CCXXXV. REFRACTOMETRIC EXAMINATION OF PURIFIED ANTITOXINS.

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Purified diphtheria and tetanus antitoxins, prepared from antitoxic horse sera by isolating the water-soluble globulins, are purer than the original sera in so far as they contain more antitoxin per g. of protein. Their higher degree of purity however does not exclude the fact that for a definite volume they often contain more protein than the original sera from which they were prepared. As the injection of concentrated protein solutions is not desirable, there are regulations fixing the maximum protein or total solid content of such purified antitoxins. For institutions concerned with the production of such antitoxins, the determination of total solids or total protein is therefore a matter of daily routine.

The gravimetric methods for determining total solids and protein, and the Kjeldahl method, are well established but rather lengthy procedures. The nephelometric method for determining proteins [Kober, 1917] is quicker, but it seems not easy to obtain reproducible results. The present paper deals with the refractometric determination of the globulin content of concentrated antitoxins.

The refractometric determination of serum-protein by Reiss [1913; 1915; 1924] differs from the method described here in that in natural sera the concentration of non-protein substances is unknown, and may in exceptional cases show great variations, so that from the refractive index alone the protein concentration can only be determined approximately. However, antitoxins, purified by a modification of Banzhaf's method of salt precipitation and heating, represent pseudoglobulin solutions free from albumin and non-protein fractions and containing known amounts of salt and preservative. The protein content of such solutions can be determined by a single refractometric reading.

Basis of the refractometric globulin determination.

First we have to prove that a linear relationship between changes in the refractive index and changes in the protein concentration, as observed by Reiss [1903] and Robertson [1909] for a number of proteins, exists also for solutions of antitoxic horse globulin.

From a solution of pseudoglobulin containing 1600 units of diphtheria antitoxin per cc., and freed from salts by prolonged dialysis against distilled water, a series of dilutions was prepared so that each dilution contained exactly 0·80 % NaCl and 0·38 % tricresol.

Table I records the globulin content of the undiluted antitoxin, the globulin content of each dilution (c) as well as the refractive index (n) determined by means of a Zeiss dipping refractometer, at 17·5°.

Table I. *Diphtheria antitoxin 512 C.*

Before dilution 1 cc. contains 1600 antitoxin units and 24·68 % globulin (by weight).
All solutions contain 0·80 % NaCl + 0·38 % tricresol.

Globulin content C* %	Refractive index n	Change of n per 1 % globulin a
6·17	1·34675	0·00185
4·63	1·34391	0·00186
4·11	1·34300	0·00187
3·08	1·34101	0·00185
1·54	1·33810	0·00181
	Average	0·00185

n' = refractive index of NaCl 0·80 % + 0·38 % tricresol = 1·33531.

* Calculated from dilutions.

If for such solutions, the relation between refractive index and globulin concentration is linear, then the following equation must hold true:

$$n = n' + ac \qquad \ldots\ldots(1),$$

where n is the refractive index of the solution, n' a constant, being the refractive index of the solvent (solution of 0·80 % NaCl + 0·38 % tricresol) and c the globulin concentration. The value a represents the supposedly constant change in the refractive index (n) due to the presence of 1 % horse pseudoglobulin.

In Table I, col. 3, are recorded the values of $a = \frac{n - n'}{c}$ calculated from equation (1). These represent the changes in the refractive index caused by the presence of 1 % globulin.

Table II shows the results of a similar series of experiments, in which the solutions examined were prepared from the same antitoxin, but contained no salt or tricresol. Here, too, the values of a show a high degree of constancy and are practically identical with those recorded in Table I. The presence in solution of 0·38 % tricresol and 0·80 % NaCl, seems, consequently, not to change the refractive properties of pseudoglobulin.

Table II. *Diphtheria antitoxin 512 C.*

Before dilution 1 cc. contains 1600 antitoxin units and 24·68 % globulin (by weight).
All dilutions are free from salt and tricresol.

