[FROM THE HARRIMAN RESEARCH LABORATORY, ROOSEVELT HOSPITAL, NEW YORK, N. Y.]

NEPHELOMETRIC DETERMINATION OF PROTEINS; CASEIN, GLOBULIN AND ALBUMIN IN MILK.[1]

BY PHILIP ADOLPH KOBER.

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CONTENTS.

I. Introduction.

Filtering and washing precipitates in physiological work is usually a long, tedious, and often decidedly inaccurate process, particularly in the quantitative estimation of proteins, nucleic acids, purine bases and other similar substances. Gelatinous and colloidal suspensions not only frequently clog the filter, but also require much washing to remove the small amounts of adsorbed mother-liquor, while some precipitates have also an appreciable solubility in the wash water. As an example, it may be mentioned that *the estimation of casein, globulin and albumin in milk requires from two to three days, if it is done according to the usual technic whereas with the method to be described in this paper, it can be done in twenty to thirty minutes.*

Precipitation, in physiological and biological, as in other branches of chemistry, has been the chief basis for quantitative analysis, and only recently Folin and others have made successful use of colorimetric reactions. And, since it is the filtering and washing that causes delay and inaccuracies in the precipitation method, any scheme whereby the precipitates can be determined with a fair degree of accuracy[2] without filtering and washing ought to find a large field of usefulness in the biological sciences.[3] The scheme worked out in previous papers[4] accomplishes this purpose, *i. e.*, the determination of the amount of precipitate in suspension, photometrically.

The success of the nephelometric method depends upon the use of the right precipitants, and, therefore, most of the work connected with this method was devoted to finding and trying out suitable precipitants. By "suitable precipitants" are meant those that precipitate 0.01% or weaker solution, quantitatively, in the form of suspensions, which do not agglu-

[1] Read in part at the Milwaukee meeting of Amer. Chem. Soc., March, 1913.

[2] The maximum accuracy is about 0.4% which can be easily obtained even in 0.002% solutions, while 1% is readily obtainable with 0.0003% solutions.

[3] The author believes that when the convenience and the accuracy of the nephelometer is more generally appreciated, applications to technical analysis will quickly multiply. It ought to be comparatively easy to determin chlorides, phosphates, etc., nephelometrically.

[4] Kober, *J. Biol. Chem.*, **13**, 485; THIS JOURNAL, **35**, 290 (1913).

tinate appreciably in less than 10 to 20 minutes, *i. e.*, time enough to permit two to five readings with the nephelometer.

For the different classes of proteins a number of such precipitants are known, but what is most needed is a specific precipitant for each protein in dilute solution. Such a specific precipitant used nephelometrically, will be equal in value to a very sensitive color reaction (like the Nessler reaction) for each substance. By suitable dilution this method can be used for large amounts of substances and with the same ease and accuracy, amounts as small as 0.00002 gram, can be determined.

It is, therefore, the object of this paper to describe the details of the nephelometric method, to compare it with other methods and to show the latest form of the nephelometer.

II. Method.

(*a*) *Instrument—The New Nephelometer.*—In the first paper of this series published, the differences between the Richards[1] and Wells instrument, and the one designed in this laboratory, were discussed. The original nephelometer was an adaptation of the Duboscq colorimeter. The new model is an improvement, in that metal is substituted for wooden parts, thereby eliminating any disadvantages due to warping. The change from one instrument to the other can be rapidly made, and the metalic parts can be easily removed, so that the plunger is accessible.

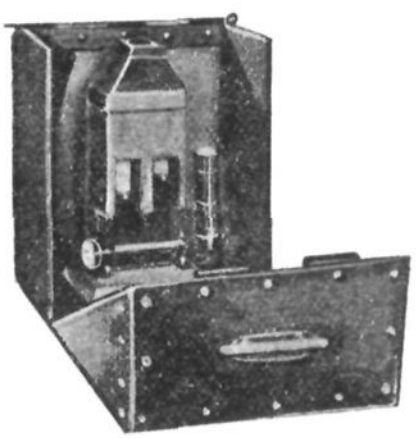

Fig. 1

The illustration given below is sufficiently clear without much further description.

