CXIX. THE DETERMINATION OF MILK- PROTEINS BY FORMALDEHYDE TITRATION.

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THE formaldehyde method for the determination of milk-proteins, originally proposed by Steineggar [1905], has been much employed since its inception on account of its ease, rapidity, and reasonable accuracy. In the original method, sodium hydroxide appears to have been the alkali employed in the titration. Richmond [1906, 1911] later recommended strontium hydroxide as giving a sharper end-point, and remarked further that the titrations obtained with this substance were considerably higher—on the average by about 10 %—than those obtained with sodium hydroxide. This difference he attributed primarily to the differing degrees of hydrolysis of the strontium and sodium salts of the milk-proteins and of the products formed from them by the action of formaldehyde, and, to a slight extent, to similar differences in the degrees of dissociation of the strontium and sodium salts of the acids usually present in commercial formaldehyde. It appears however that there is an additional and probably more important factor which plays a part in the titration differences mentioned, namely, the formation of tricalcium phosphate during the formaldehyde titration. It has long been recognised that this precipitation occurs in the determination of milk-acidity in the usual way, and that the extent to which it occurs, and therefore the resulting titratable acidity of the milk, depends greatly on the conditions of titration. Thus the diminished acidity resulting from the dilution of milk has been ascribed by Sommer and Menos [1931] to the greater solubility, *i.e.* diminished precipitation, of tricalcium phosphate in the diluted medium. Similarly the very different milk-acidities found by Roeder and Radoi [1925] according to whether sodium or calcium hydroxide had been used for the neutralisation have been attributed to the different degrees of phosphate precipitation in the two cases, the higher acidities occurring, as was to be expected, when the alkaline earth was employed, and conditions were more favourable to increased precipitation.

It is clear that such an influence, evident enough in the neutralisation of milk, must also make itself felt in the subsequent formaldehyde titration. The latter will therefore give a composite result, dependent mainly on the protein content of the milk but also influenced by the presence of phosphates

to an extent which will vary with the individual milk sample and the type of alkali used for the titration.

Confirmation of this view is furnished by determinations of the formaldehyde titration of proteins in the presence and absence of phosphates, with various alkalis (Table I) and also by determinations on some samples of milk to which phosphate had been added.

Table I.

Solution	Formaldehyde value cc. <i>N</i> alkali per 100 cc. sol.	Alkali
Egg-albumin A	1.37	Sr(OH) ₂
„ + 10 % <i>M</i> /5 alkali phosphate (<i>p_H</i> 6.6)	1.60	„
Egg-albumin B	1.80	„
„ + 20 % serum*	2.00	„
Egg-albumin C	1.20	NaOH
„ + 20 % serum*	1.27	„
Calcium caseinogenate	1.60	Sr(OH) ₂
„ + 10 % serum*	1.71	„
Milk A	1.88	„
„ + 10 % serum*	1.98	„
Milk B	1.71	NaOH
„ + 10 % serum*	1.77	„

* Prepared by adding to the protein solution in order equal volumes of (a) 0.3 *M* CaCl₂ and (b) a solution containing 0.25 *M* sodium citrate, 0.15 *M* KH₂PO₄, and 0.15 *M* Na₂HPO₄. The mixture diluted ten-fold corresponds approximately in salt content to that of average milk-serum.

It is evident from these results that the use of Sr(OH)₂ for the determination of formaldehyde values of proteins in the presence of phosphate, *e.g.* in milk, is open to objection, and it would also appear that this applies, though to a less extent, to alkali hydroxides. It would seem advisable therefore that milk before determination of its formaldehyde values should be treated so as to obviate the disturbing effects of phosphate. For this purpose, the addition of neutral potassium oxalate (2 % of a saturated aqueous solution), recommended by Van Slyke and Bosworth [1914] for similar reasons for the determination of milk-acidity, suggested itself, and its influence on the formaldehyde titration has therefore been examined.

In Table II are shown some formaldehyde values of milk determined by all three methods, *i.e.* by NaOH, with and without the addition of oxalate, and by Sr(OH)₂.

Table II.

