Sedimentation and Diffusion of Human Albumins

1. NORMAL HUMAN ALBUMINS AT A LOW CONCENTRATION

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The sedimentation and diffusion of normal human albumins have been studied as the initial part of a programme designed to investigate whether the molecular weights of the serum and urinary proteins in cases of nephrosis do differ from normal, as has been suggested by a few observers (e.g. Bourdillon, 1939). The present results are being communicated separately, as some delay appears likely before sufficient suitable pathological samples have become available for study.

Since the final object of this work is a comparison of normal and pathological materials, the limitations thus imposed have affected both the detailed technique and the scope of the experiments. Although it is well known that the albumin fraction isolated by electrophoresis contains at least two distinct components (e.g. Hoch & Morris, 1945), this method of isolation was selected, as the purpose was to determine the mean molecular weight of the albumin fraction. In any case no human albumin preparation yet obtained satisfies all criteria of purity. A particular advantage of the electrophoretic method in dealing with nephrotic sera is the possibility of separating albumin free from α-globulin, which is usually present in greatly increased amounts in such cases.

Sedimentation experiments require little protein, whereas, until recently, diffusion work needed appreciably more. The introduction of the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948; Ogston, 1949) has made it possible to obtain results with small amounts of material and much more rapidly. The limiting factors introduced by working with pathological samples are the small volumes of serum often obtainable and the low concentration of urinary protein. Electrophoretic isolation of more than a certain volume of protein solution (in this case 6 ml.) can be laborious and excessively time-consuming. It was, therefore, considered impracticable (often almost impossible) to prepare sufficient albumin to enable the values of $s^0_{20,w}$ and $D^0_{20,w}$ to be measured as a routine by extrapolation to zero concentration. Since the indications are that $s_{20,w}$ and $D_{20,w}$ vary rather slowly with moderate alterations in concentration so far as globular proteins are concerned, it was decided to make all measurements at a standard low concentration of 0·2–0·3 %, when results should all be comparable and should not differ very greatly from $s^0_{20,w}$ and $D^0_{20,w}$. All values of s are in Svedberg units and values of D in units of 10^{-7} cm.^{2} sec.$^{-1}$.

EXPERIMENTAL

Samples of blood from normal individuals were collected without any preservative, allowed to stand overnight in the refrigerator, and the sera separated by low-speed centrifugation. After removal of the few residual red cells by centrifugation at a slightly higher speed, the serum was diluted with buffer to give a total protein concentration of about 2 %, and dialysed in the cold against 2 l. of buffer for 2–3 days.

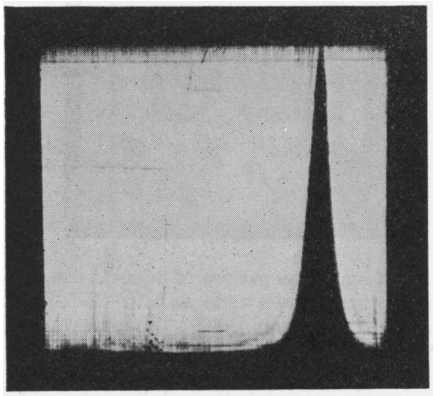

Fig. 1. Electrophoretic pattern of human serum albumin. Ascending (anode) limb. Migration is from left to right. Veronal buffer, pH 8·6, $I = 0·05$. Protein 0·7 %. Exposure after 56 min. at 6·3 V./cm.

Electrophoretic separations were carried out in the standard form of Tiselius apparatus at +0·5°, the albumin boundary in the ascending limb being held stationary at a suitable level by means of an electrolytic compensator, as described by Johnson & Shooter (1949). Phosphate buffer (pH 8·0, $I = 0·1$), used in the preliminary work, was found not adequately to resolve α_1-globulin from albumin. A number of experiments were then carried out in veronal buffer (pH 8·6, $I = 0·05$), but, since even here the resolution of α_1-globulin may not be sufficiently good, the medium finally adopted was veronal buffer (pH 8·6, $I = 0·1$). The maximum current considered safe, so far as heat convections were concerned, was about 20 ma. It was then possible after 6–10 hr. to recover from the ascending limb 3 ml. of solution containing

