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NITROGEN FACTOR FOR THE CALCIUM CASEINATE–CALCIUM PHOSPHATE COMPLEX IN MILK

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The casein colloid in milk contains a phosphoprotein which has the same phosphorus : nitrogen ratio for all the broad intermediate ranges of particle sizes. The purpose of this work was to determine the nitrogen factor. For many milks and many particle-size fractions, the average ratio of dry weight of calcium caseinate–calcium phosphate complex was about 6.89 ± 1.3%, and this applies to normal milk. This factor does not apply to the largest casein colloid particles and may not apply to the smallest. *Editor.*

To calculate densities, hydration, voluminosity, and other constants of the casein-containing particles in milk, a factor for conversion of grams of casein nitrogen to grams of dry casein complex is necessary. Experiments previously reported *(3)* indicate that the casein colloid in milk, in the broad intermediate range of sizes, contains a phosphoprotein which has the same phosphorus : nitrogen ratio in all the sizes of particles. This phosphoprotein is apparently present as a calcium salt, which is closely associated with excess calcium and phosphorus in the proportions in which these elements exist in tricalcium phosphate. The combination is frequently referred to as a complex. A definite minimum amount of such calcium phosphate seems to be always present *(3)*, and an excess amount which differs as between milks and over the particle-size range. The differences in over-all composition must be taken into account in assigning a nitrogen factor. This is here done by relating the factor to the calcium content. The factor calculated does not refer to the total micelle or particle, but only to the dry calcium caseinate–calcium prosphate complex contained in it; thus excluding the salts, lactose, and serum proteins which are also present.

EXPERIMENTAL PROCEDURE

Skimmilk samples were centrifugally fractionated, using techniques previously described *(3)*. Liquid samples progressively depleted of the casein colloids, and deposited fractions of these colloids were obtained. The deposits were washed in most cases, by redispersing them in distilled water and centrifuging.

Both gravity and centrifugally separated skimmilks were used. The milks were not treated in any way and no preservatives were added. All samples were stored at 4° C. The methods of analysis are given in the previous publication *(3)*.

Figure 1 is a typical plot showing the relationship between per cent total solids and per cent total nitrogen in skimmilk samples, as the casein-containing colloids are progressively removed by centrifuging. The serum nitrogen value shown was obtained by extrapolating the accompanying casein nitrogen : total nitrogen plot to zero casein nitrogen. This particular experiment was done in April, 1955. It has been previously reported *(3)*, but without the total solids data.

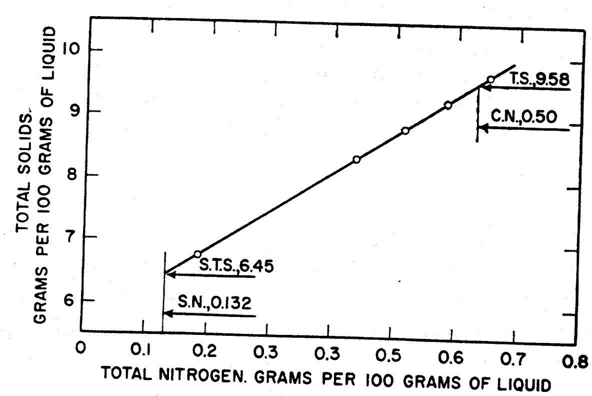

FIG. 1. Relationship of total solids to total nitrogen for skimmilk samples centrifuged for different lengths of time. *S.T.S.* = serum total solids. *S.N.* = serum nitrogen. *C.N.* = casein nitrogen. Ca = calcium. For this particular skimmilk, the ratio of casein nitrogen removed to total nitrogen removed is 1.00; the ratio of calcium removed to casein nitrogen removed is 0.181.

The method of calculating the complex : casein nitrogen factor, or ratio, from plots such as Figure 1, is similar to that used for calculation of the calcium : casein nitrogen ratio, as previously described *(3)*.

