14.15	13.05	1.10	1.654	0.914	0.740	2.83	0.0567
A-3	8	"	20	—	14.36	13.18	1.18	1.610	0.868	0.742	2.68	0.0563
A-4	60	"	50	—	14.60	12.70	1.90	1.54	0.83	0.71	—	0.056
					April, 1951. Jersey centrifugally skimmed milk							
B-1	½	Not washed	2	230	12.55	11.74	0.81	1.55	0.89	0.66	2.60	0.056
B-2	1	"	3	239	12.75	11.97	0.78	1.62	0.94	0.68	2.60	0.057
B-3	2	"	3	255	12.50	12.05	0.45	1.562	0.88	0.68	2.48	0.056
B-4	3	"	3	247	12.63	12.02	0.61	1.564	0.88	0.68	2.45	0.0565
B-5	30	"	70	237	12.70	12.30	0.40	1.52	0.83	0.69	2.42	0.056
					August, 1951. Jersey centrifugally skimmed milk							
C-1	½	Serum washed	2	(230)	12.59	11.64	0.95	1.67	1.02	0.65	2.65	0.056
C-2	30	Not washed	80	(237)	12.92	12.20	0.72	1.62	0.94	0.68	2.62	0.056
C-3	30	Water washed	80	—	14.55	13.74	0.81	1.700	0.93	0.77	2.75	0.056

[a] Details of the methods of preparation of the deposits for analysis are given in the text.
[b] Water content based on total solids determinations on the wet deposits.

TABLE 4

Calculations of various mol ratios for the colloid containing the main component of native casein

1	2	3	4	5	6	7	8	9	10	11
No.	Approx. serum H_2O in deposit	S.N. proportional to approx. serum H_2O	S.N. deposited	Serum H_2O corrected	Ca not serum Ca	A.S.P. not serum P	Mols Ca assigned to colloid per mol C.N.	Mols A.S.P. assigned to colloid per mol C.N.	Mols O.P. per mol C.N.	Mols inorganic Ca per mol A.S.P. assigned to colloid
				(g. per 100 g. vacuum dry solids)						
				January, 1951. Jersey 24-hr. gravity skimmilk						
A-1	—	—	—	—	2.81	0.886	0.0718	0.0293	0.0256	1.58
A-2	—	—	—	—	2.83	0.914	0.0758	0.0316	0.0256	1.59
A-3	—	—	—	—	2.68	0.868	0.0711	0.0297	0.0254	1.54
A-4	—	—	—	—	—	0.83	—	0.0295	0.0253	—
				April, 1951. Jersey centrifugally skimmed milk[a]						
B-1	188	0.27	0.54	186	2.53	0.79	0.0754	0.0304	0.0254	1.64
B-2	196	0.28	0.50	194	2.53	0.84	0.0739	0.0317	0.0256	1.52
B-3	212	0.30	0.15	211	2.41	0.77	0.0699	0.0288	0.0255	1.54
B-4	204	0.29	0.32	203	2.38	0.77	0.0692	0.0289	0.0255	1.51
B-5	193	0.27	0.13	193	2.35	0.73	0.0668	0.0268	0.0253	1.55
				August, 1951. Jersey centrifugally skimmed milk[b]						
C-1	(188)	0.26	0.69	186	2.56	0.91	0.0770	0.0353	0.0252	1.47
C-2	(193)	0.26	0.46	191	2.53	0.83	0.0725	0.0307	0.0252	1.54
C-3	—	—	—	—	2.75	0.93	0.0700	0.0306	0.0253	1.46

[a] The April, 1951, casein-free serum contained 0.132% S.N., 0.033% Ca, 0.050% P, 6.4% total solids, and 5.53% lactose.

[b] The August, 1951, casein-free serum contained 0.127% S.N., 0.045% Ca, and 0.0535% P. 6.4% total solids is assumed.

Column 2. Total water less C.N. times 6.89 and times 0.52, the colloid:N factor and the assumed hydration. Total water from Col. 2, Table 3, and C.N. from Col. 4, Table 3.

Column 3. Figures in Col. 2 divided by 100 and times 0.132/(1-0.064) = 0.141, for April; and times 0.127/(1-0.064) = 0.136, for August. Cf. notes *a* and *b* above.