0·5–1·0% albumin. In order to check that no accidental contamination with globulin had occurred, every sample of albumin was examined in the micro cell of the Tiselius apparatus, a typical electrophoretic pattern being that shown in Fig. 1. About 4 ml. of solution, containing all the albumin, were removed from the micro cell. The concentration was calculated from the area of the peak in the diagonal schlieren pattern, the appropriate constants of the optical system being known. After dilution to bring the concentration into the selected range the albumin solution was dialysed for 5–6 days prior to sedimentation and diffusion. The few early preparations made in phosphate buffer were dialysed against the same buffer at this stage. Samples obtained in the veronal buffer of $I = 0{\cdot}05$ were dialysed against 0·2N-sodium chloride, but in the later work the sodium chloride concentration was reduced to 0·05N for the reason discussed later.

Sedimentation measurements. These were made in a Spinco ultracentrifuge, a short description of which has been given by Smith, Brown, Fishman & Wilhelmi (1949). The cell and

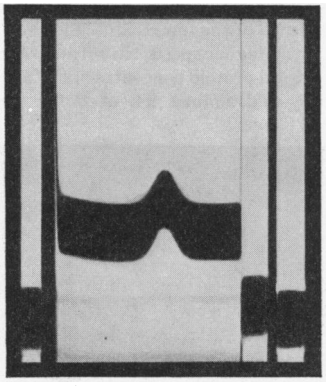

Fig. 2. Ultracentrifuge pattern of human serum albumin. Sedimentation is from right to left. Sodium chloride solution, 0·2N. Protein 0·2%. Exposure 71 min. after reaching full speed.

rotor are of conventional design and dimensions. The rotor spins in a high vacuum ($\sim 0{\cdot}5\,\mu$. of mercury) which is maintained by rotary oil and oil diffusion pumps. It is driven by an electric motor transmitting power to a special gear box (ratio 5:1) and a shaft which enters the vacuum chamber through an oil-seal of the type used in air-turbine machines. The rotor can be run at a series of set speeds, each of which is automatically maintained. Once the set speed is reached, variations are due almost entirely to fluctuations of mains frequency. A revolution counter, geared down in the ratio 6400:1, is provided which, together with a stop-watch, enables the mean speed during any interval of time to be determined accurately. A diagonal-bar schlieren optical system is incorporated, and photographic exposures can be taken either automatically at set intervals or manually. One such record is shown in Fig. 2.

The practice followed in these runs was to bring the rotor to full speed (60 000 rev./min.), to take the first photograph after about another 20 min. when the albumin peak had fully separated from the meniscus, and thereafter to take another four photographs automatically at 16 min. intervals.

As a routine the average speed of rotation was calculated from the counter readings immediately after the first and last photographs, but the speed during individual intervals could deviate from this. However, experiments in which the mean speed was also determined between each successive pair of photographs gave results almost identical. Further justification for the simpler procedure was provided by the small standard errors of the slopes of the $\log x - t$ lines (see Results). A final check on the accuracy of the counter was obtained by comparing its readings with the light impulses registered on an electronic counter fed from a photocell interposed in the optical system.

Travelling microscope measurements of the records (to the nearest 10 μ.) gave the distance of the boundary (defined as the maximum of the symmetrical peak observed) from the index marks. The boundary was thus located with adequate sharpness, and the correctness of its position was checked by verification of the magnification of the optical system ($\sim 2:1$) over different parts of the field. The known distances of the index marks from the axis of rotation, which were measured several times, allowed the distance (x) of the boundary from that axis to be calculated at different times (t).

Before the chamber was shut the rotor temperature was recorded by means of a contact thermocouple with a reference junction immersed in an intimate mixture of ice and water in a Dewar vessel. The scale of the indicating galvanometer was carefully calibrated with the measuring couple at a series of temperatures registered by a standard thermometer. The rotor temperature at the end of these runs was usually 1·0–1·5° higher than initially. The mean of initial and final rotor temperatures was taken as the temperature during the run (Pickels, 1950). In view of the importance of an accurate temperature estimation, both the contact thermocouple calibration and the standard thermometer were checked from time to time.

