CLXII. RAPID METHODS FOR THE QUANTITATIVE DETERMINATION OF TOTAL PROTEIN AND NON-PROTEIN NITROGEN IN HUMAN AND COW'S MILK.

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Most methods for the determination of protein in milk depend on the Kjeldahl analysis of the milk in the presence of the lactose and non-protein nitrogen, because there is no known convenient milk-protein precipitant that allows of two complete washings of the protein precipitate without loss of protein. The nitrogen determination is thus always too high and the presence of the non-protein nitrogen and the relatively large amount of lactose adds to the material to be oxidised. Moreover the excessive carbonisation and resulting loss of sulphur trioxide caused thereby are responsible for the formation of a cloudy digest unsuitable for direct nesslerisation. Time-consuming procedures such as distillation or aeration have therefore to be employed.

Likewise, the recent formaldehyde titration method proposed by Pyne [1932], although rapid and simple, fails to give a true total protein content of milk, in that the formaldehyde titration factor used is obtained by standardisation against the total nitrogen as estimated by the Kjeldahl process.

The method presented here overcomes these difficulties by double precipitation of the protein with zinc hydroxide, the supernatant fluid being separated by centrifuge, and, if necessary, retained for the estimation of the non-protein nitrogen. The protein is subjected to a micro-Kjeldahl digestion employing a modified Folin and Denis [1916] reagent in which the proportion of catalyst and the concentration of the digestion mixture are raised. This overcomes the inhibitory action of the fat which is partly precipitated and adsorbed on the protein precipitate, as well as the resistant physical character of the milk protein itself. The clear digest which results is diluted and directly nesslerised. By using the special digestion mixture direct determination of the non-protein nitrogen is possible, obviating the necessity of introducing elaborate procedures such as that devised by Denis and Minot [1919] to remove first lactose, fat and other interfering substances.

The present method as finally evolved provides a technique which is very convenient for the requirements of a routine laboratory, making possible a quantitative determination of proteins in less than 30 minutes.

It is necessary to emphasise the importance of using only fresh samples for analysis. Where a delay is inevitable, sodium fluoride to the extent of 1 % must be added.

Reagents.

- (1) 0.45 % zinc sulphate.
- (2) *N*/10 and *N*/100 NaOH.
- (3) 40 % NaOH.
- (4) *N*/100 H₂SO₄.
- (5) Standard ammonium sulphate 0.494 g. per litre. 10 ml. contain 1.04 mg. nitrogen.
- (6) Digestion mixture:
 - 50 ml. of 10 % copper sulphate;
 - 300 ml. of syrupy (85 %) phosphoric acid;
 - 100 ml. of pure nitrogen-free sulphuric acid.
- (7) Nessler-Folin reagent [Folin and Wu, 1919].

Method.

To 10 ml. of 0.45 % zinc sulphate in a centrifuge-tube graduated to hold about 15 ml., add 1 ml. of human or 0.5 ml. of cow's milk. Mix well by inserting a rubber stopper or by placing the thumb over the opening of the tube and shaking vigorously, and then add 2 ml. of *N*/10 sodium hydroxide. Mix again, and after standing for about one minute or until the protein flocculates centrifuge at high speed (over 2000 r.p.m.) for 5 minutes. The supernatant fluid may be slightly opalescent with specimens of human milk, if the fat content is above normal, and this is an added advantage as the excess of fat is removed with the supernatant fluid and therefore does not retard the digestion process by heavy carbonisation. Carefully pour off the supernatant fluid, and add another 10 ml. of zinc sulphate solution. Mix vigorously to obtain a uniform emulsion of precipitated protein and add 1 ml. of the *N*/10 sodium hydroxide. After thorough mixing, again centrifuge at high speed for 5 minutes. Pour off the supernatant fluid and transfer the precipitated protein to a round-bottomed clear silica flask of 50 ml. capacity, using exactly 10 ml. of distilled water for transferring. It is convenient to have the distilled water in a graduated cylinder. Measure approximately 5 ml. of water into the centrifuge-tube, insert a rubber stopper and shake vigorously. Carefully remove the stopper and transfer the extract to the silica flask. Repeat the above procedure using 3 ml. and finally 2 ml. of water, transferring all the washings to the silica flask. This procedure removes all the remaining precipitate adhering to the sides of the centrifuge-tube and the rubber stopper, with the exception of a little precipitated fat. The total volume of wash-fluid in the silica flask should not be greater than 10 ml. Finally add 5 ml. of digestion mixture and proceed with the digestion carefully so as to obtain a clear digest.

