XLVIII. AN ULTRACENTRIFUGAL INVESTIGATION OF THE SERUM PROTEINS.

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THE extensive researches into the molecular dimensions of the proteins carried out in recent years by Svedberg and collaborators² by means of the ultracentrifuge have hitherto been conducted mainly on dilute protein solutions. This has been necessitated by the fact that the sedimentation velocity in the centrifugal field is independent of the protein concentration only in dilute solution. In addition to this, various practical difficulties arise when it is desired to examine protein solutions, such as serum, which contain more than 1–2 % of protein. For instance, the light absorption in the ultraviolet of such solutions is generally too great to permit the use of the absorption method of following the protein sedimentation. Also, in the case of serum, the absorption over the whole cell would not be due to the proteins alone, as is desirable, but also to the serum pigments.

The refractive index method of Lamm [1928; 1929], which depends on photographic measurements of the light deviation caused by the sedimenting boundary, offers better possibilities of examining strong protein solutions, because in this method light may be used of a wave-length which is outside the absorption region of the proteins or of any non-centrifugible absorbing substances which may be present in the solution. Mutzenbecher has recently used this method extensively in his studies on the serum proteins [1933, 1, 2, 3]. In this method a regular ruled scale is photographed through the centrifuge cell as it rotates, and according to whether the sedimentation boundary is sharp or diffuse the scale must be placed very close to, or at some distance from, the centre of the centrifuge cell. It is at present mechanically impossible to bring the scale closer to the centre of the rotating cell than 3.5 cm.,³ and at this distance the maximum concentration of serum albumin which can be examined in the thinnest centrifuge cell available is about 4 %, and of globulin about 2 %, owing to its lower diffusion constant. In the course of experiments directed towards the design of a system which might permit satisfactory centrifugal examination of undiluted human serum it was suggested to the writer by Dr Lamm that in place of the actual scale it should be possible to project optically close to, or even inside, the rotating centrifuge cell an image of an actual scale placed at some distance from the centrifuge. This scheme was immediately successful and afforded for the first time excellent photographs of the sedimentation of the proteins in undiluted serum and in some artificial protein mixtures of even higher concentration. It was found, for instance, that with certain undiluted sera scale distances as small as 0.9 cm. were required to give successful photographs. It was also found, however, that there is a definite limit to the concentration of protein which may be examined owing to the appearance at high

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² A complete bibliography of this literature will be found in a recent article by Svedberg [1934].

³ In the new rotors which are in process of construction it will be possible to bring the scale to within 2.5 cm. of the centre of the cell.

concentrations of a black band across the scale which completely blots out the lines in the boundary area. This phenomenon, which apparently arises when the deviation of the beam is so great that the light from the mercury lamp fails to reach the camera at all, must depend on a great variety of factors for its appearance, such as the thickness of the cell, the refractive gradient in the solution and the relative positions of lamp, cell, objective lens and camera. The phenomenon is closely related to the "Totalreflexion" described by Gans [1915] in his studies on the propagation of light through inhomogeneous media. On the basis of our experience with the present optical system of the ultracentrifuge we may say that the complication does not in general occur with serum protein concentrations less than 10 % in a 1.5 mm. cell, and that higher concentrations may usually be examined unless there is an abnormally large proportion of globulin or substances of higher molecular weight. With globulin alone, the black band will appear first at a concentration of approximately 6 %.

The method would appear to open a new field, *viz.* that of very concentrated protein solutions, and promises therefore to throw light on the molecular properties of many proteins in their natural biological state. There will be reported here the results of an investigation into some new phenomena which appear in concentrated mixtures of albumin and globulin, and which affect fundamentally our present ideas of the molecular state of these proteins in untreated serum. Throughout the work the opportunity has been taken to verify experimentally some of Lamm's more fundamental equations relating to the deviation of light by diffusing systems.

GENERAL THEORETICAL CONSIDERATIONS.

(a) *The refractive index method of Lamm.* A full account of the theory of the refractive index method will be found in Lamm's original papers. As already stated, a ruled scale is photographed through the centrifuge cell as it rotates and those lines on the scale which are seen through the diffusing boundary area appear in displaced positions. A photograph obtained in a preliminary run at the same speed but with water only in the cell gives a standard undeviated scale in which, of course, the effect of the compression gradient throughout the fluid in the cell is present and may be seen by comparing the average distance between the lines with the same distance on a photograph taken at low speeds. During the subsequent run with the protein solution in the cell photographs are taken at intervals of 10–20 mins. and the position of each scale line on the photograph is then measured with an accuracy of $\pm 1\mu$ in a microscope comparator. In order to find the deviation (Z) of any line in a photograph from its position in the standard undeviated scale it is necessary to have the same comparator zero for all photographs. On each scale photograph there appear also 3 or 4 scale lines which are photographed directly through small apertures in the balancing cell of the centrifuge. Since the absolute positions of these lines in space remain unaltered during the run, one of these is chosen as reference zero and every scale is placed on the microscope stage so that the arbitrary comparator reading for this line is 0.

The photographic displacement of any line (Z) from its position on the undeviated scale is given by

$$Z = G a b \frac{dn}{dx} \quad \dots\dots(1)$$

where G = the photographic enlargement factor;

a = the thickness of the fluid layer in the cell traversed by the beam;

b = the optical distance from the scale to the middle of the cell;

and $\frac{dn}{dx}$ is the gradient of refractive index, along a radial axis, at the displaced line position in the plane of the cell.

The photographic displacement (Z) for each line is plotted against its displaced position in comparator units. In this way a curve is obtained which shows the change of refraction gradient throughout the cell (*cf.* Fig. 2). If we assume that the gradient is zero in those parts of the cell above and below the diffusion region (which is usually experimentally true) then the area enclosed by this curve and the base-line is given by

$$A = \int_{-\infty}^{+\infty} \left(G a b \frac{dn}{dx} \right) dx = G a b (n_1 - n_0) \quad \dots\dots(2),$$

where n_0 is the refractive index of the solution of non-sedimenting electrolytes which separates above and n_1 is the refractive index of the protein solution below the sedimenting boundary.

It is to be emphasised that in the derivation of this equation no assumptions are made as to the nature of the forces which give rise to the gradient, and in particular, that the validity of the formula is not dependent on the curve conforming to any particular type.

The area A , obtained by graphical integration, is multiplied by the following factors:

1. $\left(\frac{x}{x_0} \right)^2$ where x and x_0 are the distances of the ideal sedimentation boundary (*i.e.* the theoretical boundary assuming that no diffusion has taken place) and the meniscus respectively from the centre of rotation. This factor [Svedberg and Rinde, 1924] corrects for the sector shape of the cell and the fact that the protein is sedimenting in a centrifugal field the intensity of which varies with the distance from the centre of rotation.

2. A factor (F) which converts the arbitrary comparator and graph units into absolute units (cm.).

3. $\frac{l}{(l+b)G}$ where l is the distance of the camera objective lens from the centre of the cell. This factor converts the displaced line positions on the photograph into absolute positions in the plane of the cell.

$$\text{The equation} \quad n_1 - n_0 = \frac{A}{G a b} \cdot \left[\frac{x}{x_0} \right]^2 \cdot \frac{l}{(l+b)G} \cdot F \quad \dots\dots(3)$$

gives, therefore, the protein refraction increment of the solution, but on the condition already stated, *viz.* that the solution which separates above is free from centrifugible matter and that no high molecular protein is thrown to the bottom of the cell before the first photograph is taken. Such an assumption is, of course, only justifiable for approximately monodisperse protein solutions and in particular for protein solutions which are free from protein disintegration products.

