XXIV. REFRACTOMETRIC METHODS FOR DETERMINING TOTAL PROTEIN

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Subsequent to establishing a refractometric method for determining the protein content of purified antitoxins [Siebenmann, 1933], a comparative study of refractometric and gravimetric protein determinations of horse serum and of plasma, as well as of protein solutions of unknown salt content, was carried out.

Two different refractometric methods were worked out. The first one is in principle an application of Reiss's refractometric determination of serum protein [1904; 1924] by means of one single refractometer reading. This method of Reiss has been modified for horse serum and is described hereunder (A) in form of a simple graphic method. Like the method of Reiss, this graphic method can be applied to native serum and plasma only, for which it gives satisfactory agreement with gravimetric methods, except in cases where the concentration of non-protein substances is abnormally high.

To serum dilutions or other protein solutions containing unknown amounts of salt, the graphic method cannot be applied. For protein determination in such solutions a method has been worked out in which the refractometric change occurring during heat coagulation is taken as a quantitative measure of the amount of protein present. This second method, which may be called the "differential method", is described under (B). This method further allows accurate determination of total protein in native sera.

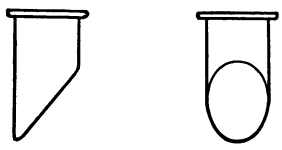

Fig. 1

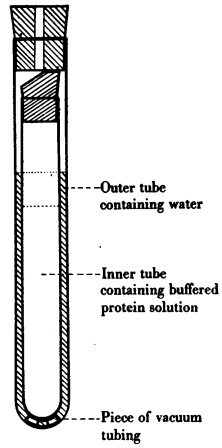

Fig. 2

Technique of refractometric measurements

All refractometer readings are carried out at 20° with a Zeiss Dipping Refractometer, using a circular water-bath (Zeiss Model A), containing 12 cylindrical refractometer cups. The constant temperature, essential for this

work, is obtained by providing the water-bath with a cold water supply from an insulated water reservoir[1] fixed on the wall above the refractometer table. An electric stirrer assures an even temperature throughout the refractometer bath. For a refractometric determination 3–4 ml. of serum are required. If less material is available, special glass cups (Fig. 1) are used which are adapted to the shape of the refractometer prism (No. I). Working with these cups, which are specially designed for serial work,[2] not more than 1 ml. of serum is required for a determination. The glass of these cups should be clear and care should be taken that during moulding no thickening of the wall occurs along the bottom edge.

Refractometric readings on serum are reproducible within less than 0·05 scale unit, corresponding to a maximum error of 0·00002 in the refractive index.

A. Graphic method

The refractometer readings of a number of horse sera and plasmata were determined and the Zeiss scale units plotted as abscissae against the corresponding protein percentages, determined by the acetone method (see gravimetric methods). The points obtained (Fig. 3) lie close to a straight line, drawn through the two points (marked as double circles) which correspond to the two horse sera with the highest and lowest protein content respectively (serum Nos. 628 and 451, Table II, col. 7).

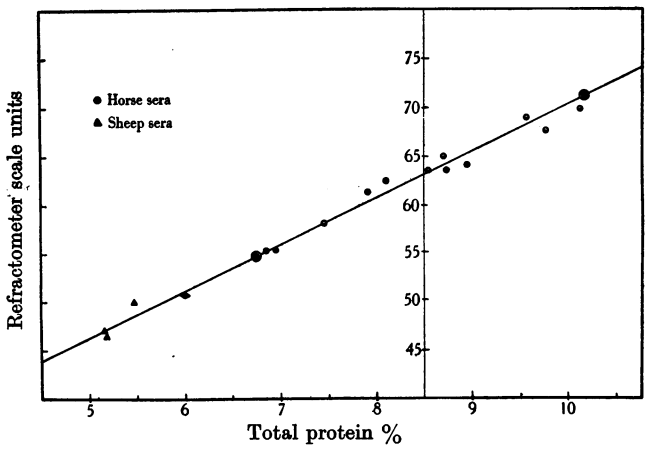

Fig. 3. Serum protein chart, 20°.

