The Use of the Gouy Diffusiometer with Dilute Protein Solutions. An Assessment of the Accuracy of the Method

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The interference optical method of observing the refractive index gradient changes occurring in an initially sharp diffusing boundary has been developed on somewhat different lines in this country and the United States. Thus, Coulson, Cox, Ogston & Philpot (1948) and Ogston (1949) have developed an apparatus (the Gouy diffusiometer) primarily for use as a very rapid micromethod, and have used it mostly for rapidly diffusing substances or reasonably concentrated protein solutions, while Kegeles & Gosting (1947) and Longsworth (1947), utilizing the optical and cell systems of the Tiselius electrophoresis apparatus, have emphasized the greater accuracy of the method, using larger quantities of material and much longer diffusion times; results on rapidly diffusing substances only have so far been published.

It is the purpose of the present paper to show that the English form of apparatus, somewhat modified in minor details, may be used to give rapid results of reasonably good accuracy on protein solutions down to a concentration of about 0·2%, using only 1 or 2 ml. of solution. This makes the method particularly suitable for use with electrophoretically isolated components of protein mixtures, on which the interest of this laboratory has been concentrated.

With a cell suitable for the above quantities, and when using dilute (0·2%) albumin solutions, the downward displacement of the outermost interference minimum, on the recording of which the method depends, is small (about 7 mm.) even when the boundary is sharp. Within 20 min. the displacement is under 2 mm. and it is not considered that values less than this figure may justifiably be used in the calculation. Accordingly, it becomes an essential feature of the present apparatus that the diffusion record shall be usable from very soon after the boundary has commenced to diffuse. This presents no particular difficulties with rapidly diffusing substances (such as sucrose), where any slight irregularity in the concentration gradients in the boundary rapidly disappears as the boundary spreads, but with the more slowly diffusing proteins, particularly when the density increment across the boundary is small, minute disturbances tend to persist proportionately very much longer, and cause anomalous diffusion coefficients to be obtained. This has also been the experience of Longsworth (1947) even with the American form of apparatus, where the later stages of diffusion may readily be observed; this work also demonstrated the difficulties involved in the use of any form of partition cell. These difficulties have been overcome, however, by certain slight refinements of experimental technique and minor modifications to components of the apparatus. As a result of this work, which has dealt with a single, well characterized protein, bovine serum albumin, a considerable amount of diffusion data have become available which permit the calculation of a diffusion coefficient for this protein possibly more accurate than values obtained by other methods.

In addition, with a few of the solutions for which diffusion coefficients were determined, ultracentrifuge experiments were performed, and the sedimentation constants calculated. The two sets of data have been combined to give a value of the molecular weight somewhat lower than the accepted value.

EXPERIMENTAL

The apparatus and its use are fundamentally the same as those described by Coulson *et al.* (1948) and Ogston (1949). The several factors which have been found to be of critical importance if reproducible results are to be obtained with dilute protein solutions have been examined. and where this has led to the modification of a component or procedure, this will be described in detail; otherwise, where the procedure does not differ significantly from that previously published, no mention is made of it.

The diffusion cell. This closely follows that described by Coulson *et al.* (1948), but the thermostating compartments are omitted, as the cell is mounted within a small water bath (see below). Possible sources of disturbance in the outflow line have been minimized by omitting a regulating tap or any other form of control; the flow rate is reduced to the correct order by passage through a 50 cm. length of 0·04 cm.-bore capillary. The latter is coiled so that it is completely mounted within the water bath; the outflow rate is about 0·8 ml./min.

The pipette. This component has a very great effect on the boundary formation; it has been found that two conditions relating to pipette size are essential: (*a*) Near equality of free surface areas of solution and solvent. This was specified by Coulson *et al.* (1948). If this condition is not fulfilled, flow rates of solution and solvent differ, the boundary becomes

sharper on one side than the other, and the position of maximum concentration gradient does not coincide with the vertical midpoint of the exit slit. Errors then arise from two sources: the boundary may never become sharp enough on its more diffuse side to give constant values of the fractional refraction increment, and, more important, such uneven sharpening produces a boundary which tends to diffuse anomalously in the early stages. (Boundary sharpening by the present method can never, of course, lead to an initially ideal (Gaussian) boundary, but it appears that a boundary sharpened symmetrically attains Gaussian form very much more rapidly.) (*b*) Avoidance of surface tension effects in the bath, through which thermostated water is pumped at a temperature within 1° of room temperature (21–25°). This bath, of cast aluminium, is about 5 cm. wide (along the optic axis) × 10 cm. deep × 15 cm. long, and has optically flat windows opposite the cell position. The cell has two short Perspex arms which serve to clamp it to the top of the bath, and a third mounting is provided by the outflow tube, which passes over the bath wall and is secured to the latter by a rubber-lined brass clip. The bath is supplied from a large (10 l.) reservoir, provided with both cooling coils and heater. A constant-level device (mounted apart from the water bath) drains the outgoing water back to the reservoir.