Possible variations in rotor temperature, due to different causes, were investigated as follows. The thermocouple leads were introduced into the vacuum chamber in such a way that the temperature of the (stationary) rotor could be observed whilst evacuation took place. This proved that no measurable change of temperature occurred during the process. It was also shown that no appreciable flow of heat took place along the driving shaft to (or from) the gear-box. Since the effect of any residual air in the chamber must be to raise the temperature of the moving rotor, the observed rise needs no other explanation. Low results (see Discussion) would make it necessary to postulate that the rotor temperature during the runs was actually lower than was believed. The only remaining possibility of error in estimating the temperature arises from the use of the refrigerating coils round the chamber to diminish or prevent the overall temperature rise in the rotor. If the refrigeration produced a drop in rotor temperature during the run, whilst admission of air to the chamber at the conclusion of the run caused a rapid rise, the estimated mean value would be too high. Although evidence against this hypothesis was quite decisive, as will be seen from the examples shown in Table 1 of duplicate experiments carried out with and without cooling, refrigeration has been dispensed with at present in order to eliminate any possibility of criticism on this point.

Diffusion measurements. The Gouy diffusiometer used here was the model described by Creeth (1952), a version of the instrument developed by Coulson *et al.* (1948) and Ogston (1949). The technique followed was exactly that

described by Creeth (1952), the only points it is necessary to emphasize being as follows. With the dilute solutions used it is vital that the value of j_m (Longsworth, 1947) for the dialysed protein solution should be measured with the greatest possible precision. Thus, it is obvious from the equation which describes the behaviour of mixtures (Ogston, 1949) that, should there be an excess or deficiency of salts in the protein solution (due to such causes as lack of dialysis equilibrium, or accidental dilution of the upper solvent layer through insufficient washing out of the diffusion cell), an incorrect result will follow. This is the reason for the ample time allowed for dialysis. To guard against evaporation, dialysis was carried out in closed vessels and the dialysis bags were not removed until immediately before the diffusion experiments. In some of the later work, as an additional precaution, the strength of the sodium chloride was reduced to 0·05 N, a value still high enough to depress anomalies under the conditions of these experiments. Although dialysis took place in the refrigerator, for the last few hours the solutions were left at room temperature to allow dissolved air to be expelled. Otherwise the value of j_m might have been affected slightly (Gosting & Morris, 1949), and air bubbles were liable to form in the diffusion cell, interfering with the optics and rendering an experiment worthless.

The pipette holds sufficient solution for three or four runs, but experience has shown that the first run may give a spuriously high result, evidently through insufficient 'clearing' of the cell. After this initial run, which was not recorded, the boundary was sharpened again, and the next two runs were recorded. Generally there was enough material to repeat the whole procedure with a further pipette-full, giving four independent determinations with each solution.

In one case a stronger solution of albumin was prepared (0·9 %) and diffusion carried out in 0·2 N-sodium chloride solution. The resulting patterns contained a large number (33) of interference minima, the relative positions of which could be analysed to show whether or not the boundary involved an essentially Gaussian distribution.

Photography. Both electrophoretic and diffusion patterns were recorded on Ilford Thin Film Half Tone Panchromatic plates, the former being processed in a caustic-quinol developer, whilst for the latter 'Wellington borax' developer was used in order to reduce the grain size. This borax developer was also used for the ultracentrifuge plates, which were Ilford Rapid Process Panchromatic.

RESULTS

Sedimentation experiments

The formula $s = \dfrac{\log_e x_2/x_1}{\omega^2(t_2 - t_1)}$ has often been used for calculating the sedimentation constant for successive time intervals (Svedberg & Pedersen, 1940). These are then corrected for temperature, etc. As the exact manner of the temperature variation of the rotor in the Spinco ultracentrifuge is not known, the mean value of s was worked out from the slope of the best straight line through the points on the graph of log x plotted against t (cf. Cecil & Ogston, 1948), ω and T being taken as the mean values for the whole run (see above). The standard error of the slope was always small (0·2–1·0 %). The sedimentation constants, all reduced to water at 20°, are shown in Table 1.