Material	Formaldehyde value cc. <i>N</i> alkali per 100 cc. milk		Alkali
	A	B	
Milk alone	1.69	1.71	NaOH
„ + 2 % sat. oxalate	1.80	1.92	„
Milk alone	1.94	2.01	Sr(OH) ₂

In these experiments the use of oxalate is seen to give values intermediate between those obtained with sodium and strontium hydroxides, but closer

perhaps to those of the latter. This result was somewhat unexpected, as it was thought likely that the oxalate values would be the lowest of the three, on account of the exclusion of any phosphate precipitation. The most obvious explanation of the values actually obtained seemed to be connected with the hydrolysis of the protein and formaldehyde-protein salts to which Richmond [1906, 1911] drew attention in this connection, the degree of hydrolysis being perhaps largely influenced by the very much increased alkali ion concentration arising from the introduction of the oxalate.

To investigate the effect of such variations in the extent of hydrolysis, experiments were next carried out with pure proteins—egg-albumin and calcium caseinogenate—under similar conditions.

Typical results are shown in Table III.

Table III.

Solution	Formaldehyde value cc. <i>N</i> alkali per 100 cc. sol.				Alkali
	A	B	C	D	
Egg-albumin	1.93	1.37	—	—	Sr(OH) ₂
"	1.83	1.31	2.00	—	NaOH
" + 4 % sat. oxalate	—	—	2.04	—	"
Calcium caseinogenate	2.00	2.03	1.22	1.42	Sr(OH) ₂
"	1.90	1.96	1.20	1.38	NaOH
" + 4 % sat. oxalate	2.00	2.00	1.22	1.43	"

From these it is seen that, in the absence of phosphates, the use of strontium hydroxide leads to somewhat higher formaldehyde values (about 3–5 %) than are obtained with sodium hydroxide, but that if much oxalate is present the values obtained with NaOH increase, and approximate to those of Sr(OH)₂. The values obtained with the two hydroxides do not differ, however, very greatly, and it is evident therefore that variations in hydrolysis resulting from their use cannot account for more than 1/3 to 1/2 of the total difference usually observed in the formaldehyde titration of milk.

The residual and greater part of the titration difference appears to arise from the presence of phosphate, as suggested earlier.

It may be remarked here that the precipitation of tribasic phosphate during titration obviously cannot account for the formaldehyde titration of oxalated milk (using NaOH) being so much higher than that of untreated milk—approximating in fact to that obtained with Sr(OH)₂. The explanation of this point will be considered later.

As it seemed of interest to determine to what extent each of the protein salts of milk contributed to the hydrolysis discussed above, some further experiments of a qualitative nature were made with egg-albumin and caseinogen. Solutions of these proteins alone and solutions containing formaldehyde were neutralised to phenolphthalein with strontium hydroxide, and gradually increasing amounts of neutral potassium oxalate were added to each. The indicator tint in the neutralised protein solution was scarcely affected by this

treatment, but that in the solution containing formaldehyde deepened considerably on the first small addition of oxalate (*i.e.* on conversion of the strontium into a potassium salt) and showed a marked tendency to lighten again as the amount of oxalate added was increased. The difference in the formaldehyde titration values with NaOH and $\text{Sr}(\text{OH})_2$ in the absence of phosphates appears therefore to be mainly due to the different degrees of hydrolysis of their respective formaldehyde-protein salts.

The experiments on hydrolysis make it clear that if oxalate is to be used, the amount employed must be definitely specified. Van Slyke and Bosworth [1914] prescribed an addition of 2 % of saturated potassium oxalate for the determination of the titratable acidity of milk, and for the determination of formaldehyde values this amount seemed adequate with most samples. An occasional sample was met with, however, in which the addition of more than this proportion of oxalate was required to develop a minimum titratable acidity—and therefore a maximum removal of calcium—and a somewhat higher proportion of saturated oxalate, *viz.* 4 %, was therefore decided on.

This amount has proved adequate for all milks met with up to the present, and has the further advantage that the formaldehyde values obtained by its use agree fairly closely with those obtained by Richmond's $\text{Sr}(\text{OH})_2$ titration.

Table IV gives the results of a number of comparative estimations of the formaldehyde value of milks, carried out with and without the addition of 4 % saturated potassium oxalate, the alkali throughout being sodium hydroxide.

Table IV.

