ULTRACENTRIFUGAL ANALYSIS OF DILUTE SOLUTIONS*

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INTRODUCTION

Evaluation and interpretation of the ultracentrifuge patterns exhibited by many macromolecules is often difficult because of the high degree of particle interaction present in solutions at the concentrations customarily employed in the ultracentrifuge[1,2,3]. This difficulty applies both to the determination of the sedimentation coefficient at infinite dilution and to the analysis of the shape of the ultracentrifuge boundary in terms of the homogeneity of the sedimenting material. For elongated macromolecules or particles, such as deoxyribonucleic acid (DNA)[4] and tobacco mosaic virus (TMV)[5], the particle interaction even at concentrations of only 0.2% is marked, resulting in a large dependence of sedimentation coefficient on concentration and in boundaries which are hyper-sharp often causing the misleading impression that the sedimenting material is homogeneous with regard to size and shape.

Several attempts have been made to predict the magnitude of the decrease in the sedimentation coefficient due to particle interaction[3,6-9]; and, as a result of these treatments, it has become common practice to plot either the sedimentation coefficient or its reciprocal as a function of concentration and to extrapolate such plots linearly to infinite dilution. For many substances these methods produce satisfactory results; but for others the extrapolated values are open to serious doubt, and conflicting results for similar preparations of the same material are not uncommon[4,10]. Moreover, profound changes occur in the shape of the sedimenting boundary of polydisperse solutions of elongated macromolecules upon dilution of the material. This effect, now known as the JOHNSTON-OGSTON effect[11,12,13], is a reflection of the changes in the sedimentation coefficients of the material across the boundary region due to the changes in concentration within the boundary. A number of workers have treated this phenomenon in great detail and several methods have been proposed for the evaluation of the ultracentrifuge patterns in terms of the distribution of sedimentation coefficients[13-16]. Unfortunately these methods require knowledge of the dependence of sedimentation coefficient on concentration for each molecular species forming the boundary. Further, the calculations are quite laborious and the resulting distributions are not rigorously correct.

All of the described effects due to molecular interaction can be obviated by performing the studies at very great dilution. However, the conventional optical methods based upon the gradient of refractive index are usually restricted to solute concentra-

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tions greater than 0.1%, thereby precluding studies at sufficiently low concentrations. To circumvent the difficulties imposed by the limitations of present schlieren optical methods, we have returned to the use of absorption optical methods similar to those first employed by SVEDBERG and his collaborators in their early work on the development of the ultracentrifuge[17]. The emphasis of the present work is directed toward a study of the factors involved in successful ultracentrifugal studies at concentrations in the neighbourhood of a few thousandths of a percent. Although there have appeared recently[18,19,20] several papers containing results of studies related in part to some reported herein, the present work deals mainly with ultracentrifugal problems rather than with considerations of the materials examined.

It is the purpose of this communication to describe the absorption optical techniques which we have been using for the determination of sedimentation and diffusion coefficients and for the evaluation of the distribution of sedimentation coefficients in polydisperse systems. For the former studies we have used the globular proteins, cytochrome and carboxyhemoglobin, whose behavior at high dilution might be anticipated from data obtained with conventional schlieren techniques. For the latter studies we have examined a sample of deoxyribonucleic acid, the molecules of which are so large that the bound aryspreading due to diffusion should be negligible. The techniques used for the evaluation of the photographic plates are discussed in detail. Since there have been raised, on many occasions, serious doubts as to the gravitational stability of boundaries across which there is a concentration change of only a few thousandths of a percent, this question is considered in detail. As a result of these studies we have concluded that convective disturbances are absent or, at least, negligible and that meaningful, quantitative ultracentrifugal analyses can be performed on certain systems with as little as a few hundredths of a milligram.

MATERIALS AND METHODS

For the analysis of boundary spreading in the ultracentrifuge in terms of diffusion, a sample of a bacterial cytochrome, *Pseudomonas aeruginosa*, kindly made available by Dr. M. KAMEN, was used because of its high extinction coefficient at 415 mμ. Similarly, human carboxyhemoglobin, freshly prepared and stored under an atmosphere of CO, was used for the sedimentation velocity experiments at very low concentrations. A phosphate buffer, 0.1 *M* and pH 6.8, was used for the experiments with cytochrome and the buffer employed by KEGELES AND GUTTER[21] was used with carboxyhemoglobin. The authors would like to thank Dr. J. VINOGRAD for the sample of human hemoglobin. A sample of DNA, prepared by the method of SCHWANDER AND SIGNER[22] and kindly supplied by Dr. S. KATZ, was dissolved in 0.2 *M* NaCl and 0.01 *M* phosphate buffer at pH 7 and used for the studies of the distribution of sedimentation coefficients employing absorption optics with ultraviolet light of 254 mμ.