Globulin content C* %	Refractive index n	Change of n per 1 % globulin a
8·23	1·34834	0·00184
6·18	1·34688	0·00187
4·63	1·34389	0·00186
3·71	1·34215	0·00184
1·85	1·33877	0·00185
	Average	0·00185

n' = refractive index of distilled water = 1·33320.

* Calculated from dilutions.

The same values of a were obtained by examining diluted antitoxin solutions prepared from another diphtheria antitoxin containing 1800 units per cc. (Table III).

Table III. *Diphtheria antitoxin 512 A.*

Before dilution 1 cc. contains 1800 antitoxin units and 18·55 % globulin (by weight).
All dilutions contain 0·80 % NaCl + 0·38 % tricresol.

Globulin content C* %	Refractive index n	Change of n per 1 % globulin a
9·27	1·35258	0·00186
6·18	1·34688	0·00187
4·63	1·34389	0·00186
3·71	1·34215	0·00184
1·85	1·33877	0·00185
	Average	0·00186

$n' = 1{\cdot}33531$.

* Calculated from dilutions.

Finally we had to show that antitoxic globulin does not differ from normal globulin with regard to refractive properties. Hurwitz and Meyer [1916] and later Meyer, Hurwitz and Taussig [1918], made refractometric globulin determinations on antitoxic horse sera, using a salt precipitation method outlined by Robertson [1915]. They apparently assumed that the refractive properties of antitoxic globulin are identical with those of normal globulin, without showing experimental proof. From normal horse plasma, pseudoglobulin was separated in exactly the same way as in the preparation of diphtheria antitoxin. From the salt-free dialysed globulin solution dilutions were made and examined by the refractometer. The results, recorded in Table IV, show that the change in

Table IV. *Solutions of normal horse globulin.*

Before dilution 1 cc. contains 14·54 % globulin (by weight).
All dilutions contain 0·80 % NaCl + 0·38 % tricresol.

Globulin content %	Refractive index n	Change of n per 1 % globulin a
3·63	1·34203	0·00185
2·42	1·33981	0·00186
1·81	1·33871	0·00187
0·45	1·33613	0·00181
	Average	0·00185

$n' = 1{\cdot}33531$.

the refractive index of a normal globulin solution due to the addition of 1 % globulin (col. 3) is exactly the same as for solutions containing antitoxic globulin.

This value ($a = 0{\cdot}00185$) is considerably lower than the corresponding value for ox serum-globulin ($a = 0{\cdot}00229$) used by Robertson [1915] but is practically identical with $a = 0{\cdot}00184$ determined by Reiss [1924] for human serum-protein and with $a = 0{\cdot}00186$ calculated by Adair and Robinson [1930] for horse globulin.

For a purified diphtheria antitoxin containing a known amount of salt and tricresol, the globulin content can consequently be determined by a single refractometric reading. From the refractive index the globulin content is calculated according to equation (1).

Technique.

Using the attachment No. 1 of the Zeiss dipping refractometer, it is possible to determine refractive indices in the range from 1·325 to 1·367. As the refractive indices of purified antitoxins are generally considerably higher, the

concentrated antitoxins must first be diluted (1:4) in order to make the determination possible.

Example: Globulin determination of diphtheria antitoxin 513 A. Exactly 6 cc. of salt solution "A" containing 0·80 % NaCl + 0·38 % tricresol are placed in a test-tube and 2 cc. of antitoxin measured with an Ostwald-Folin pipette (to contain) are added. The pipette used for the antitoxin should be rinsed out several times with the diluted antitoxin solution. This dilution (1:4) gives in the refractometer at 17·5°, a reading of 42·55, corresponding to a refractive index of 1·34371[1].

From the value (n) and the refractive index of the salt solution ($n' = 1{\cdot}33531$) $c = \frac{n-n'}{a} = \frac{1{\cdot}34371 - 1{\cdot}33531}{0{\cdot}00185} = 4{\cdot}55$ % globulin. Taking into account the dilution (1:4) of the antitoxin, the globulin content of the undiluted antitoxin is $4 \times 4{\cdot}55\ \% = 18{\cdot}20\ \%$.

Globulin determination by means of a chart.