Milk No.	Formaldehyde value cc. N alkali per 100 cc. milk		Ratio	Milk No.	Formaldehyde value cc. N alkali per 100 cc. milk		Ratio
	With oxalate	Without oxalate			With oxalate	Without oxalate	
1	1.82	1.68	1.09	16	2.26	2.04	1.11
2	2.00	1.84	1.09	17	1.81	1.63	1.11
3	1.84	1.69	1.09	18	1.94	1.75	1.11
4	1.74	1.60	1.09	19	2.02	1.82	1.11
5	1.96	1.80	1.09	20	2.61	2.35	1.11
6	2.20	2.01	1.10	21	2.25	2.02	1.11
7	1.95	1.78	1.10	22	1.93	1.73	1.12
8	2.03	1.85	1.10	23	2.38	2.13	1.12
9	1.91	1.74	1.10	24	1.90	1.70	1.12
10	2.01	1.83	1.10	25	1.84	1.64	1.12
11	2.00	1.82	1.10	26	2.13	1.90	1.12
12	2.07	1.88	1.10	27	2.30	2.05	1.12
13	1.94	1.76	1.10	28	1.92	1.71	1.12
14	2.02	1.83	1.10	29	2.36	2.09	1.13
15	1.97	1.77	1.11	30	1.98	1.76	1.13
				Mean ratio		1.107	

The titration values of the oxalated milks are seen to be considerably (9–13 %) the higher—a somewhat unexpected result. From the earlier experiments on pure protein solutions it appeared that (*a*) the formaldehyde values obtained with NaOH alone were only slightly (3–5 %) lower than those obtained in the presence of oxalate (see Table III), and that (*b*) the

addition of phosphate was capable of increasing slightly the titrations with NaOH alone (Table I). It was thought therefore that these two factors, acting in opposition when milk was under investigation, should result in the formaldehyde values being approximately the same whether oxalate were present or not. The results obtained in Table IV, showing on the contrary the oxalate titration to be about 11 % the higher, suggested that there were other and important factors affecting the formaldehyde titration of milk which had escaped consideration.

The only milk constituent besides protein and soluble phosphate which appeared in any way likely to play such a part was colloidal calcium phosphate, which from its close connection with the calcium caseinogenate of milk might be conceived to influence in some way the interaction of formaldehyde with the protein, or the degree of titratable acidity developed therefrom. An attempt was therefore made to examine its effect on the formaldehyde value of caseinogen, and for this purpose a caseinogen-calcium phosphate complex was prepared on the lines indicated by Porcher [1930]. The method used consisted briefly in dissolving caseinogen (Hammarsten) in an excess of lime water (36 mg. CaO per 1 g. caseinogen) and running *M*/20 phosphoric acid slowly and with vigorous stirring into this alkaline solution until a p_H value of slightly less than 7.0 was attained. To obviate as far as possible partial decomposition of the caseinogen by the alkali during the preparation of the complex, the protein was dissolved originally in about one-half the total quantity of lime water needed for the complex, the solution cooled in ice, and the remainder of the lime water, similarly cooled, added almost immediately before proceeding to run in the phosphoric acid. During this stage the mixing vessel was also cooled with ice.

Table V shows the formaldehyde values determined in the various ways already employed, of a series of caseinogen-calcium phosphate complexes

Table V.

Solution	Protein %	Form- aldehyde value cc. <i>N</i> alkali per 100 cc. sol.	Alkali used	Ratio Protein Formald. value
Caseinogen-calcium phosphate complex A	—	1.20	NaOH	—
	—	1.42	Oxalate and NaOH	—
	—	1.45	Sr(OH) ₂	—
Caseinogen-calcium phosphate complex B	2.24	1.10	NaOH	2.04
	2.24	1.27	Oxalate and NaOH	1.76
	2.24	1.26	Sr(OH) ₂	1.78
Caseinogen-calcium phosphate complex C	2.44	1.20	NaOH	2.03
	2.44	1.38	Oxalate and NaOH	1.77
	2.44	1.40	Sr(OH) ₂	1.76
Caseinogen-calcium oxalate complex C	2.32	1.30	NaOH	1.78
	2.32	1.32	Oxalate and NaOH	1.76

prepared in this fashion, and, for comparison, those of a similar complex in which the calcium phosphate is replaced by calcium oxalate (by neutralising

the excess calcium hydroxide with dilute oxalic acid instead of $M/20$ phosphoric acid). Some protein determinations made by the Kjeldahl method are included and assist the interpretation of the results.