The model E ultracentrifuge manufactured by the Spinco Division, Beckman Instruments, Inc., was used for all of the work to be described. A red filter (Wratten No. 25) was used together with red sensitive photographic plates (Kodak I-N) for all of the studies of the colored proteins with the conventional schlieren optical system. In this manner uniform illumination across the image of the cell is obtained.

For the ultracentrifuge studies employing absorption optics with visible light, the schlieren optical track was converted to an absorption optical system by making the following adjustments: (a)The light source slit was widened by pulling the knife edges back to their extreme positions. (b) An interference filter with a band width of 20 mμ at 410 mμ was placed over the camera lens*. Next to the interference filter was placed a blue filter (Klett No. 42). This blue filter eliminates the

* The suggestion involving the use of an interference filter of the appropriate wavelength was made by Dr. K. PAIGEN, with whom early experiments were conducted. His departure from this laboratory to the Roswell Park Memorial Institute, Buffalo, New York, unfortunately prevented the continued collaboration which was originally contemplated.

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harmonics of 410 mμ. Because of imperfections in the filters, their location in the conventional filter holder in the ultracentrifuge immediately in front of the photographic plate caused striations and uneven illumination on the resulting photographs. This difficulty was eliminated by relocation of the filter combinations as indicated. (c) The schlieren bar or phase plate was turned to the zero degree position. (d) The yellow filter was removed from the filter holder and replaced by a metal frame of the same dimensions in order to delineate sharply the top and bottom margins of the image. (e) Because of fogging of the plates by unfiltered light, a sheet of black paper was taped over the viewing window to eliminate the small amount of light reaching the film from that point. Also a strip of friction tape was placed around the edges of the filter holder cover to prevent extraneous light leaks at that site. (f) Kodak I(D)-2 photographic plates were used for these absorption runs and the exposure times were of the order of 3 seconds. Although some gain could be achieved by removal of the cylindrical lens, the disadvantages, from the point of view of the return to conventional operation of the ultracentrifuge, made such a modification undesirable.

An ultraviolet absorption optical system[19], similar to that used by SVEDBERG and collaborators[17] and installed by the manufacturer, was used for the experiments with DNA.

For the diffusion measurements on the low molecular weight colored protein, cytochrome, a synthetic boundary cell was used. This allows the formation of a sharp boundary in the center of the cell so that spreading of the boundary can readily be measured. Usually the boundary is formed at about 9000 r.p.m., when the centrifugal field becomes sufficient to cause compression of the rubber plug which acts like a valve allowing the solvent in the cup to layer onto the solution in the sectoral cavity of the cell. For the boundary formation to be successful there must be a slight difference in density between solvent and solution. In the experiments described in this communication, the protein concentration in the solution is too low to produce a density increment sufficient to insure gravitational stability during the emptying of the upper reservoir. Therefore the salt concentrations are adjusted so that the concentration of the upper solution is 0.01 M to 0.02 M less than that of the lower solution. During the period of emptying of the reservoir the schlieren optical system shows a sharp boundary due to the difference in salt concentration between the two solutions. In the first few minutes after the boundary is formed the gradient of refractive index at the boundary is so great that some of the deviated light is not retained by the ultraviolet light absorption optical system. This light, therefore, does not reach the photographic plate thereby leading to a white band on the photographs. Within a few minutes, however, the boundary has spread sufficiently by diffusion of the salt that all of the light is now contained within the optical system. The absorption patterns then represent the distribution of ultraviolet-absorbing material. During the early part of the experiment, there is a change in the viscosity and density of the solvent at the boundary, but this generally has only a negligible effect on the calculations of the ultracentrifuge patterns. Furthermore, the diffusion of the salt is so rapid that the gradient becomes very small and the average viscosity and density of the solvent suffice for the calculations.

The photographic images obtained by the absorption optical techniques are converted into plots of concentration versus distance along the axis of rotation by the use of a recording microphotodensitometer. For this purpose we used the Model R Analytrol, manufactured by the Spinco Division, Beckman Instruments, Inc., equipped with a microanalyzer attachment. This consists of an adapter for holding and moving the photographic plates at a very slow rate across a thin slit (0.004″) through which a light beam passes to a photovoltaic cell. The light intensity striking this cell is automatically balanced against that impinging on a matched cell. Balancing is achieved by means of a motor-operated special, logarithmic cam which turns in front of the control cell thereby varying the size of the aperture of the cell. Coupled with the rotation of the cam is the linear movement of a pen which traces a curve on a moving chart. The height of the tracing at each point is proportional to the blackening of the film; and, as is shown below, to the concentration of the absorbing material. The uniformity of illumination of the cell is first checked by analyzing a plate from an ultracentrifuge run with only solvent in the cell. This, in effect, gives a baseline for further measurements. To measure the boundary positions, the tracing from a pattern of the solution is superimposed over that obtained with the solvent. The height of the solution tracing above the baseline is then measured in the plateau region. Next, the point in the boundary region corresponding to 50% of that height is located, and the distance between that point and the reference hole is measured. This distance is converted by the appropriate magnification factors and geometrical distances to real distances from the axis of rotation. Finally, from plots of the logarithms of these distances as a function of time the sedimentation coefficients are calculated in the customary manner.