The protein percentages of horse sera and plasmata are read from this chart by using this straight line for converting graphically the refractometer scale units into protein percentages (Table II, cols. 3 and 4).

The same chart can be used for routine determination of protein in sheep serum (Table II).

B. Differential method

The refractometric determination of serum albumin and globulin, if carried out according to Robertson [1924], using the correct value for the constant a (0·00185), gives in the case of horse serum results which differ distinctly from

[1] For details see Siebenmann [1936].

[2] For single determinations the refractometer cup, Zeiss Model B, is suitable.

those obtained by gravimetric methods. The reason is that ammonium sulphate precipitation of globulin does not lend itself directly to quantitative refractometric evaluation, because of secondary physico-chemical processes which accompany the globulin precipitation. Our observations on this point are not reported here, as they agree essentially with those made by Deseo [1931, 2].

In the case of heat-coagulation of protein, however, the refractometric change taking place during the precipitation process can, in our experience, serve as an accurate measure of the amount of protein present.

Principle

Total protein is determined by measuring the refractometric difference between the two following solutions:

(1) Protein solution + acetate-acetic acid buffer (*p*H 4·6).

(2) Same as (1) but after heat-coagulation (100°).

For calculation of the protein percentage, the constant $a = 0{\cdot}00185$ [Siebenmann, 1933] was used, which represents the change in the refractive index of a horse protein solution due to a change in protein concentration by 1 %. Recently determined values of a for horse protein are: 0·00186 [Adair & Robinson, 1930], 0·00184 [Gerlaugh & White, 1931], 0·00187 [Deseo, 1931, 1] and 0·00185 [Siebenmann, 1933]. The last value was determined for globulin only. There is no distinct refractometric difference between normal and antitoxic horse globulin [Gerlaugh & White, 1931; Siebenmann, 1933]. The use of a correct value for the constant a is essential. A first attempt at a refractometric evaluation of heat-coagulation of protein [Homer, 1919] led to wide discrepancies between gravimetric and refractometric protein figures, because the constant a, used for calculation, was too high. In this differential method the Na acetate-acetic acid buffer (*p*H 4·6) replaces $N/25$ acetic acid, used by Robertson for refractometric determination of non-protein. Diluted acetic acid does not always lead to quantitative coagulation of serum protein. A similar change was introduced in the case of gravimetric methods.

Technique

(*a*) *Serum and plasma.* To 10 ml. of a Na acetate-acetic acid buffer 2 ml. of serum are added. On 4 ml. of this mixture the refractive index is determined. The rest is used for heat-coagulation, using a special double tube shown in Fig. 2, which allows heating of aqueous solutions at 100°, without evaporation. The size of the outer tube is 20 × 150 mm., that of the inner tube 15 × 125 mm. (pyrex No. 2370). The double tube is placed for 15 min. in boiling water. Then it is cooled and the inner tube centrifuged. Of the clear protein-free supernatant the refractive index is determined. Filtration through filter-paper can take the place of centrifuging if evaporation is carefully avoided.

The above amounts of serum and buffer can be reduced by half if the special refractometer cups (Fig. 1) are used.

Buffer contains 11·3 g. glacial acetic acid and 23·6 g. Na acetate per litre.

Calculation of protein content. The refractometer readings are converted into refractive indices and the protein percentage (c) calculated by means of the following equation:

$$c = \frac{n_1 - n_2}{a} . \text{dilution} \qquad \ldots\ldots(1),$$

in which n_1 is the refractive index before and n_2 the refractive index after heat-coagulation of the protein.