It is convenient to base the calculations on the analysis of the casein-free serum, as given by extrapolation, and on the analysis of an arbitrary skimmilk containing, for convenience, 0.5% casein nitrogen. A first approximation is given by the following equation:

$$\text{Complex}/C.N. = \left[\frac{T.S. - 0.9476\, S.T.S. - (N.C.N. - 0.9476\, S.N.)\,(6.38)}{C.N.}\right]\left[0.995\right]. \tag{1}$$

Here *C.N.* means casein nitrogen; *T.S.* means total solids, at 0.5% *C.N.*; *S.T.S.* means (centrifugal) serum total solids, at zero casein nitrogen; *S.N.* means (centrifugal) serum nitrogen, at zero casein nitrogen, and *N.C.N.* means noncasein nitrogen (total nitrogen less casein nitrogen), at 0.5% *C.N.*, a value usually identical or nearly identical with the centrifugal serum nitrogen. These values are taken from the plots at zero and 0.5% *C.N.* The numerical constant, 0.9476, represents the weight of casein-free serum in 1 g. of skimmilk, at 0.5% *C.N.*, assuming a complex : casein nitrogen factor of 6.89 and a hydration of

0.52 g. of hydrate water per g. of dry colloid.[3] The constant, 6.38, is the factor for conversion of serum nitrogen to serum protein. The multiplier, 0.995, is a correction factor for the fats, phospholipids, and other ether-extractable substances removed with the colloid by centrifugation.

Substituting the first approximate complex : nitrogen factor given by Equation 1 for 6.89, and recalculating, gives in every case a second approximation which differs negligibly from the first.

Complex : casein nitrogen factors calculated as indicated for several milks, including the one for which the data are shown (Figure 1), are listed (Table 1)

TABLE 1

Relationship of the complex : casein nitrogen factor to the calcium : casein nitrogen ratio, based on analysis of progressively depleted samples of skimmilks; together with the serum nitrogen and the total solids values used in the calculations

Date	*S.N.*[a]	*S.T.S.*	*TS* at 0.50% *C.N.*	Ca/*C.N.*	Complex /*C.N.*	Milk
	g/100 g.	*g/100 g.*		*g/g*	*g/g*	
October, 1946	0.165	6.86	9.99	0.182	6.83	Jersey
December, 1948	0.134	6.68	9.84	0.207	6.90	Herd
April, 1951	0.132	6.45	9.58	0.181	6.81	Jersey
August, 1951	0.127	7.06	10.26	0.198	7.02	Jersey
June, 1954	0.139	7.10	10.24	——	6.91[b]	Jersey
March, 1955	0.119	6.75	9.84	——	6.78[b]	Jersey
August, 1955	0.119	6.29	9.40	0.182	6.77	Jersey[c]
August, 1955	0.140	6.26	9.45	0.184	6.90	Holstein[c]
Averages	0.134	6.68	9.83	0.189	6.872[d]	

[a] *S.N.* = serum nitrogen; *S.T.S.* = serum total solids; *C.N.* = casein nitrogen.
[b] Omitted in averaging, because calcium was not determined.
[c] About 20% of the colloid was removed from these two skimmilks by preliminary centrifugation. These results, therefore, apply to intermediate and small particle sizes only.
[d] Standard deviation, 0.081.

together with the serum nitrogen and total solids values on which the calculations were based, and together with the calcium : nitrogen ratios for the complex, where these were determined. Since the total solids : total nitrogen plots are in every case straight lines within the accuracy of the data, the factors given are averages for the ranges of particle sizes removed. The first four experiments, which include the April, 1951, experiment, have all been reported previously, in part *(3)*.

FACTORS BASED ON ANALYSES OF DEPOSITS

Complex : casein nitrogen factors and calcium : casein nitrogen ratios, based on analyses of several centrifugally deposited casein colloids, are given (Table 2). The original data for the first two sets of values are given in the paper referred to previously.

[3] 100-(0.5)(6.89)(1.52) = 94.76. The hydration, 0.52 g. per g. of dry complex, is based on results by deKadt and van Minnen *(1)*, who, however, refer their values to casein. Their average hydration, 0.55 g. per g., multiplied by the ratio of the casein : nitrogen factor, 6.45, to the assumed complex : nitrogen factor, 6.89, is 0.52 g. per g. DeKadt and van Minnen's values vary quite widely, but we have repeated their experiments and obtained about the same results. The effect of variations in hydration will be considered in the discussion.