Apparent diffusion coefficients are calculated by measuring the distance between the 25% point in the boundary region and the 75% point. The square of this distance (corrected for the appropriate magnification factors) is then plotted against time and the slope of the line so obtained is equal to $3.64\ D$[23] where D is the diffusion coefficient in cm^2/sec.

For large elongated macromolecules the diffusion coefficients are so small that spreading of the boundary due to diffusion is negligible during the length of an ultracentrifuge run. Hence the

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spreading of the boundary must be attributed to polydispersity of sedimentation coefficients within the sample. In such studies it is useful to transform the photographic records into plots of the weight distribution of sedimentation coefficients. To calculate the apparent distribution of sedimentation coefficients for systems in which diffusion can be neglected, it is necessary to evaluate the quantity $(1/c_0^{obs})\ dc_0^{obs}/ds^\circ_{20,w}$ which is the normalized distribution function[24]. The symbol, $s^\circ_{20,w}$ is the sedimentation coefficient at infinite dilution corrected to the value it would have in a solvent with the viscosity and density of water at 20°C. The symbol c_0^{obs} is the observed concentration corrected for radial dilution[25]. From the photodensitometer tracing of the absorption pattern, the concentration as a function of position in the ultracentrifuge cell is immediately obtained. Hence the quantity $\Delta c^{obs}/\Delta x$ may be calculated across the boundary region by measuring the increment in concentration between $(x - \Delta x/2)$ and $(x + \Delta x/2)$ for each value of x, the distance in cm from the axis of rotation. The square law correction is then applied by multiplication of each $\Delta c^{obs}/\Delta x$ by its $(x/x_0)^2$ which converts the quantity to $(\Delta c_0^{obs}/\Delta x)$. In this computation, x_0 is the position of the meniscus in cm from the axis of rotation. Multiplication by the factor $dx/ds^\circ) = \omega^2 x t$, where t is the time in seconds, then gives $\Delta c_0^{obs}/\Delta s^\circ$. (The sedimentation coefficients measured at these very low concentrations are effectively s°.) Finally $\Delta c_0^{obs}/\Delta s^\circ_{20,w}$ is obtained by multiplication by $ds^\circ/ds^\circ_{20,w} = \frac{(1-\bar{V}\varrho)}{(1-\bar{V}\varrho_{20,w})} \cdot \frac{\eta_{20,w}}{\eta}$. Normalization of the distribution is performed by a process of summation and division, hence

$$(1/c_0^{obs})\frac{dc_0^{obs}}{ds^\circ_{20,w}} = \frac{\frac{\Delta c_0^{obs}}{\Delta s^\circ_{20,w}}}{\sum_i \left(\frac{\Delta c_0^{obs}}{\Delta s^\circ_{20,w}}\right)_i (\Delta s^\circ_{20,w})_i} = \frac{\frac{\Delta c_0^{obs}}{\Delta s^\circ_{20,w}}}{\overline{\Delta s^\circ_{20,w}} \sum_i \left(\frac{\Delta c_0^{obs}}{\Delta s^\circ_{20,w}}\right)_i} \qquad (1)$$

where $\overline{\Delta s^\circ_{20,w}}$ is the average of the largest and smallers values of $\Delta s^\circ_{20,w}$.

The approximations are very good. Combining all of these expressions, the following formula is obtained

$$(1/c_0^{obs})\frac{dc_0^{obs}}{ds^\circ_{20,w}} = \frac{\frac{\Delta c^{obs}}{\Delta x}\left(\frac{x}{x_0}\right)^3_i}{\overline{\Delta s^\circ_{20,w}} \sum_i \left(\frac{\Delta c^{obs}}{\Delta x}\right)_i \left(\frac{x}{x_0}\right)^3_i} \qquad (2)$$

The values of $s^\circ_{20,w}$ may be calculated at each point x from the expression

$$s^\circ_{20,w} = \left(\frac{1-\bar{V}\varrho_{20,w}}{1-\bar{V}\varrho}\right)\left(\frac{\eta}{\eta_{20,w}}\right)\frac{2.303}{\omega^2 t}\log\left(\frac{x}{x_0}\right) \qquad (3)$$

The only quantity which remains to be determined is the effective time of sedimentation, t. This time may be calculated by plotting the logarithms of the 50% points from several exposures as a function of the time between the exposures, and extrapolating the straight line so obtained back until it intersects the meniscus position. The effective time can also be obtained by using measured times after reaching speed plus $^1/_3$ of the time required to attain the operating speed.