The conversion of the refractometer readings into refractive indices can be avoided by substituting equation (1) by equation

$$c = (R_1 - R_2) . k . \text{dilution} \qquad \ldots\ldots(2).$$

Here R_1 and R_2 are the scale readings before and after protein coagulation respectively. k is the amount of protein in % which on heat-coagulation leads to a drop in the refractometer reading of one scale unit (Zeiss Dipping Refractometer). This value k can be calculated from the refractometer tables (Reiss [1924] or Zeiss, Mess 165 G/XI) and the constant $a = 0{\cdot}00185$. The k values slightly decrease with increasing scale units. One scale unit indicates 0·207% horse protein in the range of 20–30 scale units and 0·205% protein between 30 and 40 scale units.

Example: Serum 628, dilution: 2 ml. serum plus 10 ml. acetate buffer.

$$R_1 = 27{\cdot}00 \ (n_1 = 1{\cdot}33781); \ R_2 = 21{\cdot}50 \ (n_2 = 1{\cdot}33570).$$
$$c = (R_1 - R_2) . k . \text{dilution} = 5{\cdot}50 \times 0{\cdot}207 \times 6 = 6{\cdot}83\ \% \text{ protein}.$$

The same result is obtained using equation (1).

(*b*) *Protein solutions other than serum and plasma.* This differential method can be extended to horse protein solutions, the salt content of which is not exactly known. The volume ratio of protein solution : buffer is so chosen that heat-coagulation of the buffered dilution leads to a drop in the refractometer reading of from 1 to 10 Zeiss scale units. In the case of solutions with low protein content (less than 1%) the *p*H adjustment is carried out using a minimum of acetate buffer (1 ml. per 10 ml. of protein solution). The buffer (*p*H 4·6) used for this purpose contains 56·6 g. glacial acetic acid and 118 g. Na acetate per litre. Table I contains the data concerning the volumes to be used. Here again, the volume of serum and buffer can be reduced if the special refractometer cups (Fig. 1) are used. In the case of solutions with low protein content (less than 1%) the heat-coagulated protein should always be separated by centrifuging and not by filtration.

Table I

Protein percentage range	Acetate buffer *p*H 4·6		Protein solution ml.	Dilution
4–12	10	+	2	6
1– 4	5	+	5	2
0·2– 1	1	+	10	1·1

Example: Plasma dilution (Tetanus horse 453) containing unknown amount of Na citrate. 5 ml. are diluted with 5 ml. of acetate buffer.

R_1 (before heat coagulation) = 26·52.
R_2 (after heat coagulation) = 24·00.
$c = (R_1 - R_2) . k . \text{dilution} = 2{\cdot}52 \times 0{\cdot}207 \times 2 = 1{\cdot}04\ \%$.

C. Gravimetric methods

The refractometric protein figures were compared with the results of gravimetric protein determinations, using the following two methods.

Heat-coagulation (at *p*H 4·6). From 2 to 5 ml. of serum or protein solution are diluted in a porcelain dish with acetate buffer (*p*H 4·6) so that the dilution contains not more than 1% protein (2 ml. serum + 18 ml. buffer). The covered dish is placed for 20 min. on the steam-bath. The protein precipitate is then

collected on a paper filter and is thoroughly washed with hot water. The filter-paper is then placed in a flat aluminium dish, dried for 16 hours at 100° and weighed (with lid), the weight of the empty filter plus aluminium dish having been determined previously under identical conditions.

Acetone method. The method of Bierry & Vivarro [1923] modified by Guillaumin *et al.* [1929] was used. Only those phases of the procedure are described which differ from the technique used by the above authors [see Peters & Van Slyke, 1932].

Table II. *Comparison of refractometric and gravimetric protein determinations of sera*