(b) *The calculation of sedimentation velocity.* The sedimentation constant is given by

$$S = \frac{dx}{dt} \cdot \frac{1}{x_m \omega^2} \quad \dots\dots(4),$$

where the boundary has moved through a cell depth dx in time dt under a mean centrifugal force of $x_m \omega^2$. In every case we have corrected this value to a medium of pure water at 20° by means of the formula

$$S_{W, 20} = S_{S, T} \cdot \left[\frac{\eta_{S, T}}{\eta_{W, 20}} \right] \left[\frac{\left(\frac{1}{\bar{V}} - \rho_{W, 20} \right)}{\left(\frac{1}{\bar{V}} - \rho_{S, T} \right)} \right] \quad \dots\dots(5),$$

where $S_{w,20}$ is the sedimentation constant in water at 20° ;
 $S_{s,T}$ is the sedimentation constant in the salt solution at T° ;
 $\eta_{w,20}$ is the viscosity of water at 20° ;
 $\eta_{s,T}$ is the viscosity of the salt solution at T° ;
 V is the partial specific volume of the protein ($=0.745$ for horse serum albumin and globulin [Svedberg and Sjögren, 1928]);
 $\rho_{s,T}$ is the density of the salt solution at T° ;
 $\rho_{w,20}$ is the density of water at 20° .

Tiselius [1932] has considered the electroviscous effect which appears when charged particles are caused to move in relation to the surrounding fluid by centrifugal forces and which in certain circumstances may give rise to abnormally small values of S . He gives the approximate formula

$$S^1 = S(1 - t_k) \quad \text{.....(6),}$$

where S^1 is the apparent sedimentation constant, S is the true sedimentation constant, and t_k is that fraction of the total conductivity (colloid + all other electrolytes) which is due to the colloidal ions. t_k varies widely with the protein, salt and hydrogen ion concentrations. In spite of the unusually high protein concentrations which we have examined in this work the value of t_k is always small because of the high conductivity of the 1 % sodium chloride or Ringer solution used as dispersion medium for the protein. No electroviscous correction need therefore be applied to the sedimentation constant values.

(c) *The base-line of the sedimentation diagram.* A phenomenon of rather frequent occurrence in the sedimentation graphs consists in a shift of the apparent base-line of the curve, i.e. the line joining the two horizontal limbs, so that it lies above or below the true base-line or x -axis. It appears that a displaced base-line is always associated with the use of a large centrifuge cell or a large scale distance or the two together. With water alone in the cell it may be seen from the scale photographs that the light beam passing through the cell is deviated as a whole (in relation to the undeviated beam passing through the apertures in the balancing cell) even at low speeds, and that the angle of deviation changes progressively with changing speed of the rotor. The amount of deviation is too great to be due to the compression gradient in the water. It is possible that the deviation is caused by a lack of true parallelism in the quartz end-plates of the cell. A consideration of the cell as a prismatic deviator of small angle shows that the deviation will increase with an increase of a or b , and this has been observed in practice. It is also possible that changes in the quartz plates under the influence of the high centrifugal forces used may contribute in large part to the deviation.

Pedersen [1934] has found that this deviation is strictly reproducible in the same cell when great care is taken to maintain the speed of the rotor constant to within ± 100 r.p.m. When this and other precautions which we need not mention here are observed it is possible to determine the true base-line, as Pedersen has done in his sedimentation equilibrium experiments with inorganic salts. In a prolonged series of experiments such as we are concerned with here, and in which it is proposed to examine a wide range of concentrations, it is not possible to use the same cell throughout and to observe all the precautions which Pedersen has been able to take. It will be apparent that if an insidious shift of the base-line is allowed to occur, then the area enclosed by the curve and the true base-line, or x -axis, will be abnormal and a serious error will be introduced in the calculations of $n_1 - n_0$ (equation (3)). If instead we measure the area enclosed by the curve and the apparent base-line this will still be accurately

related to $n_1 - n_0$ in accordance with the terms of equation (3), provided that by $n_1 - n_0$ we understand the difference in the refractive indices at two levels in the cell just outside the area of the diffusing boundary—one above and one below, and not necessarily as in the original definition, the difference between the refractive indices of the protein solution and of the salt solution in which the protein is dispersed. We have, therefore, throughout this work adopted the convenient and safe procedure of basing all measurements on the apparent base-line. It should, however, be pointed out that in thus abandoning temporarily the true base-line we have no longer an absolute reference point for our optical measurements, and in particular, that it is impossible to make measurements either of absolute refractive index or of absolute refraction gradient in the cell. This means that we cannot compare directly the refractive index of the solution which separates above with that of the inorganic solution in which the protein is dispersed, which we would wish to do in order to verify the absence of so-called non-centrifugible matter. Neither is it possible to compare the refractive index of the solution below the boundary with that of the original protein solution in order to verify the absence of any molecules which might be sedimenting in advance of the protein. In both these cases, however, it is very unlikely that such material would be present in the cell without giving rise to irregularities on the base-line of the curve or without causing it to slope. We have indeed found, especially with globulin solutions, base-line irregularities of this nature, and we have always been able to confirm indirectly the presence of contaminating matter in these cases by comparing the calculated value of $n_1 - n_0$ with the measured value of the total protein refraction increment after dialysis. The difference between the two represents the amount of colloidal matter which is retained in the dialyser and which nevertheless does not appear in the apparent area of the diffusion curve.

The error involved in the sedimentation velocity measurements in using the apparent base-line instead of the true base-line is negligible.

(d) *The measurement of refraction increment.* Before each experiment the protein solution is dialysed against 1 % sodium chloride or Ringer solution until ionic equilibrium is reached, and the refractive indices of the solution and its dialysate are then measured. With the high concentrations of protein which we have used, and notwithstanding the relatively high concentration of diffusible electrolytes, considerable inequality in the distribution of diffusible ions is present at equilibrium such as is capable of giving rise to appreciable osmotic pressure effects. Since, however, the refractivity of one molecule of albumin or globulin is so much greater than the corresponding value for sodium chloride (more than 1000 times) the effect on the refraction increment is very small. Adair and Robinson [1930, 1], in a series of experiments on horse serum albumin using concentrations up to 10 g. per 100 ml. of solution in *M*/15 phosphate buffer at p_H 7.4, found the effect of the uneven distribution of diffusible ions to be within the limits of their experimental error, viz. 3 %. We have always used higher concentrations of diffusible electrolytes than *M*/15 and p_H values usually less than 7.4, so that the effect in our case must be even smaller. In calculating protein concentrations from these refractometric measurements we have used Adair and Robinson's values for the specific refraction increments of horse serum albumin and globulin, viz. 0.00183 and 0.00186 respectively. These are corrected values and we have not endeavoured to apply to our measurements any corrections for Donnan effect or the volume of the protein molecules. Our concentration values may therefore be in error, but by not more than 3 %. For the total protein of horse serum we have assumed a mean value, viz. 0.001845.

In the process of sedimentation a protein boundary is set up at which the same uneven distribution of electrolytes must exist, and which, so far as we know, is subject in every way to the same Donnan considerations as the boundary at the collodion bag in the preliminary dialysis [cf. Tiselius, 1926]. We are, therefore, strictly justified, subject to certain considerations governing the conditions under which the two measurements are made, in comparing directly the refraction increment from measurements of the refractive indices of the solution and its dialysate with the value obtained from the sedimentation diagram.