The results with the caseinogen-calcium phosphate complex are seen to be somewhat similar to those obtained with milk and make it evident that the presence of colloidal calcium phosphate can depress the formaldehyde titration of caseinogen with NaOH to a value much below that found for caseinogen in its absence. The titrations obtained on addition of potassium oxalate are practically identical whether colloidal phosphates were originally present or not, as is to be expected. Those obtained with $\text{Sr}(\text{OH})_2$ as alkali seem also to be relatively independent of the presence of colloidal phosphates, but the observations in this case must be interpreted to mean that the reduction in the formaldehyde titration through the presence of colloidal phosphates and the influence of these on caseinogen is approximately counterbalanced by the additional alkaline earth used in precipitating some of the soluble, and also some of the colloidal, dicalcium hydrogen phosphate as tricalcium phosphate.

The results obtained with sodium hydroxide, which show that the acidity developed on adding formaldehyde to a solution of calcium caseinogenate is markedly reduced in the presence of colloidal calcium phosphate, appear to favour the view that the caseinogen and colloidal phosphate in the complex and also in milk are not present as isolated substances, but exist in some kind of chemical union, the exact nature of which is not yet clear. That their relation however is something more specific than one of mere colloidal protection of the phosphate by the protein seems to be inferable from the entirely different behaviour of the caseinogen complex containing protected calcium oxalate instead of phosphate, and from further experiments made on colloidal calcium phosphate protected by other proteins. Thus, a 5 % gelatin solution containing colloidal phosphate, whether prepared by the interaction of disodium hydrogen phosphate and calcium chloride, or by that of calcium hydroxide and phosphoric acid, gives practically identical formaldehyde values with NaOH, with or without addition of potassium oxalate.

The general practical conclusion to be drawn from these results is that the formaldehyde value of milk, as hitherto estimated, is a composite value, determined largely by the protein content of the milk, but affected in various directions by both colloidal and soluble phosphates—to an extent which depends on the type of alkali used for the titration. It seems advisable therefore in using this value for the determination of protein in milk, to eliminate these disturbing features as far as possible, a result easily attained by the use of oxalate. Not only is the correlation between protein and formaldehyde value greatly improved by this means (see Table VI), but the titration is also facilitated by the improved sharpness of the end-point and the practical elimination of that fading (due to slow formation of tribasic phosphate) which otherwise accompanies the use of NaOH and more particularly $\text{Sr}(\text{OH})_2$.

In Table VI will be found the results of a number of protein determinations ($N \times 6.38$), and formaldehyde values of mixed milks, and in Table VII the results in abridged form of a somewhat similar but more extensive series of estimations made on the milks of individual cows (Shorthorn breed).

Table VI.

Milk mixed	Protein per 100 cc. milk	Formaldehyde value cc. <i>N</i> alkali per 100 cc. milk		Ratio $\frac{\text{Protein}}{\text{Formald. value}}$	
		With oxalate	Without oxalate	With oxalate	Without oxalate
1	3.44	2.00	1.82	1.72	1.89
2	3.40	1.97	1.76	1.73	1.93
3	3.43	1.98	1.76	1.73	1.95
4	3.36	1.94	1.76	1.73	1.91
5	3.19	1.84	1.64	1.73	1.94
6	3.14	1.81	1.63	1.74	1.93
7	3.41	1.95	1.78	1.75	1.92
8	3.33	1.90	1.70	1.75	1.96
9	3.05	1.75	1.60	1.75	1.90
10	3.53	2.01	1.83	1.76	1.93
Mean				1.74	1.926

Table VII.