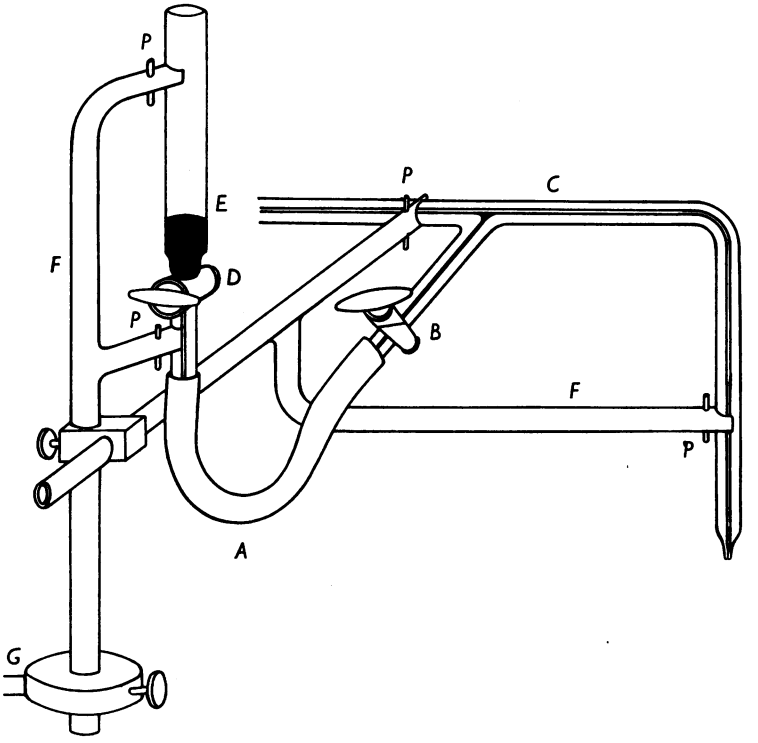

Fig. 1. Boundary-forming device. *A*, Polyvinylchloride reservoir of mercury; *B*, *D*, taps closed during diffusion; *C*, outflow capillary from cell; *E*, secondary reservoir of mercury. Rubber bands (not shown) round the pegs *P* clamp the glass tubes to the supporting frame *F* (aluminium tubing), which is fixed to the cell thermostating bath by the mounting *G*.

cell. A pipette of size conforming to the above condition leaves very little clearance between pipette and cell walls, and consequently surface tension effects become pronounced. Such effects, which may be a prolific source of boundary disturbances, disappear if the clearance is greater than about 1·2 mm.

Consideration of these conditions has led to the use of a thin-walled (0·4 mm.) pipette, of radius such that the ratio of free cell to pipette surface areas is 3:2. After insertion in the cell, the pipette is clamped in a central and vertical position; with this system, if boundary sharpening is rapid, the errors listed above are not encountered.

Thermostating. In view of the importance of maintaining accurate temperature control, the cell is thermostated on all surfaces; this is done by mounting it within a small water

Boundary former. This device (see Fig. 1) is similar to, but somewhat simpler than, that described by Ogston (1949), and has proved very convenient in operation. The mercury reservoir is contained in stout polyvinylchloride tubing (*A*) connected by a capillary tube, fitted with a tap *B* to the outflow capillary *C*. To form a boundary, the tap *B* is opened, and the tube gently squeezed until a bead of mercury about 1 mm. long has been forced into the exit capillary; the tap is closed before the mercury bead reaches the end of the outflow capillary, thus sealing off the mercury reservoir from the diffusing boundary. After four or five runs the reservoir is refilled by opening a tap *D* at its upper end which connects it to a vertically supported wide-bore tube *E* containing several ml. of mercury. The advantages of this system, apart from its speed and simplicity, are that the

compressible reservoir is sealed off from the diffusion cell, and the pressures on either side of the tap *B* are so nearly equal that the possibility of leakage is remote. Although some 12 cm. of the outflow capillary are not thermostated in this arrangement, by working within one degree of room temperature, no anomalies traceable to this cause have been detected.

Several self-breaking siphon boundary-forming systems were tried. These have the advantage that they could be mounted within the water bath and thus completely thermostated, and also that the operation of boundary forming could be made completely automatic; none, however, was found to give the necessary abrupt cessation of flow which is characteristic of the mercury bead type.

