28. The Estimation of the Plasma Proteins by the Salicylsulphonic Acid Reaction

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During recent years the increased use of intravenous therapy has led to a development of laboratory control of the more important inorganic constituents of the plasma. It has been customary in this hospital to perform serial estimations of plasma chlorides and alkali reserve in conjunction with frequent determinations of the blood urea when patients have been receiving saline infusions, particularly if duodenal suction, vomiting or copious sweating seemed likely to add to the difficulty of ensuring a correct salt balance. Estimations of the plasma proteins were made from time to time by the micro-Kjeldahl method of King *et al.* [1937], but they were not done as a routine, as the method was not rapid enough to give results at the rate demanded by the large number of samples received. It became evident, however, that a high proportion of the patients had plasma proteins of the order of 4 %, below which concentration oedema becomes a serious complication.

Trials were made of several rapid methods for the estimation of plasma proteins and the measurement of the 'optical density' of the cloud produced by the addition of salicylsulphonic acid to a saline dilution of plasma was selected for investigation. Serious objections raised by Plötner [1936, 1] against nephelometric methods based on the use of salicylsulphonic acid have not been found to be applicable to the method adopted, which, though it does not give results comparable in accuracy with the Kjeldahl procedure, is sufficient for clinical purposes and may be applied to serum, plasma, cerebrospinal fluid and urine.

EXPERIMENTAL

Standardization of protein solutions. The protein solutions used in this investigation have all been standardized by a slight modification of the micro-Kjeldahl method of King *et al.* [1937]. It had been found that the porous pot used for the prevention of bumping often formed a fine cloud upon which the Nessler colour was adsorbed. In order to avoid this source of error the digest, after dilution with water, was centrifuged and an aliquot taken for nesslerization. Owing to difficulty in obtaining nitrogen-free reagents, reference curves were constructed for total proteins and non-protein nitrogen by taking nitrogen standards through the complete series of operations.

Apparatus. The 'optical density' of the cloud produced by addition of salicylsulphonic acid to a protein solution was measured in a Klett colorimeter by the photometric method of King *et al.* [1937]. The standard was an Ilford neutral grey screen, and an orange Ilford 'Spectrum' filter was used on the eyepiece of the colorimeter. With the standard screen on the left-hand rack of the colorimeter, the suspension was placed in the micro-cup on the right and the depth of the suspension adjusted until the brightness of the two fields seen in the eyepiece appeared equal. Then

$$\text{'Optical density'} = D_{\text{or.}} = \frac{\text{Density of standard screen}}{\text{Depth (cm.) of suspension}}.$$

The illumination of both sides of the instrument must be equal and the right-hand scale reading must be exactly zero when the cup is racked up into contact with the plunger.

Absorption curve of cloudy suspension. 3 ml. 3 % salicylsulphonic acid solution was added to 1 ml. of a 1/100 dilution of plasma in normal physiological saline (0.85 % NaCl).

The mixture was shaken and allowed to stand for 10 min. The 'optical density' of the cloudy suspension was determined in the colorimeter, using each of the 8 'Spectrum' filters on the eyepiece. The results are shown in Fig. 1, curve 1. Mr S. Y. Thompson, of

Fig. 1. Absorption curves for salicylsulphonic acid-protein suspensions. Curve 1, using colorimeter and Ilford 'Spectrum' filters; curve 2, using photoelectric spectrophotometer.

Fig. 2.

Fig. 3.

Fig. 2. Effect of time on optical density of salicylsulphonic acid-protein suspensions. Curves 1 and 2, plasma albumin; curves 3 and 4, total proteins.

Fig. 3. Influence of temperature on optical density of salicylsulphonic acid-protein suspensions. Time between addition of salicylsulphonic acid and reading of density: circles, 10 min.; crosses, 70 min.

the National Institute for Research in Dairying, Shinfield, kindly determined the extinction coefficients of a similar suspension, using monochromatic light of various wavelengths, in his photoelectric spectrophotometer (Fig. 1, curve 2). The two curves follow one another closely and show a minimum absorption at the red end of the spectrum. As it was desired to eliminate the effect of colour as far as possible, an orange filter was

selected for use on the eyepiece. This colour was preferred to red, as it was found to be less tiring to the eyes when a long series of readings was necessary.