The labor involved in calculating several sedimentation coefficient distributions is very great. Fortunately, two of the functions may be tabulated and the time required can be reduced to about one hour per run. The quantities $(x/x_0)^3$ and $\log(x/x_0)$ are almost independent of small differences in x_0 for constant values of $(x\text{-}x_0) = \delta$. Therefore, tables of $(x/x_0)^3$ and $\log(x/x_0)$ as a function of δ may be constructed. To facilitate the calculations and measurements, the photodensitometer patterns are retraced on two coordinate paper so that the meniscus lies on a heavy vertical line and the distance, δ, may be measured directly. With these tables, the calculation of $(1/c_0^{obs})\ dc_0^{obs}/ds^\circ_{20,w}$ and $s_{20,w}$ from equations 2 and 3 becomes a simple and rapid operation. It should be noted that the ultracentrifuge cells should be filled reproducibly from run to run so that x_0 in all runs is within $\pm$ 0.02 cm, otherwise the tables of $(x/x_0)^3$ and $\log(x/x_0)$ lose their general utility.

For small molecules with large diffusion coefficients, the above calculations are inappropriate since the distribution function would also be a function of the time of sedimentation unless the patterns have been previously corrected for the effect of diffusion. Since this can not, in general, be done for unknown mixtures, it is recommended instead that the quantity

$$\frac{1}{c_0^{obs}}\frac{dc_0^{obs}}{dx} = \frac{\left(\frac{x}{x_0}\right)^2\left(\frac{\Delta c^{obs}}{\Delta x}\right)}{\sum_i \left(\frac{x}{x_0}\right)^2_i \left(\frac{\Delta c^{obs}}{\Delta x}\right)_i \Delta x_i}$$

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be plotted against x to give curves analogous to those obtained with the schlieren optical system but corrected for radial dilution.

Some preliminary fractionation experiments were performed on DNA by the technique of zone centrifugation in the swinging bucket rotor described by HOGEBOOM[27]. A density gradient is made by careful layering in centrifuge tubes 1 ml of each of a series of solutions of 20%, 15%, 10% and 5% sucrose, each of which is 0.2 M in sodium chloride. To allow the gradient to become nearly linear by diffusion of the sucrose, the tubes are stored overnight in the cold room. One-half ml of a 0.04% DNA solution, 0.2 M in sodium chloride, is then layered onto the top of the sucrose gradient column. The centrifuge rotor is spun at 27,000 r.p.m. for 2 hours, and fractions are removed by syphoning from the bottom of each tube. A piece of "melting point" capillary may conveniently be used for the syphon. In this manner a series of fractions are obtained containing macromolecules from different parts of the distribution.

RESULTS AND DISCUSSION

With only solvent in the ultracentrifuge cell the photodensitometer tracing should be a horizontal line called the baseline. Actually there are generally small imperfections in the optical system, such as dust on the lenses, which cause vertical or horizontal

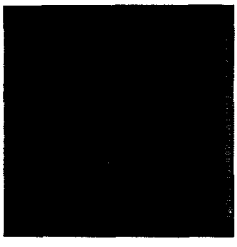

Fig. 1a.

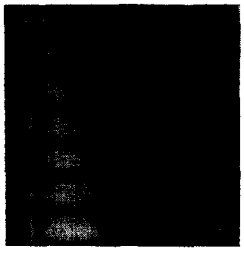

Fig. 1b.

Fig. 1. Ultracentrifugal analysis by absorption optical methods. The photographic patterns are shown on the left and the photodensitometer tracings on the right. (a) Cytochrome at a concentration of 0.005%. Solvent 0.1 M phosphate buffer at pH 7. Photograph taken 16 min after boundary was formed near the center of the cell. Speed was 44,770 r.p.m. Schlieren optical system was used as an absorption optical system with appropriate filters to isolate 415 mμ line. (b) Calf thymus DNA at a concentration of 0.004%. Solvent 0.2 M NaCl at pH 7. Photograph taken 2 min after reaching speed of 59,780 r.p.m. All patterns taken with ultraviolet absorption optical system.

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streaks on the photographs. These will cause fluctuations in the baseline tracing and also in the tracing of the sedimenting macromolecules. Therefore, in order to obtain the most accurate data, the base line should be subtracted from the pattern of the solution. This will also correct for uneven illumination of the cell which causes sloping baselines. In Fig. 1 are shown some typical patterns and the photodensitometer tracings of the sedimenting boundaries and the corresponding baselines for both the ultraviolet and visible light absorption optical systems. The baselines for the ultraviolet system are reproducible from run to run. However, the baselines with the optical system using visible light varied somewhat even within a single run owing to fluctuations in the uniformity of illumination from the high pressure AH-6 mercury light source.