Serum or plasma*	No.	I. Graphic method: R in Zeiss units	I. Graphic method: Protein %	II. Differential method: $R_1 - R_2$† Zeiss units	II. Differential method: Protein %	Gravimetric methods (Protein %): Acetone method	Gravimetric methods (Protein %): Acetate buffer method
Horse sera							
Tetanus plasma	470	64·25	8·70	7·05	8·75	8·72	8·92
Normal plasma	635	58·05	7·48	6·05	7·51	7·46	7·28
Normal serum	628	54·70	6·74	5·50	6·83	6·74	6·83
Normal plasma	551	55·40	6·88	5·46	6·78	6·95	7·02
Normal serum	551	55·35	6·87	5·53	6·87	6·85	6·90
Tetanus serum	451	71·21	10·15	8·10	10·10	10·15	9·99
Sheep sera							
Sheep serum	1	46·90	5·26	4·18	5·21	5·19	5·00
Sheep plasma	1	47·45	5·15	4·19	5·22	5·17	5·17
Sheep serum	2	50·97	5·88	5·03	6·25	6·05	6·03
Sheep plasma	2	51·00	5·89	4·90	6·07	5·99	6·02
Sheep plasma	3	50·38	5·75	4·64	5·76	5·47	5·68

* 20 ml. blood + 0·1 ml. saturated K oxalate.
† Dilution: 2 ml. serum + 10 ml. acetate buffer.

Table III. *Protein determinations in solutions containing horse proteins and salt*

Protein solution	I. Differential method. pH 4·6 (Zeiss Dipping Refractometer) 20°: Protein solution + buffer ml.	Final dilution	$R_1 - R_2$ Zeiss units	c_I protein %	II. Gravimetric method. Heat coagulated, pH 4·6: c_{II} protein %	$c_I - c_{II}$
Tetanus serum No. 444:						
Undiluted	2 + 10	6	8·08	10·05	10·10, 10·10*	−0·05
Diluted 1 : 10	10 + 1	1·1	4·41	1·005	1·010	−0·005
,, 1 : 20	10 + 1	1·1	2·18	0·496	0·505	−0·009
,, 1 : 50	10 + 1	1·1	0·88	0·200	0·202	−0·002
,, 1 : 100	10 + 1	1·1	0·36	0·082	0·101	−0·019
Diluted horse plasma† No. 453:						
Bottle No. 1	2 + 10	6	5·54	6·87	6·98	−0·11
No. 7	5 + 5	2	13·35	5·52	5·54	−0·02
No. 12	5 + 5	2	2·52	1·04	0·98	+0·06
Horse albumin solution 0·80% NaCl	10 + 1	1·1	1·22	0·278	0·312, 0·282 } 0·297	−0·019
Horse protein solution containing $(NH_4)_2SO_4$ corresponding to 33% saturation	3 + 7	3·33	2·60	1·79	1·82	0·03

* Acetone method. † Containing Na citrate.

The heat-coagulation of the lipoid-free protein was carried out by means of acetate buffer (*p*H 4·6) (instead of diluted acetic acid), by heating the buffered protein solution for 20 min. in a covered porcelain dish on the steam-bath. Instead of using Gooch crucibles, the protein was collected, washed and weighed on filter-paper as described under "heat-coagulation". This technique gives sharply reproducible results. For serial work, the porcelain dishes, used for heat-coagulation of protein, can be substituted to advantage by wide test-tubes (25×100 mm. pyrex No. 2370).

Discussion

In Tables II and III the protein figures obtained with the refractometric and the gravimetric methods are tabulated for comparison.

(*a*) *Serum and plasma* (Table II)

All gravimetric figures are averages of two determinations agreeing within less than 3%. For sera and plasmata of horse and sheep there is no distinct difference between the two gravimetric methods (cols. 7 and 8). The lipoid contents of these sera are therefore too small to influence markedly the protein figures obtained by heat coagulation at *p*H 4·6 (col. 8). Both gravimetric methods agree closely with the refractometric protein figures arrived at by the differential method. This good agreement confirms the correctness of the numerical value of the constant $a=0{\cdot}00185$ for horse serum protein and corroborates the finding of Gerlaugh & White [1931] that albumin and globulin have the same refractive properties.

The protein figures for sera, arrived at by means of the differential method, are sharply reproducible. For a definite serum the (R_1-R_2) values of single determinations agree within less than 0·05 scale unit. This corresponds to an experimental error of less than 1% of the protein figure.