Optical system. This closely follows the one described by Coulson *et al.* (1948). A 250 W. compact-source mercury lamp is used, and a combination of a green and a 'minus-red' filter proved adequate to isolate the $\lambda = 5461$ A. line. The light source slit was kept at a constant width.

The stop diaphragm is mounted in front of the cell. The construction of this is of some importance as the sharpness of the diffracting edges is a determining factor in image definition. Two types were found satisfactory and have been used in this investigation: (*a*) Photographic type. This was made by photographing on to maximum-resolution plates, with a magnification of 1/10, a drawing of the stops arrangement ten times the desired size. By over-exposure and over-development, followed by reduction, a negative of extremely high contrast was obtained, and the definition of the stop images with this diaphragm was very good. (*b*) Built-up type. The disadvantage of the photographic type of diaphragm is that it introduces two more glass-air interfaces into the system, with consequent loss of intensity. The best results were obtained with a diaphragm which was built up of small pieces of razor blade, assembled in the correct positions under a measuring microscope. Completely straight, very sharp, interference lines were observed with this diaphragm.

Deviating block and prisms. The plane glass deviating block in the path of the light traversing the double stop (forming the reference trace in the recorded image) is of thickness 1·2 cm. The emergent light from the block, which is deviated vertically by a distance of 3·8 mm., is then deviated laterally, by an amount equal to the lateral separation of the stops, by a pair of prisms arranged horizontally in the light path. By this means the light forming the reference trace is made to record exactly vertically above the light traversing the cell. This is very important, as the distance between the traces is measured at right angles to the reference trace, and it is thus essential that the two traces should have been recorded simultaneously. In the absence of the prisms any small disturbance caused by an irregularity in the drive of the scanning camera would affect the two traces to different extents.

Camera. The lens arrangement differs from that of Coulson *et al.* (1948) only in that a cylindrical lens of focal length 2·5 cm. is used to focus the final image. The scanning arrangement consists of a lead screw (0·25 in. British Standard Fine) mounted in twin ball races at one end, and driven via a two-speed reduction (1/3 and 1/9) gear box mounted directly on the camera back. The gear box is driven by a flexible drive from a 112 rev./min. (max.) motor, the speed of which is also controllable by a variable resistance. By this means, scanning times of from 90 sec. to 45 min. are obtainable. As the camera is thus subjected to slight mechanical vibration, it is not mounted on the optical bench, but directly on to the main concrete bench. There is no direct contact between the camera tube and the lens cover box mounted on the cell platform, and no vibration was visible on the latter when the drive was on at full speed. A micro-switch on the camera back is actuated by the plate holder when the latter has completed its traverse of the diffusing image, and breaks the drive motor circuit.

Mounted vertically above the optic axis, and as close as possible to it, is the time-marking device. A small bulb blackened except for a narrow strip on one side, illuminates a very narrow vertical slit, which is focused on the camera back by a short focus (0·5 in.) cylindrical lens. When the bulb is alight a very narrow vertical line is produced, directly above the stop images. The time marker itself utilizes the mechanism of a 'Camera control' unit used for aerial photography control (R.A.F.). This consists of an electrically actuated mechanical escapement, the period of which is independent of input fluctuations. By re-wiring the unit, and making some mechanical adjustments, the escapement unit was made to provide a pulse of about 0·1 sec. duration to the marker bulb. The interval between pulses can be set at any value between 2 and 50 sec., but for greater reproducibility, two stops were attached to the setting dial so that time intervals of either 10 or 45 sec. could be provided. The accuracy and reproducibility of this unit are quite good; once it is warm (i.e. after running for 20 min.) the time mark interval varied by less than 0·1 %.

Photography. After many trials it was found that best results were obtained by using a fine grain panchromatic plate (Ilford Thin Film Half Tone) and a mildly contrasting developer ('Wellington Borax'). More rapid emulsions produce a loss in definition, and are more liable to shrinkage during processing.

Measurement. With this apparatus the intensity on the focal plane is high, and hence the interference minima narrow, so that precise location of the minima is possible when observed under fairly high ($\times 50$) magnification. The travelling microscope used has a lead screw (tested by the National Physical Laboratory) of 1 mm. pitch with a drum graduated in tens of microns; all readings are estimated to the nearest micron. Illumination of the plates was from below using a glazed screen above the lamp to provide a diffuse source. The stage of the microscope is rotatable, so that after the plate has been clamped down, one traverse of the microscope can be accurately lined up along the reference trace.