Calculation of the distribution of sedimentation coefficients and of diffusion coefficients from the absorption photographs requires that the height of the tracing above the baseline be directly proportional to the concentration of absorbing material at that point. The validity of this relationship is tested by filling the cell with different concentrations of light-absorbing macromolecules and taking pictures of the rotating cell at various exposures. In Fig. 2 are shown the optical densities of solutions of DNA and cytochrome plotted against the distance of pen travel above the baseline for the representative tracings. The plot is seen to be linear at optical densities below 0.5 for cytochrome and 0.9 for DNA. Therefore, the height of pen travel, within the linear range, gives a faithful indication of concentration of the absorbing material.

Stability of the sedimenting and diffusing boundaries is an essential requirement for the study of macromolecular systems at the very low concentrations employed in the present investigation. There are at least two types of disturbances which may cause convection in the ultracentrifuge cell: mechanical vibration from the drive shaft and temperature gradients across the cell. Stabilizing the boundary against these disturbing influences are two concentration gradients, that due to the sedimentng macromolecules themselves and also that arising from the redistribution of salt in

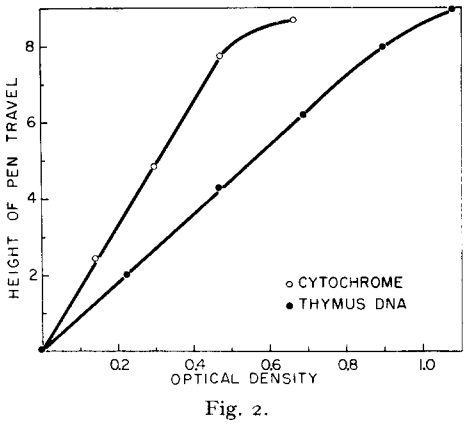

Fig. 2.

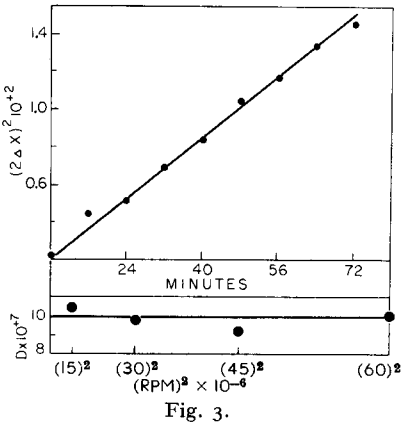

Fig. 3.

Fig. 2. Calibration of the blackening of photographic plates as measured by the height of the photodensitometer pen travel when scanning ultracentrifuge patterns of solutions of different optical densities.

Fig. 3. Diffusion of bacterial cytochrome at a concentration of 0.005 %. The top curve is a plot of the square of the measured boundary width from 25 % to 75 % concentration against time for the 44,770 r.p.m. run. The bottom plot is that of the apparent diffusion coefficient against the square of the speed of the rotor in r.p.m.

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the solution. The density gradient due to compression of the liquid is not a stabilizing factor because no net work is required to interchange different regions of liquid and hence there exists no force tending to prevent convective transport. Whereas increasing the centrifugal field, through its effective magnification of small density differences due to concentration gradients, tends to offset the effect of mechanical vibrations, it does not offer any stabilizing force over temperature gradients. It is important, therefore, to consider the effect of centrifugal field in consideration of convection in studies at low concentrations. This is especially important since any existing temperature gradients in ultracentrifuges in common use are likely to be very small.

A sensitive test of the stability of a boundary formed in the ultracentrifuge is afforded by analyzing the spreading of the boundary in terms of the diffusion coefficient. Since the cytochrome was found to have a small sedimentation coefficient, the boundary spreading in a run of only 1.5 hours duration is attributable almost completely to diffusion. Furthermore, at the concentration, 0.004%, employed in these experiments such boundary spreading would be uncomplicated by artificial sharpening due to a change in sedimentation rate across the boundary. In Fig. 3 are shown the data for the boundary spreading as a function of time in an individual experiment. Also shown in Fig. 3 are the results of measurements of the apparent diffusion coefficients as a function of the square of the angular velocity. It is seen that reasonably constant values of the apparent diffusion coefficient are obtained, indicating that the boundaries are stable over the time interval of the experiments.