TABLE 2

Relationship of the complex:casein nitrogen factor to the calcium:casein nitrogen ratio, for various centrifugally deposited casein colloids

Ca/*C.N.*[a]	Complex/*C.N.*	Milk	Particle-size fraction	Approximate per cent of total complex
		Colloids not washed		
0.216	7.18	Jersey 1	Largest	2
0.211	7.01	Jersey 1	Large	3
0.200	7.06	Jersey 1	Intermediate	3
0.198	7.03	Jersey 1	Intermediate	3
0.191	7.02	Jersey 1	Intermediate and small	70
0.207	6.92	Jersey 2	Composite	80
0.204 av.	7.04 av.			
		Colloids washed once with distilled water		
0.205	6.95	Jersey 3	Large	7
0.217	7.12	Jersey 3	Intermediate	10
0.203	7.02	Jersey 3	Intermediate	20
——	6.92	Jersey 3	Intermediate and small	50
0.200	6.90	Jersey 4	Composite	80
0.206 av.	7.00 av.			
		Colloids washed twice with distilled water		
0.190	6.81	Herd 1	Intermediate	60
0.207	6.91	Herd 2	Intermediate	60
0.190	6.75	Jersey 5	Intermediate	60
0.194	6.94	Jersey 6	Composite	80
0.200	6.80	Jersey 7	Composite	80
0.209	6.85	Jersey 8—24 hr.[b]	Composite	80
0.215	6.91	Jersey 8—48 hr.[b]	Composite	80
0.202	6.83	Jersey 8—1 wk.[b]	Composite	80
0.204	6.96	Jersey 9	Intermediate	60
0.206	6.87	Jersey 9	Composite	80
0.232	6.95	Jersey 9	Large	10
0.204 av.	6.87 av.[c]			

[a] *C.N.* = casein nitrogen.
[b] The same Jersey milk held for the times indicated at 4° C.
[c] Standard deviation, 0.056.

In the calculations for the first set, *i.e.*, for the unwashed deposits, the weight of dry complex was obtained by diminishing the total solids by the contained serum protein, by serum salts and lactose proportional to the free (not hydrate) water evaporated from the deposits, and by an additional 0.5% as an approximate correction for ether-extractable substances. The weight of calcium in the complex was obtained by diminishing the total calcium by calcium proportional to the free water evaporated.

In the calculations for the second set—the once-washed deposits—since analyses showed lactose, fat, and chloride to be negligible, the total solids value was diminished only by the contained serum protein, and no other corrections were used.

The colloid samples on which the third set of ratios is based were washed twice, because the once-washed deposits, despite negligible lactose by analysis, nevertheless showed slight yellowing in total solids determinations. The second washing seems permissible, because the results for Sets One and Two fail to show any significant change in the nitrogen factor or the calcium : casein nitrogen ratio due to washing, and the complete analyses *(3)* similarly show no significant change in inorganic phosphorus. With these twice-washed samples, no yellowing in total solids determinations was noted. The dried colloids were colorless, glassy solids. The total solids values were corrected for contained serum protein as shown by analysis, as for the unwashed and once-washed deposits.

All of the skimmilks used for the preparation of the twice-washed deposits were submitted to sufficient preliminary centrifugation to remove those extremely large particles which apparently differ in composition from the rest *(3)*. These may represent from zero to about 15% of the total colloid. Although size classes are indicated in a general way (Table 2), close size fractionations were not attempted.

CALCULATED FACTORS BASED ON COMPOSITION OF THE COMPLEX

Differential and direct analyses of the centrifugally separated casein colloid for total nitrogen, casein nitrogen, calcium, total phosphorus, and total solids indicate that the unit particle of serum-free casein colloid, *i.e.*, casein complex, may be considered as composed of calcium caseinate and tricalcium phosphate in the proportions of one phosphorus equivalent of the former to one-half mol of the latter; and that the weight ratio of organic phosphorus to nitrogen is 0.0563 *(3)*. The weight ratio of casein to nitrogen calculated from analyses of casein by Ramsdell and Whittier *(4)* is 6.45. This casein : nitrogen factor is also that used by deKadt and van Minnen *(1)*. Assuming the composition indicated, an aggregate of particles of casein complex containing 1 g. of nitrogen would be composed as follows:

Component	*g.*
Casein	6.45
Calcium equivalent to organic phosphorus	0.0727
½ $Ca_3(PO_4)_2$ equivalent to organic phosphorus	0.2815
Hydrogen replaced by calcium (subtracted)	−.0037
Total	6.8005

This total weight is a theoretical nitrogen factor for the complex, stripped of all calcium phosphate in excess of that assumed for the unit particle. The assumption that calcium in calcium caseinate is equivalent to the organic phosphorus is the same assumption made by deKadt and van Minnen *(1)*, and is based on various published experiments *(1, 4)*.