Fig. 1 shows the instrument in a portable case which in Figs. 2 and 3 are used as a shade for the eye-piece. In Fig. 2 on the right of the instrument are shown the regular Duboscq colorimeter attachments, while on the left are shown the new nephelometric arrangements. As may be observed the only changes[2] necessary, are a coat of black

[1] *Am. Chem. J.*, **31**, 235 (1913). The Vereinigte Fabriken für Laboratoriums-Bedarf seems to be the only firm which keeps the Richards instrument in stock.

[2] Eimer & Amend manufacture the necessary additions to the Duboscq colorimeter, given above. In ordering one should specify whether the additions are to be fitted to an old type Duboscq colorimeter with movable plungers, or to the new type with stationary plungers. Cost including 4 tubes without combination shade and case $13.50, with case $20.00 and including Duboscq colorimeter $100.00. The author is indebted to Mr. H. E. Blomquist, of the Mechanical Department of Eimer & Amend, for supervising the construction of the instrument and for many useful suggestions as to practical details.

asphaltum paint on the plungers,[1] a special nephelometer tube, and a metallic receptacle for the same. Fig. 3 shows the instrument set up and ready for use, with the colorimeter tube on one side and a nephelometer tube on the other.

The shade surrounding the eye-piece is indispensable for accurate nephelometric work, and it has also proved useful in colorimetric estimations. By shutting out the glare of extraneous and conflicting lights and colors, it permits the use of both eyes in reading the instrument, which in a long series of experiments tends to lessen the usual eye-strain and fatigue.

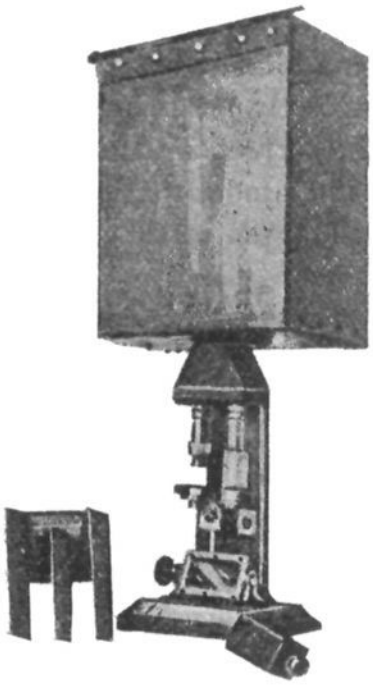

Fig. 2.

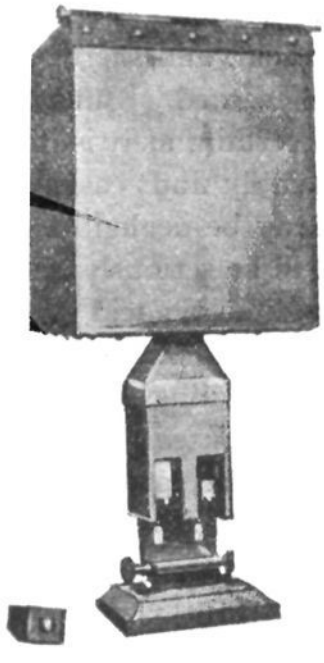

Fig. 3.

Although in other scientific work similar precautions are used, this seems to be the first application to colorimetric and nephelometric work. In working with optical instruments it is as essential to eliminate errors of vision as to eliminate errors of the instrument.

(*b*) *General Considerations in Regard to the Use of the Instrument.*—These may be found in the previous papers.[2]

(*c*) *Precipitants for Milk Proteins.*—The correct choice of precipitants for proteins in general has been a matter of great difficulty. Recent

[1] It is best to paint the plungers oneself, and also to have on hand a supply of paint so that the metallic parts can be repainted when necessary. To paint the plungers, it is best to tip them in asphaltum paint and allow them to dry. Then by means of a sharp knife, held at an angle, the circular end may be freed from paint. By painting the plungers in the evening, and drying over night, they are ready to clean and to use the next morning.