The satisfactory agreement existing between the protein percentages, determined graphically by means of one single refractometric reading (Table II, cols. 3 and 4), and the gravimetric protein figures justifies the use of the graphic method for routine purposes. The basis of this method is purely empirical in contrast to the graphic method introduced for refractometric protein determinations of purified antitoxins [Siebenmann, 1933].

The average variation between the gravimetric and the refractometric (graphic) protein figures, calculated for 15 horse sera and plasmata, is less than ±2%; the maximum variations observed are ±3%.

A simple refractometric (graphic) method can be applied for determining total solids of sera by means of a single refractometer reading.

The differential refractometric method for determining protein of sera offers the advantage over the graphic method that it gives protein figures which are not influenced by the amount of non-protein substances present.

(*b*) *Serum dilutions and other protein solutions*

For protein determinations of serum and plasma dilutions as well as of other horse protein solutions, the salt content of which is not exactly known, the differential method is the only refractometric method available. Representative results obtained by this method are shown in Table III.

Of a horse serum (Tetanus No. 444) with a known protein content (col. 6) a dilution series was prepared (Table III, col. 1). Of these serum dilutions the protein was determined by means of the differential method. The results (col. 5) are compared with the gravimetric protein figures calculated from the dilutions

(col. 6). There is good agreement between the refractometric results and the calculated protein figures down to a concentration of 0·2% protein. For still lower protein concentrations the differential method gives too low results. It seems that at such low protein concentrations heat-coagulation is not a quantitative process.

The differential method has proved of value for protein determination in citrated plasma, obtained at the end-bleeding of antitoxin horses by means of the perfusion method (see Table III, Plasma 453).

The application of the differential method for protein determinations in presence of ammonium sulphate (using the constant $a=0{\cdot}00185$) is limited to solutions containing not more than 18% of this salt (corresponding to 1/3 saturation). For horse protein solutions containing more ammonium sulphate, the change in the refractive properties of the horse serum protein, due to the presence of large amounts of salt, has to be taken into account. This point requires further investigation as it is of special interest for the application of refractometric methods in the quantitative study of antitoxin distribution in horse sera.

The application of the differential method for determination of total protein of human serum is under investigation.

Summary

A rapid and accurate refractometric method for determining horse serum protein is described, in which the refractometric change taking place during heat-coagulation (*p*H 4·6) serves as a quantitative measure of the amount of protein in solution.

This method has so far been used for total protein determination of horse serum, plasma and horse protein solutions, the salt content of which is not exactly known. For solutions containing from 0·2 to 10% protein the results obtained are in close agreement with gravimetric protein figures. For calculation the constant $a=0{\cdot}00185$ for horse protein is used.

A simple pressure device is described for carrying out heat-coagulation at 100°, avoiding evaporation of water.

Reiss's method for determining serum protein by means of a single refractometer reading has been worked out for horse sera in the form of a graphic method. As an approximate method, it gives for routine purposes satisfactory agreement with gravimetric methods.

References

Adair & Robinson (1930). *Biochem. J.* **24**, 993.
Bierry & Vivarro (1923). *C.R. Soc. Biol., Paris*, **89**, 13.
Deseo (1931, 1). *Biochem. Z.* **230**, 373.
—— (1931, 2). *Biochem. Z.* **239**, 329.
Gerlaugh & White (1931). *J. phys. Chem.* **35**, 1328.
Guillaumin, Wahl & Laurencin (1929). *Bull. Soc. Chim. biol., Paris*, p. 387.
Homer (1919). *J. Soc. chem. Ind., Lond.*, **38**, 145 T.
Peters & Van Slyke (1932). Quantitative Clinical Chemistry. (London: Baillière, Tyndall and Co.)
Reiss (1904). *Arch. exp. Path. Pharmak.* **51**, 18–29.
—— (1924). In Abderhalden, *Hdb. d. Biol. Arbeitsmeth.* Abt. 4, T. 3.
Robertson (1924). Physical Chemistry of Proteins.
Siebenmann (1933). *Biochem. J.* **27**, 1745.
—— (1936). *J. Immunol.* **31**, 257.