In the above analysis, the ratio of total calcium to nitrogen is 0.1817. Centrifugal analyses of many milk samples *(3)* give calcium : nitrogen ratios ranging from 0.181 to 0.232, and an average ratio of 0.206. Adjusting the tricalcium phosphate in the above analysis to make the calcium : nitrogen ratios 0.181, 0.206, and 0.232, the factors calculated are 6.798, 6.867, and 6.934. However, the separated colloid contains also about 1/15th mol of magnesium per mol of calcium. This does not affect the theoretical factor, 6.80, but, assuming all the magnesium to be present as $Mg_3(PO_4)_2$, it increases the experimental minimum, average, and maximum complex : nitrogen factors to 6.814, 6.883, and 6.953, respectively.

According to the analysis given, the nitrogen factor for calcium caseinate is $6.45 + 0.0727 - 0.0037 = 6.519$. This is identical with the factor calculated from data by Ramsdell and Whittier *(4)*, who analyzed the washed complex, and then deducted tricalcium phosphate equivalent to the inorganic phosphorus from this analysis.

DISCUSSION

Comparison with factors calculated from data by others. De Kadt and van Minnen *(1)* give analyses of successive liquid fractions and of unwashed deposits from which complex : casein nitrogen factors can be calculated. Handling these data as outlined here, for 11 sets of liquid samples, the average nitrogen factor obtained is 6.97, and the corresponding average calcium : nitrogen ratio is 0.201; for seven fractional deposits from three milks, the average nitrogen factor is 7.06, and the corresponding average calcium : nitrogen ratio, 0.221. These factors are about 1.5% higher than those here reported. It is presumed that de Kadt and van Minnen's milk was from Friesian cows. They used a Sharples Supercentrifuge.

Importance of the numerical value for hydration, and possible errors arising from the corrections for nonproteinaceous solids: reliability of results by different methods. The calculations based on analyses of liquid samples and on analyses of unwashed deposits both involve a numerical value, 0.52 g. per g., for hydration. Higher values for hydration would give higher nitrogen factors. The effect is appreciable. If a hydration of 1.00 g. per g. were assumed, for example, the average factor for liquids would be raised from 6.87 to 7.06; if the hydration were assumed to be zero, the factor would be lowered to 6.66. By comparison with the results for washed deposits, for which hydration is not a factor, it would appear that the true hydration is probably not far from the value used. The correction for included fatty substances (0.5% of the total solids removed) is an average correction, based on many ether extractions of unwashed dried deposits; but individual samples give ether extracts which vary widely, from zero to about 1%. Therefore, for individual samples, this correction may introduce an error of $\pm$ 0.5%. The corrections for serum total solids, in Equation 1 for liquid samples, and for unwashed deposits, assume that no more such solids, except serum proteins, are deposited as part of the colloid than would be contained in the free or serum water. It is possible that the amount of nonprotein serum solids in the deposits may be slightly more than this. The effect of excess serum proteins is eliminated in Equation 1, and is taken into account in the calculations for deposits.

The calculations based on analyses of washed deposits involve the assumption that the composition of the casein complex is unchanged by the washing. This assumption appears to be justified. Lactose, and presumably other serum solids, except serum proteins, are removed from the deposits by washing. The ether extract is reduced to a negligible value, as has been stated. Therefore, data for washed colloids need be corrected only for retained serum proteins and, since this correction is based on analyses, it is presumed to be accurate. The results for washed deposits are, therefore, probably more reliable than those for liquid samples or unwashed deposits.

Collected results. Although arithmetic averages are given throughout this paper, such averages are not adequate, since a trend rather than a central tendency is involved.