[2] THIS JOURNAL, **35**, 290 (1913). *J. Biol. Chem.*, **13**, 485 (1913).

work[1] has shown that the precipitates obtained by various salt solutions are not single substances, but mixtures and that a definit line of demarcation has not been found for them. Therefore, no definit statement can be made as to the nature of these fractions but to call attention to an easy method of estimating them accurately seems distinctly worth while. Not only can the suspensoids from the various salt solutions like NaCl, $MgCl_2$ and $(NH_4)_2SO_4$ be estimated, but other precipitants like the one recommended in this paper for casein, sulfosalicylic acid, can be employed. Heat coagulation and nuclein precipitation with the various nucleic acids can be used after the necessary standardizations are made.

The most common reagent for casein in milk has been dilute solutions of acetic acid, and there seems to be no doubt that it is specific as far as milk is concerned. But whether it will completely precipitate the protein is not so certain, as it is difficult to gauge the correct amount of acid, and one invariably finds casein in the filtrate. The amount of error produced by this may be negligible, but the estimation of the other proteins in the filtrate will be seriously affected.

Although acetic acid is apparently the only specific precipitant used for casein, many are known that throw down the greater portion of coagulable proteins, including casein, globulin and albumin. As a reagent of this kind we have used a 3% solution of sulfosalicylic acid which precipitates casein and other proteins quantitatively. Whether or not it precipitates all of the lacto-albumin is a question, as coagulable proteins appear even after using this reagent. The following figures indicate the amount of albumin and other proteins precipitated with various salt solutions. The milk was precipitated with acetic acid; in the diluted filtrates were found:

Source. (Dealer).	Half saturated magnesium sulfate. Per cent.	Saturated magnesium sulfate. Per cent.	Half saturated ammonium sulfate. Per cent.	Saturated ammonium sulfate. Per cent.	Albumin. Per cent.
Sheffield.....	0.056	0.089	0.177	0.112	0.088
McDermot.....	0.052	0.078	0.149	0.093	0.071
Borden.....	0.042	0.061	0.142	0.092	0.081

These figures were obtained by diluting a measured portion of the filtrate, from the official agricultural method, with an equal volume of water. To 10 cc. of this solution, 10 cc. of saturated magnesium sulfate solution were added, making it a half saturated solution which gave the values in column 1. To 10 cc. of the diluted filtrate 10 cc. of saturated ammonium sulfate solution were added, thus making a half saturated solution of ammonium sulfate, giving the results in column 3. By adding some finely divided salts in excess to diluted portions of the filtrate (after measuring

[1] Obermayer and Willheim. *Biochem. Z.*, **50**, 372 (1913), give data and literature on this subject.

the resulting volume) the saturated solutions were obtained which gave the results shown in columns 2 and 4.

As the tables show, some of the protein precipitated out by half saturated ammonium sulfate is redissolved on saturating the solution with the salt. The values given for albumin in the last column were obtained as the difference in the amount of protein obtained by saturated magnesium sulfate, which is supposed to precipitate the globulin, and that obtained by half saturated solution of ammonium sulfate. The latter gave the maximum precipitate and, therefore, probably contains most of the albumin.

(*d*) *The Estimation of Casein.*—The main problem, in applying the nephelometer to the estimation of proteins in milk, besides the finding of suitable precipitants, was to remove the fat, so that it would not produce any turbidity and, therefore, make a nephelometric error. Centrifuging, even after acidification, is not sufficient to remove the very fine suspensions of fat, but the following procedure gave very satisfactory results.