The degree of reproducibility, illustrated by preparations 2 and 3, was quite satisfactory, particularly in view of the fact that in the alternate runs refrigeration was used. Any real differences between the values of $s_{20,w}$ for different solutions due to concentration effects are estimated as at most 1 % and therefore less than the errors involved. The mean value, 4·25, for preparations made in 0·1 I veronal is statistically just different from the mean of the rest (taking the probability limit as 0·05). Nevertheless, as this might have been due to a small error in the original calibration of the thermocouple, the mean value of all results, 4·28, is used in the discussion.

Diffusion experiments

Mean values of the diffusion constants for each sample, reduced to water at 20°, are recorded in Table 2. Except in one case, all determinations on

Table 1. *Sedimentation constants of normal human serum-albumin preparations*

Serum no.	Buffer used in isolation	Solvent for sedimentation	Protein concn. (%)	$s_{20,w}$
1	Phosphate (I = 0·1)	Phosphate (I = 0·1)	0·29	4·30*
2	Veronal (I = 0·05)	0·2 N-Sodium chloride	0·20	4·27*
2			0·20	4·28
3			0·21	4·33*
3			0·21	4·23
4			0·27	4·34
5			0·24	4·28
6			0·27	4·32
8	Veronal (I = 0·1)	0·05 N-Sodium chloride	0·24	4·20
9			0·26	4·25
10			0·20	4·29
11			0·25	4·25
12			0·24	4·27
13			0·21	4·24

* Refrigeration used during these runs.

Table 2. *Diffusion constants of normal human serum-albumin preparations*

Serum no.	Buffer used in isolation	Solvent for diffusion	Protein concn. (%)	No. of experiments	$D_{20,w}$
4	Veronal ($I=0{\cdot}05$)	0·2 N-Sodium chloride	0·27	4	6·44
5			0·24	2	6·23
6			0·27	4	6·34
7			0·23	2	6·26
8	Veronal ($I=0{\cdot}1$)	0·05 N-Sodium chloride	0·24	4	6·44
9			0·26	4	6·30
10			0·20	4	6·27
11			0·25	4	6·16
12			0·24	4	6·40
13			0·21	4	6·28

the same sample agreed within ± 2 %, in accordance with the observations of Creeth (1952) at similar concentrations. The final mean of all measurements was 6·32. Although there was no correlation between diffusion constant and concentration, which was not surprising in such a narrow range and with relatively high errors for individual measurements, detailed statistical analysis showed that the differences between the various preparations were nevertheless just significant on the basis of a probability of 0·05; these differences were independent of the buffer used in isolating the samples.

Table 3. *Diffusion of normal human serum albumin in 0·2 N-sodium chloride; variation of C_t with j*

(Time—approximately 10 min. after the beginning of diffusion. $j_m=33{\cdot}59$.)

j	Y_j (cm.)	C_t (cm.)
0	0·9677	1·0700
1	0·8905	1·0677
2	0·8306	1·0685
3	0·7771	1·0676
4	0·7290	1·0670
5	0·6836	1·0646
6	0·6420	1·0634
7	0·6032	1·0625
8	0·5671	1·0627
9	0·5331	1·0639
10	0·4998	1·0633
12	0·4377	1·0630
14	0·3804	1·0632
16	0·3266	1·0621
18	0·2755	1·0577
20	0·2278	1·0523

The sample at 0·9 % concentration gave for $D_{20,w}$ 6·14, 6·11, 6·06 and 6·08 (mean 6·10), reproducibility being much better than at the lower concentrations. However, the main point of these experiments was to obtain records for examination of homogeneity. Measurements were made at two different time marks on each record, at about 3 and 10 min. respectively after the beginning of diffusion. The equivalent treatments of Longsworth (1947), as modified by Gosting & Morris (1949), and Ogston (1949) were applied. Typical results are shown in Table 3. The values of C_t (defined by Longsworth, 1947) show a pronounced, but fairly small, tendency to decrease as j increases, proving that there is some departure from the ideal Gaussian form.