With regard to the conditions of measurement it will be seen that the value for $n_1 - n_0$ which is measured with the refractometer is for a wave-length in the region of $590m\mu$ and at room temperature and atmospheric pressure. From the ultracentrifugal data, on the other hand, we compute $n_1 - n_0$ for the $366m\mu$ mercury line, at a cell temperature which varies between 30° and 36° and under a hydrostatic pressure of up to 300 atmospheres. The writer has measured the refraction increment of serum albumin over the range 15° to 30° and has found the maximum change to be less than 1 %, so that this factor can be neglected. The pressure factor may be neglected also since the hydrostatic pressure inside the centrifuge cell varies from about 300 atmospheres at the bottom to practically zero at the meniscus, and there is no evidence of the calculated values for $n_1 - n_0$ being significantly different at the bottom of the cell and at the top. It is not possible to neglect the wave-length factor, however. Pedersen and Andersson have recently undertaken an investigation of the variation of the specific refraction increments of various proteins and inorganic salts with the wave-length of the light used, and I am greatly indebted to them for permission to give here some of their preliminary results. They have used a differential prism apparatus arranged for photographic registration of the undeviated and refracted beams. The values in Table I are calculated relative to Adair and

Table I. *The specific refraction increments (α) of horse serum albumin and globulin at different wave-lengths (Pedersen and Andersson).*

Wave-length $m\mu$	α_{albumin}	α_{globulin}
366	0.00198	0.00202
436	0.00190	0.00195
546	0.00185	0.00187
579	0.00183	0.00186
589	0.00183	0.00186
656	0.00179	0.00182

Robinson's values for horse serum albumin and globulin. They were obtained in the examination of various preparations of horse serum proteins made by Pedersen and McFarlane according to the technique which will be described later in this paper. Some of the albumin preparations had been electro-dialysed and even dried and redissolved, but the agreement between all values for the same wave-length was always within 1 %. The solutions were examined at 20° with the dialysate (water, 1 % sodium chloride or Ringer solution) in the other compartment of the prism. These experiments of Pedersen and Andersson are still in progress and may lead to the publication of new absolute values for α_{albumin} and α_{globulin} , but there is no reason to expect that further investigation will lead to any change in the relative values at different wave-lengths, and in this particular case it is only the relative values which matter. It will be seen that the value for α at $366m\mu$ is greater than the value at $589m\mu$ by 0.00015 in the case of albumin, and 0.00016 in the case of globulin. We have,

therefore, corrected all our values for $n_1 - n_0$ obtained by measurements either with the dipping refractometer, or the Pulfrich refractometer using the sodium D line, to the wave-length $366m\mu$ by means of the following relationship:

$$n_1 - n_0 \text{ (measured at } 590m\mu) = [n_1 - n_0 \text{ (measured at } 366m\mu)] \times 1.08.$$

This holds with sufficient accuracy both for horse serum albumin and globulin, and we assume it to hold also for the total protein of horse serum.

It would, of course, have been a more direct procedure to use light of the same wave-length for measuring the refractive indices of solution and dialysate and for photographing the scale. Unfortunately, the technique for measuring refractive index in invisible, or poorly visible, regions of the spectrum is not nearly so convenient as with easily visible light, and on the other hand, the line at $366m\mu$ is much to be preferred for taking centrifuge photographs. It is conveniently present in the mercury arc spectrum in great intensity and almost monochromatic filters are available for its isolation; it is not absorbed to any appreciable degree by most proteins, and it lies in a spectral region in which photographic plates are highly sensitive. While it is possible to use lines in the green or red regions, in general the photographs with these do not show the same degree of contrast as with the mercury line at $366m\mu$, and we have, therefore, preferred to use this line throughout and to apply the correction factor given above.

(e) *The measurement of "diffusion" constants.* The diffusion constant may be calculated in various ways from measurements taken from the sedimentation diffusion curve [Lamm, 1928; 1929]. There are, however, a number of factors which reduce materially the accuracy with which diffusion coefficients may be thus obtained from centrifugal data. The exact time at which the diffusion process starts is not known and is almost certainly variable between experiments according to the degree of convectional disturbance at the meniscus during the initial acceleration period. Whereas in an ideal diffusion experiment with proteins extending usually over a number of days it is possible to eliminate the unknown starting time between the equations of two exposures [*e.g.* see Tiselius and Gross, 1934], this cannot be done here with any degree of accuracy because of the very short time during which the diffusion process may proceed and because the diffusing boundary is sedimenting in a cell the walls of which are not parallel. Also, it is not possible to have the temperature of the diffusing solution the same in all experiments. Finally, in the case of protein solutions more concentrated than about 0.5 % the sedimentation velocity is markedly influenced by the protein concentration. This means that as the protein molecules at the boundary diffuse into the supernatant fluid they enter a region of diminished protein concentration in which their sedimentation velocity is increased. The effect of the centrifugal force, therefore, is to cause a "sharpening" of the boundary in opposition to the natural process of diffusion. This sharpening effect will be slightly greater at the bottom of the cell than at the top owing to the increase in sedimentation velocity as we proceed away from the centre of rotation. As will be shown later, it is even possible, with undiluted sera and concentrated globulin solutions, to have the diffusion process so completely counteracted by the differential sedimentation that the boundary sediments to the bottom of the cell with an apparent decrease in the breadth of the diffusion area.

Even a very approximate idea of the extent of "spreading" or "blurring" of the boundary may be of some value, however. Where we are concerned with a mixture of two or more proteins differing so little in molecular weight that a

separation of the maxima cannot be obtained with the centrifugal forces available, or with a protein contaminated with numerous molecules slightly larger and slightly smaller than its own (a state of affairs which we have reason to believe exists in certain circumstances), then the only evidence that we are not dealing with a monodisperse protein lies in an abnormal spreading of the boundary. We have therefore endeavoured in this work to calculate a value for this in each case. Now it is clear from the foregoing that we are concerned with a composite effect in which the most that may be said meantime is that the process of diffusion is, in general, a major influence. For this reason, and in the absence of any complete theoretical treatment of the phenomenon, we propose to apply to our concentrated solutions the recognised methods of calculating diffusion coefficients in dilute solutions in the centrifuge cell and to call the resulting figure the spreading coefficient. In doing so we wish it to be understood that spreading coefficients are purely arbitrary measures of the apparent diffusion at a boundary which is sedimenting under non-ideal conditions, and that they may only be compared with each other under strictly comparable conditions of sedimentation. In a private communication Lamm has given the following equation for an ideal diffusion process

$$K \cdot t = \frac{A^2}{Z_{\max}^2} \cdot \frac{1}{4\pi} \quad \text{.....(7),}$$

where K is the diffusion coefficient, t is the time of diffusion, A is the area of the diffusion curve at time t , and $Z_{\max}$ is the height of the curve. Therefore, for any two exposures, at times t_a and t_b , we have

$$K(t_a - t_b) = \frac{1}{4\pi} \left[\frac{A_a^2}{Z_{a\max}^2} - \frac{A_b^2}{Z_{b\max}^2} \right] \quad \text{.....(8).}$$

This equation is used throughout to calculate the spreading coefficient. The exposures are chosen as far as possible the second from the beginning and the second from the end of the run. Errors arising in the calculation of individual boundary areas may give rise to large errors in the spreading coefficient because in equation (5) we are concerned with the difference between the squares of the areas. Now the value of each boundary area multiplied by Svedberg and Rinde's factor $(x/x_0)^2$ [1924] is a constant independent of the time at which the exposure was taken. We have, therefore, avoided the above source of error by obtaining firstly an average value for $A(x/x_0)^2$ over all exposures and dividing this by $(x_a/x_0)^2$ and $(x_b/x_0)^2$ to obtain the values for A_a and A_b respectively. The x values are usually obtainable with a much higher degree of accuracy than the A values.

GENERAL EXPERIMENTAL TECHNIQUE.