Instead of calculating the globulin content in the way described, it is simpler to read the results directly from a chart. Such a chart (Fig. 1) is worked out

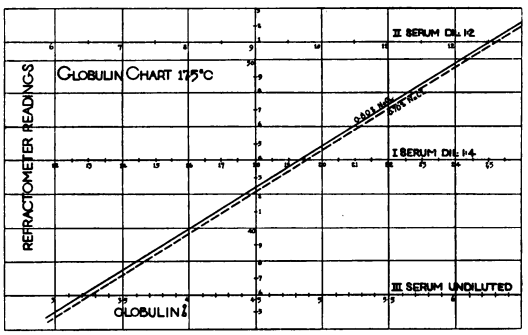

Fig. 1. Ordinates: refractometer scale readings at 17·5°. Abscissae: globulin percentages of the undiluted antitoxin. Abscissae I, for antitoxin examined at dilution 1:4. Abscissae II, for antitoxin examined at dilution 1:2. Abscissae III, for antitoxin examined undiluted.

——— Solutions containing 0·80 % NaCl + 0·38 % tricresol.
– – – – Solutions containing 0·70 % NaCl + 0·38 % tricresol.

on the basis of equation (1), using for the constant a the value 0·00185. The globulin percentages are recorded as abscissae, the refractometer readings as ordinates. Using this chart, it is not necessary to convert the refractometer readings into refractive indices. The chart records directly the scale readings of the Zeiss refractometer obtained by examining the antitoxins at the dilution 1:4[2]. These direct readings represent points all lying on a straight line. The fixed points of this chart calculated from equation 1 are tabulated in Table V.

For the determination of globulins with the technique described, it is necessary that the purified antitoxins contain exactly 0·8 % NaCl + 0·38 % tricresol, but the method may be worked out for any definite salt or tricresol concentration by introducing into equation (1) corresponding values of n'. For antitoxins containing *e.g.* 0·7 % NaCl instead of 0·8 %, n' becomes 1·33519 (20·16) and

[1] Using the conversion table of Reiss [1924].

[2] Antitoxins with less than 12 % total solids should be examined undiluted or diluted 1:2. Corresponding abscissae are entered in Fig. 1.

Table V.

Fixed points of Fig. 1.
Calculated for antitoxins containing 0·80 % NaCl + 0·38 % tricresol.

Globulin % of undiluted antitoxin	n Calculated for dilution 1:4	Corresponding refractometer reading (17·5°)
12	1·34086	35·00
18	1·34363	42·35
20	1·34456	44·81
24	1·34641	49·71
26	1·34733	52·25

Example. Globulin determination of diphtheria antitoxin 512 A. Dilution 1:4 with salt solution A. Refractometer reading at 17·5° = 42·55 (n = 1·34371) indicating on Fig. 1, 18·17 % globulin (against 18·20 % by calculation).

the refractometric readings lie according to equation (1) on a straight line which runs parallel with the 0·8 % line (Fig. 1).

For research work it is often desirable to determine the globulin content of freshly dialysed, salt-free antitoxins. For such cases the same chart can be used, but the dilution (1:4) has to be carried out in the following way. Instead of using saline "A" a special saline "B" containing 1·07 % NaCl and 0·506 % tricresol is needed, bringing the final concentration to 0·8 % NaCl and 0·38 % tricresol. Then the determination can be carried out by means of Fig. 1.

This dilution technique has the advantage over refractometric examination of salt-free globulin solutions in that it applies not only to pseudoglobulin solutions, but also to mixtures of pseudoglobulin and euglobulin, the latter being brought into solution by the saline used for dilution.

Conversion of globulin percentages into total solid figures.

The technique of determining total solids by weight, carried out in connection with this work, is as follows.

In a wide weighing bottle with ground glass stopper, 6 cc. of distilled water are placed. To this exactly 2 cc. of antitoxin are added, using an Ostwald pipette (to contain), the pipette being washed out at least 6 times with the diluted antitoxin. The pipette is finally filled with distilled water and this is added to the diluted antitoxin. The bottle is first heated for 2 hours at 90° and then at 100–105° until its weight becomes constant. By using this technique the total solids can be determined with a maximum error of 0·20 %.