Since the calculation of an approximate diffusion coefficient for DNA from the molecular weight and average sedimentation coefficient indicates that boundary spreading from diffusion would be trivial, the ultracentrifuge experiments should measure the intrinsic inhomogeneity with respect to sedimentation coefficient. Therefore measurement of the distribution of sedimentation coefficients exhibited by a preparation of DNA would give positive information about the presence or absence of convective disturbances which affect the sedimenting boundary. If the calculated distributions were independent both of the time of sedimentation and the centrifugal

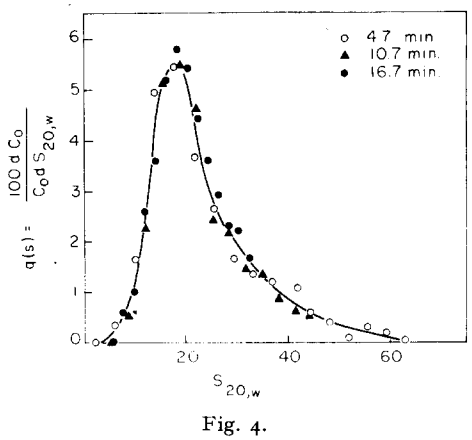

Fig. 4.

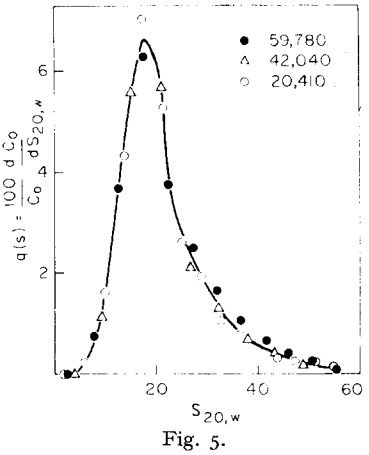

Fig. 5.

Fig. 4. Distribution of sedimentation coefficients in thymus DNA at 0.004%. The points are from three different exposures in one ultracentrifuge run at the times noted.

Fig. 5. Distribution of sedimentation coefficients in thymus DNA at different r.p.m.

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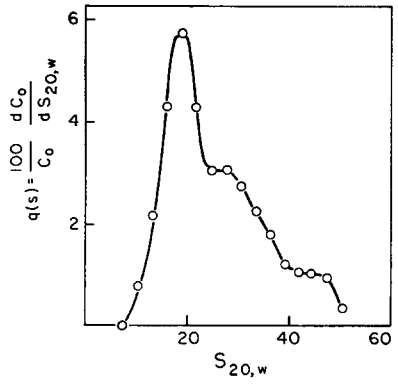

Fig. 6

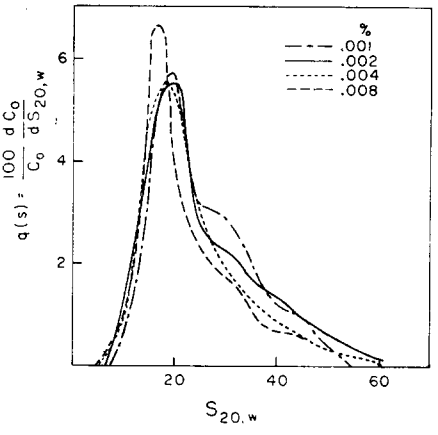

Fig. 7.

Fig. 6. Distribution of sedimentation coefficients in thymus DNA at a concentration of 0.001 %.
Fig. 7. Concentration dependence of sedimentation coefficient distributions for thymus DNA.

field, the absence of significant convection would have been demonstrated. It is seen in Fig. 4 that the calculated distribution is independent of time even though the boundary width increased from 0.2 cm to 1.0 cm in the different photographs. Additional evidence that the observed heterogeneity in sedimentation coefficients is characteristic of the macromolecules and does not represent boundary spreading due to convective forces is shown by the results presented in Fig. 5. It is seen that the calculated distribution is essentially independent of centrifugal field over a broad range of field.

Any convection caused by thermal gradients in the cell should begin to have a disruptive effect on the boundary as the concentration of the sedimenting material is decreased. It is of interest, therefore, to see in Fig. 6 that even at a concentration of only 0.001% sufficient data are obtained from which the distribution curve can be evaluated. In Fig. 7 are shown the distributions calculated from experiments at different concentrations.

When it is considered that these plots represent a point by point differentiation of the photodensitometer tracings, the agreement among the runs at 0.001, 0.002 and 0.004% is excellent. Boundary sharpening with a concomitant apparent reduction in the concentration of the faster moving species and an increase in the amount of the more slowly moving components is apparent in the run at 0.008%. Little change is evident, however, for the patterns at concentrations below 0.004%, and it is likely that macromolecular interactions, as well as convective disturbances, are unimportant at these dilutions.

In those experiments in which the boundary movement was appreciable, the concentration in the plateau region, as indicated by the height of the pen travel above the baseline, was found to decrease in accord with the radial dilution square law[25]. This is still further indication that there is not any significant disturbance of ideal sedimentation.