Fig. 1 shows schematically the optical system fitted to the high-speed oil turbine centrifuge which was used throughout this work. The scale and its associated projection lens are mounted together in fixed relationship on a metal base which slides on an optical bench. The position, in relation to a fixed point on the metal base, and the size of the scale image were first determined by photographic means using the mercury line at $366m\mu$ and allowing for the presence of the quartz windows in the centrifuge housing, but neglecting the effect of the rarified hydrogen atmosphere inside the centrifuge. The optical bench was then graduated in terms of the distance from the scale image to the mid-plane of the rotor (or centre of cell). By means of a fine screw adjustment the scale-lens combination could be set at any position on the bench and the corresponding distance from image to centre of cell read off to 0.1 mm. In

addition to enabling much smaller scale distances to be used, this arrangement offers a number of valuable practical advantages over the original method in which the scale was fixed inside a steel tube in the centrifuge housing. For instance, in the course of an experiment using the refraction method the scale-lens combination may be temporarily removed from the optical path and a light-absorption exposure taken in order to measure the concentration distribution of any light-absorbing substance in the cell. Also, since the scale position can be altered during an experiment it is not necessary to know beforehand the value of b which, in association with the boundary gradient to be expected in the cell, will give scale photographs with the requisite degree of definition. The determination of this scale position was formerly a matter requiring some experience. Now it is only necessary to view the scale from the camera by means

Fig. 1. Showing diagrammatically the new optical system fitted to the high-speed oil turbine centrifuge, which was used throughout this work.

of a hand lens immediately before the first photograph is to be taken and to judge with the eye which scale position gives the maximum line displacement in the diffusion area consistent with good definition of the displaced lines. Where a mixture of two or more proteins is under examination, one of which is present in much smaller amount than the other, it is also possible, when sedimentation has proceeded long enough to effect a clear separation of the two diffusion boundaries, to photograph the line displacement due to each boundary separately, using the best scale position for each photograph. In this way, where mixtures are concerned, the analytical sensitivity of the method may be increased many times.

Throughout the work to be described in this and following papers efforts were made to employ as small a number of centrifuge cells as possible and to run the centrifuge at a speed in the neighbourhood of 60,000 r.p.m., which corresponds to a mean force of 255,000 times gravity in the cell. In a preliminary run at 60,000 r.p.m. with each cell filled with water two photographs were obtained for each of a series of scale positions, 3 mm. between each—one just after starting the centrifuge and the other on reaching full speed. The latter served as the standard undeviated scale for that particular scale position and cell, and the former was used to obtain the photographic enlargement factor. The mercury line at $366m\mu$ was used in all cases and was isolated by means of a nickel oxide glass filter. Imperial Process plates were used in the earlier work but later plates made by G. Cramer and Co., St Louis, Mo., U.S.A., were used, which, although requiring longer exposures, had the advantage of giving better contrast.

According to the concentration of protein, a period of 1–3 hours after reaching 60,000 r.p.m. was required to produce complete sedimentation of albumin and globulin. During this time six to eight photographs were taken at equally spaced intervals and with suitably chosen stops over the objective lens according to the degree of definition of the displaced scale lines. The temperature inside the cell at reaching full speed was usually 28–32° and did not rise by more than 4° during the whole run. The height of the column of solution in the cell was 10–11 mm. and the solution was always covered by a layer of paraffin to prevent evaporation into the rarified hydrogen atmosphere in the centrifuge housing.

The general plan of the investigation was to prepare and centrifuge separately solutions of purified albumin and globulin in various concentrations and then to examine a series of mixtures of the two in various proportions. The preliminary refractive index measurements after dialysis were made at first with the Zeiss dipping refractometer, but subsequently with the Pulfrich refractometer using the “difference” prism and the sodium D line as light source. The two methods of measurement are in essential agreement here but the latter is more convenient in practice, and in later work with highly coloured untreated serum, the use of monochromatic light was found strictly desirable. The hydrogen ion concentrations were measured by means of the glass electrode using a valve potentiometer already described by the writer [1932; 1933, 1, 2]. A few experiments—particularly with dilute solutions—in which the apparent base-line did not run parallel to the x -axis, or the horizontal limbs of the curve differed markedly in height above the x -axis (for reasons probably associated with the establishment of convection gradients in the cell), were discarded and are not reported. But, for the great majority of runs, the apparent base-line was satisfactory and enabled an accurate determination of A and the position of the maximum (corresponding to the position of the “ideal” sedimentation boundary) to be made. In the case of mixtures of two proteins the component curve with the most clearly defined maximum was completed by symmetry first and the other constructed by a process of graphical subtraction from the original.

Methods of preparing serum proteins.

Preparation of horse serum albumin. To fresh horse serum an equal volume of saturated ammonium sulphate was added, and after standing overnight the supernatant fluid was filtered off, treated with $N/10$ sulphuric acid in half-saturated ammonium sulphate to slight permanent cloudiness and given 24 hours to crystallise. The crystals were dissolved in water and recrystallisation induced by half-saturation with ammonium sulphate and careful addition of M acetic acid and M sodium acetate according to the procedure described by Young [1922]. The preparation was recrystallised four times in all and washed between each crystallisation with a solution of approximately the same inorganic composition as the mother-liquor. The final aqueous solution was dialysed and then electrodialysed at 440 v. until the current density was steady at 0.5 ma. per sq. cm. Finally the solution was made quite clear by centrifuging in an ordinary laboratory centrifuge at 2000 r.p.m. and filled into a number of small sterile bottles. The solution had a pale amber colour and was quite odourless.

Preparation of horse serum globulin. The globulin precipitate (above) was dissolved from the filter-paper with water and reprecipitated by half-saturating the solution with ammonium sulphate. The precipitate was washed with half-saturated ammonium sulphate. The precipitation and washing were repeated three times and the final solution dialysed against frequent changes of Ringer solution and under pressure until the product was quite free of sulphate ions

and had a concentration of approximately 5 % globulin. This solution was centrifuged clear and stored in the same way as the albumin. The solution was pale blue, faintly opalescent and also quite odourless.

DESCRIPTION AND RESULTS OF A TYPICAL EXPERIMENT.

A solution of horse serum albumin after prolonged dialysis against Ringer had a refractive index of 1.34193, and the dialysate 1.33500. Therefore

$$n_1 - n_0 = 6.93 \cdot 10^{-3},$$

and the concentration of albumin is $\frac{0.00693}{0.00183}$, or 3.79 g. per 100 ml. solution. The p_H of solution (measured) = 5.26. The solution was placed in a centrifuge cell of thickness (a) 1.5 mm. and with quartz end plates 5 mm. thick. The scale distance was 4.0 cm. Refractive index of crystalline quartz = 1.56.

b (optical distance from scale to centre of cell)

$$= 4.0 - \left(0.5 + \frac{0.15}{2} \right) + \frac{0.5}{1.56} + \frac{0.15}{2 \times 1.34} = +3.80 \text{ cm.}$$

The centrifuge was started and reached 60,000 r.p.m. in 30 mins. The first exposure was taken 20 mins. later and thereafter five additional exposures were taken at intervals of approximately 10 mins. The scale photograph of each exposure was then measured in the comparator, the line displacements were obtained by subtraction, and the curve showing photographic displacement of each line against its displaced position was plotted. Figs. 2 *a* and 2 *b* show the curves corresponding to the second and fifth exposures. The high degree of symmetry of these curves and the almost complete absence of irregularities of the base-line testify in large measure to the monodispersity of the serum

Table II. *Results of a typical experiment with horse serum albumin.*

$G = 0.984.$		$\frac{l}{(l+b)} = 0.987.$		$x_0 = 6.266 \text{ cm.}$	$F = 1.10^{-4}.$	
Exposure no.	Time (mins.)	$x \text{ cm.}$	$(x/x_0)^2$	$A \text{ sq. cm.}$	$(n_1 - n_0) \cdot 10^3$	$S_{w,20} \cdot 10^{13} \text{ cm./sec.}$
1	0	6.421	1.050	35.30	6.63*	—
2	10	6.482	1.070	32.77	6.27	3.18
3	20	6.533	1.093	31.88	6.23	3.58
4	30	6.627	1.119	31.23	6.25	3.66
5	40	6.717	1.149	30.14	6.19	3.57
6	51	6.778	1.170	29.73	6.22	3.44