Comparisons of total solid determinations on antitoxins free from tricresol with determinations on the same products, but containing 0·38 % tricresol, show that during the heating all tricresol is volatilised. For converting the globulin content, determined with the refractometer, into total solid figures, we have consequently to add only 0·80 % in weight, taking into account the salt content of the solutions. For salt-free antitoxins, the globulin figures should, within the limits of experimental error, be identical with the total solid figures determined by weight. The mineral content of the tap water, used for dialysis, has no influence on the results, if its total solid content is below 0·1 %, as in our case. This possible cause of error has however to be checked for each place where such determinations are carried out.

Temperature coefficient of the refractometer readings.

As the refractive index of any solution varies greatly with temperature, it is necessary to carry out refractometric measurements at a constant temperature.

In order to be able to carry out our determinations at any given room temperature, the temperature coefficient (K) of the refractometer readings (*i.e.* the change of the scale reading due to a temperature change of 1°) was determined. For the temperature range 17–25°, K was found to be 0·260 for an antitoxin[1] containing 19·20 % total solids and 0·267 for an antitoxin[1] with 25·38 % total solids, while for distilled water K is lower (0·236). K seems consequently not to be independent of the concentration. For the type of globulin solutions, however, which are in question, the coefficient K = 0·26 is sufficiently accurate.

Using this temperature coefficient the globulin contents and total solids of a number of antitoxins were determined by the refractometer at room temperature[2]. The results obtained (Table VI, col. 6) are compared with determina-

Table VI. *Determinations at room temperature.*

Antitoxin* Seitz-filtered	Temp. ° C.	Scale reading	Temp. corrections (K = 0·26)	Reading calculated for 17·5°	Total solid (Fig. 1)	Total solid† determined at 17·5°	Total solid† by weight
Erysipelas:							
2 A	24·30	40·62	+1·78	=42·40	18·85	18·77	18·79
3 A	24·20	40·97	+1·75	=42·72	19·11	19·10	18·94
Tetanus:							
60 A	24·00	41·87	+1·70	=43·57	19·77	19·77	19·85
B	24·10	41·99	+1·73	=43·72	19·90	19·90	19·79

* Containing 0·80 % NaCl + 0·38 % tricresol. † Compare Table VII B.

tions done on the same material but carried out at exactly 17·5° (col. 7). The measurements carried out at room temperature yield total solid figures which show satisfactory agreement with the results calculated from the readings obtained at 17·5° and lie very close to the total solid figures determined by weight.

For carrying out the refractometric determinations in the way just described, temperatures above 25° should be avoided and care should be taken that the single determination does not take more than 10 minutes because of possible evaporation of the solutions.

Discussion.

Refractometric globulin determinations (17·5°), using Fig. 1, were carried out on a number of diphtheria, tetanus and scarlet fever antitoxins. The results are shown in Tables VII A and VII B.

The total solid figures in col. 4 are calculated from the refractometric globulin values (col. 3), taking into account the salt content of the antitoxins (see p. 1749). These calculated figures based on the refractometric method show a satisfactory agreement with the total solid figures obtained by weight (col. 5). Of interest is that whatever antitoxin these solutions of horse globulin contain, their refractive properties do not differ from those of normal horse globulin.

The possibility of the turbidity giving rise to error was studied in the case of diphtheria antitoxin (Table VII A). The turbidity (col. 7) of the undiluted bag material, was determined by comparison with turbidity standard suspensions containing definite amounts of fuller's earth (expressed in parts per 1 million parts of the solution). The total solid figures (col. 4) based on the

[1] Containing 0·80 % NaCl + 0·38 % tricresol, dilution 1:4 with saline A.

[2] Using the water-bath (No. 418111 Zeiss Catalogue on Dipping Refractometer) which was used for all other experiments but without the constant water level (No. 418101 and No. 418133) designed for maintaining the bath temperature at 17·5°.