A final demonstration of the heterogeneity of the DNA with respect to sedimentation rate is afforded by the comparison of the distribution of the unfractionated prep-

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aration with that of one of the fractions obtained by zone centrifugation. These results can be compared with those of SHOOTER AND BUTLER[18] who fractionated DNA not by zone centrifugation but by the classical technique of moving boundary centrifugation.

The validity of sedimentation velocity experiments at very low concentration is also shown by the data in Fig. 9 on human carboxyhemoglobin. Boundary movement at the concentrations of 1.0, 0.5, and 0.25% was followed by schlieren optics. At a concentration of 0.05% the boundary was followed by absorption optics using light at 254 μ, and light absorption at 410 μ was employed for experiments at 0.005%. The line on the graph is the least squares line taken from KEGELES AND GUTTER[21] after correcting their data for the adiabatic cooling of the rotor due to stretching at high speed. It is clear from the figure that accuracy of about 0.05 Svedbergs is attainable even at these great dilutions. Under these experimental conditions there is no evidence of dissociation of the hemoglobin with an accompanying decrease in sedimentation coefficient.

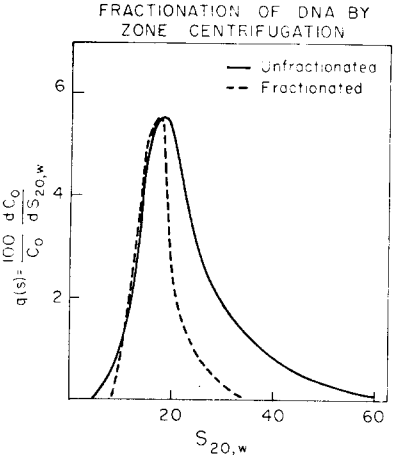

Fig. 8.

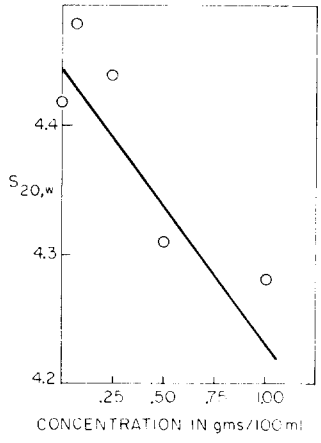

Fig. 9.

Fig. 8. Sedimentation coefficient distributions of DNA fractionated by zone centrifugation.

Fig. 9. Sedimentation of human carboxyhemoglobin. The data at concentrations of 0.25%, 0.9% and 1.0% are obtained with the schlieren optical system. The value at 0.05% was obtained with ultraviolet absorption optics, and absorption at 415 mμ was employed for measurements at 0.005%.

GENERAL CONSIDERATIONS

The problems of boundary stability at concentrations of only a few thousandths of a percent has been investigated by several authors. SHARP *et al.* found, with both T_2 bacteriophage[28] and polystyrene latex particles[29], boundary instability as exhibited by an abrupt decrease in the sedimentation coefficient and deterioration of the boundary at concentrations of 0.004% and 0.02%, respectively. Essentially identical results were reported by the same group with T_4 bacteriophage[30]. SINGER AND SIEGEL[31] found that a small fraction, 1% to 7% of the infectivity, remained in the upper compartment of a partition cell[32] after times of sedimentation sufficient to cause a bacteriophage boundary to have passed the perforated, separation plate. The presence of this residual

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activity has been attributed by them to convective disturbances. It should be noted, first of all, that these studies were performed on very rapidly sedimenting materials at low centrifugal fields where the stabilizing effect of the field against mechanical vibrations is likely to be insufficient to prevent convective disturbances. Secondly, the change in density across the boundary is important. This is especially noticeable with the latex particles of density, 1.052 g/ml, sedimenting in a solvent of density, 1.01 g/ml. Here the change in density across the boundary is much smaller than in experiments with proteins of density about 1.3 g/ml or nucleic acids of density, 1.8 g/ml sedimenting through a dilute buffer solution. For proteins and nucleic acids we would expect the boundaries to be 7 to 20 times more stable than that for the low density latex particles. Due allowance must be made for the sharpness of the boundary, since the density gradient involves both the change in density across and the width of the boundary. It is of interest, therefore, to recall that convection did not occur in the bacteriophage experiment until the concentrations were 5 fold lower than those with the latex particles. In both of these experiments the boundaries are very sharp. With regard to the experiments of SINGER AND SIEGEL it is significant to note that the fraction of the activity remaining in the upper compartment at the conclusion of the run is almost independent of the concentration of the original solution. If convective transport of liquid played a significant role in these experiments it might be expected that the fraction of the residual activity would depend, to some extent, on the concentration of the sedimenting substance. Indeed, it seems more likely, as EPSTEIN AND LAUFFER[33] have suggested, that the presence of residual activity in the upper compartment is more the result of contamination with a small amount of bacteriophage solution than the product of convective transport. Finally, it should be noted that the partition in this separation cell does interfere, in part, with normal sedimentation; and the presence of convection in such a cell, if conclusively demonstrated, does not justify the conclusion that there would also be similar convection in a conventional ultracentrifuge cell. In the cell designed by YPHANTIS AND WAUGH[34] there is occasional evidence of boundary instability at very low concentrations; but this can be attributed to the slight disturbances caused by the movement of the partition from the cell bottom to its rest position during the deceleration of the rotor. These workers, it should be noted, have performed successful ultracentrifugal analyses at very low concentrations.