Average value (excluding the first) of $(n_1 - n_0)$ = $6.23 \cdot 10^{-3}$,

Average value (excluding the first) of $S_{w,20}$ = $3.56 \cdot 10^{-13} \text{ cm./sec.}$,

Spreading coeff. (calculated between Exps. 2 and 5) = $1.6 \cdot 10^{-7} \text{ cm.}^2/\text{sec.}$

* An abnormally large area in the first exposure occurred frequently throughout the present series of experiments. It may be due to optical effects in the neighbourhood of the meniscus, or to the fact that separation from the meniscus was not complete when the photograph was taken. On the other hand, a more probable explanation is that the preparation contains some poly-disperse protein molecules which at the beginning of sedimentation give rise to a relatively sharp boundary, but in the later stages the molecules are so separated by the centrifugal field as to give rise to a very diffuse boundary the curve of which merges with the apparent base-line. While it would be of some interest to know in each experiment the maximum area of the sedimentation curve in the early part of the run, we cannot record this because we do not know when to take the photograph, and also very early photographs are often impossible because the gradient at the meniscus is so great. We must, therefore, confine our measurements to those exposures for which the values of the protein refraction increment are constant, and in the process of averaging the refraction increment values for one complete experiment we have accordingly omitted the value for the first exposure where this was clearly abnormal.

albumin preparation. The abscissa of the vertical axis of symmetry of each curve was read off and from it the distance (x) of the boundary from the centre of rotation was calculated. The distance (x_0) of the meniscus from the centre of

Fig. 2 (a). $a=1.50$ mm.; $b=3.80$ cm. Centrifugal force = 255,000 times gravity. Exposure taken 30 mins. after reaching full speed.

Fig. 2 (c). $a=2.00$ mm.; $b=2.41$ cm. Centrifugal force = 210,000 times gravity. Exposure taken 46 mins. after reaching full speed.

Fig. 2 (b). From same experiment as (a). Exposure taken 60 mins. after reaching full speed.

Fig. 2 (d). $a=2.00$ mm.; $b=2.56$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 55 mins. after reaching full speed.

Fig. 2. *Ultracentrifugal sedimentation diagrams obtained by the refraction method.* The abscissae (x) represent distances from the centre of rotation, and the ordinates (Z) represent the scale-line displacements which are proportional to the gradient of refractive index (dn/dx). A comparison of (a) and (b), from the same experiment, shows how the curve moves parallel with the x -axis during the run, and also shows the symmetry and regularity typical of the albumin curves. A comparison of (c) and (d) shows the differences which may be expected in the sedimentation diagrams of different globulin preparations.

rotation was similarly calculated and the value of the sedimentation constant obtained by means of equations (4) and (5). Finally, the area of each curve on the graph paper was obtained by counting the equivalent millimetre squares and the value of $n_1 - n_0$ calculated using equation (3). Table II shows the results of this experiment.

DISCUSSIONS OF RESULTS OBTAINED WITH ALBUMIN
AND GLOBULIN SOLUTIONS.

In Table III are presented the results obtained from the investigation of various horse serum albumin and globulin solutions. Preparations A2, A3, A4 are all dilutions of the same stock solution of albumin, and the curves all show the same degree of symmetry and absence of base-line irregularities as are shown in Figs. 2 *a* and 2 *b* (from preparation A2). Preparation A1 is also from the same stock solution which, in this case, had been preserved for 6 months in the

Table III. *Results obtained in a series of experiments with solutions of horse serum albumin and globulin.*

Protein solution	Protein conc. g./100 ml.	Medium	p_H	Cell thickness mm.	Optical scale dist. cm.	Average $S_{w,20} \cdot 10^{13}$ cm./sec.	Measured $(n_1 - n_0) \cdot 10^8$ $\lambda = 366 m\mu$ (a)	Calculated average $(n_1 - n_0) \cdot 10^8 \cdot \lambda = 366 m\mu$			Boundary spreading coefficient $\times 10^7$ cm. ² /sec	
								Principal molecule	Other molecules	Total (b)		
Albumin experiments:												
A 1	5.40	1 % NaCl	—	2.00	1.96	3.80	10.7	8.62	0.16	8.78	—17.9	1.3
A 2	3.79	Ringer	5.26	1.50	3.80	3.56	7.48	6.23	0	6.23	—16.7	1.6
A 3	0.47	„	5.46	6.00	7.57	4.56	0.93	0.81	0	0.81	—12.9	7.4
A 4	0.173	„	6.40	12.0	9.0	4.60	0.349	0.305	0	0.305	—12.6	8.3
Globulin experiments:												
G 1	3.82	1 % NaCl	—	2.00	2.56	5.83	7.68	5.42	1.15	6.57	—14.5	0.1
G 2	3.02	„	6.54	2.00	2.41	6.44	6.07	5.10	0.25	5.35	—11.9	0.2
G 3	2.45	Ringer	6.54	1.50	3.80	6.27	4.91	4.10	0.30	4.40	—10.4	1.7
G 4	1.48	„	6.54	1.50	3.80	6.69	2.97	2.16	0.22	2.38	—19.9	5.2
G 5	0.52	„	6.54	4.0	11.6	6.82	1.05	0.82	0.04	0.86	—18.1	8.6
G 6	0.174	„	6.54	12.0	11.5	7.20	0.349	0.250	0.040	0.290	—16.9	7.6

electrolyte-free state and adjusted to 1 % NaCl just before the experiment. Slight base-line irregularities were present here, suggesting the presence of about 2 % of larger molecules. Also, the value of the sedimentation constant is unexpectedly high. In Fig. 2 *c* is shown one of the curves obtained in the investigation of the globulin preparation G4. The irregularity of the base-line of this curve was also present in the curves of G2, G3, G5 and G6, which are all dilutions of the same stock solution. By constructing the symmetrical curve (shown in dotted lines) it is estimated in this case that the preparation is only 90 % monodisperse, the remaining 10 % consisting of molecules both larger and smaller than the principal molecule. Preparation G1 is from a different stock solution prepared in exactly the same way as the stock solutions of G2, G3 *etc.*, but from a different horse serum. Fig. 2 *d* shows a late exposure from this experiment and it will be seen that the preparation is contaminated with about 20 % of a lighter molecule, presumably albumin. There seems to be no explanation of this other than a possible difference in the constitution of the parent horse serum in the two cases. Data to be presented in a subsequent paper relative to a variety of globulin preparations from human, horse and cow serum will show these preparations to be contaminated to a variable extent with larger or smaller molecules. It does not seem to be possible, merely by a process of repeated precipitation and washing, to obtain a globulin preparation with the degree of monodispersity shown by recrystallised albumin. This observation is one of considerable significance because there has been in the past, and still exists, considerable variation in the physico-chemical properties of serum globulin as recorded by different observers—particularly in regard to solubility, osmotic pressure and isoelectric point. In so far as the methods adopted to measure

these properties are only capable of giving a statistical mean result for all the molecules present, the lack of agreement among different observers may be explained by variations in the composition of different globulin preparations, such as we have found here by ultracentrifugal analysis.

The marked diminution of sedimentation constant with increasing concentration of albumin and globulin will be at once evident. Also at globulin concentrations greater than 3 % practically no active process of spreading at the boundary can be detected.