Table VII A. *Refractometric globulin determination by means of Fig. 1 (17·5°).*

Product*	Refr. reading at 17·5° (dilution 1:4)	Globulin (refr.) %	Total solid (refr.) %	Total solid (by weight) %	Difference†	Turbidity
Diph. ant. Seitz-filtered:						
507,2 B	41·43	17·23	18·03	17·83	+0·20	<100
509 A	41·82	17·53	18·33	18·35	−0·02	<100
510 A	42·98	18·52	19·32	19·34	−0·02	—
511 A	42·73	18·30	19·10	19·21	−0·11	<100
512 A	42·93	18·45	19·25	19·08	+0·18	<100
513 A	42·55	18·17	18·97	18·84	+0·13	<100
513 B	42·75	18·30	19·10	18·90	+0·20	<100
514 A	43·41	18·83	19·63	19·52	+0·11	<100
Bag material:						
509	51·55	25·42	26·22	26·41	−0·19	600
515	51·60	25·45	26·25	26·37	−0·12	500
516	50·61	24·58	25·38	25·38	0	500
517	49·55	23·82	24·62	24·83	−0·21	300

* All products examined contain 0·80 % NaCl + 0·38 % tricresol unless otherwise stated.
† Total solids by refractometer *minus* total solids by weight.

Table VII B.

Product*	Refr. reading at 17·5° (dilution 1:4)	Globulin (refr.) %	Total solid (refr.) %	Total solid (by weight) %	Difference
Tetanus ant. Seitz-filtered:					
58 A	42·92	18·47	19·27	19·18	+0·09
58 B	42·74	18·30	19·10	19·31	−0·21
56 A	43·15	18·65	19·45	19·12	+0·33
Repeat	43·00	18·52	19·32	19·29	+0·03
57 A	43·20	18·68	19·48	19·31	+0·17
60 A	43·58	18·97	19·77	19·85	+0·08
60 B	43·75	19·10	19·90	19·79	+0·11
Scarlet fever ant. Seitz-filtered:					
116 A	40·26	16·31	17·11	16·86	+0·25
112 A	42·37	18·02	18·82	19·00	−0·18
Repeat	42·46	18·08	18·88	(19·00)	−0·12
120 A	39·45	15·62	16·42	16·61	−0·19
123 A	40·30	16·35	17·15	16·91	+0·24
121 A	39·75	15·87	16·67	18·81	−0·14
Bag material:					
†124 I	46·67	21·47	21·47	21·37	+0·10
Repeat	46·70	21·53	21·53	(21·37)	+0·16
†124 II	48·52	22·98	22·98	22·96	+0·02
Erysipelas ant. Seitz-filtered:					
2 A	42·30	17·97	18·77	18·79	−0·02
3 A	42·78	18·30	19·10	18·94	+0·16

* All products contain 0·80 % NaCl + 0·38 % tricresol unless otherwise stated.
† Products free from salt and tricresol.

refractometric globulin determinations, are, on the average, slightly lower than the total solid figures determined by weight. However, the differences (max. = −0·21 %) lie within the limits of experimental error encountered with the total solid determinations by weight. The refractometric globulin determinations as such show a high degree of accuracy (see repeats in Table VII B). Using calibrated pipettes for preparing the antitoxin dilutions, the refractometric readings are reproducible within less than 0·2 division of the refractometer scale corresponding to a variation of less than 0·2 % in the globulin figure (see Fig. 1).

SUMMARY.

1. The refractive properties of globulin solutions containing diphtheria antitoxin were investigated. No distinct differences were found between these and the refractive properties of normal horse globulin solutions. These findings were confirmed for purified tetanus, scarlet fever and erysipelas antitoxins.

2. A refractometric method for routine determination of globulins and total solids of purified antitoxins by a single refractometer reading is described. From this refractometer reading the globulin concentration is obtained either by calculation or by using a reference chart.

Instead of carrying out the determinations at 17·5°, the readings can be made at any definite room temperature (17–25°) taking into account the temperature coefficient of the refractometric readings.

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