Five cc. of milk are carefully measured into a 250 cc. graduated flask and after adding 200 cc. of distilled water and 10 cc. of decinormal sodium hydroxide solution, water is added to the mark and the solution thoroughly shaken. Ten cc. are put with exactly two cc. of ether[1] in a centrifuge tube, which is then tightly stoppered with a cork and vigorously shaken. After allowing the mixture to stand until the layers have separated, or after centrifuging for one to two minutes, the cork is removed and 5 cc. of the aqueous layer withdrawn. This is done by closing the top of the pipet with the finger and inserting it quickly into the centrifuge tube. If this is done correctly the ether solution will not contaminate the sample. This 5 cc. aqueous layer is then diluted in a graduated flask to exactly 50 cc.

The milk treated in this way is quite clear (see page 1592) and ready for nephelometric estimation. Taking 10 cc. of this solution and adding 10 cc. of 3% sulfosalicylic acid, a suspension of casein is obtained which can be matched accurately with the following standard: One volume (5 cc.) of a 0.01% casein solution to which is added two volumes (10 cc.) of 3% sulfosalicylic acid.

The protein obtained with this reagent is not all casein, and in order to obtain the exact amount of casein, the following procedure is followed. The casein from a fresh portion of milk is precipitated according to the "official" method and the amount of precipitate obtained in an aliquot portion of filtrate, by adding four volumes of the reagent, is then determined nephelometrically. This fraction, for the want of a better name, we will call "globulin and albumin fraction" which, subtracted from the gross casein, gives the amount of casein precipitated by the "official" method.

[1] Previously washed with a 10% aqueous solution of sodium hydroxide.

As the ether used in extracting the fat, increases the volume of the solution, a factor allowing for this, must be used. For 10 cc. of diluted milk and 2 cc. of ether, this factor is 0.910 (see page 1591).

(*e*) *Results with the New Method.*—The standard given for the figures below was one volume of 0.00921% of casein solution with two volumes of sulfosalicylic acid (3%), which gave a 0.00307% standard. The height of "s" was in each case 14.8 mm. For the 5 cc. of milk taken for the "gross" casein determination the total volume of dilution was equivalent to 5,000 cc.

The filtrate from the precipitation with acetic acid was, for 10 cc. of milk, in each case 133 cc. The total dilution (with 20 cc. of reagent to 5 cc. of filtrate) was, therefore, 650 cc.

	"Gross" casein.			Globulin albumin frac.				
Source of milk (Dealer).	Mm. of "Y".	Ratio to stand. according to formula.	Casein volume. Per cent.	Mm. of "Y".	Ratio to stand. according to formula.	Amount of "globulin and albumin" volume. Per cent.	Net casein volume. Per cent.	"Official" agricultural method volume. Per cent.
June 3, 1913:				Not determined, assumed an average value				
Sheffield	16.9	0.841	2.84	of		0.17	2.67	2.56
	17.0	...	..			..	..	2.58
				Not determined, assumed an average value				
Borden	17.2	0.825	2.79	of		0.17	2.62	2.49
	17.1	...	..			..	..	2.54
				Not determined, assumed an average value				
McDermont	16.5	0.873	2.94	of		0.17	2.77	2.68
	16.6	...	..			..	..	2.60
June 4, 1913:								
Sheffield	17.1	0.842	2.84	17.0	0.841	0.17	2.67	2.58
	17.0	...	..	14.2	1.056	0.21	2.63	2.60
Green Lake Co.	18.3	0.763	2.58	18.8	0.734	0.15	2.43	2.39
	18.1	...	..	18.0	0.778	0.16	2.42	..
McDermot	17.2	0.825	2.79	17.0	0.841	0.17	2.62	2.62
	17.1	...	..	18.8	0.734	0.15	2.64	2.58
June 5, 1913:								
Sheffield	16.6	0.873	2.94	16.3	0.886	0.18	2.76	2.66
	16.4	...	..	16.3	0.886	0.18	2.76	2.76
Mutual	17.1	0.842	2.84	15.9	0.918	0.18	2.66	2.58
	16.9	...	..	17.2	0.825	0.16	2.68	2.57
Warwick	16.4	0.881	(2.98)	15.2	0.970	0.19	(2.79)	2.60
	16.5	...	..	..	...	..	..	2.57