The column of Table III headed “% diff.” expresses the difference between the measured and calculated values of $n_1 - n_0$ as a percentage of the measured values. These values show an average loss of approximately 15 % over all the experiments, and a variation of individual values about the mean “% diff.” of never more than ± 5 %. This systematic discrepancy of -15 % is difficult to explain. It may be due to basing all our calculations on the apparent instead of the true base-line. If so, it is probable that all our albumin and globulin preparations contain about 15 % of protein which is so completely heterodisperse as not to affect the base-line of the curve. Another source of error, but only in the experiments with the smaller cells, lies in the fact that we cannot measure the thickness of the cell to less than 0.1 mm. because periodical tightening of the assembly rings, which is necessary to prevent leakage, causes small changes in the distance apart of the plates, and also the plates are not absolutely parallel. Changes in the refractive index of the quartz plates under high pressure may be a contributory cause. It is conceivable, also, that the sedimenting asymmetrical protein molecules are orientated in a particular plane in relation to the light beam and thus show an abnormal refractivity.

More important, however, than the extent of the discrepancy between measured and calculated $n_1 - n_0$ is the fact that the error appears to be almost entirely systematic and reproducible. It is considered that the agreement of the individual results among themselves, extending, as the experiments do, over a wide range of protein concentration, affords strong support for the validity of Lamm's fundamental equations and demonstrates the practicability of estimating protein concentrations from the scale photographs. When one considers the practical difficulties which beset these measurements of protein refraction increment, using 0.1–0.5 ml. of solution inside an optically imperfect cell and rotating at a speed of 60,000 r.p.m., the whole being observed from a minimum distance of 6 metres, it will be appreciated that the figure of ± 5 % for the error in individual experiments is a very satisfactory one. When, further, one considers the possibilities which are presented to estimate the molecular fractions in untreated native protein mixtures and mixtures of non-absorbing substances, even with an error of ± 5 %, it will be realised also what considerable satisfaction this substantiation of Lamm's equations gives rise to.

EXPERIMENTS WITH MIXTURES OF ALBUMIN AND GLOBULIN.

The general scheme underlying the preparation and investigation of these mixtures was as follows. A fresh horse serum (undiluted horse serum 1) was examined in the ultracentrifuge without previous treatment of any kind. The curve obtained (Fig. 3 *a*) showed the presence of two molecules corresponding in sedimentation constants approximately to albumin and globulin. The “albumin” component curve is unsymmetrical and represents in its total area 79.1 % of the total protein. The “globulin” curve, representing 20.9 %, is quite sym-

Fig. 3 (a). $a = 2.00$ mm.; $b = 2.14$ cm. Centrifugal force = 235,000 times gravity. Exposure taken 117 mins. after reaching full speed.

Fig. 3 (c). $a = 2.00$ mm.; $b = 3.62$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 57 mins. after reaching full speed.

Fig. 3 (b). $a = 2.00$ mm.; $b = 2.26$ cm. Centrifugal force = 250,000 times gravity. Exposure taken 92 mins. after reaching full speed.

Fig. 3 (d). $a = 3.0$ mm.; $b = 11.10$ cm. Centrifugal force = 235,000 times gravity. Exposure taken 60 mins. after reaching full speed.

Fig. 3. Ultracentrifugal sedimentation diagrams by the refraction method showing the dilution effect in serum and albumin-globulin mixtures. (a) shows the surprisingly small amount of "globulin" present in undiluted horse serum; (b) is from an experiment using a mixture of albumin and globulin, both prepared from the serum used in (a) and subjected to the usual purification procedures. They were mixed together in the same ratio and approximately the same concentration as in the original serum; (c) is from mixture 6, containing 1.53 g. albumin/100 ml. and 3.03 g. globulin/100 ml.; and (d) is from mixture 4, containing 0.61 g. albumin/100 ml. and 0.50 g. globulin/100 ml.

metrical, and the base-line is free from irregularities. An analysis of the serum by precipitation with ammonium sulphate gave 51 % albumin and 49 % globulin. The original serum was then used to prepare recrystallised albumin and reprecipitated globulin by methods identical with those already described

(p. 416), and a mixture of the two preparations in the same ratio and of very roughly the same total protein concentration as in the original serum was made up ("synthetic" horse serum 1). Here again the proportion of "albumin" was found to be abnormally high (72.7 %) (*cf.* Fig. 3 *b*). One or two tentative experiments were now tried to suggest a reason for the abnormal albumin/globulin ratio and in one of these the original serum was examined after dilution with 3 volumes of 1 % NaCl. It was noticed that the ratio had now fallen very considerably. Mixtures of albumin and globulin covering a wide range of concentrations were then made up using the same stock albumin and globulin solutions as had been used for A2, A3, A4, and G2, G3, G4, G5 and G6 (Table III) and the behaviour of which on dilution had, therefore, been examined in the ultracentrifuge. The results are shown in Table IV.

Discussion of results.

(*a*) *Sedimentation constants.* The values for $S_{w, 20}$ for the "albumin" fraction show a marked concentration effect similar to that shown by pure serum albumin. Also, if we plot the "albumin" values against the calculated value of $n_1 - n_0$ (column D), and show on the same diagram the same values for pure albumin (from Table III) it appears within the rather wide limits of comparability that the sedimentation velocity of the "albumin" fraction in a concentrated mixture is the same as the sedimentation velocity of a pure solution of albumin of the same concentration. In the case of the "globulin" values a marked concentration effect is present, and also the values from the concentrated mixtures as a whole are uniformly smaller than the values for the same concentration of globulin in pure solution. It is impossible to say whether this effect is entirely due to the viscosity and density of the albumin solution in which the globulin molecules are sedimenting, or whether any additional changes in the shape and dimensions of the "globulin" molecule have occurred. It is only at concentrations less than 0.5 % of each that the two proteins in a mixture appear to sediment independently of each other.

(*b*) *Refraction increment.* The figures in the column headed "'% diff.' (total $n_1 - n_0$)" represent the difference between the measured and calculated values of the total protein refraction increment of the mixture, expressed again as a percentage of the former. The average difference is -12.4 %, and the individual figures may be represented by the approximate formula

$$n_1 - n_0 \text{ (measured)} = n_1 - n_0 \text{ (calculated)} - (13 \% \pm 9 \%).$$

The systematic error agrees well with the value found in the experiments reported in Table III. The range of individual variation in the mixtures is, however, greater than in the case of the pure solutions. This is probably due to the greater difficulty in locating accurately the apparent base-line where, as in the mixtures, only comparatively short horizontal portions of curve can be seen. It is to be noted also that, in spite of the fact that the original globulin of these mixtures is only approximately 90 % monodisperse, the mixtures appear practically free from base-line irregularities (*cf.* Figs. 3 *c* and 3 *d*). No explanation can be offered of this. In view of the agreement between the average "% diff." values of Tables III and IV we conclude that when albumin and globulin are mixed together the total concentration of the added proteins is represented by the sum of the areas of the component curves in the diagram of the mixture. This simple conclusion assumes its full significance when we consider the refractive equivalents of the individual components in the diagram of the mixture.

Table IV. Results obtained in the investigation of horse serum and mixtures of horse serum albumin and globulin.