In general, four measurements of the double stop records were made, followed by about ten measurements of the diffusion trace.

General. The whole apparatus was mounted on a stone bench in a sub-basement laboratory, which is some 20 ft. below ground level. Vibration, even during the day, was very slight and did not appear to have appreciable effect. All the electric motors, pumps, etc., were mounted together on a movable chassis, away from the main bench. Finally, the whole apparatus was supplied with stabilized electric power so that fluctuations in scanning speed, light intensity, etc., were obviated.

Protein. Throughout this work crystallized bovine plasma albumin (Armour) has been used. This substance contains less than 0·01 % globulins and has been used in many physical chemical investigations. (See, for example, Longsworth & Jacobsen, 1949.) Its electrophoretic pattern, obtained in the same buffer (sodium phosphates, *I* 0·10,

pH 6·8) as was used for the majority of the diffusion experiments, is shown in Fig. 2. The lack of enantiography between the ascending and descending boundaries is very marked, and resembles the behaviour below the isoelectric point reported by Longsworth & Jacobsen (1949), and ascribed by them to interaction between the different forms of the protein. Alberty, Anderson & Williams (1948), in an isoelectric electrophoresis spreading experiment, concluded that about 15% of a rather heterogeneous component was present.

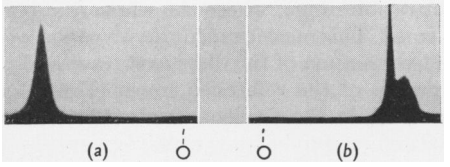

Fig. 2. Electrophoresis of bovine serum albumin. (*a*) Ascending (anode limb), (*b*) descending (cathode limb) boundaries after 207 min. at 3·64 V./cm. Protein concentration 0·8 % (w/v), in sodium phosphate buffer, *I* 0·10, pH 6·80. (○, ○, position of original boundaries before passage of current.)

For diffusion, the solutions were prepared by dissolving the protein directly in the buffer and then dialysing against a large excess of buffer for 2–4 days at 2–3°. Before running, the dialysis vessel and contents were allowed to come to room temperature.

Experimental technique. The distances relating to the separation of the reference trace from the optic axis (m_1, m_2, m_3, in the notation of Coulson *et al.* 1948) have been found, in agreement with theory, to be independent of the buffer used, and consequently are not recorded for every diffusion run. The cell and contents are allowed to come to temperature equilibrium over about 30 min., after which a preliminary run is performed in order to check the height of the stop diaphragm (adjustment of this is seldom necessary): the boundary is sharpened for not less than 60 sec. before diffusion is allowed to start. This amount of outflow ensures that the contents of the lower part of the cell are renewed at least twice; thorough clearing of the cell has also been recognized by Gosting & Morris (1949) to be necessary for the removal of any minor anomalous concentration gradient adjacent to the boundary.

As the boundary is sharpened on one side of the cell only, the interference bands produced in the focal plane during boundary sharpening are greatly tilted; as diffusion proceeds they flatten out, a process which, with albumins, is complete after about 100 sec. The first 2 min. recording of the diffusion trace are accordingly not used for measurement.

In general, diffusion experiments have been recorded in duplicate (the pipette contains sufficient for four runs), about 30 sec. recording of the fractional part of the refraction increment (sharp boundary) and from 15 to 25 min. of diffusion being obtained in each case.

Calculation of the diffusion coefficient

D is calculated from the equation (Coulson *et al.* 1948)

$$D=\frac{\Delta(1/X^2)}{\Delta t}\,v^2\beta^2\,\frac{F^2\lambda^2}{4\pi}, \qquad (1)$$

where X is the distance of the outermost interference minimum (sometimes referred to as a 'dark band') from the optic axis, t the time, v the refraction increment across the boundary, and β a function, varying with v, the computation of which has been described and will be discussed below. F is the distance of the spherical lens used to focus the interference pattern from the screen, and should be equal to its focal length; λ is the wavelength of the light used. The first (slope) term in the equation is determined from approximately ten measurements of X at suitably spaced time marks, and either plotting the points graphically or by calculating the best straight line through them. The whole number part of the refraction increment v is equal to the number of interference minima (dark bands) in the diffusion record. When the fractional part is small (i.e. less than 0·2) the double stop record consists of three equally spaced interference maxima, the displacement of this trace is positive, and the first dark band in the single stop record is narrow and very close to the optic axis. Where the fractional part is about 0·5, the double stop record consists of four interference maxima, and the displacement is positive or negative depending on which pair of minima are measured. When the fractional part approaches unity, the trace reverts to the equally spaced, three maximum type, but the displacement is negative; the first dark band is now quite broad and well separated from the optic axis, and indeed it is frequently possible to detect the next band more or less on the optic axis. (The presence of this band, the position of which, in common with the one next to it, is not defined accurately by the quarter-wave relationship (Kegeles & Gosting, 1947), is rather arbitrary, as it depends on the relation between the intensity distribution about the optic axis when the boundary is sharp, and the latitude of the photographic plate.) When negative displacements of the double-stop record are encountered, therefore, the first band is counted as that one which is well separated from the optic axis, and the fractional part as the difference between unity and the negative relative displacement; this gives the same result as determining the fractional part from the less well defined positively displaced minimum in the double stop record.