Influence of time. The effect of time on the optical density of the suspension will be seen in Fig. 2. When a 1/100 dilution of plasma in 'normal' saline was used ('Total proteins') the maximum density was attained in 5 min., after which it remained constant. Sedimentation was slow, and though a deposit was present after 24 hr., reconstitution of the suspension by shaking gave a cloud of the original density. This confirms the observation of Plötner [1936, 2].

For the estimation of albumin the Na_2SO_4 filtrate of Howe [1921] was used. 3 ml. 3% salicylsulphonic acid were added to 0.5 ml. Howe filtrate diluted with 0.5 ml. water. The development of the cloud was slower, but the density remained constant after 30 min. Sedimentation was more rapid, but again reconstitution by shaking was effective.

Influence of temperature. Between 30° and 40° the density attained in 10 min. was independent of the temperature (Fig. 3), but at lower temperatures it was considerably reduced. This was not due to failure to attain maximum density within the time given, as the same result was obtained if the development of the suspension was allowed to proceed for 70 min. The best temperature to select for the reaction would be 38°, but this might introduce certain inconveniences. Laboratory temperatures in England usually range between 17° and 25°, and it will be seen from Fig. 3 that a rise in temperature from 17° to 25° leads to an increase in optical density of about 9%. It was considered that for most purposes the use of 'laboratory temperature' would be sufficiently accurate, for, if necessary, a correction could be applied from the curve.

Order of addition of reagents. When salicylsulphonic acid was added to a saline dilution of plasma the optical density of the suspension was consistently somewhat higher than when the protein solution was added to the acid. In the case of Howe's albumin filtrate no such consistent difference was observed.

Size of tubes. The reaction was carried out in a series of 10 tubes varying in internal diameter from 2.39 to 0.66 cm. To ensure mixing, the two narrowest tubes (0.80 and 0.66 cm.) had to be reversed twice, while the others were shaken. The same optical density was obtained in all tubes except the two narrowest, in which the results were 8% higher.

Influence of neutral salts. The presence of 5% NaCl, Na_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$ had relatively little effect on the optical density of the suspension, but at higher concentrations a significant reduction occurred. A protein solution giving $D_{\text{or.}}$ of 0.370 in normal saline solution gave the following optical densities when salts were added:

	Added salt (%)		
	5	8	11
NaCl	0.361	0.349	0.335
Na_2SO_4	0.349	0.323	0.308
$(\text{NH}_4)_2\text{SO}_4$	0.341	0.336	0.314

It will be seen from Fig. 4, however, that the cloud produced by globulin is increased by the presence of 11% Na_2SO_4 .

Effect of abnormal albumin/globulin ratio. Since it is not possible to rely upon receiving many samples of plasma having abnormal albumin/globulin ratios in the ordinary course of laboratory practice, a series of mixtures was prepared from several different specimens of plasma or Howe albumin filtrate, to which globulin was added. In this way specimens were obtained with albumin/globulin ratios ranging from 0.16 to 21.6.

The globulin was precipitated from 4 ml. plasma or serum by diluting to 100 ml. with 22.2% Na_2SO_4 . The mixture was kept overnight at 38° and filtered through a 3 G4 Jena sintered glass filter. After being thoroughly drained, the globulin was scraped off the filter and dissolved in 20 ml. normal saline at 38°. The solution was kept at this temperature for 4 hr. and then overnight in the refrigerator, after which it was passed repeatedly

through a 3 G4 filter. A typical product contained 300 mg./100 ml. globulin, 6.0 mg./100 ml. non-protein nitrogen (both by Kjeldahl), and a trace of sulphate.