As the results indicate, good agreement was found between the two methods. It should be noted, however, that no duplicate determinations were made on the "gross" casein and that only two readings for this estimation were made with the nephelometer, while duplicates were made with the "official method" and with the "globulin and albumin" fraction on the filtrates of these official estimations. The variations in this fraction are no doubt due to variations in the "official" method.

In removing fat from milk, hydrochloric acid cannot replace sodium hydroxide to dissolve the membrane from the fat globules, as free hydrochloric acid seems to interfere with the precipitation of casein by sulfosalicylic acid (see page 1592). Furthermore the residual turbidity with HCl is much greater than with NaOH. The former is appreciable, while the latter is inappreciable (see page 1592).

(*f*) *Standard Solutions.*—The method of preparing the standard solution of casein in these experiments was practically the same as stated in the previous paper.[1] For the purpose of estimating proteins, the standard must be controlled by a total nitrogen estimation, using the factor 6.38. 0.1000 gram of casein, or its equivalent, was dissolved with stirring in one cc. of 0.1 *N* NaOH; after adding about 95 cc. of distilled water and 2 cc. toluene and shaking thoroughly, the solution was made up to 100 cc. This served as a stock solution and kept unchanged for 3 or 4 days, and even longer. Ten cc. of this stock solution made up to 100 cc. with distilled water, fresh every day, served as a standard solution. In reference to the toluene, attention should be called to an oversight in previous papers. It was stated that the stock solutions should be "made up to a 100 cc." . . "after adding chloroform and shaking, the solution was ready for use." This, of course, may produce a volume change, due to the solubility of the chloroform or toluene, of 1 to 2%. This error, however, did not influence previous results since the volume change affected both the standard and unknown solution exactly the same way.

III. Experimental.

(*a*) *Change of Volume Due to Ether.*—Ten cc. of a 0.1% solution of sodium caseinate, made by dissolving 0.1000 gram of pure casein—in 1 cc. of *N* NaOH and making up to a volume of 100 cc.—were shaken in a graduated centrifuge tube with exactly 2 cc. of ether. The aqueous layer after centrifuging increased to 10.80 cc. in each case, which alone would indicate a factor of 0.925. Assuming the ether layer contains 5% of aqueous solution in suspension, it would make 0.006 less in the factor, or 0.919.

The agreement between observed factor 0.910 and that calculated approximately 0.919 is sufficiently close to show that the change in solution is due exclusively to the change in volume.

[1] THIS JOURNAL, **35**, 292 (1913).

Ether.	Standard "S." 3 per cent. sulfosalicylic acid. Cc.	Standard "S." 0.01 per cent. casein. Cc.	Unknown "Y." 3 per cent. sulfosalicylic acid. Cc.	Unknown "Y." 0.01 per cent. casein after treating with ether.	Mm. of "S."	Mm. of "Y."	Ratio according to formula.	Average.
Unwashed	10.0	5.0	10.0	5.0	14.8	15.9	91.4	..
Unwashed	10.0	5.0	10.0	5.0	14.8	16.0	90.7	..
Unwashed	10.0	5.0	10.0	5.0	14.8	16.1	89.7	90.6
Washed with 10% soln. of NaOH	10.0	5.0	10.0	5.0	14.8	15.9	91.4	..
Washed with 10% soln. of NaOH	10.0	5.0	10.0	5.0	14.8	16.0	90.7	91.1
Washed with H_2O	10.0	5.0	10.0	5.0	14.8	15.9	91.4	..
Washed with H_2O	10.0	5.0	10.0	5.0	14.8	15.9	91.4	91.4
							Average,	91.0