Column 4. Figures in Col. 5, Table 3, less figures in Col. 3, this table. This serum nitrogen could be in part centrifugally deposited, in part trapped in the deposited jelly.

Column 5. Approximate serum water from Col. 2 less serum nitrogen deposited times 6.38 and times 0.52, the serum protein:N factor and an assumed hydration for the serum proteins.

Column 6. Total Ca from Col. 9, Table 3, less Ca proportional to serum water; i.e., less serum water from Col. 5 divided by 100 and times 0.033/(1-0.064) = 0.035, for April, and times 0.045/(1-0.064) = 0.048, for August. Cf. notes *a* and *b* above.

Column 7. A.S.P. from Col. 7, Table 3, less P proportional to serum water; i.e., less serum water from Col. 5 divided by 100 and times 0.050/(1-0.064) = 0.053, for April, and times 0.0535/(1-0.064) = 0.057, for August. Cf. notes *a* and *b* above.

Columns 8, 9, and 10. Ca and A.S.P. from Cols. 6 and 7 divided by C.N. from Col. 4, Table 3, and O.P./C.N. from Col. 10, Table 3, all expressed as mol ratios.

Column 11. Mols Ca per mol C.N. from Col. 8 less mols O.P. per mol C.N. from Col. 10, divided by mols A.S.P. per mol C.N. from Col. 9.

contained in the liquid part of the unwashed deposits be subtracted from the total calcuim and phosphorus. To this end certain assumptions are made: that the serum protein that may be considered a part of the liquid phase in the deposit is proportional to the total water less hydrate water; that the hydration of the casein colloid, and of the serum protein also, is 0.52 g. of hydrate water per gram of dry material; and that the amounts of calcium and phosphorus not part of the casein colloid are proportional to the free or serum water in the concentrations in which these elements are found in the casein-free serum. Details of the methods of calculation are given in the footnotes to the table. The several values for the calculated mol ratio of inorganic calcium to inorganic phosphorus are given in the last column. The average of all of these is 1.54. The average, omitting the first value for April, 1951, for the largest particles, is 1.52.

DISCUSSION

Comparisons with results of similar centrifugal analyses by others. De Kadt and van Minnen *(3)*, using the Sharples supercentrifuge, obtained variously depleted liquid samples and deposits. In the cases in which more than two liquid fractions were obtained, plots of calcium and total phosphorus against casein nitrogen constructed from their data are straight lines. The ratios of calcium removed to casein nitrogen removed range from 0.188 to 0.212, and the average is 0.202 as compared with the average ratio 0.206 given by the plots of Figure 4. The ratios of total phosphorus to casein nitrogen range from 0.122 to 0.128, and the average is 0.125 as compared with 0.128 given by Figure 5. Their average calcium:total phosphorus weight ratio for liquids is therefore 1.62; the present data give 1.61.

From analyses of their unwashed deposits by calculations similar to those used here, de Kadt and van Minnen obtain an average mol ratio of calcium less calcium equivalent to organic phosphorus, to inorganic phosphorus, of 1.53. These authors did not determine acid-soluble or inorganic phosphorus. They assumed a weight ratio of organic phosphorus to casein nitrogen of 0.053.

Von Hostettler *et al.* *(12, 13)*, using an angle centrifuge, separated two-thirds to three-fourths of the casein colloids from milk in fractions. Their analyses of deposits show a decrease in the calcium and total phosphorus content from the largest particles to the smallest. This result is in agreement with the present data (cf. Table 3), but their over-all decrease is greater. This could be because they started with whole milk, which would have contained all of the very largest particles. De Kadt and van Minnen, who, like Hostettler *et al.*, base their conclusions on their analyses of deposits, state (loc. cit.) that the fractions exhibit practically no differences in composition, but they leave open the possibility that slightly more calcium phosphate may be associated with the large particles than with the smaller particles. This indefinite result could be attributed to appreciable removal of large particles in the preparation of their skimmilks. Their original casein contents are all low, averaging 2.61%; the average original casein content of the skimmilks used here was 3.23%.