Difficulty in counting the whole number of minima (single-stop record) has been encountered by English & Dole (1950), who adopted a graphical extrapolation procedure. Gosting, Hanson, Kegeles & Morris (1949) take the whole number to be one less than the number of interference maxima wholly or partly resolved from the optic axis. This procedure gives the same result as the one outlined above.

The function β. This quantity is defined (Ogston, 1949) as

$$\beta=e^{-z^2}, \quad \text{where} \quad z=\frac{x}{2\sqrt{Dt}};$$

x is the vertical distance from the centre of the boundary and t is the time since diffusion commenced. The relation between v and z for the outermost minimum is (Kegeles & Gosting, 1947; Ogston, 1949)

$$v\left[\frac{2}{\sqrt{\pi}}\int_0^z e^{-z^2}\,dz-\frac{2ze^{-z^2}}{\sqrt{\pi}}\right]=\frac{3}{4}.$$

A table of corresponding values of v and β was constructed, using values of the probability functions obtained from *Tables of Probability Functions* (Vol. I, Federal Works Agency, Works Project Administration, City of New York, 1941); in this computation, the Airy integral refinement of

the quarter-wave theory (Gosting & Morris, 1949) was introduced, the value 0·75867 being substituted for '$\frac{3}{4}$'.

Diffusion coefficients were corrected to water at 20° by the factor $\frac{293 \cdot 1}{T} \times \frac{\eta_{T,s}}{\eta_{20,w}}$, using the data of Svedberg & Pedersen (1940) and Landolt-Bornstein, supplemented, in the case of veronal buffers, by relative viscosity measurements in a B.S. Ostwald viscometer.

Sedimentation constant determination

Sedimentation constants were determined in a 'Spinco' electrically driven ultracentrifuge, which has been described in the literature (see, for example, Smith, Brown, Fishman & Wilhelmi, 1949). Runs were made over a range of concentration, five photographs of the sedimenting boundary being obtained at regular intervals in each case. Temperature measurement of the rotor was by contact thermocouple at the beginning and end of each run; another couple in the bearing of the drive provided a continuous check on the temperature at this point: this is probably closely related to the rotor temperature (Pickels, 1950). In some runs (on very hot days) the rotor chamber was refrigerated. In no case in this work did the rotor show more than 1·5° temperature difference between the beginning and end of the run, and accordingly the effective rotor temperature was taken as the mean of initial and final values.

The sedimentation constant S was calculated from the equation

$$S = \frac{\Delta \ln x}{\Delta t \omega^2},$$

where x is the distance of the sedimenting boundary (taken as the peak of the schlieren diagram, this being symmetrical) from the centre of rotation, t the time, and ω the angular velocity.

The experimental records were measured up with the travelling microscope described earlier, using a magnification of 25, the positions of reference marks and boundary maxima being determined to the nearest 10 μ. The term $\frac{\Delta \ln x}{\Delta t}$ in the equation was then determined from the slope of the graph of ln x against t; the points obtained always lay on very good straight lines.

Sedimentation constant values were corrected to water at 20° in the usual way.

Possible accuracy of the Gouy method

Previous values of the diffusion coefficient of bovine serum albumin, in common with those referring to most other proteins, have been obtained either by the Lamm (1937) scale, or the diagonal schlieren method. Both of these have been criticized (Kegeles & Gosting, 1947), and although the former method is probably more reliable, it appears likely that results obtained by such methods, depending on simplified optical theory, may be somewhat high. Now that the theory of the interference optical method has been shown to be adequate (Longsworth, 1947; Ogston, 1949) it has theoretically greater possibilities of accuracy; by surveying the errors likely to be encountered in the experimental determination of the variables v, X, and t it is possible to assess how far these possibilities are fulfilled with the present form of apparatus.