Ordinarily the digestion is carried out in a fume chamber, or better under a hood to which suction is applied, as the fumes by this method are exceedingly heavy. With the flask somewhat inclined, boil vigorously over a micro-burner, using a long thin flame of about 4 in. in height which just touches the flask and spreads out slightly over the round bottom. To prevent bumping keep the flask moving so that the fluid spins round vigorously. At the first signs of boiling, heat the flask very cautiously, removing the flame if necessary, until the initial foaming subsides. This ceases after only a few seconds. Vigorous boiling is continued until the organic matter begins to carbonise. This usually occurs about 2 minutes after commencement of boiling. It is now necessary to place the neck of the flask inside the fume hood. On continued heating the charred material changes in colour from brown to almost black and frothing

usually occurs. ~~Remove the micro-burner, and heat very cautiously by just touching the bottom of the flask with the flame and quickly removing it.~~ Overheating at this stage should be avoided otherwise the charred material may creep up into the neck of the flask. After a further few seconds when the frothing will have subsided replace the flame for vigorous boiling to continue. When the frothing ceases dense white fumes fill the flask, condensing on the sides and serving to wash down and digest any carbonised material adhering to the sides of the flask. The mixture becomes lighter in colour and clears in about 5-5.5 min. from commencement of boiling. The end-point is reached when the digest turns a bright greenish-blue. Prolonged heating will always cause a precipitate to form, although this can be removed by centrifuging after nesslerisation, without absorbing any colour. As soon as the digestion is complete the flask should be removed from the flame and cooled in running water. After cooling the digest darkens slightly in colour. Quantitative figures recorded in the appended table show that the slight carbonisation is not due to incomplete digestion of the protein.

Add about 30 ml. of water to the flask as soon as possible and warm gently over the micro-burner to dissolve the thick syrup. Transfer the extract to a 250 ml. flask, and wash out the digestion-flask with liberal quantities of water, transferring the washings to the 250 ml. flask. Dilute to the mark with distilled water and mix well. Measure 50 ml. into a 100 ml. graduated flask, and add 30 ml. of Nessler's reagent. Dilute to the mark with water and mix thoroughly.

Prepare the standard at the same time by adding to about 60 ml. of water in a 100 ml. flask 3 ml. of the standard ammonium sulphate. Finally 1 ml. of digestion mixture is added, and after mixing, 30 ml. of Nessler's reagent. Dilute to 100 ml. with water and again mix well.

Compare in the colorimeter setting the standard at about 25 mm.

Calculation :

$$\frac{\text{Standard}}{\text{Unknown}} \times 157 = \text{mg. N/100 ml. human milk.}$$

Since 1 mg. of nitrogen is equivalent to 6.38 mg. of milk protein, the above equation multiplied by $\frac{6.38}{1000}$ gives the percentage of protein in human milk. For cow's milk multiply final result by two.

In order to prove the efficiency of the zinc hydroxide precipitant, the results obtained were checked by estimating the non-protein nitrogen, and by comparing with values for protein and non-protein nitrogen ascertained by using the tungstic acid precipitation in a form which is well known to have a wide application to biological fluids generally, particularly in regard to the estimation of the non-protein nitrogenous constituents.

The tungstic acid precipitation was carried out as follows:

To 5 ml. of a saturated solution of sodium sulphate in a centrifuge-tube add 1 ml. of human milk or 0.5 ml. of cow's milk. Add 2 ml. of 10 % sodium tungstate followed by 2 ml. of $\frac{3}{4}$ N sulphuric acid and shake vigorously. Immediately centrifuge at high speed (over 2000 r.p.m.) for 15-20 minutes, otherwise if the precipitate is allowed to flocculate before spinning it is impossible to obtain a clear supernatant fluid after centrifuging. Pour off the supernatant fluid. A little scum of precipitated fat may collect on the surface of the supernatant fluid, but this is of no consequence. Break up the precipitate by tapping the tube firmly with the finger, and then add 5 ml. of the saturated sodium sulphate *plus* 2 ml. of sodium tungstate and 2 ml. of the sulphuric acid. Mix by shaking vigorously and immediately centrifuge for 20 minutes. Carefully

pour off the supernatant fluid and transfer the precipitated protein to the silica flask as recommended in the standard technique. Proceed then as for the recorded method.