Ramsdell and Whittier *(10)* also separated and analyzed the casein-containing colloids from milk, thoroughly washing the deposits with water. They did not separate the colloids in fractions, and their samples probably contained a high proportion of very large particles. The organic phosphorus:nitrogen ratio given by their data is quite low, 0.051, which can probably be attributed to the inclusion of these large particles.

The very large and very small particles. More refined separations and more elaborate analyses than here attempted are required definitely to establish the compositions of the particles at both extreme ends of the size range.

In the region of very large particle sizes the low organic phosphorus indicated suggests the existence of a phosphoprotein which differs in composition from that in the particles of the broad intermediate size range, but it is not proof. This requires assurance that the large particles may not be merely aggregates of smaller ones with inclusion of such noncasein proteinaceous substances as would lower the organic phosphorus:nitrogen ratio.

In the region of very small particles the last 5 to 10% not removed is, of course, not included in the present analysis. These particles may or may not contain the same phosphoprotein as found in the main component. They also may not be associated with calcium phosphate, or at least not in the same way.

Composition of the main component of native casein in terms of α-, β-, and γ-caseins. Perfect linearity of both the total phosphorus and acid-soluble phosphorus plots over all or part of their length would require a perfectly constant organic phosphorus:nitrogen ratio for all of the casein complex particles over the range of linearity. Within the accuracy of the present data, such linearity is indicated over most of the size range. As a first approximation the plots show the existence of a native casein of constant composition in these particles. This conclusion is supported by the analyses of deposits.

The data on liquids give an average phosphorus:nitrogen ratio of 0.056, and the data on deposits give the ratio 0.0563. If this ratio is correct, then the native casein represented cannot be α-, β-, or γ-casein alone. These have the phosphorus:nitrogen ratios 0.0637, 0.0398 and about 0.0032 *(6, 9)*. The ratio found could, however, result from the presence of all three of these particular caseins in definite proportions.

Warner *(14)* originally reported the proportions of α- and β-casein in acid-precipitated casein as about 4:1, and electrophoresis and analyses indicate that γ-casein cannot amount to more than 5% of the total *(9)*. These figures suggest over-all proportions of α-; β-: γ-caseins of about 16:4:1. In such a combination the ratio of phosphorus to nitrogen would be 0.0563. The agreement with the experimental values 0.056 and 0.0563 is strong evidence for the existence in milk of such a definite mixture or combination of simpler phosphoproteins.

The implication is that each individual particle contains α-, β-, and γ-caseins in the proportions indicated. An alternative possibility, which is not ruled out by the data, is that α-, β-, and γ-caseins exist in separate particles and that the α-particles, β-particles, and γ-particles have comparable or identical size distributions.

Correction of the inorganic calcium:phosphorus ratio for magnesium in the casein colloid, and for a possible error in hydration. De Kadt and van Minnen *(3)* found magnesium to be removed with the casein colloids. Experiments not here reported confirm this, and give a mol ratio of magnesium to calcium removed of about 1 to 15. If the inorganic calcium:inorganic phosphorus ratios, 1.40 for liquids, and 1.52 for deposits, are increased by 1/15, they become 1.49 and 1.62, respectively.

The calculations for the liquid samples and for the unwashed deposits and the one serum-washed deposit, but not for the water-washed deposits, involve the factor 0.52 g. per gram, for hydration. This numerical value, due to de Kadt and van Minnen, is based on analyses of deposits, on the assumption that lactose is proportional to serum water, i.e., that it is not removed with the particles except as a part of the solvate liquid. Actually, as has been stated, excess lactose is removed with the large particles, and it is therefore not a valid reference substance, at least for these particles. Although de Kadt and van Minnen's deposits were perhaps free of large particles, still the possibility is suggested that the smaller particle-size fractions also might contain some lactose in excess of that in the solvate liquid. The necessary correction for inclusion of even a small amount of such nonsolvate lactose would give a hydration appreciably greater than 0.52 g. per gram. Any increase in the hydration would tend to lower the combined calcium-magnesium:acid-soluble phosphorus mol ratios. If the hydration were 1.00 g. per gram, for example, the average ratios corrected for both magnesium and hydration would be, for the liquid samples, 1.47; for all of the deposits omitting the largest particle size fraction for April, 1951, 1.50.