Errors of measurement. (*a*) *The refraction increment.* As described above, the fractional part of the increment is obtained by using the double stop-sharpened boundary system as a Rayleigh refractometer. The error in v depends, therefore, solely on the accuracy with which the relative displacement of the Rayleigh pattern can be measured, and of course will be much more important in the low concentration range, where the whole number part of v is small. This measurement involves the location of the two minima of the displaced trace and one of the minima of the reference trace. (The reference distance, m_3 in the Coulson *et al.* (1948) notation, may be assumed to be known very accurately as the symmetrical, undisplaced, pattern is very easy to locate precisely.) Experience has shown that under the best conditions of definition, the minima may be located to about 1–2 μ., so the maximum error to be expected is ± 4 μ. The separation of the displaced minima is 123–129 μ., so the error in v when the whole number part is, for example, 8, is about $\pm 0 \cdot 03$ (eight bands in the present cell corresponds to a protein concentration of about 0·2 %, which is probably approaching the lower useful limit of concentration). The corresponding error in β is 0·0006, so the final error in the squared term of Eqn. 1 has a maximum of ± 1 %. By making four determinations of the fractional part, this error will be reduced to about $\pm \frac{1}{2}$ %.

(*b*) *The slope.* The useful X values for this type of solution range from about 5 to 2 mm., with an estimated maximum error of location of 10 and 3 μ. respectively, under ideal relative exposure conditions. The maximum error in the slope, for diffusion times of 15–20 min., will then be about $\pm 0 \cdot 4$ %.

(*c*) *The constant term.* Only errors in F affect this term. Small errors in F may arise owing to the focusing procedure; the focal length of the lens having been determined, the camera back was set at this distance (50·15 cm.) from the centre of the lens by manual adjustment, then the first lens (that producing parallel light) was moved until the image definition on the camera screen was maximal. Positioning errors of up to 1 mm. are possible in this procedure, so that the maximum error in the constant term will be approximately $\frac{1}{4}$ %. Thus the total error to be expected in the determination of D from a plate on which all the relevant information has been recorded exactly is about 1·1–1·2 % (when $v \sim 8$).

In practice, further errors must occur from sources such as small temperature variations (affecting both v and the slope of the diffusion curve), minor vibrations in the building and shrinkage of the photographic emulsion during processing. With this system, errors in the location

of the outermost interference minimum in the early stages of diffusion, owing to the superposition of the Fraunhofer pattern from the stop (see Ogston, 1949) are very slight, because of the very high relative intensity of the diffusion pattern.

RESULTS

As interest accrues in the accuracy of the method rather than in the actual value of the diffusion coefficient obtained, some of the results are presented in statistical form. Table 1 shows the reproducibility of measurement of the fractional refraction increment in one particular experiment, Table 2 the accuracy of the slope determination for three different values of v, and Table 3 the general

Table 1. *Results of twenty-five measurements on double-stop record displacement*

Plate P26
Distances in microns
Reference distance 3746 μ.
$v = 7+$
Mean measurement 3762·6
Extremes 3760, 3764
S.D. 1·18
Estimate of fractional increment 0·14 ± 0·02

Table 2. *Accuracy of slope determination: ten measurements of X in each case*

Exp. no.	P27	N88	N100
v	7·16	24·00	8·78
Slope	1·7995	0·111003	1·1094
Standard error of slope	0·0110	0·000406	0·0044
Estimate of slope	1·7995 ± 0·0220	0·1110 ± 0·0008	1·1094 ± 0·0088

Table 3. *Results of ten experiments on solution* N II

(Phosphate, I 0·10. Temp. = 25·0°)

	v	Slope	$D_{20,w}$
Mean	13·62	0·436	6·12
Extremes	13·61, 13·64	0·432, 0·441	6·07, 6·18
S.D.	$0{\cdot}10_5$	0·0030	$0{\cdot}040_1$

S.D. of mean diffusion coefficient = 0·0127. Estimate of diffusion coefficient = 6·12 ± 0·03.

Table 4. *Diffusion coefficients of bovine serum albumin over concentration range* 0·17 (v = 6·16) *to* 0·89 % (v = 32·4)

(NaP refers to (sodium) phosphate buffer, pH 6·8, NaV to veronal buffer, pH 8·6. Figures after solvent composition indicate ionic strength.)