DISCUSSION

Sedimentation constants

The trend of results in the Spinco ultracentrifuge has been mentioned in a recent paper from this laboratory (Creeth, 1952). In Fig. 3 the results for normal human serum albumin are compared with the values available in the literature. Comparisons must take into account: (*a*) the different methods of

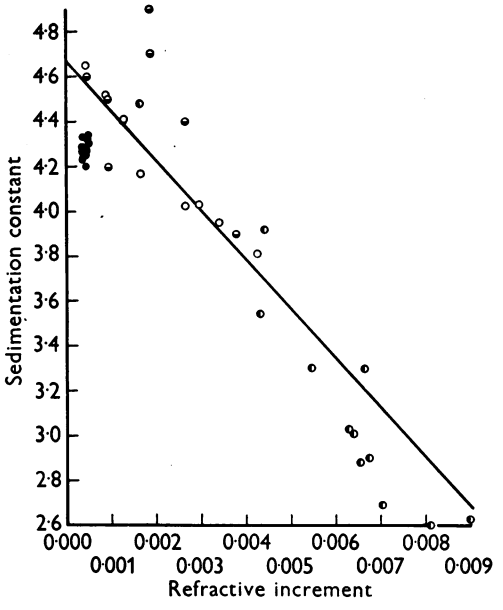

Fig. 3. Values of $s_{20,w}$ for human serum albumin. Points correspond to observations as follows ◐, McFarlane (1935*a*); ○, Pedersen (1945); ◒, Oncley, Scatchard & Brown (1947); ●, this work. The line shown is Pedersen's line of regression.

preparation of the materials, and (*b*) the reproducibility of the individual techniques of measurement. The figures published by the American workers show a surprising degree of variation, the cause of which is not evident. Although the oil-turbine ultracentrifuge yields a series of sedimentation constants which increase fairly uniformly with decreasing protein concentration, McFarlane's (1935*a*) values are scattered more widely than those of Pedersen (1945). Possible reasons for this are: (*a*) McFarlane's measurements were made on the albumin peak in serum patterns, not on isolated albumin, and (*b*) it does not appear that these particular samples were dialysed by him prior to ultracentrifugation. McFarlane (1935*a*) commented that his values did not agree to within the full experimental accuracy, suggesting the presence in serum of a factor which affected the sedimentation constant. This merely expressed the fact that the medium was not sufficiently well defined with respect to its inorganic constituents. The presence of the other proteins in the initial solution was probably not such a great objection since most of the globulins sediment away from the more slowly moving albumin. Although McFarlane's results are generally lower than Pedersen's, one sample of normal human albumin, isolated electrophoretically by McFarlane (1935*b*) and dialysed against 0·1 N-sodium chloride, gave a sedimentation constant (at 1 % concentration) of 4·39, about 2 % higher than Pedersen's results would suggest.

At the concentrations used in the Spinco, Pedersen's regression line corresponds to a constant of 4·6. The discrepancy between this and the mean value of 4·28 for the preparations in Table 1 requires careful consideration. It must either be due to errors in the determination or reside in the protein samples themselves. As a check on the latter point a sample of crystalline bovine plasma albumin (Armour), made up to about 0·5 % in, and dialysed against, 0·2 N-sodium chloride solution was used for parallel runs in the Spinco and oil-turbine machines. Mr J. W. Lyttleton at the Lister Institute obtained 4·49 in the oil-turbine, as compared with 4·22 in the Spinco ultracentrifuge.

The factors involved in the determinations have all been considered and possible sources of error checked (see Experimental section). This applies particularly to the temperature, the least satisfactorily defined variable in ultracentrifuge work.

These sedimentation measurements, therefore, whilst individually still subject to errors of about ± 2 %, give a mean value which must be very close to the correct values for this concentration range, and the oil-turbine figures quoted are too high. This is in accord with the conclusions of Cecil & Ogston (1948), who found that, unless the conditions of running of the oil-turbine ultracentrifuge were carefully standardized, both reproducibility and accuracy could suffer. They decided that in one case (β-lactoglobulin) previous figures were as much as 10 % high. A sample of their material (1·2 % β-lactoglobulin in a medium consisting of 0·1 N-sodium chloride, 0·1 N-sodium acetate, 0·04 N-acetic acid) was obtained from Oxford and its sedimentation constant measured as additional support for the conclusions formed during this work. The value of 2·78 from the Spinco, compared with 2·80 by the Oxford workers, completely confirmed previous deductions. It is also interesting to note that Johnston & Ogston (1946) obtained sedimentation constants for horse serum albumin which indicated a value of 4·45 at 0·25 % concentration. When corrected by the factor 0·978 (Cecil & Ogston, 1948) this becomes 4·35, only slightly different from the Spinco value for human serum albumin.