As indicated in the preliminary experiments with cytochrome, diffusion coefficients can be measured in the ultracentrifuge with the use of the synthetic boundary cell and absorption optics. Thus far detailed studies of the quality of the initial boundaries, as judged by the zero time correction, have not been made. In view of the encouraging preliminary results more critical tests would seem to be warranted because the use of absorption optics in conjunction with the ultracentrifuge would allow measurements of both the sedimentation and diffusion coefficients, and hence the molecular weight, on as little as 0.05 mg of material. A tentative value of about 12,000 is calculated from the data obtained with solutions of cytochrome, at a concentration of 0.005%, and with an assumed value of 0.72 cc/g for the partial specific volume. The total movement of the boundary for such small molecules is not sufficiently large to permit reliable sedimentation coefficient determinations with the absorption technique as presently used. With more rapidly sedimenting material like hemoglobin, however, accuracy comparable to that given by schlieren optical techniques may be

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anticipated as shown by Fig. 9. It seems at present that the calculated derivatives of the photodensitometer tracing are not sufficiently accurate, particularly at the meniscus and bottom of the cell, to permit use of the ARCHIBALD method[35–38] for the direct determination of molecular weights in the ultracentrifuge. However, the tracings may be sufficiently accurate for calculations of solute distributions throughout the cell which would then be used in the expressions obtained by YPHANTIS AND WAUGH[39] for molecular weight determinations.

Analyses of complex mixtures containing several light absorbing, sedimenting components is, of course, readily performed by these techniques. Especially valuable information concerning purity can be obtained by simultaneous examination of the sedimenting material at different wave lengths. In the examination of a preparation of *Chromatium* D-cytochrome-552, kindly supplied by Dr. M. KAMEN, we observed two different sedimentation coefficients when the movement of the boundary was evaluated from absorption diagrams at 410 mμ and 280 mμ.

SUMMARY

Absorption optics both in the visible and ultraviolet regions of the spectrum have been used in ultracentrifugal studies at concentrations of about 0.001 %. The experimental techniques have been described, and methods of handling the photographic plates to determine sedimentation coefficients, apparent diffusion coefficients and distribution of sedimentation coefficients are illustrated in detail. Different types of experiments have been performed with preparations of a bacterial cytochrome, human hemoglobin and calf thymus deoxyribonucleic acid. In view of the risks that convective disturbances may invalidate ultracentrifuge experiments at very low concentrations, special attention was directed toward testing the reliability of the studies reported herein. It is shown that a cytochrome boundary behaved ideally and the apparent diffusion coefficient was independent of centrifugal field. Similarly the spreading of the DNA boundary gave a calculated distribution curve which was independent of time of sedimentation, speed of the rotor and of concentration of the material. The polydispersity was conclusively demonstrated by comparison of the patterns of a fractionated sample and the original preparation of DNA. Measurement of accurate sedimentation coefficients at 0.05 and 0.005% was illustrated with a preparation of carboxyhemoglobin. These studies taken together show the reliability of the measurements at very high dilutions. Other applications of absorption techniques in ultracentrifugation are discussed.

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A NEW COLOR REACTION FOR THE DETERMINATION OF ALDOPENTOSE IN PRESENCE OF OTHER SACCHARIDES*

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The occurrence of heptose- and hexose-phosphate esters as intermediates in the breakdown of ribose-5-phosphate in living cells may create considerable difficulty in the determination of pentose in extracts of living tissues. In the widely used Bial's orcinol reaction, the colored product from aldoheptoses[1] shows an absorption curve similar in its shape to that from pentose. Furthermore, heptoses, as well as large amounts of hexoses, influence to a considerable extent the absorption curve of pentoses, and this makes the quantitative determination of the latter sometimes rather difficult.

The phloroglucinol reaction of Tollens does not appear useful for quantitative determinations in its original from, because of the strong interference by other sugars and the instability of the color. The modification of this reaction by VON EULER AND HAHN[2] recommended for the determination of RNA appears insufficiently sensitive and its specificity has not been adequately investigated. The present report deals with another modification of the phloroglucinol reaction which permits the differentiation between aldo- and ketopentose and the determination of small amounts of pentoses and their nucleotides in presence of larger amounts of other sugars.

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