Protein solution	Protein conc. g./100 ml.	p _H	Cell thickness mm.	Optical scale distance cm.	Average S _w , 10 ¹³ cm./sec.	Measured $(n_1 - n_0) \cdot 10^3$ $\lambda = 366 m\mu$		Calculated average $(n_1 - n_0) \cdot 10^3$ $\lambda = 366 m\mu$		“% diff.” total $\left(\frac{a-b}{a}\right) \times 100$	Theoretical expected individual $(n_1 - n_0) \cdot 100$ $\left(\frac{c-d}{c}\right)$	“% diff.” individual $\left(\frac{e-d}{c}\right) \times 100$	Boundary spreading coefficient $\times 10^7$ cm. ² /sec.
						Individual	Total	Individual	Total				
Undiluted horse serum	A 3.75	—	2.00	2.11	2.94	7.42	14.46	10.43	13.18	— 8.9	6.76	+54.3	0.8
	G 3.50	—	2.00	2.11	4.35	7.04	7.04	2.75	7.04	— 8.9	6.42	-57.2	2.6
“Synthetic” horse serum	A 4.00	1 % NaCl	2.00	2.26	3.34	8.00	15.77	9.95	13.69	-13.2	7.02	+41.8	0.5
	G 3.82	1 % NaCl	2.00	2.26	4.72	7.68	7.68	3.74	13.69	-13.2	6.67	-43.9	3.1
1	A 2.04	Ringer	2.00	3.62	3.72	4.03	8.06	4.21	6.48	-19.6	3.24	+30.0	2.0
	G 2.00	Ringer	2.00	3.62	5.49	4.03	4.03	2.27	6.48	-19.6	3.24	-30.0	1.9
2	A 1.02	M/2.6 acetate buffer	3.00	5.00	4.12	2.02	4.04	2.56	3.85	— 4.7	1.92	+33.3	5.1
	G 1.00	M/2.6 acetate buffer	3.00	5.00	5.87	2.02	2.02	1.29	3.85	— 4.7	1.92	-33.3	3.7
3	A 1.02	M/6.7 phosphate buffer	3.00	5.00	4.16	2.02	4.15	2.18	3.50	-15.7	1.70	+28.2	4.0
	G 1.06	M/6.7 phosphate buffer	3.00	5.00	6.38	2.13	2.13	1.32	3.50	-15.7	1.80	-36.3	2.0
4	A 0.61	Ringer	3.00	11.1	4.43	1.21	2.22	1.27	2.13	— 4.1	1.16	+9.48	7.7
	G 0.50	Ringer	3.00	11.1	6.63	1.01	1.01	0.86	2.13	— 4.1	0.97	-11.3	5.0
5	A 0.127	“	12.0	11.5	4.81	0.250	0.50	0.218	0.428	-14.0	0.215	+1.87	9.1
	G 0.125	“	12.0	11.5	7.31	0.250	0.250	0.210	0.428	-14.0	0.215	-1.87	7.4
6	A 1.53	“	2.00	3.62	3.77	3.02	9.11	4.08	7.58	-16.8	2.52	+62.6	2.4
	G 3.03	“	2.00	3.62	5.14	6.09	6.09	3.50	7.58	-16.8	5.07	-44.8	1.3
7	A 2.15	“	1.50	3.80	3.79	4.24	6.77	3.91	5.30	-21.7	3.32	+17.8	2.1
	G 1.26	“	1.50	3.80	6.18	2.53	2.53	1.39	5.30	-21.7	1.98	-29.8	5.0
8	A 0.48	“	3.00	11.6	4.57	0.95	1.42	0.98	1.36	— 4.2	0.91	+7.7	7.8
	G 0.23	“	3.00	11.6	6.75	0.47	0.47	0.379	1.36	— 4.2	0.45	-15.6	4.8

G = Globulin.

A = Albumin.

It is at once apparent in all the more concentrated mixtures that the concentration of the "globulin" component is much less than corresponds to the globulin added, and that the concentration of the "albumin" is correspondingly much greater. In an attempt to express equitably the relative loss of globulin and increase of albumin we have firstly divided the discrepancy between measured and calculated total $n_1 - n_0$ between the two components in the ratio in which they were originally added together and thus we obtain a "theoretically expected" value for the refraction equivalent of the area of each component curve in the diagram (*cf.* column headed "theoretically expected" individual $n_1 - n_0$). The difference between this value and the value calculated for the refraction equivalent of the actual area of the component curve, expressed as a percentage of the former, is shown under "'% diff.' (individual $n_1 - n_0$)". A consideration of the figures in this column leads to the following general conclusions. The "albumin"/"globulin" ratio is always shifted in the direction of increased "albumin". The extent of shift increases markedly with increasing concentration of total protein and is particularly marked with high relative concentrations of globulin (*cf.* mixture 6, Fig. 3 *c*). The phenomenon seems to appear only at concentrations greater than 0.25 % total protein. From a comparison of mixtures 2 and 3 it is deduced that hydrogen ion concentration and the nature of the inorganic ions present seem to have little, if any, effect. The results suffice to demonstrate the existence in mixtures of albumin and globulin of a type of molecular dimensional equilibrium the nature of which is primarily dependent on the concentrations in which the two proteins are present. Before discussing some theoretical aspects of the phenomenon we may consider briefly the boundary spreading coefficients shown also in Table IV.

(*c*) *Boundary spreading coefficients.* In Fig. 4 we have plotted the value of the spreading coefficients of the "albumin" boundaries in each mixture against the calculated values of $n_1 - n_0$ for the same fraction. A mean line has been

Fig. 4.

drawn through the points and its course demonstrates the marked suppression of boundary diffusion which takes place at high protein concentrations and under the roughly standardised sedimentation conditions of our experiments. We have also shown on the same diagram as ringed points the values from Table III for the four albumin solutions, and it will be seen that these points lie tolerably well on the curve. It appears, therefore, that the degree of spreading at the boundary of the "albumin" fraction in a mixture is not significantly greater than the degree of spreading at a sedimenting boundary of pure albumin

of the same concentration. This we consider to be strong contributory evidence in favour of the "albumin" fraction in a mixture being dimensionally homogeneous. We can say nothing with regard to the homogeneity of the globulin fractions because the spreading coefficients cannot be compared with each other owing to the variable effect of the albumin concentration.

GENERAL DISCUSSION.

In the first place we may summarise many of the above data by the statement that only when the total protein concentration is less than 0.25 % does a solution of albumin and globulin behave ideally. At all higher concentrations the diffusion, sedimentation and relative concentration of the component proteins are abnormal.

Throughout this work we have referred consistently, when speaking of the mixtures, to the "albumin" and "globulin" components. This terminology is merely a matter of convenience since the available data are insufficient to define accurately the true nature of either molecular fraction. It can be said from a consideration of the sedimentation constants that the average molecular weight of the lighter fraction corresponds in all mixtures approximately to that of albumin, and from considerations of the degree of boundary spreading, that the fraction is probably homogeneous. On the other hand, in concentrated mixtures it is present in much greater amount than the monodisperse albumin which may be obtained from the same mixture by treatment with ammonium sulphate. With regard to the heavier fraction we can only say that in diluted mixtures it has all the characteristics of salted-out globulin and in concentrated mixtures it may still be the same substance. In the latter case, however, it is present in amount much less than the practically monodisperse globulin which may be obtained by precipitation.

The question has often been asked whether in a solution of two proteins of different isoelectric points mutual neutralisation of the electrical charges on the colloidal particles may not take place, such as occurs with lyophobic colloids of opposite charge and leads to precipitation. Hardy [1905-06] says "The proteids of serum are electrically inactive. Neither the whole nor any fraction moves in a field. It is not possible to detect a trace of 'ionic' protein. Dialysis or dilution disturbs the equilibrium and 'ionic' globulin appears...". For some reason Hardy's views seem to have been largely neglected. Adair and Robinson [1930, 2], however, say that it is possible that Hardy failed to detect any ionic globulin because of the low ratio of the charge to the mass and the effects of the ion atmosphere. Tiselius [1930] using a considerably more accurate cataphoresis technique than Hardy has examined various artificial mixtures of egg albumin, Bence Jones protein, phycocyan, phycoerythrin and also some native mixtures and concludes that oppositely charged proteins can exist together in solution without influencing each other to any great extent. The concentrations of proteins examined by Tiselius were all less than 0.5 % and it is not possible with his method to examine solutions much stronger than this. The possibility of interaction between proteins at higher concentrations, such as we have been examining in the ultracentrifuge, therefore, still remains and indeed becomes more of a probability in view of Hardy's carefully executed, although somewhat crude, electrophoresis experiments. It is indeed difficult to visualise any other method by which albumin and globulin may influence each other, as they do in the above mixtures, than by virtue of their electric charges, and the fact that the p_H of the medium has, if any, a minor effect does not alter the possibility

because the force operative in producing the union would be more a function of the difference between the isoelectric points than of the hydrogen ion concentration of the solution.