Relationship between calcium phosphate and particle size. The analyses of deposits (Tables 3 and 4) show a decrease in calcium and acid-soluble phosphorus, i.e., a decrease in calcium phosphate, with decreasing particle size. As has been stated, this is in agreement with results by Hostettler *et al. (12, 13)*.

The drift in calcium phosphate content with particle size, for the main casein-homogeneous component, is at first sight inconsistent with the linear plots of Figures 5 and 6. For some of these figures, slightly sagging lines might have been drawn, and more accurate data possibly would justify such curved lines. It seems likely, however, that usually, over appreciable distances at least, the lines should in fact be straight and represent single size classes of constant, or essentially constant, composition. For some of these milks the depletion plots disclose very few different particle sizes. It is to be noted also that with liquid samples the influence of the smaller particles predominates, since large amounts of these are included in all the fractions analyzed.

There is, in addition to the drift noted for individual milks, an over-all relationship between the calcium, i.e., calcium phosphate, content and particle size. For the August, 1946, milk, for which Figure 5 gives the calcium:nitrogen ratio 0.192, the depletion plot shows that less than 13% of the colloid was present as particles having sedimentation constants greater than 470×10^{-13}; and for the August, 1947, milk, for which the calcium:nitrogen ratio is 0.224, the depletion

plot shows that 50% of the colloid was present as particles having sedimentation constants greater than 470×10^{-13}.

Nature and condition of the inorganic phosphate in the casein-containing particles. Since the inorganic phosphate is removed with the casein in essentially constant proportions over most of the particle size range, it is unlikely that it exists to any appreciable extent as a separate colloid in fresh untreated milk. It must be present in all the particles as molecules combined in some way with calcium caseinate as a complex. This conclusion is the same as that of de Kadt and van Minnen and of Ramsdell and Whittier.

In Tables 2 and 4 the arbitrary assumption that the organic calcium is equivalent to the organic phosphorus is the same assumption made by de Kadt and van Minnen. As pointed out by these authors, this simplification is consistent with results of various more or less direct determinations of the combining power of casein for calcium. Ramsdell and Whittier used a value for organic calcium obtained from pH-calcium plots, but the end result is the same; and the conclusion by both de Kadt and van Minnen and Ramsdell and Whittier that the so-called inorganic phosphate is tricalcium phosphate is supported by the present data—with the important reservation that these data apply only to the particles in the intermediate size range, and not to the very largest nor the smallest particles.

The data given here indicate mol proportions of calcium to organic phosphorus to acid-soluble phosphorus, in the size fractions analyzed, of 5:2:2. This ratio is approached very closely when the calcium content is low and for the smallest particles (cf. columns 2, 4, and 5 of Table 2 and columns 8, 9, and 10 of Table 4). It may be considered to represent the mol relationships in a unit particle of the casein colloid, or complex, from which larger particles are formed by aggregation. The ratio suggests a combination of two mols of calcium caseinate with one mol of tricalcium phosphate.

SUMMARY

1. Roughly 75 to 90% of the casein in fresh untreated skimmilk appears to exist as a single phosphoprotein, which is probably a definite mixture or combination of α-, β-, and γ-caseins. The organic phosphorus:nitrogen ratio for this phosphoprotein is in close agreement with that for a mixture of these caseins in the proportions 16:4:1. This phosphoprotein is presumed to be contained in all the colloidal particles comprising the broad intermediate size range. An alternative assumption, that these individual caseins exist in separate particles having comparable or identical size ranges, is possible, however.

2. In the range of sizes referred to, the phosphoprotein may be considered to be combined with calcium and tricalcium phosphate as a complex. The proportion of inorganic phosphorus is variable as between milks and decreases with decreasing particle size. For milks of low calcium content, however, and for the smallest particles removed, a definite minimum proportion of inorganic phosphorus is indicated, such that the mol proportion of calcium to organic phosphorus to inorganic phosphorus is approximately 5:2:2.

3. The very largest casein-containing particles in skimmilk, which are not included in the size range above referred to, differ in composition. They appear to contain less organic phosphorus, but they also contain nonproteinaceous substances, and more refined analyses are required to characterize either the phosphoprotein or the inorganic components.

4. A residual fraction of the casein-containing particles, representing from 5 to 10% of the total casein, was not removed in the centrifuging times used.

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