Ratio $\frac{\text{Protein}}{\text{Formald. value}}$ (using oxalate)	No. of samples (milk of single cows)	Ratio $\frac{\text{Protein}}{\text{Formald. value}}$ (using oxalate)	No. of samples (milk of single cows)
1.63	1	1.75	6
1.64	1	1.76	3
1.65	1	1.77	3
1.67	1	1.78	3
1.69	3	1.79	1
1.70	5	1.80	2
1.71	2	1.82	1
1.72	3	1.84	1
1.73	3	1.87	1
1.74	5	Mean	46

The formaldehyde titrations (using oxalate) were made with *N*/10 sodium hydroxide on 10 cc. samples of milk to which 0.4 cc. saturated aqueous potassium oxalate, and 0.5 cc. of 0.5 % phenolphthalein as indicator, were added. After standing for about 2 minutes the milk was neutralised, the end-point being a faint pink which was matched with that of a similar quantity of milk and oxalate tinted by addition of a drop of dilute alcoholic magenta¹. 2 cc. 40 % formaldehyde were then run in and the titration with NaOH continued to the same tint as before. The acidity of the formaldehyde must of course be subtracted from the milk titration and may be determined by similarly titrating 2 cc. formaldehyde in 10 cc. water with *N*/20 alkali. The recorded values in Tables VI and VII are in all cases the mean of two or more estimations. Where oxalate was used the agreement of duplicates was almost exact—the difference in no case amounting to as much as 0.05 cc.

¹ The depth of tint given by one drop of this magenta solution to 10 cc. water was approximately the same as that developed in 10 cc. of a borate buffer (p_H 7.9) containing 0.5 cc. of 0.5 % phenolphthalein.

The results with mixed milks (Table VI) and with individual milks (Table VII) agree very well, giving an almost identical ratio $\frac{\text{protein}}{\text{formald. value}}$ of approximately 1.74 where oxalate is employed. The average value obtained with NaOH alone (without oxalate) for mixed milks may be expressed as 1 cc. *N* NaOH = 1.926 g. milk-protein, and agrees very well with that found by Steineggar for NaOH, quoted by Richmond [1906], namely 1 cc. *N* NaOH = 1.933 g. milk-protein. It will be noted from Table VI that the ratios $\frac{\text{protein}}{\text{formald. value}}$ obtained by the use of oxalate show much less variation from sample to sample than those for NaOH alone. This is in line with the somewhat considerable degree of variation in the ratios of the values obtained with these two types of titration (see Table IV) and is, of course, to be expected from what has been seen of the various factors which influence the formaldehyde titration in the absence of oxalate.

The protein values of 1 cc. *N* alkali, using oxalate, obtained with the milks of individual cows vary more, naturally, than those from mixed milks, as may be seen by comparing the data of Tables VI and VII. Some of the individual samples, which deviated most from the mean, were from cows approaching the end of lactation, and in these the percentage of protein was unusually high, and presumably the ratio of caseinogen to albumin also abnormal. As the acidities developed by equal weights of albumin and caseinogen on addition of formaldehyde are very different [cf. Richmond, 1906], marked variation in their relative amounts in milk is bound to cause a displacement of the protein/formald. value ratio, which cannot be obviated by the use of oxalate. The formaldehyde method in either its original form or the modified one suggested here is thus suited best to average normal or mixed milk. The question of further modification to cover abnormal samples is however receiving attention.

The formaldehyde and protein values given in the foregoing tables have all been expressed in terms of the same volume. For practical use the factor 1.74 requires to be modified slightly to express the protein as a percentage by weight. The standard 10 cc. pipette used to measure the milk for both formaldehyde and Kjeldahl determinations was found to deliver 10.25 g. of average milk (sp. gr. 1.032). The factor for converting the observed formaldehyde value (expressed as cc. *N*/10 alkali per quantity of milk delivered by a 10 cc. pipette) into percentage protein by weight therefore becomes $\frac{1.740}{1.025}$ or 1.70 for milk of average density.

SUMMARY.

1. The formaldehyde titration for the determination of milk-proteins, as usually carried out, has been shown to be considerably affected in different directions by the presence in milk of (a) soluble or easily reacting phosphates, and (b) colloidal phosphates. The former bring about an increase in the

formaldehyde value, particularly if an alkaline earth hydroxide is employed for the titration, and the latter a decrease.

2. The effect of the colloidal phosphate on the formaldehyde titration seems to favour the view that the calcium caseinogenate and colloidal phosphate of milk exist in some form of chemical combination.

3. The disturbing effects of both classes of phosphate can be eliminated by treating milk with an oxalate, prior to carrying out the formaldehyde titration. A suitable procedure is suggested for the purpose.

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