By whatever means the two molecules influence each other the important fact which is revealed in the above experiments remains, *viz.* that in some manner the interaction involves a reduction in the molecular size of part of the globulin to approximately the size of albumin. It is impossible to determine on the basis of the available data the real mechanism of this. We may say, however, that the work of Svedberg and collaborators in recent years on the structure of the protein molecule and on the p_H stability region of the proteins makes the idea of a reduction in the size of the globulin molecule, under the conditions with which we are dealing here, not at all such an improbability. When the molecules of both albumin and globulin at p_H values below 4 and above 9 disintegrate in a reversible manner into smaller molecular units [Svedberg and Sjögren, 1930] it is clearly a possibility that in circumstances which may cause an electrochemical union of albumin and globulin the same molecular dimensional forces may be operative and ensure that the resulting product has a molecular weight no greater than 68,000.

There are one or two interesting theoretical aspects of the phenomenon which we have described. If there exists in undiluted serum and concentrated mixtures of albumin and globulin a form of equilibrium between a low molecular fraction and a high molecular fraction, then in the process of centrifugal separation of the two a state of unstable equilibrium must exist. Globulin molecules ought to be continually produced in the solution between the two boundaries, and as these sediment more rapidly than the "albumin" molecules among which they originate, the net result ought to be a steady fall in the area of the "albumin" curve. This has never been found. We do not know, however, what order of velocity constant might be associated with the equilibrium reaction or with the process of aggregation by which the globulin is subsequently formed, and it is conceivable that more time than the centrifuge allows is required for an appreciable formation of globulin in the cell.

If we consider the state of affairs at the "globulin" boundary in undiluted serum it is by no means certain that the concentration of albumin is uniform here. It has been shown that when globulin sediments in a medium containing albumin the sedimentation velocity of the globulin is markedly diminished by the viscous effect of the albumin. It is possible, if not probable, that in the same circumstances the rapidly sedimenting globulin molecules exert a viscous traction on the albumin molecules to cause them to sediment more rapidly. If this should be the case more albumin would cross in unit time a unit cross sectional area in the lower ("globulin" + "albumin") part of the cell than in the middle ("albumin" only) part. The net effect would be again a steady diminution in the area of the "albumin" curve analogous to the effect of the increasing centrifugal force on the protein molecules as they sediment away from the centre of rotation.

As already stated, no such abnormal diminution of the area of the "albumin" curve has been recorded. Without some independent evidence of the existence and extent of such a viscous-attractive force it is, of course, impossible to have any exact idea of the concentration disturbance which it might be expected to give rise to. It is also possible that it might give rise to rather low values for the "globulin" areas by superimposing on the positive globulin concentration gradient a negative "albumin" gradient, but it is not conceivable that it could give rise to effects sufficiently great to furnish an explanation of the

phenomenon described here. In addition, any apparent dilution of the "globulin" fraction due to an extraneous cause of this type would also represent a loss in the total protein represented in the sedimentation diagram, and this we know to be constant. Also, no concentration disturbance at the "globulin" boundary can explain the abnormally high concentration and apparent homogeneity of the "albumin" fraction. We can find no explanation for all the facts other than the one advanced here, *viz.* that we are concerned with a truly molecular phenomenon governed by some unknown form of interaction between albumin and globulin.

We have thought it wise to postpone to the end of this paper a consideration of the relationship which the results bear to those of Mutzenbecher, who preceded the writer in this field of work at Upsala. The first experiments which we made with the ultracentrifuge were designed to extend some of Mutzenbecher's experiments, and it was during the course of these experiments that we realised that the phenomenon of a shifting base-line, which was a continual source of trouble to Mutzenbecher, was, at least in part, of mechanical origin. Mutzenbecher believed that it was due to the presence in the solution of low-molecular products, and therefore based all his calculations on the true base-line or x -axis without, at the same time, observing all the technical precautions which Pedersen has shown to be necessary if this is done. Also, whereas the conclusions of the present paper are based on determinations of the individual refraction increment of each fraction in a mixture and depend crucially on the preliminary experimental verification of Lamm's relationships, Mutzenbecher, who had no such experimental verification to rely on, has based his conclusions entirely on calculations of the concentration ratio of the fractions. We believe that these, in the main, are the explanations of the different conclusions arrived at because it is clear that the experimental results in the two cases are in essential agreement.

Mutzenbecher has concluded that there exist in normal horse serum two fractions "identisch mit den rein darstellbaren Fraktionen Albumin und Globulin". We prefer to draw the conclusion that there exist in normal horse serum two fractions of very approximately the same molecular dimensional properties as isolated horse serum albumin and globulin, but considerations of the concentrations in which the two fractions are present suggest strongly that at least the lighter fraction cannot be identical from a chemical structural point of view with pure albumin.

SUMMARY.

1. It has been found possible by means of a modification of Lamm's refractive index method of following protein sedimentation in the ultracentrifuge to obtain satisfactory sedimentation photographs using very concentrated protein solutions.

2. It has been shown that recrystallised horse serum albumin in solution exists in a monodisperse state over a wide range of concentration. Reprecipitated horse serum globulin, on the other hand, has not been obtained in a completely monodisperse form, being always associated to a variable extent with molecules both larger and smaller than itself. It is suggested that much of the disagreement in the literature over the characteristic physico-chemical properties of globulin may be attributed to the variable composition of different preparations of globulin. The composition, from a molecular dimensional point of view, of a particular specimen of globulin remains sensibly the same over a wide range of concentration.

3. A sample of native horse serum, quite untreated in any way, has been examined in the ultracentrifuge and shown to contain two molecular fractions. The lighter fraction represents nearly 80 % of the total protein and the heavier fraction 20 %, despite the fact that by ordinary precipitation analysis the albumin/globulin ratio of the serum is approximately 1.

4. It has been found that on simple dilution of this horse serum the ratio of the two fractions obtained by ultracentrifugal analysis approaches the analytical value for the ratio of albumin to globulin.

5. A series of artificial mixtures of albumin and globulin has been investigated in the ultracentrifuge and it has been shown that only at very low concentrations does the mixture behave like an ideal solution. At all concentrations greater than 0.25 % of total protein the sedimentation constants, boundary spreading coefficients, and concentrations of the two fractions are abnormal. With increasing total concentration there is a steady change in the concentrations of the two fractions so that the lighter fraction always increases in amount and the heavier fraction decreases correspondingly—until the state of affairs present in the original serum is reproduced. The phenomenon is primarily a concentration effect and apparently reversible. The hydrogen ion concentration and nature of the salts in the solution appear to have little, if any, effect.

6. A consideration of the sedimentation rate and degree of spreading at the sedimenting boundaries in this series of mixtures indicates that the lighter species in a concentrated mixture has approximately the same molecular weight as albumin and appears to be homogeneous in the centrifugal field. It is only possible to obtain a very rough idea of the average molecular weight and homogeneity of the heavier species, and the most that the results are able to indicate is that this fraction may be identical with pure globulin.

7. The results indicate that it is not possible to regard the lighter molecular fraction in undiluted horse serum as identical with isolated horse serum albumin.

8. Throughout the work the opportunity has been taken to verify experimentally the basic equations of Lamm's refractive index method.

There will be reported in subsequent papers experiments which employ the new method to investigate the structure of the proteins in normal and pathological sera of various types. Throughout the whole of the work I have been greatly indebted to Prof. Svedberg for his inspiring interest and advice and for the innumerable facilities which he has readily placed at my disposal and which have made the research possible. It gives me the greatest pleasure also to express my gratitude to his assistants, in particular to Dr Pedersen and to Dr Lamm, for much kind help and valuable criticism.

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