Estimation of non-protein nitrogen.

Pour off the first and second supernatant fluids obtained from the zinc hydroxide or tungstic acid precipitation into one of the silica flasks. Add 5 ml. of digestion reagent and digest in the usual way until the mixture is a bright greenish-blue and free from all carbon. Cool the flask in running water, and after dissolving the syrupy residue in about 10 ml. of water, transfer to a heat-resistant pyrex test-tube (22 × 200 mm.). Rinse out the flask with two 5 ml. portions of water adding these to the pyrex tube.

Liberate the ammonia by the addition of 20–25 ml. of 40 % sodium hydroxide. The aeration process follows that of the blood-urea method employed in this laboratory [Patterson, 1925], except that to prevent loss of ammonia during the addition of the alkali the tube containing the caustic soda is placed with its inlet tubes reversed, between the 5 % sulphuric acid tube used for removing the ammonia from the air, and the large boiling-tube containing the digestion extract, so that when the aeration is started the caustic soda is drawn over into the digested extract. The ammonia is drawn over into 5 ml. of *N*/100 sulphuric acid containing 3–4 drops of an indicator consisting of 100 ml. of 0.02 % alcoholic methyl red and 10 ml. of 0.1 % methylene blue.

Continue the aeration for half an hour, and after the first 5 minutes turn on the pump until 1 in. of foam is produced in the titration-tube. Back-titrate with *N*/100 sodium hydroxide.

Using the amounts specified in the methods, every 1 ml. of *N*/100 acid neutralised represents 0.00014 g. nitrogen for human milk, and 0.0028 g. for cow's milk. Table I illustrates the results of a few experiments on human and cow's milk.

Table I.

Specimen No.	Direct digestion mg. protein per 100 ml.	Tungstic acid precipitant mg. protein per 100 ml.	Zinc hydroxide precipitant mg. protein per 100 ml.	Slight carbonisation of digest (see p. 1197). mg. protein per 100 ml.	Non-protein nitrogen estimated directly in supernatant fluid from protein precipitant		N.P.N. calculated from difference between direct digestion and precipitation by tungstic acid and zinc hydroxide		Urea (including pre-formed ammonia) mg. per 100 ml.	Free ammonia in terms of urea mg. per 100 ml.	Age of specimen before analysis hrs.
					mg. per 100 ml.		mg. per 100 ml.				
					Tungstic acid	Zinc hydroxide	Tungstic acid	Zinc hydroxide			
Human milk :											
1	1356	1017	1020	—	54	53	53	52	47	23	24
2	1130	714	700	—	63	63	65	67	43	16	48
3	1236	1012	1008	1003	33	39	35	36	25	0	2
On 2 ml. milk	1156	—	—	—	—	—	—	—	—	—	—
Cow's milk :											
1	2800	2260	2220	—	70	96	84	90	70	37	72
2	2702	2564	2526	—	28	29	25	27	22	0	2
	2732	2380	2414	—	42	38	55	49	39	17	48
3	2631	2362	2372	2353	36	44	42	40	30	0	2
On 1 ml. milk	2629	—	—	—	—	—	—	—	—	—	—

Experimental results show the close agreement between the zinc hydroxide and tungstic acid precipitations. The zinc process has the advantage of being less time-consuming and also less cumbersome. The marked increase with the

directly digested milk is due to the presence of the non-protein nitrogen, showing the inaccuracy of the older methods based on total nitrogen.

As already stated even prolonged heating of the digestion mixture does not prevent a very slight darkening developing after cooling of the digest. The results recorded in the column represented by "slight carbonisation of digest" were obtained by discontinuing heating just before the end-point of the digestion process, and this gave rise to greater carbonisation of the digest than that obtained by cooling after full digestion. Despite this fact the figures practically correspond with those obtained by the authentic digestion.

The actually determined non-protein nitrogen results are compared with the figures obtained by subtracting the total protein estimated by either tungstic acid or zinc hydroxide precipitation, from that found by the direct digestion of the milk and dividing by 6.38.

SUMMARY.

1. A quantitative method is described for the routine estimation of protein and non-protein nitrogen in human and cow's milk employing zinc hydroxide as protein precipitant.
2. By choice of selected proportions and sequence of reagents direct nesslerisation is possible, thus avoiding time-consuming methods such as distillation or aeration.
3. A true total protein is obtained and not a total nitrogen estimation.

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