In Figure 2 the various complex : casein nitrogen factors based on analyses

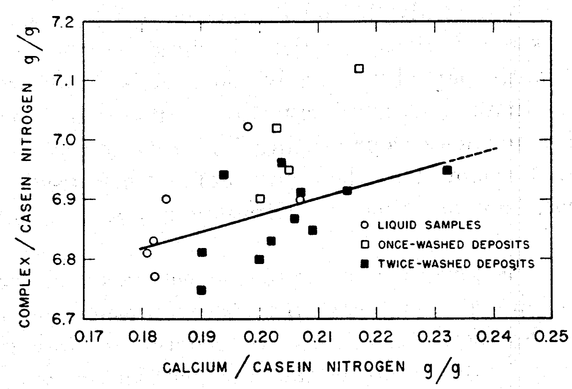

FIG. 2. Relationship of the colloid : casein nitrogen factor to the calcium : casein nitrogen ratio in the colloid, as determined by various methods.

of liquid samples, of once-washed deposits, and of twice-washed deposits, are collected and plotted against the corresponding calcium : casein nitrogen ratios. The deviations indicated may be in part attributable to experimental errors, but they also reflect the variability in composition of milks. The theoretical line, based on the composition of the complex as indicated by complete analyses, is drawn across the plot. The equation of this line is:

$$\text{Complex}/C.N. = 6.814 + (2.73)(\text{Ca}/C.N. - 0.181).$$

Here the constant, 6.814, is the calculated minimum factor for the complex, and the constant 2.73 represents g. of calcium phosphate plus g. of magnesium phosphate, per g. of calcium in excess of the experimental minimum. In the equation for the corresponding least squares line, giving equal weight to all the values, the constants are 6.820 and 3.90; omitting the values for the once-washed deposits, the constants are 6.823 and 2.70. In the first case, the standard error of estimate is 0.075, in the second case, 0.062. Considering the weight to be given the different sets of values, the relationship between the complex : nitrogen factor and the calcium : nitrogen ratio would appear to be adequately expressed by the equation:

$$\text{Complex}/C.N. = 6.82 + (2.7)(\text{Ca}/C.N. - 0.181). \quad (2)$$

This equation gives minimum, average, and maximum factors, at the calcium : casein nitrogen ratios 0.181, 0.206, and 0.232, of 6.82, 6.89, and 6.96, respectively. The probable error is about 0.04, or 0.6%.

The calcium : casein nitrogen ratio for any milk, or fraction of the colloid, can be found by either direct or differential analysis of the separated colloid *(3)*. If only the analysis of the milk is known, the calcium : casein nitrogen ratio may be estimated, by assuming a serum calcium content (in the milk) of 0.038 g. per 100 g. For any particular milk sample of unknown analysis, the average factor 6.89 could be in error by roughly 1.3%.

Except in four cases, the results here presented are for Jersey milk. However, the values for the other milks and those calculated from de Kadt and van Minnen's data suggest that for all milks the nitrogen factor may be about the same as for Jersey milks, if not identical. It should also be noted that the results given do not apply to the very largest casein-containing particles in milk. In the preparation of the skimmilks, these large particles were, in most cases, and for all the washed deposits, purposely eliminated. Similarly, the results do not necessarily apply to the very smallest particles, representing 5 to 10% of the casein colloid: these particles were not removed in the centrifuging times used. As much as 99% of the colloid has, however, been centrifugally removed in other experiments.

Although the correlation here is primarily between the complex : nitrogen factor and the calcium : nitrogen ratio, a correlation between the calcium : nitrogen ratio and particle size has been shown *(3)*. In general, for any particular milk, the calcium content and, therefore, the nitrogen factor, will be greater for the large casein-containing particles than for the small ones.

The trapped serum protein. In all the calculations given in this paper, the serum protein deposited with the casein colloid on centrifuging is excluded. The amount of this serum protein may be twice or three times as much as the amount which can be accounted for as present in the entrained solvate liquid. The excess is carried down with the colloid, by sweeping action, or otherwise, and it cannot be removed from the deposit by washing. Whether the attachment occurs on deposition or exists in the liquid milk can not be decided from present data. In the calculations, it is assumed that the attachment occurs on deposition, and that none of the serum protein in deposits is properly to be considered a part of the complex.