Diffusion constants

The literature reveals that the diffusion constants of proteins are not known with the accuracy that is desirable. Thus Neurath (1942) commented that, with the Lamm scale method and conventional cells, determinations have standard deviations from the mean of 2–3 % for a number of protein preparations.

References to the diffusion of normal human serum albumin are surprisingly few. A determination of the diffusion constant at an unspecified concentration by Longsworth & MacInnes (1940) gave a value of 3·34 for $D_{0,w}$, which becomes 6·39 when corrected to 20°. Values of 6·00 (at 0·5 % concentration) and 5·85 (at 0·25 %) were quoted by Pedersen (1945) for $D_{20,w}$. According to Oncley *et al.* (1947) preliminary measurements have led them to a tentative value of 6·1 for $D_{20,w}$, but no details were given. The values obtained here in the range of concentration 0·2–0·3 % have a mean of 6·32, but it has been shown that the differences between the preparations are significant. The experiment at 0·9 % ($D_{20,w} = 6·10$) showed that diffusion was not quite Gaussian. Since the process is not highly concentration-dependent this must be attributed to the presence of more than one molecular species in the albumin, a fact already known from electrophoretic work (e.g. Luetscher, 1939; Hoch-Ligeti & Hoch, 1948). Presumably this also accounts for the relatively small differences among the various preparations. Unfortunately, it was not possible to obtain for comparison a sample of human albumin prepared by crystallization in the presence of decanol (Cohn, Hughes & Weare, 1947).

Molecular weights

The mean values for the sedimentation and diffusion constants probably differ by no more than 2 % from $s^0_{20,w}$ and $D^0_{20,w}$ respectively. Since both constants increase as the protein concentration

decreases, the error involved in substituting them for $s^0_{20,w}$ and $D^0_{20,w}$ in the calculation of a molecular weight will be even lower. Unfortunately, molecular weights calculated from the combined data of sedimentation and diffusion constants are dependent to a considerable extent on the value of the partial specific volume, $\bar{V}$, an error of 1 % in $\bar{V}$ causing an error of about 3 % in the final result. The values of 4·6 for $s^0_{20,w}$ and 6·1 for $D^0_{20,w}$, often quoted (e.g. Oncley *et al.* 1947), give a result of 68 500, but the present work gives 61 500 if we assume the same partial specific volume, 0·733.

SUMMARY

1. The albumin fractions of a number of normal human sera have been isolated electrophoretically.

2. Sedimentation constants for these preparations in the concentration range 0·2–0·3 % are considerably lower than the hitherto accepted values. The reasons for this have been thoroughly explored.

3. Diffusion measurements in the Gouy diffusiometer show small, statistically significant variations from one preparation to another.

The author thanks Prof. E. C. Dodds, F.R.S., for the help, advice and encouragement which he has given during this work. He is indebted to Dr J. M. Creeth for many stimulating discussions, particularly in regard to the diffusion measurements. Thanks are due to Dr R. A. Kekwick for making possible the ultracentrifuge experiment at the Lister Institute, to Dr A. G. Ogston for the β-lactoglobulin solution, and to Drs D. N. Baron and J. M. Johnstone for providing the sera. The earlier part of this research was carried out whilst the author was in receipt of a personal grant from the Medical Research Council, the final phases being completed during the tenure of an I.C.I. Research Fellowship. The work described in this paper formed part of a thesis approved for the degree of Ph.D. in the University of London.

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The Proteins of *Arachis hypogaea* and Fibre Formation

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In the course of development of a textile fibre from the proteins of the groundnut (*Arachis hypogaea*) these proteins have recently been the subject of considerable study. Included in this has been the laboratory fractionation of the proteins and an examination of the fractions as fibre-forming agents.

The protein of the groundnut appears to have been first investigated with other vegetable protein systems by Ritthausen (1880), who extracted the proteins from the oil-free groundnut meal with aqueous sodium chloride and weakly basic solutions, and precipitated them by acidification. Ritthausen considered the solids so obtained to be identical. The investigations of Johns & Jones (1916) have indicated that the total protein of the nut consists of globulins and a very small amount of heat-coagulable albumin. They found it was possible, by