(*b*) *On the Influence of Hydrochloric Acid.*—The following table shows the influence of small amounts of hydrochloric acid and sodium hydroxide on the precipitation of sodium caseinate with 3% sulfosalicylic acid:

0.01 *N* HCl in 50 cc. 0.01 per cent. Na caseinate.	"Y."	Standard "S."	Ratio according to formula.	Average.
0.00	18.6	18.6	1.000	...
0.00	18.6	18.6	1.000	1.000
0.625	Agglutinated	18.6	...	...
1.25	21.4	18.6	0.750	...
1.25	20.9	18.6	0.774	...
1.25	22.1	18.6	0.716	...
1.25	19.6	18.6	0.842	...
5.00	20.5	18.6	0.792	...
5.00	20.1	18.6	0.815	0.781
0.01 *N* NaOH in 50 cc. 0.01 per cent. Na caseinate.	"Y."	Standard "S."	Ratio.	
0.00	14.8	14.8	1.000	...
0.00	14.9	14.8	0.994	0.997
5.00	14.9	14.8	0.994	...
10.00	14.9	14.8	0.994	...
10.00	14.8	14.8	1.000	...
10.00	14.9	14.8	0.994	0.996

This shows that even small amounts of hydrochloric acid interfere, but that five times more sodium hydroxide than is necessary for this method does not cause any deviations. At present I have no explanation to offer as to the cause of this phenomenon with hydrochloric acid.

(*c*) *Experiments on Removing Fat from the Milk.*—As to the efficiency of ether in clearing up the milk, after acidifying with 0.1 *N* HCl, the table below shows the amount of residual turbidity. The results apply to milk treated according to directions given with the exception that 10 cc. of 0.1 *N* HCl were substituted for the sodium hydroxide.

5 to 2500 milk solution with equal volume of 0.005 N HCl.	Mm. of solution.	Mm. of standard solution.	Concentration of standard solution.
Sheffield milk	28.2	14.0	0.0010
McDermot milk	31.5	14.0	0.0010

Repeated extraction with ether did not appreciably reduce the turbidity, while small amounts of acids cleared it up considerably. It, therefore, seems probable that the suspension is a protein of the nature of a globulin.

Using 10 cc. 0.1 N sodium hydroxide, as recommended in the directions, the residual turbidity is about ten times less than with the same amount of hydrochloric acid.

5 to 2500 milk.	Mm. of solution.	Mm. of standard solution.	Concentration of standard solution.
Sheffield	15.0	14.8	0.0001
Green Lake	18.3	14.8	0.0001
McDermot	16.5	14.8	0.0001

The amount of residual turbidity in actual work therefore, in not more than one part in 2,000,000, or an error of not more than 1.6%; but since this turbidity is about the same as that of the standard solution, the probabilities are that the greater portion of the *turbidity* using sodium hydroxide is due to *casein* and *therefore would produce no error.* It is of interest to compare this amount of residual turbidity with that of various potable waters.

Water with an equal volume of 3 per cent. sulfosalicylic acid.	Mm. of solution.	Mm. of standard solution.	Concentration of standard solution.
Croton water (surface)	15.2	27.0	0.0001
Westchester water (surface)	16.5	27.0	0.0001
Spring water	20.0	27.0	0.0001
Distilled water	29.0	13.5	0.0001

The last values on the various waters indicate the possibility of estimating proteins nephelometrically, in potable waters, directly.

IV. Summary.

1. A new method has been developed for the estimation of protein in milk which reduces the time required for such work from two or three days to twenty to thirty minutes.

2. An improved nephelometer is described. Directions and precautions for using the instrument are given.

3. It is shown that the results with the new method compare favorably with those obtained with "official" agricultural method.

4. The possibility of estimating proteins in potable waters, nephelometrically, is suggested.

My thanks are due to Mr. Walther Eberlein for the faithful and accurate assistance given throughout this work, for making and standardizing the necessary solutions, and for the casein estimations according to the "official" method.