Nature of the actual colloidal particle in milk, and nitrogen factors for the total particle. It is implied in the title of this paper, and stated in the introduction, that the nitrogen factor as calculated here refers only to the calcium caseinate–calcium phosphate portion of the casein-containing particles, or micelles, in milk. This substance may well represent a definite chemical complex; it may at least be considered the framework of the particle. These particles are spherical in shape and are very large, ranging in size upward from about 520 Å diameter. They may be visualized as loose aggregates of casein complex units, possibly coiled chains, to which hydrate water molecules are attached, and which are interpenetrated by solvate liquid containing salts, lactose, and serum proteins, in the same proportions in which these substances exist in the casein-free serum. The ratio of the volume of the total particle to the weight of contained dry complex is about 3.1 to 1 *(2)*. It can be calculated from this ratio and other data that if the hydration is 0.52 g. per g., the remaining water present as solvate liquid should contain on the average about 0.124 g. of serum solids. Therefore, for the minimum and maximum factors, 6.82 and 6.96, for example, the corresponding minimum and maximum *colloid* : casein nitrogen factors are about 7.7 and 7.8, respectively. Again, the excess serum protein found in deposits is excluded.

The analyses of unwashed centrifugal deposits given in the previous paper *(3)* give over-all *total solids* : casein nitrogen ratios of 8.13 to 8.52, and when the serum protein in excess of that proportional to the serum water in the wet deposits is deducted from the total solids, the ratios 7.96 to 8.22 are obtained. Such factors depend upon the degree of compaction, however. By centrifuging at increasingly higher centrifugal forces, maximum compaction can be attained, and analyses of these deposits give ratios of total solids less trapped serum protein, *i.e.*, colloid : casein nitrogen factors, in satisfactory agreement with the calculated values, 7.7 to 7.8. Detailed discussion of such derived factors is premature here, since data on apparent specific volumes and voluminosity are involved. Determinations of these constants will be reported later. The purpose of this section is to make an explicit distinction between the total colloidal particle and the contained casein complex for which the factors here given are directly applicable.

SUMMARY

1. Skimmilks were progressively depleted of casein colloids by centrifuging, and the supernatant liquids and deposited fractions analyzed. The dry weights of calcium caseinate–calcium phosphate complex contained in the separated colloids were calculated from the analyses. For many milks and many particle-size fractions the average ratio of dry weight of complex to casein nitrogen found is approximately $6.89 \pm 1.3\%$. This nitrogen factor is applicable to any milk sample of unknown composition.

2. The complex : nitrogen factor is shown to be correlated with the calcium : nitrogen ratio for the separated colloid, *i.e.*, with the ratio of complex bound calcium removed to casein nitrogen removed with the colloid. When this ratio is known, a more accurate nitrogen factor can be calculated by means of the statistically determined equation:

$$\text{Complex}/C.N. = 6.82 + (2.7)\,(\text{Ca}/C.N. - 0.181). \tag{2}$$

The probable error when this equation is used is about 0.6%.

3. Since it has already been shown that the calcium : casein nitrogen ratio tends to increase with particle size, it follows that the nitrogen factor does also. The range of calcium : nitrogen ratios found experimentally is from 0.181 to 0.232; the corresponding range of nitrogen factors given by the equation is 6.82 to 6.96.

4. The experimental minimum nitrogen factor, 6.82, is in close agreement with a theoretical factor, 6.8005, calculated for a casein complex unit considered as composed of one mol of calcium caseinate and one-half mol of tricalcium phosphate. Adjusting this by adding trimagnesium phosphate in the proportion

in which magnesium is known to be present, the theoretical minimum factor becomes 6.814.

5. It is emphasized that the factors calculated are for the casein complex and not for the total colloidal particle, which also contains those serum solids associated with the solvate liquid. Colloid factors can be calculated from the complex factors by adding these additional solids. They range from approximately 7.7 to 7.8, in agreement with experimental results.

6. The nitrogen factors given do not apply to the very largest casein colloid particles in milk, and may not apply to the very smallest. The largest particles are known to differ in composition from the rest and were, therefore, in most cases purposely removed before the fractionations were made. The smallest particles were not all removed in the centrifuging times used, and no close fractionations of small particles only were attempted.

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