Solvent	v	$D_{20,w}$	Solvent	v	$D_{20,w}$
NaCl, 0·20	6·16	6·14	NaCl, 0·20	8·78	6·15
	6·16	6·13		8·79	6·11
NaP, 0·10	6·55	6·24	NaP, 0·10	8·86	6·14
	6·55	6·24			
	6·95	6·17	NaCl, 0·20	8·90	6·20
NaCl, 0·20	6·99	6·17		8·90	6·20
	7·00	6·08	NaV, 0·10	9·30	6·11
NaCl, 0·05	7·03	6·04		9·31	6·10
	7·04	6·23	NaP, 0·10	9·88	6·20
	7·05	6·15		12·58	6·19
	7·05	6·19		13·62*	
	7·07	6·13	NaP, 0·10	16·40	6·13
	7·07	6·07		16·38	6·09
NaCl, 0·20	7·12	6·17		16·50	6·19
	7·12	6·16		16·52	6·16
	7·12	6·14		19·29	6·17
NaP, 0·10	7·14	6·06		22·51	6·10
	7·16	6·08		22·50	6·10
NaCl, 0·20	7·24	6·18		23·17	6·10
	7·26	6·14		23·54	6·05
NaP, 0·10	7·31	6·14		23·54	6·05
NaV, 0·10	7·45	6·03		24·00	6·15
	7·52	6·12		24·00	6·14
NaP, 0·10	8·02	6·13		30·75	6·13
	8·08	6·09		30·77	6·13
	8·54	6·21		32·40	6·13
				32·40	6·17

* See Table 3.

reproducibility of the method as exemplified by the results obtained in ten consecutive experiments on one particular protein solution; the values in the column headed $D_{20,w}$ refer to the ten individually calculated values. Finally, Table 4 shows the value of the diffusion coefficient obtained over the whole range of concentration investigated; the values show little apparent dependence on concentration, the slope of the regression line of D upon v having the value $-9{\cdot}984 \times 10^{-4}$, with a standard error of $8{\cdot}50 \times 10^{-4}$. These values give the extrapolated value at infinite dilution,

$$D^0_{20,w} = (6{\cdot}14_8 \pm 0{\cdot}02_2) \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$$

(95 % fiducial limits).

Table 5. *Sedimentation constants for bovine serum albumin*

Protein concn. (%, w/v)	Medium	$S_{20,w}$ (Svedberg units)
0·17	NaCl, 0·2 I	4·34
0·24	NaCl, 0·2 I	4·29
0·26	Phosphate, 0·1 I	4·22
0·26	Phosphate, 0·1 I	4·26
0·49	Phosphate, 0·1 I	4·28
0·49	Phosphate, 0·1 I	4·28
0·60	NaCl, 0·2 I	4·22

The values of $S_{20,w}$ obtained in this investigation are shown in Table 5. The experimental record of a typical run, showing the symmetrical peak always observed with this substance, is shown in Fig. 3.

about ± 0·3 % (Table 1), while for this solution, the standard error of the slope is 0·6 % (Table 2). Thus, allowing for the squaring of the v term, the error in the diffusion coefficient is ± 1·8 % (20:1 probability), to which must be added not more than 0·2 % for the possible error arising from variations of the time-mark interval; the overall error of the diffusion coefficient when determined from one experiment becomes, accordingly, ± 2 % in this concentration range. The accuracy is somewhat improved at higher concentrations (at 0·7 % protein concentration (Table 2) the error is ± 1·3 %) while, by performing several experiments on one solution, the error may be reduced to 0·5 % at a protein concentration of 0·3 % (Table 3).

Under the less exhaustive analysis of the experimental record performed as routine, the accuracy is not significantly lower, as shown by the values in Table 4, which show a spread not exceeding ± 2 % about the mean. Even if extraordinary measures were taken to ensure maximum definition of both reference and diffusion traces by over-exposing to the same degree, it is considered unlikely that the accuracy of the method, in its present form, could be greatly improved. For most purposes, the present standard of accuracy is probably sufficient, but, if desired, further refinement can probably best be obtained by increasing the length of the diffusing boundary along the optic axis (Longsworth, 1947; Coulson *et al.* 1948) when greater X values, more

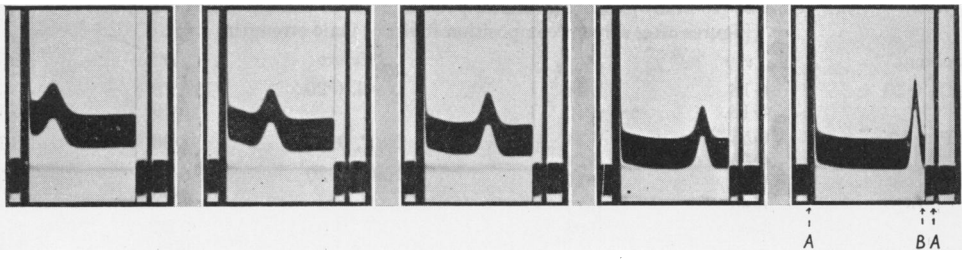

Fig. 3. Sedimentation of bovine serum albumin. Migration from right to left. Interval between exposures 32 min. Speed 59780 rev./min. Protein concentration 0·49 % (w/v) in sodium phosphate buffer, I 0·10, pH 6·8. A, A, cell indexes, B meniscus.

