THE ULTRACENTRIFUGE

PRACTICAL ASPECTS OF THE ULTRACENTRIFUGAL ANALYSIS OF PROTEINS[1]

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The Svedberg equations for determining molecular weights by ultracentrifugal analysis are shown to be valid only if the proper significance is attached to certain of the involved terms. Emphasis is placed on the physical principles involved in the method, with special reference to the effects of hydration. Probable reasons are given for the formerly unsuccessful attempts to make accurate diffusion measurements in the ultracentrifuge, and a new method of increasing the precision of such measurements is presented. Various conditions of experimentation are discussed, and hydrostatic compression of the fluid being centrifuged is shown to exert no stabilizing action with respect to convective disturbances. Various types of ultracentrifuges are reviewed briefly. An explanation is presented of the working principles involved in recently developed optical methods based on the refraction of light.

I. INTRODUCTION

The history of ultracentrifugation dates back to the development of a small "optical centrifuge" by Svedberg and Nichols at the University of Wisconsin in 1923 (2). In the following year Svedberg and Rinde described improvements in the apparatus and its application to the determination of size-distribution among gold sols (3). They proposed the name "ultracentrifuge" for the new research tool and recommended that the term be reserved to denote an instrument by means of which sedimentation in a centrifugal field is measured quantitatively. They suggested that an ordinary laboratory centrifuge of high speed might be called a "supercentrifuge." However, in recent years it has become the practice of many investigators to associate the term "ultracentrifuge" with any type of high-speed centrifuge rather than with the original connotation of quantitative measurement.

A thorough bibliography of the literature pertaining to the construction and applications of ultracentrifuges of all types prior to 1940 has been included in *The Ultracentrifuge* by Svedberg, Pedersen, and their collaborators (1). The purpose of this paper is to review briefly from both a theoretical and a technical standpoint the principal points of the ultracentrifugal method, and to discuss some of its less generally recognized aspects which have been given attention in our laboratory.

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II. THEORY

Svedberg (12) has derived mathematical equations which show the relationship existing between the molecular weight of homogeneous particles and their sedimentation characteristics under certain conditions in an ultracentrifuge. These equations apply strictly only for dilute solutions of particles which are large in comparison with the molecules constituting the medium and which are electrically neutral with respect to the medium. Consider a suspension of particles of molecular weight M revolving in a centrifuge at an angular velocity of ω radians per second. Assume that the particles under observation are far enough from the bounding surfaces of the fluid to be uninfluenced by alterations of particle concentration in these regions. The net force acting on a mole of these particles at a distance x from the axis of rotation is simply the difference between their combined centrifugal weight and the buoyancy exerted by the displaced medium, i.e.,

$$F_c = M\omega^2 x - M\frac{\rho\omega^2 x}{\sigma} \quad \text{or} \quad F_c = M\omega^2 x - MV\rho\omega^2 x \tag{1}$$

where ρ is the density of the medium and σ is the density of the particles. The latter is equivalent to $1/V$, where V is the partial specific volume of the particles. Disregarding the negligible forces due to the slight acceleration in the speed of the particles with time, the force F_c is exactly counterbalanced by a frictional force F_s, which is proportional only to the sedimentation velocity of the particles and to a factor dependent upon the extent and shape of the surface of the particles. The frictional force on each mole per unit speed is called the frictional coefficient, f_s. Thus,

$$F_c = M\left(1 - \frac{\rho}{\sigma}\right)\omega^2 x = F_s = f_s\, \mathrm{d}x/\mathrm{d}t \tag{2}$$

where t is the time. In this expression $\frac{\mathrm{d}x}{\mathrm{d}t}/\omega^2 x$ is the sedimentation velocity per unit field of force and is usually denoted by s. Rewriting the equation, we have

$$s = \frac{M\left(1 - \frac{\rho}{\sigma}\right)}{f_s} \quad \text{or} \quad sf_s = M\left(1 - \frac{\rho}{\sigma}\right) \tag{3}$$

The diffusion constant D, which expresses the rate at which the particles will diffuse, is also inversely proportional to a molar frictional coefficient f_D:

$$D = \frac{RT}{f_D} \tag{4}$$

R is the gas constant and T the absolute temperature. It should be noted that a change in the mass of particles does not affect the diffusion rate directly but only in so far as it is associated with a change in the frictional coefficient. This is true by reason of the fact that the kinetic energy of a particle is independent of its mass. Theoretical and experimental evidence justify the assumptions that the random orientation of molecular particles during either diffusion or sedi-

mentation is not appreciably altered and that, for the same medium at the same temperature T, $f_s = f_D$ to a very close approximation. Eliminating the frictional coefficient from equations 3 and 4, one obtains Svedberg's formula for the molecular weight as determined by the sedimentation velocity method:

$$M = \frac{RTs}{D\left(1 - \frac{\rho}{\sigma}\right)} \tag{5}$$

In general, sedimentation and diffusion experiments are performed at different temperatures and, for the sake of comparisons, it is customary to correct measured values of D and s to some standard temperature, usually $T = 293°$ Absolute. Svedberg has standardized conditions even further and expresses each measurement of sedimentation in terms of a "sedimentation constant," s_{20}, which is the rate the material would have in a hypothetical medium having the density and viscosity of water at 20°C. or 293° Absolute:

$$s_{20} = \frac{s\eta(\sigma - \rho_{w20})}{\eta_{w20}(\sigma - \rho)} \tag{6}$$

where η denotes viscosity.

If the particles under consideration are known to be spherical and unhydrated, the molecular weight can be determined without the diffusion constant by substituting in equation 2 the value of the molar frictional coefficient for spherical particles:

$$f_0 = 6\pi\eta N\left(\frac{3M}{4\pi N\sigma}\right)^{1/3} \tag{7}$$

in which N is the Avogadro constant. This method of estimating the size is often useful in the case of non-spherical molecules for which diffusion data are not available. It yields a minimum possible molecular weight regardless of the particle shape or the degree of hydration. When the Svedberg formula (equation 5) can be applied, some idea of the departure of the molecular species from the spherical shape can be obtained by comparing f_0 with f as determined from equation 2. f/f_0 is called the frictional ratio and always has a value equal to or greater than 1. However, values above unity may be caused by hydration, as well as by a lack of spherical symmetry, and experiments of another nature would be required to settle the question. Almost all the proteins studied by Svedberg (13) and his associates have shown frictional ratios between 1 and 2.

The sedimentation equations given above refer strictly only to particles isolated from the bounding surfaces of the fluid. However, Svedberg and Rinde (3) have shown that for the purposes of measurement use can be made of a small sector-shaped cell which has two flat walls oriented at right angles to the axis of rotation and two others which, if extended, would intersect along the axis. Particles originally close to any of these walls continue during sedimentation to pursue an average course parallel to the wall so that their radial movement proceeds just as if the bounding surfaces were further removed. However, with the passage of time the concentration of particles does progressively increase near the bottom of the cell and decrease at the top, but if the centrifugal force

is sufficiently high, measurements of rate can still be made, as will be shown. The particles originally at the top or inner surface of the fluid migrate together, forming a "rear line of march" or moving boundary which demarcates the supernatant fluid and sedimenting solute. The sedimentation rate is determined by measuring the position of this boundary at intervals. Generally the boundary becomes progressively less well defined with time, because of a superimposed diffusion of particles across the boundary. However, the position which the boundary would have had in the absence of diffusion is simply the level at which the concentration is one-half that in the lower unaffected sections. When the rate of sedimentation is rapid enough in comparison to the diffusion process there exists for some time, between the diffuse boundary and the section of the cell in which the particles are accumulating against the wall, a region in which the concentration is nearly uniform throughout. The concentration in this region decreases at a steady rate during a constant-speed centrifugation because of diverging radial movements of the particles and because of the fact that the centrifugal force, and consequently the speed of migration, increases with the distance from the axis of rotation. The relation between this concentration C_t at any time t and the original concentration C_0 has been shown by Svedberg and Rinde (3) to be given by:

$$C_t = C_0\left(\frac{x_0}{x_t}\right)^2 \tag{8}$$

where x_0 and x_t are the respective radial distances of the meniscus and the boundary at time t.

If sedimentation is allowed to progress for a sufficiently long time, usually at a rotational speed considerably lower than that used for the sedimentation velocity method, a state of equilibrium is reached in which the tendency of the particles to sediment is exactly counterbalanced by their tendency to diffuse back into the medium. The molecular weight can then be determined by comparing the concentrations C_1 and C_2 of particles at two radial distances x_1 and x_2. The formula for the sedimentation equilibrium method as given by Svedberg is:

$$M = \frac{2RT \ln (C_2/C_1)}{\left(1 - \frac{\rho}{\sigma}\right)\omega^2(x_2^2 - x_1^2)} \tag{9}$$

The sedimentation equilibrium method is most useful for the investigation of purified preparations which are known to be homogeneous, since in a mixture the contributions of several species to the distribution of total concentration are not easily differentiated. The sedimentation velocity method, on the other hand, is ideal for the study of mixtures, since an individual boundary is obtained for each species the rate of which is sufficiently different from that of the other components. The method is of great assistance in judging the purity and homogeneity of a sedimentable material.

In the equations given for the determination of molecular weight it has been assumed, as already stated, that the particles are electrically neutral with respect

to the medium, i.e., that they are not electrostatically attracted and influenced by smaller diffusible ions which sediment more slowly and diffuse faster. However, Tiselius (17) has shown that where electrical dissociation is present, the effect on the sedimentation rate of the principal particle can be rendered negligible by the addition of sufficient non-sedimenting electrolyte. About 1 per cent sodium chloride is usually used to suppress this Donnan effect.

III. HYDRATION

Of the several quantities which enter into the computation of molecular weight from sedimentation data, the density of the particles is the one which has aroused the greatest uncertainty, because it does not lend itself readily to direct measurement. The density of dried or crystallized material can be determined by measuring with a pycnometer the volume displaced by a known weight of substance. However, because of the possibility of hydration there is considerable doubt as to whether this represents the effective density of the particles sedimenting in a centrifugal field. This problem has been treated mathematically by Kraemer (15), who showed that even a comparatively large amount of hydration should not introduce a serious error into the determination of molecular weight according to the Svedberg equations. Because of the importance of hydration, it is considered worth while to discuss the physical principles which form the basis of the mathematical results. Usually the density of a suspending aqueous medium is not greatly different from that of water. As a simplification, consider a case in which they are equal. Even though the volume of a particle is then increased by the attachment or inclusion of water molecules, the net force tending to sediment the particle will not be altered, assuming that the average density of the fluid is not changed by the attachment. This is true by reason of the fact that the force acting on the bound water is exactly balanced by the buoyancy of the surrounding medium. It will thus be seen that the hydration has produced no alteration in equation 1, even though M is the molecular weight and σ the density of the unhydrated particle.

Although the net centrifugal force acting on the particle under consideration is unchanged by hydration, the size of the particle, and hence its frictional coefficient, is increased. Let the subscript h be used to differentiate quantities which refer to the hydrated state. If the same net force acts on the particle regardless of whether it is in the hydrated or unhydrated state, then, since $F_c = f\,dx/dt$, the sedimentation rates for the two cases will compare as the reciprocals of the respective frictional coefficients; i.e.,

$$\frac{s_h}{s} = \frac{f}{f_h} \quad \text{or} \quad sf = s_h f_h$$

This substitution for sf can be made into equation 3, giving

$$s_h f_h = M\left(1 - \frac{\rho}{\sigma}\right)$$

Equation 4 can, of course, be rewritten immediately for hydrated particles:

$$D_h = \frac{RT}{f_h}$$

Eliminating the frictional coefficient between these equations as before, we obtain for the molecular weight of the unhydrated molecule,

$$M = \frac{RTs_h}{D_h\left(1 - \frac{\rho}{\sigma}\right)} \tag{10}$$

which differs from equation 5 only in that the experimentally determined values of the diffusion and sedimentation rates have been obtained with hydrated particles. σ still refers to the unhydrated state. Thus the molecular weight of the unhydrated molecule is given by the Svedberg equation regardless of whether the corresponding sedimentation and diffusion measurements are made on material existing in a hydrated or unhydrated state.

Where the material bound from the medium by the particle is of a density different from that of the medium itself, we may proceed with the same argument, except that in starting, equation 2 is written as

$$F_c = \frac{M}{Z}\left(1 - \frac{\rho}{\sigma}\right)\omega^2 x$$

Here, Z is simply the ratio between the true weight of a mole and the true weight increased by the difference between the weight of the bound material and the weight of an equal volume of the suspending medium. The proportional error in a molecular weight determination is then $Z - 1$, which in most experimental cases is quite negligible.

Consideration of the principles outlined emphasizes the importance of performing the sedimentation and diffusion measurements in identical media under similar experimental conditions, in order that the respective frictional ratios will have the same value. Furthermore, it should be noted that the molecular weight determined by equation 10 is the molecular weight of particles in such a state that their density is the same as that which characterizes the material when the weight of a sample is determined. This is true whether the determination is based on chemical composition or on direct measurements with dried or crystalline material. It can be shown that the above considerations with respect to hydration and particle density apply in exactly the same manner when molecular-weight determinations are made by the sedimentation equilibrium method.

IV. APPARATUS

The Svedberg high-speed ultracentrifuge (5) rotor is driven by oil under pressure and rotates in a reduced atmosphere of hydrogen gas. The sector-shaped cells generally accommodate a column of solution about 16 mm. long and are spun in the rotor at speeds up to approximately 70,000 R.P.M. The thickness of the solution column parallel to the axis of rotation is usually less than 12 mm.,

and the width is only a few millimeters. The center of the cell is 65 mm. from the axis of rotation. The cell is provided with plane-parallel quartz windows which make it possible to direct light rays through the revolving solution in a direction parallel to the axis of rotation. Light passing through openings in a counterbalance or dummy cell at radial levels above or below the solution column is unaffected by the sedimentation and furnishes a convenient reference. Lower speed electrically driven centrifuges with smaller cells are used for making measurements of sedimentation equilibrium (1).

A simplified method of spinning rotors at high speed in a vacuum chamber by means of compressed air has been described by Pickels and Beams (6). Biscoe, Pickels, and Wyckoff (8) and also Bauer and Pickels (9) have incorporated this principle in the design of air-driven ultracentrifuges with rotor dimensions patterned after those of Svedberg. Alternative methods of spinning large rotors by compressed air at high speed have been described by Beams and Pickels (7) and by Pickels (10). More recently an electrical driving mechanism has been developed by Beams and Skarstrom (11).

V. OPTICAL METHODS

All the earlier sedimentation studies conducted by Svedberg and his collaborators were based on the light-absorption method first described by Svedberg and Rinde (3). Light of some suitable wave length which is absorbed by the sedimenting solute, but not appreciably by the solvent, is directed through the cell. Ultraviolet light from a mercury arc is usually employed for protein solutions. With visible light passing through a hemoglobin solution within the revolving cell, for example, the unaided eye sees a bright red annular band extending about the axis of rotation. The meniscus of the fluid appears as a fine line with the same center of curvature. The sedimenting boundary demarcating the clear supernatant and the solution is also concentric about the axis. To obtain a record of the sedimentation, it is only necessary to photograph at intervals a narrow cross-sectional strip of the total annular band of light. A camera of long focal length is used in order to avoid errors of parallax and to give good focusing for all depths in the cell. A typical sedimentation of a large homogeneous protein is illustrated by the series of photographs in figure 1. An absorption photograph showing two sedimenting components is shown in figure 2. These illustrations are discussed in more detail later in this paper.

Other optical methods which have to a great extent superseded the absorption method are based on the detection or measurement of deviations suffered by light rays passing through regions of refractive-index gradient such as exist at sedimenting boundaries. The method which can probably be made subject to the fewest optical errors is the refractive-scale method of Lamm (20).

Increasing attention, however, is being given to the considerably more convenient cylindrical-lens methods, the origin of which can be traced to the work of Thovert (19). Improvements and adaptations for the measurement of migrating boundaries have been described by Philpot, Svensson, and Andersson (21–23). Experience in our laboratory with a modification of Svensson's method

has indicated that even with comparatively inexpensive optical parts a precision of measurement can be attained which is quite sufficient in view of the many minor uncertainties involved in the ultracentrifugal method. Special tests have shown that deviations of light produced at any level of the cell, at least deviations up to 0.02 radian, can be registered at the photographic plate with an error of less than 1 per cent, and that for all deviations in this range the image positions of every level in the cell can be produced with an accuracy of less than 1 per cent of the total cell height.

A schematic representation of the optical principles involved is given in the perspective drawing of figure 3. A camera lens is placed so as to form on a screen the images A′, B′, C′ of the three small holes A, B, and C in a thin diaphragm. A condensing lens is placed so as to focus the image of a horizontal

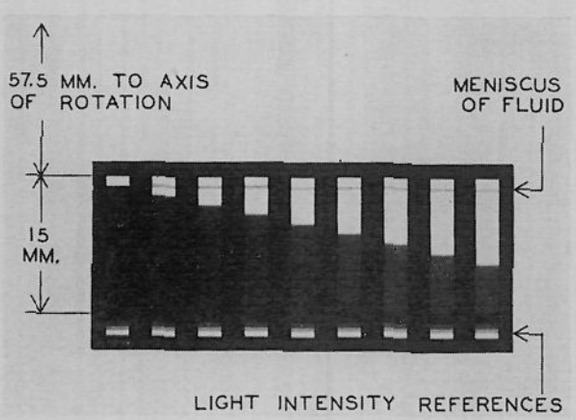

FIG. 1

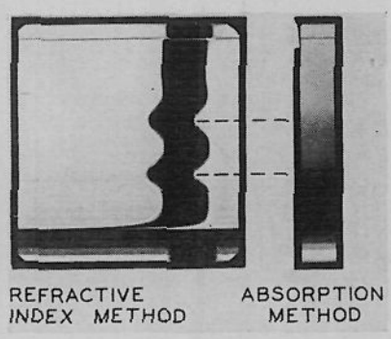

FIG. 2

FIG. 1. Sedimentation of monodisperse hemocyanin in the serum of *Limulus polyphemus*, as shown by the absorption method. Speed, 18,000 R.P.M.; interval between photographs, 15 min.; illumination, 3650 Å. line of mercury; cell thickness, 3 mm.; approximate concentration, 1.45 per cent.

FIG. 2. Sedimentation photographs illustrating correlation between absorption method and refractive-index method. Material, polydisperse hemocyanin in diluted, dialyzed serum of *Limulus polyphemus;* speed, 16,200 R.P.M.; time, 2.26 hr. after full speed; cell thickness, 1 cm.; total protein concentration, 0.41 per cent.

line source on the front surface of the camera lens. Limit consideration to those light rays which pass through the small holes. It can be seen that the light eventually constituting each of the images A′, B′, C′ consists of rays which alternately diverge and converge in accordance with simple optical principles. Each set of rays may be thought of as making up a "light sheet" that varies in width but is always parallel to the horizontal line source. If the light sheet diverging from A toward the camera lens were deviated downward from the normal course at A, as indicated by the dotted lines, it would nevertheless converge at A′ by reason of the fact that the camera lens is focused to produce such a result. An analogy holds for all other images. Suppose that a cylindrical lens with vertical axis is added to the system in such a position that a point light source at the front surface of the camera lens would be focused as a vertical line image at the screen. The effect of this lens on each of the original light sheets will be to converge it

prematurely, so that it again diverges and forms a horizontal line image $A_1'A_2'A_3'$ on the screen instead of a point image. The vertical positions of the images are not altered by the cylindrical lens. A downward deviation (dotted lines) of the sheet at A would not alter the position of the line image. Suppose that over the front surface of the camera lens is placed a mask provided with a narrow inclined slit. It can be seen that of the undeviated rays through A, only the central one is now able to pass through the camera lens and reach the screen, where it will appear as a point of light A_2', as contrasted to the previous horizontal line image $A_1'A_2'A_3'$. Since the undeviated light sheets through A, B, and C all converge

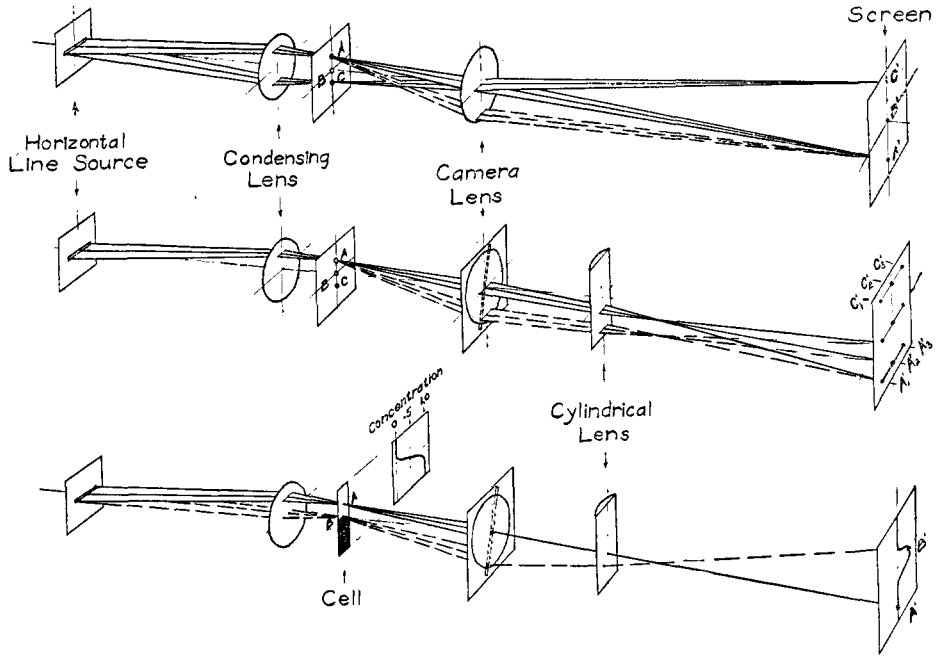

FIG. 3. Perspective schematic drawing illustrating the working principle of a refractive-index method employing a cylindrical lens.

in a common image of the light source at the mask, it follows that B and C will also be represented at the screen, when there is no deviation at the diaphragm, by light points B_2' and C_2', which are situated along a central vertical line passing through A_2'. However, if the light sheet from A, for example, is deviated downward sufficiently from its normal course, then only the ray at one edge of the light sheet will pass through the camera lens, and the light point corresponding to A will appear on the screen at A_3'. The horizontal distance by which any light point will be displaced from the central line $C_2'A_2'$ is directly proportional to the downward displacement suffered by the corresponding light sheet at the diaphragm. The same holds true for light coming from B or from C, and thus the system is capable of translating a vertical deviation of light into a horizontal

displacement of a light point on the screen without altering the point's vertical height which is characteristic of a certain level in the cell.

Consideration will show that the action of the system is not altered by substituting narrow horizontal slits for the holes A, B, and C. For any given deviation of a light sheet, more rays will pass through the camera lens, but since they do pass through at the same point the cylindrical lens converges them into a point focus again at the screen. Now suppose that a cell containing an immobilized sedimentation boundary is substituted for the diaphragm in such a way that the maximum refractive-index gradient, i.e., the center of the boundary, is in the position B. The corresponding point B′ will be the one suffering the maximum horizontal displacement from the center line on the screen. Light coming through the cell below and above the boundary, as at A, is not deviated and the corresponding light points fall on the center line. Displacements of light points corresponding to successive positions within the boundary are proportional to the respective refractive-index gradients, with the result that the light points form a smooth continuous curve on the screen. The peak of the curve, if symmetrical, determines the position of the boundary; the area between the center line and the curve is proportional to the concentration of the material forming the boundary, assuming it is the only source of refractive gradients. With the ultracentrifuge there are extraneous gradients due to hydrostatic compression and distortion of the cell, and a curve incorporating these alone and called the base line must be obtained. If heavy salt ions are present in sufficient strength in the medium, they too produce gradients which must be taken into account by making separate ultracentrifugation runs with the medium alone (1). In applying the method to the centrifuge it is important to limit the light passing through the revolving cell to a zone having a width of not more than a few millimeters in a tangential direction.

In our laboratory the substitution of an opaque strip for the inclined open slit has produced certain advantages which will be described in a later paper. In place of a line of light as described above, a dark band against a bright background indicates deviations of light at various depths in the cell. Measurements are taken from either or preferably from both edges of this band.

The photographs of figure 4 illustrate an application of the refractive-index method employing a cylindrical lens. These were obtained during the same run as were those of figure 1. