CXIII. THE SPECIFIC REFRACTION INCREMENTS OF SERUM-ALBUMIN AND SERUM-GLOBULIN.

By GILBERT SMITHSON ADAIR and MURIEL ELAINE ROBINSON.

From the Low Temperature Station and the Biochemical Laboratory, Cambridge.

(*Received July 1st, 1930.*)

(1) Introduction.

The refractive index of a protein solution is greater than that of pure water, and, according to the observations of Reiss [1904] and Robertson [1912], the difference between the refractions of the protein solution and the solvent is equal to the protein concentration in g. per 100 cc. of solution, multiplied by a constant. This constant is termed the specific refraction increment or the specific refraction of the protein.

A critical account of the work of previous investigators on the refractions of the proteins in normal and in pathological sera has been published by Schretter [1926] who has drawn attention to the question as to whether the specific refraction increments show physiological or pathological variations. The results obtained by different observers show a wide range of variation in normal as well as in pathological sera, but further investigation is required in view of the risks of error due to the relatively low stability of the proteins and their association with lipins and other impurities.

The first problem investigated in this work is the preparation of a purified solution of crystalline horse serum-albumin under conditions which minimise the risk of error due to alterations in the protein. The purity and stability of the preparations have been tested by comparing their specific refraction increments after successive recrystallisations and after different periods of dialysis in accordance with the procedure adopted by Hopkins [1900] in establishing the individuality of egg-albumin by measurements of the optical rotation of the protein after successive recrystallisations.

The second problem is the application of Donnan's theory of membrane equilibrium to measurements of the refractive indices of serum-albumin and serum-globulin solutions which have been enclosed in collodion membranes and dialysed against phosphate mixtures of well-defined hydrogen ion concentration. An experimental investigation of the effects of the unequal distribution of salts on refraction is of value in determinations of the specific refraction increment of globulin, which cannot be dissolved in the absence of electrolytes.

The theoretical interpretation of the specific refraction increment of a protein is discussed in Section 11 in this work, and compared with determinations on amino-acids and calculations based on the refractivities of the constituent atoms.

(2) *A rapid method for the recrystallisation of horse serum-albumin.*

According to Svedberg and Sjögren [1928], serum-albumin once crystallised is a homogeneous substance of molecular weight 68,000, whereas recrystallised serum-albumin is a mixture of particles of different sizes, due to the decomposition of the protein. Their suggestion that the earlier determinations of 62,000 [Adair 1926], and 45,000 [Sørensen 1925, 1] refer to material partially decomposed will be discussed in a later communication.

In this work, an attempt has been made to determine the conditions for recrystallisation in which the protein remains unaltered. It is quite certain that the decomposition products referred to by Svedberg are not formed under all conditions, because preparations have been made in which the rotation of egg-albumin and of serum-albumin remains constant after a number of successive recrystallisations [Hopkins, 1900; Young, 1922]. This constancy of the rotation is of great importance as a proof of the stability as well as the purity of the preparations.

The methods used in this work have been tested by comparing the specific refractions of material crystallised from one to four times, instead of the specific rotations. Refractometric measurements are not quite so sensitive as the measurements of rotation, but they can be made on smaller volumes of material.

Recrystallised horse serum-albumin was prepared by a slightly modified form of the method of Hopkins. The preparations made under the conditions outlined below gave constant values for the specific refraction.

Normal sterile horse serum was mixed with an equal volume of saturated ammonium sulphate solution and stored for 1 day at 0°. The globulin precipitate was removed by centrifuging or filtration. The first crystallisation was brought about by the addition of acetic acid as described in detail by Young [1922], who recommends a preliminary washing of the serum with ether, a procedure which was followed in some of our preparations.

The solution was left to crystallise over-night at room temperature. If it is placed at 0°, amorphous matter may be deposited.

The following day the crystals were separated from the mother liquor by centrifuging, and then mixed with an approximately equal volume of a fluid prepared by adding 60 cc. of *M* sodium acetate solution and 40 cc. of *M* acetic acid to 100 cc. of a saturated solution of ammonium sulphate and again centrifuged. It is possible to wash the crystals with this fluid two or three times without much loss.

The quantities of reagents used in the process of recrystallisation given below refer to a preparation of about 2 g. of washed crystals, yielded by 100 cc.

of serum. The volume of water used to dissolve these crystals is about 50 cc. After filtration of the solution of albumin in order to remove the insoluble residue, which may consist of lipins [Young, 1922], 8 cc. of *M* sodium or ammonium acetate were added, followed by a volume of saturated ammonium sulphate solution equal to the sum of the volumes of the water and the sodium acetate (about 58 cc.). Finally, the acidity was adjusted by the addition of 10·6 cc. of a mixture composed of equal volumes of *M* acetic acid and saturated ammonium sulphate (1·33 cc. of this mixture per cc. of sodium acetate). The only reagent which need be added slowly is the mixture of acetic acid and ammonium sulphate, which may be mixed with the albumin solution in a period of about 15 minutes. Crystallisation takes place within 2 hours, and it may occur in a few minutes if the amounts of water and of ammonium sulphate are reduced, but under these conditions the crystals are very small. The solution must be stirred during the addition of acid and it is advisable to stir the solution during crystallisation.

The yield can be increased by leaving the preparation over-night at room temperature. The crystals are separated from the mother-liquor by centrifuging and redissolved and recrystallised by the same method. The yield after four crystallisations is about 1 g. per 100 cc. of serum if the process is completed in a period of 3 or 4 days, a period only slightly longer than that allotted for a single crystallisation in the experiment described by Svedberg.

The process of recrystallisation adopted in this work necessitates the preparation of more reagents than does Hopkins's original method, but it shortens the time required, because the volume of ammonium sulphate required can be calculated and added rapidly. In the original method, the ammonium sulphate must be added with caution, for a slight error of judgment in estimating the amount required may lead to the formation of amorphous precipitates.

(3) *The purification of albumin by dialysis against standard buffer solutions.*

The solutions of recrystallised albumin obtained by the method described above contained ammonium sulphate and other crystalloids which were removed by dialysis in collodion membranes. The method of dialysis adopted in this work differed from that described by previous workers, in that the solutions were subjected to a preliminary dialysis with a buffer mixture composed of sodium and potassium phosphates, before they were dialysed against distilled water, in order to convert ammonium albuminates into sodium or potassium albuminates. After the ammonia has been replaced by non-volatile bases, it is legitimate to use determinations of the ash content of the solutions as a test for the degree of purification effected by dialysis. The ash contents of solutions dialysed in different membranes for different periods are recorded in Table II, and it will be seen that the amounts of ash, which vary from 0·01 to 0·06 %, are too small to cause significant errors in refractometric measurements. For comparison with the experiments on aqueous solutions of albumin, a number of preparations at the physiological hydrogen ion concentration were made by

dialysing the protein against a phosphate mixture containing 0·8/15 g.-mol. of Na_2HPO_4 and 0·2/15 g.-mol. of KH_2PO_4 per litre of solution, at 0°.

(4) *Methods for the determination of the specific refraction increment of a protein.*

The specific refraction increment, α, is calculated from measurements of refraction and of the protein concentration as defined in formula (1):

$$[R' - R_s]/C = \alpha \qquad \text{.....(1).}$$

R' = the refractive index of the protein solution.

R_s = the refractive index of the solvent, at the same temperature.

C = g. of dry protein per 100 cc. of solution.

This method of defining the concentration is of significance, for if the concentration is expressed in g. per 100 g. of water or per 100 g. of solution, the specific refraction increment is not a constant, but a variable which is a function of the protein concentration.

The determinations of refraction were made by means of a Zeiss dipping-refractometer, which was immersed in the protein solution after the temperature of the fluid had become constant within 0·1°. The refraction of water was measured first, in all experiments, followed by determinations of the refraction of the outer fluid R'' and of the protein solution R'. In some experiments, the temperature of these solutions differed by 0·1° to 0·3° from the temperature of the water, and a correction was made of 0·02 refractometer scale division, or 0·0000078 in the refractive index, per 0·1°. The neglect of this correction would cause an error of 0·43 % in estimating the refraction difference $R' - R''$ for a 1 % protein solution.

In our observations on aqueous solutions of serum-albumin, it was found advisable to make readings of the refractometer for a period of at least 15 minutes. Solutions of albumin or of globulin containing salts usually gave accurate readings 1 or 2 minutes after the immersion of the refractometer in the beaker of solution. In the aqueous solutions, the refractometer readings in the first few minutes, before the boundary grows distinct, may be about 0·2 unit below the final steady values.

It should be observed that the temperature of the protein solution and of the control solution should agree within 0·1°; the absolute value of the temperature is not of great importance, for Robertson has found that the value of α is independent of temperature, over a range of 20°. Our determinations of the value of α, at temperatures between 15° and 22°, agreed within the limits of experimental error.

The following methods for the estimation of the protein concentration have been utilised in this work. For the purified solutions of serum-albumin, a known weight of the solution, contained in a platinum crucible, was dried to constant weight at 110° in a Lothar-Meyer air-oven. The weight of protein was calculated by deducting the weight of the ash. The concentration of the protein

was calculated from these measurements and from measurements of the density of the protein solution.

For mixtures of albumin and phosphates this method is inaccurate, because the orthophosphates may be converted into pyrophosphates in the process of ashing. In such cases the protein concentration was calculated from Kjeldahl nitrogen determinations. The calculation depends upon an accurate determination of the percentage of nitrogen in the protein, and in view of the discrepancies of about 2 % recorded by different workers, the nitrogen content has been redetermined on specially purified material as stated in Section 5.

(5) *The nitrogen content of serum-albumin.*

The elementary analyses of serum-albumin recorded by different observers show a range of variation in the percentage of nitrogen which is considerably greater than the probable error of Kjeldahl determinations. Values which have been obtained are recorded in Table I.

Table I. *Percentage of nitrogen in serum-albumin.*

Author	% nitrogen	Method
Dumas and Cahours*	15·7–16·5	—
Brittener*	15·6	—
Stärke [1881]	16·04	Washed with alcohol and ether
Michel [1896]	15·8–16·0	Washed with alcohol and ether
Michel [1896]	15·6–15·8	Dialysed against distilled water
Maximowitsch [1901]	15·9	Dialysed against distilled water
Goldschmidt and Kahn [1929]	15·9	Washed with alcohol and ether
Piettre and Vila [1921]	15·1–15·2	Washed with acetone and ether

* Cited by Michel.

In this work, the percentage of nitrogen in horse serum-albumin was determined by the following method. Solutions of crystalline albumin were dialysed under pressure against distilled water at 0° for periods of 9 to 21 days, glass-distilled water being used in the final stages. The density of the protein solution was then measured, and a known weight of the solution was dried to constant weight in a platinum crucible in a Lothar-Meyer air-oven at 110°. After the final weighing, the protein was ashed and the weight of ash was obtained. The nitrogen content of the solution was estimated by Kjeldahl determinations on aliquot portions. The results obtained are recorded in Table II.

Table II. *Percentage of nitrogen in horse serum-albumin, purified by crystallisation and dialysis.*

Preparation no.	No. of crystallisations	No. of days dialysis against water at 0°	Protein g. per 100 cc. solution	Ash g. per 100 cc. solution	Nitrogen g. per 100 cc. protein
3·1	1	9	8·47	0·0592	15·54
3·2 *a*	1	18	7·06	0·0372	15·67
3·2 *b*	1	18	7·05	0·0391	15·51
3·3	1	18	3·11	0·0142	15·80
4·0	4	10	2·90	0·0116	15·54
4·5	4	21	3·57	0·0097	15·62
				Mean	15·60

Our results agree therefore with those obtained by Michel [1896] and by Maximowitsch [1901] on protein purified by dialysis.

As stated by Michel, further investigation is required to elucidate the cause of the discrepancy between the ratios of carbon and nitrogen in the materials purified by washing with alcohol and by dialysis. For the purpose of refractometric investigations, it seems desirable to adopt the figure obtained on dialysed protein, because the specific refraction increments calculated on the assumption that the protein concentration equals the nitrogen concentration multiplied by $\frac{100}{15{\cdot}60}$ are then comparable with determinations on aqueous solutions of albumin, in which the protein content is determined by drying the solution to constant weight.

(6) *The refractions of aqueous solutions of crystalline horse serum-albumin.*

Measurements of the density, the refraction and the concentrations of solutions of horse serum-albumin purified by different methods are summarised in Table III. The ash content of the preparations ranged from 0·06 to 0·01 %. In the preparation with the highest ash content, the correction for the refraction of the ash is less than 0·7 % of the refraction of the protein. It is not practicable to make an exact allowance for the refraction of the ash, and the true values of the corrections may be appreciably smaller than the figures given in Table III, which represent the maximum values rather than the most probable values. The ash must have contained traces of sodium and potassium phosphates, since these salts were used in order to displace ammonium compounds by substances which could be estimated at least approximately by ash analysis. The maximum correction is obtained by assuming that the ash is entirely due to the potassium and sodium phosphates, in which case the refraction of the ash equals 0·00178 times its concentration. If it is assumed that the ash is carbonate, produced by the incineration of sodium or potassium salts of the protein, the correction is reduced.

In comparing the refractions at protein concentrations from 3 to 8 % and at temperatures from 14·7° to 20·1°, it will be seen that the results confirm Robertson's conclusion that the specific refraction increment α is independent of the protein concentration.

The most important point illustrated in Table III is the agreement of the values of α for preparations subjected to different degrees of purification. Young states that the rotation of once crystallised serum-albumin is different from that of material crystallised two or three times. The once crystallised material used in this work differed from Young's preparation in that it was washed with the acetate buffer mixture referred to in Section 1, and dialysed in collodion membranes of an improved type. A certain amount of precipitation took place during dialysis, which indicates that once crystallised serum-albumin is contaminated with impurities which are insoluble when the inorganic salts are removed by dialysis in highly permeable membranes.

Since the results for material crystallised once and four times are in close agreement, it appears that under favourable conditions the stability of serum-albumin is great enough to justify recrystallisation. The agreement of the results for material dialysed for 10 to 21 days affords additional evidence in favour of the stability of the preparations.

Table III. *Refractions of aqueous solutions of horse serum-albumin.*

Reference no.	HA 3·1	HA 3·2 a	HA 3·2 b	HA 3·3	HA 4·0	HA 4·5
No. of crystallisations	1	1*	1*	1	4	4
Dialysis time (days)	9	18	18	18	10	21
Density (g. per ml.)	1·0208	1·0173	1·0173	1·0064	1·0069	1·0087
Temp. of pyknometer	20·1°	18·2°	18·2°	20·1°	14·9°	14·7°
Dry protein (g. per 100 cc. solution)	8·4734	7·0594	7·0547	3·1053	2·902	3·5721
Ash (g. per 100 cc. solution)	0·0592	0·0372	0·0391	0·0142	0·0117	0·0097
Temp. of refracto-meter	18·7°	16·7°	16·95°	20·5°	14·7°	14·5°
Refractive index of protein solution, minus refractive index of water	0·015521	0·013011	0·012989	0·005664	0·005317	0·006633
Refraction due to ash	0·000105	0·000066	0·000070	0·000025	0·000021	0·000017
Refraction due to protein	0·015416	0·012945	0·012919	0·006539	0·005296	0·006616
a (average = 0·0018295)	0·001819	0·001834	0·001831	0·001816	0·001825	0·001852
Percentage deviation (average = 0·41 %)	0·575	0·246	0·082	0·0738	0·245	1·23

* Treated with ether.

The results of previous observers are summarised in Table IV for comparison with those obtained in this work.

Table IV. *Refractions of serum-albumin obtained by different workers.*

Author	Mean value	Range	Species	Fraction
Reiss [1904]	0·00201	—	Horse	Crystalline
	0·00183	—	Horse	Non-crystalline
Robertson [1912]	0·00177	—	Ox	Total
Rohrer [1922]	0·00177	0·00173–0·00180	Human and ox	Total
Schorer [1913]	0·00188	0·00175–0·00198	Human	Total
Starlinger and Hartl [1925]	0·00166	0·00137–0·00204	Human	Total
Schretter [1926]	0·00200	0·00191–0·00212	Human	Total

The range of variation in the determinations of the specific refraction increments summarised in Table IV is considerably larger than the probable error of the measurements of the refractive index, but there are other sources of error which deserve consideration. The preparation used by Reiss was dialysed for many days at room temperature, and it may have contained the protein derivatives of low molecular weight referred to by Svedberg. The other determinations in Table IV refer to uncrystallised albumin, from pathological as well as from normal sera. The methods adopted by Robertson, Rohrer and Starlinger and Hartl have been criticised by Schretter. Schorer and Schretter calculated the protein concentration from nitrogen determinations; it is

probable that their calculations are based on Stärke's determination that albumin contains 16 % of nitrogen. Guillaumin, Wahl and Laurencin [1929] adopt the figure 15·27 rather than 16. In this work the value 15·60 has been adopted. If Schretter's determination of α is recalculated on the assumption that the nitrogen content is 15·6 instead of 16 %, the result obtained is 0·00195. The experiments described below suggest that the difference between Schretter's value for total albumin and the value for the crystalline fraction obtained in this work is due partly to the existence of a non-crystalline fraction with a higher value for α and partly to impurities.

Sørensen [1925, 1] has stated that it is possible to separate crystalline serum-albumin into three fractions, characterised by differences in solubility. In one of our experiments, about half of the product was dissolved by washing with a diluted solution of ammonium sulphate, but this fractionation produced no appreciable change in specific refraction increment.

(7) *The refractions of solutions of sodium albuminate equilibrated with phosphate buffers.*

Serum-albumin in blood exists in the form of a sodium salt, and therefore it is desirable to supplement the measurements of the refraction of pure albumin with measurements of the refractions of albuminates. Preparations of albumin at the physiological hydrogen ion concentration were obtained by dialysis of the protein solutions against a standard phosphate buffer mixture, containing 0·8/15 g.-mol. of disodium phosphate plus 0·2/15 g.-mol. of potassium dihydrogen phosphate per litre of solution. The p_H value of this solution is 7·38 at room temperature and 7·41 at 0°.

The protein solutions were enclosed in osmometers and dialysis was continued at 0° until the manometers gave constant pressures. The measurements of the refractive indices of the protein solution and of the dialysate were made at about 17·5°.

The relationship between the refractive index and the concentration of the protein can be determined by the calculation of the empirical coefficient α' defined by formula 2:

$$R' - R'' = \alpha' C \qquad \text{.....(2).}$$

R' = the refractive index of the protein solution.

R'' = the refractive index of the dialysate.

C = g. protein per 100 cc. solution, calculated from nitrogen determinations.

α' = an empirical coefficient, which may differ from the specific refraction increment α, on account of the unequal distribution of ions across the membrane. The mean values of α' and α are 0·00177 and 0·00183 respectively.

The results summarised in Table V and in Fig. 1 show that α' appears to have the same value for preparations from different sources and with different concentrations of the protein. Donnan's formulae, referred to below, show that α' may be increased by an increase in the protein concentration, but

Table V. *Refractions of different preparations of crystalline horse serum-albumin dialysed against a phosphate buffer* (p_H *7·4*). *In all these preparations the albumin was crystallised from solutions which had been extracted with purified ether to remove lipins.*

No.	*C* g. protein per 100 cc. solution	*a′* refraction increment	No. of crystal-lisations	Source
1	1·72	0·001757	1	Field labs. plasma
2	2·08	0·001760	1	,,
9	1·07	0·001798	1	Field labs. serum
8	2·47	0·001742	1	,,
7	2·49	0·001739	1	,,
6	3·54	0·001816	1	,,
5	4·86	0·001775	1	,,
19	1·48	0·001752	1	Burroughs Wellcome serum
18	1·50	0·001777	1	,, ,,
16	2·83	0·001811	1	,, ,,
17	2·99	0·001753	1	,, ,,
41	2·39	0·001755	4	Knacker serum
40	3·84	0·001770	4	,,
39	5·64	0·001759	4	,,
38	6·65	0·001769	4	,,
37	9·97	0·001791	4	,,

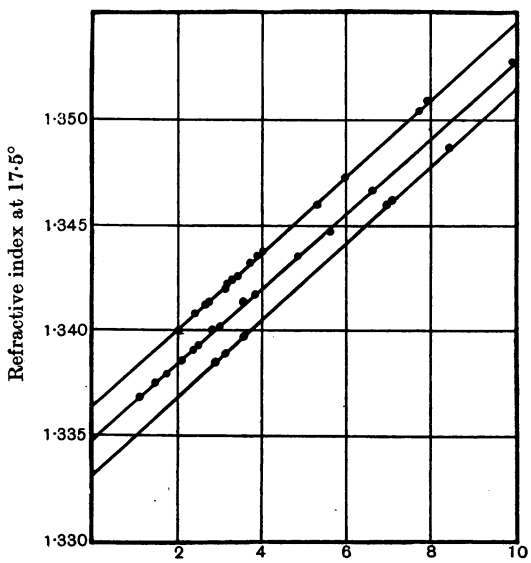

Fig. 1. Refractive indices of protein solutions at 17·5°.
Upper curve. Globulin, with 0·19 *M* KH_2PO_4 plus 0·009 *M* Na_2HPO_4.
Middle curve. Albumin, with 0·0133 *M* KH_2PO_4 plus 0·0533 *M* Na_2HPO_4.
Lower curve. Albumin dialysed against distilled water.

The lines in Fig. 1 were calculated by the following empirical formulae, in which the first constant equals the refractive index of the dialysate, and the second represents the value of *a′* or *a*.

Upper curve $R = 1{\cdot}33639 + 0{\cdot}001815C$.
Middle curve. $R = 1{\cdot}33488 + 0{\cdot}00177C$.
Lower curve $R = 1{\cdot}33320 + 0{\cdot}0018295C$.

within the range from 1 to 10 % of protein it appears that the variation is within the limits of the experimental error.

The degree of constancy in the values of α' shows that it is possible to calculate the concentration of the protein from measurements of the refractive index of the protein solution and of the dialysate by the application of formula (2). This calculation is independent of the theoretical interpretation of α'.

Schretter has pointed out that on account of the unequal distribution of ions across the membrane, it is not justifiable to assume that the refraction due to the protein can be calculated by subtracting the refractive index of the dialysate from the refractive index of the protein solution. He criticises the calculations of Rohrer [1922] which were based on the assumption that the concentration of salt is the same on both sides of the membrane. The error involved by the neglect of the salt distribution can be measured by comparing the values of α and α'. Under the conditions referred to in Table V, the difference between α and α' is about 3 %. The magnitude of the difference must depend upon the concentrations of hydrogen ions, of salts and of the protein.

The results of one experiment may be given, to show that under certain conditions, the difference α and α' must be relatively small. In this experiment, no. SA 43, a 10 % solution of sheep's serum-albumin was equilibrated with an ammonium phosphate buffer mixture at p_H 7·38 and 0°. The dialysate contained 0·115 g.-mol. of ammonia plus 0·0666 g.-mol. of phosphoric acid per litre. The protein solution contained 0·121 g.-mol. of ammonia plus 0·058 g.-mol. of phosphoric acid, plus 100 g. of protein per litre of solution.

The difference between the refractive indices of the dialysate and of water is equal to 0·00166 at 17·5°. The refraction of the ammonium phosphate inside the membrane must be approximately equal to 0·00166, multiplied by the distribution ratio, 0·058/0·0666, or 0·87. The total refraction due to inorganic ions must be greater than 0·0166 × 0·87, but it cannot exceed 0·00166 × 1·05, where 1·05 is the distribution ratio for ammonium ions. The difference between the refractive indices of the protein solution and of water is 0·02022, and by subtracting the maximum and minimum values for inorganic ions referred to above, it appears that the refraction due to the pure protein lies between 0·01848 and 0·01878. This conclusion was verified by a determination of α on an aqueous solution of sheep's serum-albumin, which gave the value 0·001856, or a refraction difference of 0·01856 for a 10 % solution. In this experiment, the value of α' is almost exactly equal to the value of α.

In the case of the experiments referred to in Table V, in which the distribution of ions was not determined by direct methods, approximate calculations of the concentrations of ions inside the membrane were made by a formula based on Donnan's theory.

$$S_{\text{inner}} = S_{\text{outer}}\,(1 - vx)\,.\,10^{-\gamma E/54\cdot2}.$$

S_{inner} and S_{outer} denote the molar concentrations of a diffusible ion in the protein solution and in the dialysate, x, the number of g. protein per cc. of solution, v is equal to the effective volume of 1 g. of the protein (according to Weber and Nachmannson [1929] $v = 1$ cc)., n = the valency of the ion, and E is the membrane potential in millivolts at 0°. In the case of a 1 % solution of serum-albumin, the membrane potential is about 0·32 millivolt. The degree of accuracy of the formula has been investigated in a previous paper [Adair, 1928].

An approximate statement of the relationship between α' and α can be obtained by applying Donnan's equations for the distribution of ions across membranes, supplemented by the assumptions formulated in equation (3).

$$R' - R'' = (\alpha + \beta)\, C + \alpha_K\, (K_{inner} - K_{outer}) + \alpha_{Na}\, (Na_{inner} - Na_{outer}) \ldots (3).$$

C = g. pure protein per 100 cc. of solution.

β is a factor approximately equal to 0·00003, which represents the effect of the sodium or potassium combined with the protein at p_H 7·4 (approximately 0·00043 equivalents per g. protein). The factor β was determined by comparing the refractions of aqueous solutions of the pure protein and of sodium proteinates.

α_K is an empirical coefficient, = 0·016, defined by a formula resembling equation 1.

K_{inner} = the concentration of KH_2PO_4 in the protein solution in g.-mols. per litre of solution.

K_{outer} = the concentration of KH_2PO_4 in g.-mols. in the dialysate.

α_{Na} is an empirical coefficient = 0·0279.

Na_{inner} and Na_{outer} refer to the concentrations of Na_2HPO_4. In the dialysate the molar concentrations of KH_2PO_4 and Na_2HPO_4 are equal to 0·01333 and 0·05333 respectively. In the protein solution their concentrations are a function of the protein concentration. In the case of a 1 % solution ($C = 1{\cdot}0$), the concentrations of KH_2PO_4 and Na_2HPO_4 are approximately equal to 0·01303 and 0·05142 respectively. These figures are provisional estimates based on the application of Donnan's formulae.

The value of $R' - R''$ is equal to 0·0177 in the case of a 1 % solution, and by applying formula (3) to the experimental data given above, it is possible to calculate the value of α. The result obtained, 0·00180, is in fairly close agreement with the more accurate value 0·00183 obtained by direct measurement. This agreement is of significance in the determination of the specific refraction increment of globulin, in which it is necessary to rely upon indirect methods for the determination of the specific refraction increment.

(8) *The effects of purification processes on the refraction of sodium albuminates.*

In the previous section it has been shown that the refraction increment α', defined by formula (2), is a constant in the case of solutions of albumin which

have been purified by washing with ether. The measurements on material which has not been purified by ether, recorded in Table VI, show a wider range of variation. The mean value of α' for the once crystallised albumin is 0·00183. The mean for twice crystallised and four times crystallised albumin is 0·00180. Hopkins [1900] and Young [1922] have observed that crystals of serum-albumin are associated with lipins, and it is not improbable that the value 0·00183, which exceeds by 3·3 % the refraction of the protein purified by ether, is due to the association of the protein with traces of lipins. The observations on protein crystallised four times agree better among themselves, and the average value 0·00180 is closer to 0·00177, the result for material treated with ether. Theoretically, the use of ether may be open to objection on the ground that it may alter the properties of the protein, but the results recorded above indicate that its use is justifiable.

Table VI. *Refractions of different preparations of crystalline horse serum-albumin dialysed against phosphate buffer at* p_H *7·4. The preparations differ from those in Table V in that the solutions were not treated with ether.*

No.	C g. protein per 100 cc. solution	α' refraction increment	No. of crystallisations	Source
13	1·94	0·00184	1	Burroughs Wellcome serum
12	2·19	0·00184	1	,, ,,
15 *a*	3·66	0·001842	1	,, ,,
14	4·64	0·001775	1	,, ,,
21 *a*	1·96	0·00184	1	,, ,,
22 *a*	1·97	0·00179	1	,, ,,
20	3·62	0·001816	1	,, ,,
35	2·44	0·001821	1	,, ,,
34	4·61	0·001823	1	,, ,,
33	6·60	0·001872	1	,, ,,
36	7·41	0·001843	1	,, ,,
32	9·14	0·001845	1	,, ,,
23	2·05	0·00177	2	,, ,,
24	1·86	0·001795	4	,, ,,
25	1·96	0·001812	4	,, ,,
27	3·81	0·001804	4	,, ,,
26	3·87	0·001813	4	,, ,,
28	1·58	0·001812	4	,, ,,
29	1·60	0·001820	4	,, ,,
30	2·82	0·001784	4	,, ,,
31	3·13	0·001809	4	,, ,,

(9) *The refraction of uncrystallised albumins from horse serum and sheep serum.*

The mother-liquor from two preparations of albumin crystals was dialysed against the phosphate buffer mixture defined in section (3). The value of α' for the first preparation was 0·001821. In the second preparation, the mean value of α' was 0·00188, a figure which is 6·2 % greater than the refraction increment of the crystallised albumin. Since a part of the protein in the mother-liquor must be crystalline albumin remaining in solution, it may be inferred

that the mother-liquor contains a protein with a refraction increment greater than 0·00188.

One experiment was made on albumin from sheep's plasma. The globulin was removed by half-saturation with ammonium sulphate and acetic acid was added. The albumin formed an amorphous precipitate instead of the crystals which appear when horse serum is used. The precipitate was redissolved and reprecipitated and then dialysed against distilled water. The specific refraction increment α, defined by formula (1), was 0·00185. The protein concentration in this experiment was calculated from nitrogen determinations. Within the limits of experimental error, this agrees with the results for crystalline horse serum-albumin recorded in Table IV.

Sheep serum, like horse serum, may contain substances with a higher refraction than the protein crystallised or precipitated by acid, for the total albumin in one preparation gave a value of 0·00194 for α', the refraction against a phosphate mixture. This preparation was not treated with ether, and therefore an appreciable fraction of the refraction increment may be due to lipins.

(10) *The refractions of isoelectric globulins and of sodium globulinates equilibrated with phosphate buffers.*

Direct determinations of the specific refraction increment for globulin have been made by Robertson and other workers, who have precipitated globulin by dilution or dialysis and redissolved the precipitated globulin by the addition of electrolytes. Robertson worked with very dilute solutions, as the opalescence of his preparations made it impossible to determine the refractive indices in concentrated solutions.

Preliminary experiments made in this work indicated that clear solutions of globulin could be obtained from material precipitated by ammonium sulphate and purified by dialysis against standard phosphate mixtures. The coefficient α' can be determined directly by measuring the refractive indices of the protein solution and the dialysate, and the value of α can then be calculated by formula (3), which allows for the effects of the unequal distribution of ions.

Svedberg and Sjögren [1928] state that globulin is an unstable substance, rapidly decomposed during the processes of fractionation into "euglobulin" and "pseudoglobulin." In order to minimise the risk of error due to decomposition of the protein, a rapid method of preparation was adopted in this work. The precipitate of globulin obtained by the addition of an equal volume of saturated ammonium sulphate to normal horse serum was separated by centrifuging. It was purified by the addition of water and reprecipitation with ammonium sulphate not more than three or four times, then cooled to 0° and dialysed in collodion membranes against standard phosphate mixtures. The protein appears to be fairly stable at 0°, because the values of α' for different preparations dialysed for different periods are in close agreement.

Table VII. *Nitrogen content of horse serum-globulin.*

No.	C g. dry protein per 100 cc.	g. nitrogen per 100 g. dry protein	Difference. Refractive index protein solution minus dialysate	α' calculated from dry weight determinations
G 7	3·162	15·24, 15·19	0·00582	0·00184
G 16	12·069	15·05, 15·06, 15·00	0·02219	0·00184
G 17	7·96	15·07, 15·06, 15·06	0·01452	0·00183
G 18	5·94	15·22, 15·24	0·01086	0·00183
G 21	10·421	15·16, 15·18	0·01909	0·00183

In five experiments, given in Table VII, the protein concentration C in g. dry protein per 100 cc. of solution was determined by the method of Devoto, as described by Hopkins and by Young. The removal of the last traces of sulphate from the heat-coagulated protein may require 10 days' washing in distilled water. For most of the time the material was kept at 0°. The nitrogen content of these solutions was determined by Kjeldahl's method and the percentages of nitrogen in the dry protein are given in Table VII. The mean value obtained for total globulin is 15·13 g. nitrogen per 100 g. dry protein.

Table VII gives the values of α' calculated from the dry weight determinations and measurements of refractive indices of solutions of globulins equilibrated with a mixture containing 0·19 g.-mol. of KH_2PO_4 plus 0·009 g.-mol. of Na_2HPO_4 per litre of solution. The p_H of this solution, 5·35, is in the isoelectric region.

Table VIII. *The refractions of globulin from horse serum equilibrated with phosphate buffer* p_H *5·35.*

No.	C	α'	No.	C	α'
1	3·30	0·001813	11	7·74	0·001817
2	3·39	0·001803	16	12·00	0·001848
3	4·02	0·001828	17	7·93	0·001832
6	5·33	0·001796	18	5·98	0·001817
7	16·98	0·001827	19	3·92	0·001825
8	15·91	0·001826	20	4·01	0·001805
9	11·92	0·001762	21	10·45	0·001828
10	9·84	0·001797	22	2·43	0·001813

In the experiments summarised in Table VIII the values of α' have been calculated from nitrogen determinations.

The mean value of α' for the isoelectric protein is equal to 0·001815. According to Donnan's theory, the concentration of the diffusible ions should be the same on both sides of the membrane at the isoelectric point. It is, however, necessary to make a correction for the volume of the protein. In the case of an ideal solution of an isoelectric protein, the correction can be made by the following formula:

$$\frac{R' - R''}{C} = \alpha' = \alpha - 0{\cdot}01 v_1 (R'' - R_{H_2O}).$$

v_1 = the volume occupied by 1 g. protein, plus the volume of its water of hydration. According to Weber and Nachmannson [1929], the effective volume of 1 g. globulin is 1·3 cc.

$R'' = 1{\cdot}33639$ at 17·5°.

$R_{H_2O} = 1{\cdot}33320$ at 17·5°.

In a 1 % solution of the protein the concentration of the diffusible salts is diminished by 1·3 %. By applying the formula given above, it appears that $\alpha = \alpha' + 0{\cdot}000041 = 0{\cdot}001857$.

In addition to the measurements of the isoelectric point, observations have been made on globulin equilibrated with the phosphate mixture used in the work on albumin, as stated in Table IX.

The mean value of α' for this medium is 0·00181. If it is assumed that the difference between α and α' for globulin is the same as that observed for albumin (0·00006) it follows that $\alpha = 0{\cdot}00187$.

Table IX. *The specific refraction increments of globulin from horse serum and sheep serum, equilibrated with phosphate buffer mixture.* $\frac{0{\cdot}8}{15}$ *M* Na_2HPO_4 *plus* $\frac{0{\cdot}2}{15}$ *M* KH_2PO_4. p_H *7·4.*

No.	g. protein per 100 cc. solution	α'	Species	Notes
100	7·80	0·00178	Sheep	—
101	14·00	0·00180	Sheep	—
G 4	2·75	0·00182	Horse	Serum treated with ether
G 5	2·72	0·00181	Horse	Serum treated with ether
G 24	1·64	0·00181	Horse	Stored 8 months

Table X. *Determinations of the specific refraction increment of globulin.*

Author	Range	Mean	Mean re-calculated	Species
Reiss	—	0·0023	—	Horse
Robertson	—	0·00229	—	Ox
Rohrer	0·00172–0·00180	0·00177	—	Human and ox
Schorer	0·0023 –0·0027	0·00245	0·00232*	Human
Starlinger and Hartl	0·00214–0·00312	0·00265	—	Human
Schretter	0·00206–0·00234	0·00219	0·00208*	Human
Adair and Robinson	0·00180–0·00189	0·00186	—	Horse

* These recalculations have been made because it is probable that Schorer and Schretter used Hammarsten's estimate that globulin contains 16 % of nitrogen in calculating the protein concentrations from Kjeldahl determinations. The value found in this work is 15·13 %.

(11) *The theoretical significance of the specific refraction increment of a protein.*

An approximate statement can be made of the theoretical correlation of the specific refraction increment and the refractive index of the pure protein. The refractive index has been computed by Robertson [1918], whose formula is based on the assumption that Gladstone's formula [Gladstone and Dale, 1863] for the refractions of mixtures is applicable to protein solutions, and that the specific volume of a dilute protein solution is equal to unity.

Gladstone's formula is

$$(R - 1)\, v = (R_p - 1)\, v_p x + (R_s - 1)\, v_s (1 - x).$$

R, R_p and R_s denote the refractive indices of the solution, the pure protein and the solvent.

v, v_p and v_s denote the specific volumes of the solution, the protein, and the pure solvent.

x = g. of protein per g. of solution.

Robertson's assumption that $v = 1{\cdot}0$ leads to the conclusion that

$$\alpha = 0{\cdot}01\,(R_p v_p - v_p - R_s + 1).$$

A more accurate formula, numbered (4) below, can be obtained by assuming that the specific volume of the solution is equal to $xv_p + (1 - x)\,v_s$. Gladstone's formula is then equivalent to the following simple expression:

$$R - R_s = 0{\cdot}01 C v_p\,(R_p - R_s) \qquad \text{.....(4).}$$

C = g. solute per 100 cc. of solution = $x \times 100/v$.

If it is assumed that the solute is combined with g g. of water per g. of dry material, the formula requires modification

$$R - R_s = 0{\cdot}01 c\,(1 + g)\,v_p'\,(R_p' - R_s) \qquad \text{.....(5).}$$

R_p' = refractive index of hydrated solute of specific volume v_p'.

C = g. anhydrous solute per 100 cc. of solution.

According to the data of Sørensen [1925, 2], $g = 0{\cdot}303$ for serum-albumin and 0·343 for serum-globulin.

By applying formula (5) to the values of α for albumin and globulin, 0·00183 and 0·00186 respectively, it appears that the refractive indices of the pure protein hydrates are approximately 1·514 and 1·511. These figures are approximations, because it is not yet possible to make allowances for solution-contraction volumes and for alterations in the refractivity of water [Fajans, 1927]. In the case of inorganic salts, the refractive index calculated from observations on solutions may differ by more than 3 % from the refractive index of the crystalline solid.

It is possible that the water of hydration in the protein crystals differs according to the composition of the mother-liquor, and the refractive indices of the crystals should show corresponding variations.

(12) *The specific refractivity of amino-acids and proteins.*

Gladstone and Dale define the specific refractivity of a substance by the formula $(R_p - 1)\,v_p$. An alternative definition, based on the formula of Lorentz and Lorenz, has been used by Brühl [1891], but the simpler equation of Gladstone and Dale is sufficient for the purpose of this work. The materials required for the calculations of the specific refractions of certain amino-acids are given in Table XI. The values of α have been calculated for comparison with the values of α given for the proteins. It was observed that the amino-acids differ from the proteins in that α is not a constant, but tends to increase as the degree of dilution is increased. A similar increase takes place

Table XI. *The refractions of solutions of amino-acids.*

Acid	Acid weight (g.)	Solvent weight (g.)	Temp. ° C.	Density	Refraction difference	a
Glycine	0·7505	10·0086	15·3	1·02924	0·01286	0·00179
Glycine	0·7505	9·9899	17·8	1·02886	0·01282	0·00178
Glycine	0·7500	9·9806	15·5	1·0294	0·01285	0·00179
Alanine	0·8908	9·9983	16·4	1·02564	0·01429	0·00171
Alanine	0·8908	9·9807	14·9	1·0260	0·01431	0·00171
Valine	0·188	9·9827	21·1	1·00216	0·00326	0·00176
Valine	0·3904	9·9872	21·5	1·00626	0·00664	0·00175
Tryptophan	0·102	9·9723	17·8	1·00166	0·002547	0·00251
Tryptophan	0·072	9·9810	20·9	1·00004	0·001813	0·00253

in solutions of electrolytes such as sodium chloride, in which the partial specific volume of the solute diminishes as the solution is diluted.

It is known that under certain conditions, the specific refractivity of an organic compound can be calculated from the specific refractivities of its constituent atoms. The calculated values for the amino-acids and for anhydrous serum albumin are given in Table XII, col. 1, for comparison with the observed values, based on determinations of refraction and density of the solutions recorded in Table XI.

Table XII. *The specific refractivities of amino-acids and of serum-albumin.*

Acid	Calculated specific refractivity	Observed specific refractivity
Glycine	0·37	0·37
Alanine	0·40	0·40
Valine	0·43	0·43
Tryptophan	0·42	0·49
Albumin	0·42	0·43

The calculated values are based on the values for the elements given by Smiles [1910], namely, C = 0·383, H = 1·488, N = 0·343, O = 0·203, S = 0·422. The calculation for albumin is based on an analysis given by Michel: C, 53·04 %; H, 7·1 %; N, 15·71 %; O, 22·29 %; and S, 1·86 %; these figures refer to the anhydrous protein. The calculation of the specific refractivity of the anhydrous protein is based on a determination of its apparent specific volume in an aqueous solution, 0·739 cc., computed from the density determinations given in Table III.

The results for glycine, alanine and valine agree with the theoretical calculation. The figure for tryptophan is approximately 17 % in excess. This excess is characteristic of unsaturated linkages in general. In benzene, for example, the observed specific refractivity is nearly 25 % greater than that calculated from the refractivity of the atoms. In the case of serum-albumin the observed value is about 2·9 % greater than the calculated. It is not improbable that the proportion of phenylalanine and other acids containing the benzene ring is sufficient to account for the discrepancy.

SUMMARY.

1. Horse serum-albumin has been prepared and recrystallised by a rapid method based on the original method of Hopkins. The nitrogen content has been redetermined as 15·6 %, a figure agreeing with the earlier estimates of Maximowitsch and of Michel for dialysed albumin.

2. The purity and stability of the preparations have been tested by measurements of their refractive indices, and it has been found that the specific refraction increment is a constant, unaffected by recrystallisation. In aqueous solutions the specific refraction increment of crystalline serum-albumin is 0·00183.

3. The refractive indices of solutions of alkali albuminates, equilibrated with phosphate buffers, have been measured and represented by a simple empirical formula, which can be used for calculations of the protein concentration from refractometric measurements. A calculation of the specific refraction increment of the protein from measurements of the refractive indices of the solution and the dialysate has been made by the application of Donnan's theory of membrane equilibrium.

4. The specific refraction increments of the uncrystallised fractions of albumin are larger than 0·00183.

5. The preparation of clear solutions of horse serum-globulin is described. The nitrogen content of total globulin has been redetermined as 15·13 %. The refractive indices of solutions of isoelectric and of alkaline globulins equilibrated with phosphate buffers have been measured and the specific refraction increment of the protein calculated, after making corrections for the effects due to the unequal distribution of ions. The mean value obtained for total globulin is 0·00186.

6. Preliminary determinations indicate that the specific refraction increments of both albumin and globulin prepared from sheep's serum are approximately equal to 0·00185.

7. Measurements have been made of the refractive indices of solutions of four amino-acids, namely, glycine, alanine, valine and tryptophan.

8. The theoretical significance of the specific refraction increment of a protein is discussed.

One of us (G. S. A.) is specially indebted to Sir F. G. Hopkins for permission to work in the Biochemical Laboratory, Cambridge, and to the Provost and Fellows of King's College, Cambridge, for a grant from the Stringer Fund.

REFERENCES.

Adair (1926). *Skand. Arch. Physiol.* **49**, 76.
—— (1928). *Proc. Roy. Soc. Lond.* A **120**, 573.
Brühl (1891). *Z. physikal. Chem.* **7**, 179.
Fajans (1927). *Trans. Farad. Soc.* **23**, 357.
Gladstone and Dale (1863). *Phil. Trans.* **153**, 317.
Goldschmidt and Kahn (1929). *Z. physiol. Chem.* **183**, 19.
Guillaumin, Wahl and Laurencin (1929). *Bull. Soc. Chim. Biol.* **11**, 387.
Hopkins (1900). *J. Physiol.* **25**, 306.
Maximowitsch (1901). *J. russ. physik. chem. Ges.* **6**, 460.
Michel (1896). *Verhand. würzburger phys. med. Ges.* **29**, 117.
Piettre and Vila (1921). *Bull. Soc. Chim. Biol.* **3**, 483.
Reiss (1904). *Hofmeister's Beitr.* **4**, 150.
Robertson (1912). *J. Biol. Chem.* **11**, 179.
—— (1918). Physical chemistry of the proteins, p. 367. (Longmans, New York and London.)
Rohrer (1922). *Schweiz. med. Woch.* **22**, 555.
Schorer (1913). *Korrespondenzbl. schweiz. Ärzte*, **43**, 1523.
Schretter (1926). *Biochem. Z.* **177**, 349.
Smiles (1910). The relations between chemical constitution and some physical properties. (Longmans, London.)
Sørensen (1925, 1). Proteins. (The Fleischmann Co., New York.)
—— (1925, 2). *Compt. Rend. Carlsberg Lab.* **15**, No. 11, 21.
Stärke (1881). *Maly's Jahresber.* **11**, 17.
Starlinger and Hartl (1925). *Biochem. Z.* **160**, 113.
Svedberg and Sjögren (1928). *J. Amer. Chem. Soc.* **50**, 3318.
Weber and Nachmannson (1929). *Biochem. Z.* **204**, 215.
Young (1922). *Proc. Roy. Soc. Lond.* B **93**, 15.