The slope of the line expressing the regression of the sedimentation constant upon the concentration has the value $-1{\cdot}18 \times 10^{-2}$, with standard error $8{\cdot}85 \times 10^{-3}$. These values give an extrapolated value of the sedimentation constant at infinite dilution, $S^0_{20,w} = 4{\cdot}31 \pm 0{\cdot}07$ (95 % fiducial limits).

DISCUSSION

It is evident from the diffusion results above that the accuracy obtainable in practice approaches that expected from theoretical considerations. Thus v for a dilute (0·19 %) solution may be determined to readily measurable, would be obtained. Such a cell, however, might well be complicated by inequalities of flow through the exit slit (Ogston, 1950).

The accuracy obtainable, therefore, is not very much better than that of the Lamm scale method, but the twin advantages of rapidity and small quantities of material required, weigh greatly in its favour. The extrapolated value of $D^0_{20,w}$ of $6{\cdot}15 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ may be compared with the figure 6·0 (referring to a 1 % solution) which was obtained by Stern, Singer & Davis (1947) by correcting from the value determined at 1°. The diagonal

schlieren method was used to observe the spread of the boundary, which was formed in a modified electrophoresis cell. Oncley (quoted in Cohn, 1942) obtained the value 6·4 for this protein, but no details were given of the method employed. Pedersen (1945) quotes 6·49 and 6·21 respectively for the values obtained by the height-area and second moment methods, using salt-fractionated albumin from cow foetus and calf. He used the former value only for molecular weight calculation. In the light of the present results, it appears that both Oncley's and Pedersen's values are somewhat high, while the validity of the extrapolated figure obtained by Stern *et al.* (1947) may be somewhat lessened by the magnitude of the correction factor involved.

The value of $4{\cdot}31 \pm 0{\cdot}07$ *S* for the sedimentation constant is distinctly lower than previous published values for this substance: e.g. 4·50 (Svedberg & Pedersen, 1940), 4·5 (Oncley, unpublished, referred to in Cohn, 1942), 4·6–4·7 in the concentration range 0·2–0·3% (Pedersen, 1945) and 4·4–4·8, giving the relation $S = 4{\cdot}73 - 0{\cdot}25c$ (Koenig & Pedersen, 1950).

Recent criticism (Cecil & Ogston, 1949) has indicated that the earlier S_{20} values obtained with the oil turbine may be somewhat high; as, however, an examination of the various factors involved in the accuracy of a sedimentation constant determination with the present apparatus will form the subject of another communication shortly to be published from this laboratory, no further discussion of this question will be presented here.

Molecular weight of bovine serum albumin. Using the above values for the diffusion and sedimentation constants at infinite dilution and the value of 0·74 for the partial specific volume (Pedersen, 1945) one obtains the value of 65360 for the molecular weight, and a frictional ratio of 1·30. In the absence of precise information concerning the hydration, the axial ratio of the molecule cannot be determined accurately, but a value between 3 and 4 seems very probable (see Alexander & Johnson, 1949).

The molecular weight value may be compared with the figures of 68000 quoted by Cohn (1942) and 69000 obtained by Scatchard, Batchelder & Brown (1946) from osmotic studies; the former figure only differs from the value obtained in this investigation by virtue of the use of a different value for the partial specific volume, the ratio of *S* to *D* being very nearly the same.

SUMMARY

1. The construction, use, and possible accuracy of a slightly modified form of Gouy diffusiometer are described; results with bovine serum albumin show that an accuracy of ±2% may be obtained down to concentrations of 0·2% or less, using only 1 or 2 ml. of solution.

2. A short series of ultracentrifuge experiments has been performed; these lead to a value of the sedimentation constant of bovine serum albumin somewhat lower than the present accepted value.

3. The diffusion and sedimentation results have been combined to give a molecular weight of 65360 for this protein.

The help and advice of Dr A. G. Ogston in the design and construction of the apparatus and the interpretation of results is gladly acknowledged. The author is also greatly indebted to Dr P. A. Charlwood, of this Institute, for his assistance in the measurement of experimental records, in computation of various tables, and for advice in many discussions; to Mr R. L. Warren, for technical assistance in the construction of some parts of the apparatus; and to Prof. E. C. Dodds, F.R.S., for the continual encouragement he has given to this work.

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