On addition of salicylsulphonic acid to globulin the density of the suspension was of the same order of magnitude as that obtained with diluted plasma or albumin filtrate, though the straight-line relationship no longer held when the globulin concentration was below 0.1 mg./ml. (Fig. 4). Owing to this fact, addition of very small amounts of globulin in an

Fig. 4. Addition of globulin to albumin and total plasma protein solutions. Curve 1, addition of globulin in 11.1% Na_2SO_4 solution to 0.760 mg. albumin; curve 2, addition of globulin in normal saline solution to 0.319 mg. total plasma proteins; curve 3, globulin alone, in 11.1% Na_2SO_4 solution; curve 4, globulin alone, in normal saline solution.

Table 1. *Addition of globulin to diluted plasma (total protein)*

Protein content = 0.445 mg./ml. Albumin/globulin ratio = 2.18.

Total globulin (mg.)	0.140	0.205	0.269	0.399	0.658	1.012
Added globulin (mg.)	0	0.065	0.129	0.259	0.518	0.962
Total proteins (mg.)	0.445	0.510	0.574	0.704	0.963	1.407
Albumin/globulin ratio	2.18	1.49	1.14	0.765	0.464	0.276
Optical density (D_{or})	0.202	0.230	0.249	0.317	0.450	0.704
Relative density (mg. total proteins/ D_{or})	2.21	2.22	2.31	2.22	2.12	2.00

Table 2. *Addition of globulin to Howe albumin filtrate*

(a) Albumin content 0.908 mg.

Added globulin (mg.)	0	0.042	0.084	0.168	0.252	0.336
Total proteins (mg.)	0.908	0.950	0.992	1.076	1.160	1.244
Albumin/globulin ratio	∞	21.6	10.8	5.39	3.59	2.69
D_{or}	0.428	0.422	0.414	0.469	0.509	0.528
Relative density	2.11	2.25	2.39	2.29	2.48	2.36

(b) Albumin content 0.405 mg.

Added globulin (mg.)	0.168	0.336	0.672	1.344	2.016	2.520
Total proteins (mg.)	0.573	0.741	1.077	1.749	2.421	2.925
Albumin/globulin ratio	2.41	1.21	0.601	0.301	0.188	0.161
D_{or}	0.250	0.298	0.454	0.801	1.038	1.275
Relative density	2.29	2.49	2.36	2.18	2.34	2.29

albumin solution may cause no appreciable increase in the density of the cloud obtained with salicylsulphonic acid (Table 2a). When, however, globulin is added to a dilution of plasma this 'lag' effect is not seen owing to the fact that the original globulin of the plasma is present.

Fig. 4 shows that the optical density/concentration curve for globulin in saline solution is parallel with that obtained for 'total protein' plus globulin, while the curve for globulin containing 11.1 % Na_2SO_4 is parallel with that obtained for globulin plus Howe albumin filtrate. Tables 1 and 2 illustrate the fact that very large variations in the albumin/globulin ratio cause little alteration in the relative density of the suspension when measured by the photometric technique.

Method

Total plasma proteins. 0.2 ml. plasma was diluted with normal (0.85 %) NaCl to 20 ml. To 1 ml. of this solution were added 3 ml. 3 % salicylsulphonic acid solution. The tube was shaken and allowed to stand for 10 min. It was then shaken again and the optical density of the suspension read in a Klett colorimeter using a neutral grey screen (usually of density 0.50) as standard, with an orange filter on the eyepiece.

$$\text{Optical density} = D_{\text{or.}} = \frac{\text{Density of screen}}{\text{Reading of colorimeter (cm.)}};$$

$$\text{Total plasma proteins} = D_{\text{or.}} \times 22.2 \%$$

A standardization curve prepared from numerous samples of plasma is shown in Fig. 5. The lowest point was obtained by using a 1/200 plasma dilution. A similar curve was prepared by Mr Thompson from results obtained with the spectrophotometer, and in this case there was considerably less scatter about the straight line.

Fig. 5.

Fig. 6.

Fig. 5. Relation between percentage total plasma proteins and optical density of salicylsulphonic acid-protein suspensions.

Fig. 6. Relation between percentage plasma albumin and optical density of salicylsulphonic acid-protein suspensions.

Albumin. 0.2 ml. plasma was diluted to 5 ml. with 22.2 % Na_2SO_4 solution. After remaining for 3 hr. at 38° the mixture was filtered at 38° through two no. 2 Whatman filter papers. 0.5 ml. filtrate was diluted with 0.5 ml. water, and 3 ml. 3 % salicylsulphonic acid solution were added. The tube was shaken, left to stand for 40 min. and shaken again. The optical density was then read as before:

$$\text{Plasma albumin} = D_{\text{or.}} \times 10.3 \%$$

A standardization curve for albumin, prepared from numerous Howe albumin filtrates, is shown in Fig. 6. In a few cases low results for albumin have been observed with the above procedure. Improved results were obtained by diluting 1 ml. plasma to 25 ml. with 22.2 % Na_2SO_4 , the mixture being kept at 38° overnight. Owing to the extra time involved this has not been adopted as a routine.

Total proteins of cerebrospinal fluid or urine. 1 ml. of cerebrospinal fluid or urine (diluted if necessary with normal saline) was used instead of the diluted plasma. If the urine after filtration was cloudy or highly coloured, a blank was run using urine alone in the colorimeter cup. The optical density so obtained was divided by 4 and subtracted from that of the suspension. For this purpose a neutral grey screen of density 0.125 was used as a standard.

$$\text{Total proteins} = D_{\text{or.}} \times 222 \text{ mg./100 ml.}$$

DISCUSSION

When it became necessary to find a rapid micro-method for the serial estimation of proteins in large numbers of specimens of plasma or serum, the biuret method of Robinson & Hogden [1940] was tried with promising results. It is rapid and gives a colour of good optical properties which was measured photometrically, using a yellow-green filter. Results were highly reproducible in the sense that duplicates were as a rule almost identical, but when the method was standardized against the Kjeldahl procedure the variations observed were disappointing.

King & Haslewood [1936] have described a turbidometric method for the estimation of proteins using the turbidity produced by salicylsulphonic acid and comparing this with a series of permanent standards prepared from a suspension of formazin in gelatin. While this procedure was not quite accurate enough for the estimation of plasma proteins, the method has been adapted for use in the colorimeter and this modification has increased the accuracy sufficiently to make it suitable for clinical investigations. The curve obtained (Fig. 5) relating the optical density of the suspension to the protein content found by the micro-Kjeldahl method was a straight line passing through the origin, which compares favourably with the sigmoid curve reported by Looney & Walsh [1939, 1] for the salicylsulphonic acid reaction in presence of gum ghatti.

The latter authors determine globulin by turbidometric estimation of the cloud produced by $(\text{NH}_4)_2\text{SO}_4$ in presence of gum ghatti [Looney & Walsh, 1939, 2], but although their reference curve is a straight line, it cuts the globulin axis slightly below 1 %. In the present investigation it has been found that the reference curve for globulin solutions plus salicylsulphonic acid also cuts the globulin axis at about 0.4 %. Dulière [1937] has stated that the use of $(\text{NH}_4)_2\text{SO}_4$ for the estimation of globulin is undesirable, as it does not give the same results as the classical method of Howe which he regards as the standard procedure. It has been found that the Howe albumin filtrate can be used for the estimation of albumin by the salicylsulphonic acid technique, although a slightly different relative optical density is obtained in these circumstances, necessitating the use of a different reference curve for the estimation of albumin (Fig. 6).

The chief difficulty in the way of the use of the salicylsulphonic acid reaction for the estimation of proteins lies in an observation of Plötner [1936, 1] who found that the addition of globulin to an albumin solution led to a decrease in the turbidity produced by salicylsulphonic acid, as measured by the nephelometer. This decrease was progressive as more globulin was added, until a minimum density was reached when the albumin/globulin ratio was unity. From this minimum the turbidity increased in proportion to the added globulin, the reference curve becoming parallel with that obtained for globulin solution alone. Plötner also observed that the turbidity produced by globulin alone was only one-fourth of that produced by the same amount of albumin. When Plötner [1936, 2] determined the density of the suspension, per unit weight of protein, in a series of patho-

logical plasmas of widely different albumin/globulin ratio, variations up to 300 % were observed. For the method to be valid for clinical use this relative density must be constant for all specimens of plasma, and Plötner therefore concluded that the salicylsulphonic acid method was useless for the estimation of the plasma proteins.

It has been shown in the experimental section that when the salicylsulphonic acid reaction is carried out in the manner described, and the density of the cloud is measured photometrically in a colorimeter, the objections raised by Plötner's observations do not apply. This can be seen in Fig. 4, where it is evident that no tendency exists to form a minimum in the optical density/added globulin curve at the point at which the concentrations of albumin and globulin are equal. This is confirmed in Tables 1 and 2, where fairly constant figures are shown for the relative density of the cloud over a very wide range of albumin/globulin ratios. It must be admitted that the globulin used was not pure, but it was of about the same quality as that used by Plötner, and the density of the suspension formed by globulin alone was of the same order of magnitude as that due to albumin or to diluted plasma of the same concentration.

Table 3. *Estimation of proteins by the salicylsulphonic acid method; effect of variation of albumin/globulin ratio*

Patient	Diagnosis	Total proteins %		Albumin %		Albumin/globulin ratio	
		Kj.	S.S.A.	Kj.	S.S.A.	Kj.	S.S.A.
Br (1)	Eosinophilia and anaemia	9.9	9.9	1.5	0.5	0.18	0.05
(2)	of unknown origin;	8.8	8.4	1.4	1.0	0.19	0.13
(3)	(? trichiniasis)	12.0	10.9	1.8	1.0	0.18	0.10
Co (1)	Nephrosis with lipaemia	3.7	3.6	0.7	0.7	0.23	0.26
(2)		3.9	3.7	0.8	0.7	0.25	0.24
Ha	Multiple myelomatosis	10.5	9.7	2.2	2.0	0.26	0.25
Ga	Chronic nephritis	4.6	4.4	1.4	1.2	0.43	0.42
Gr	Nephrosis with lipaemia	2.9	3.2	1.2	0.4	0.66	0.13
Mo	Acute retention (E.P.)	6.1	6.0	3.3	3.3	1.2	1.2
Os	Acute retention (E.P.)	5.9	5.9	3.2	3.4	1.2	1.3
Do	(Stored plasma)	4.8	4.9	2.6	2.7	1.2	1.2
To	(Stored plasma)	6.4	6.2	4.0	3.7	1.7	1.4
St	Chronic retention	6.1	6.3	4.0	3.7	1.9	1.4
Gn	Anastomotic ulcer	7.0	7.2	4.9	5.2	2.4	2.6
Cl	Pyloric stenosis	7.2	7.4	5.2	5.2	2.7	2.4

Kj. = Kjeldahl method; S.S.A. = salicylsulphonic acid method.

The results of the estimation of total proteins and albumin in 15 specimens of plasma are given in Table 3. The albumin/globulin ratios range from 0.18 to 2.7, and it is evident that discrepancies between the Kjeldahl and salicylsulphonic acid methods for the estimation of total proteins are not correlated with variations in this ratio. However, in two cases ('Br', with 3 different samples taken over a period of one month, and 'Gr') very low results were obtained for plasma albumin by the salicylsulphonic acid method. In both cases the albumin/globulin ratio was low, but the errors occurred in the albumin determination, not in the total proteins, as would be expected from Plötner's results. No explanation for these results has been found, since correct results have been obtained with other plasmas of apparently similar composition.

It must be emphasized that no high degree of accuracy is claimed for the method described, which is designed for the rapid assessment of the protein needs of a patient receiving intravenous therapy, where precise results are not necessary. When exact figures are required the Kjeldahl method should be used. In a series of 29 determinations of total plasma proteins and 24 determinations of plasma albumin the following results were obtained:

	Conversion factor (% protein by Kjeldahl)	Standard error	Standard deviation
Total plasma proteins	D_{cr} 22.2	± 0.22	± 1.21
Plasma albumin	10.3	± 0.13	± 0.64

SUMMARY

1. The suitability of the salicylsulphonic acid reaction for the photometric estimation of total plasma proteins and plasma albumin has been investigated.
2. Methods are described for the use of this reaction for the estimation of the proteins of plasma, cerebrospinal fluid and urine.

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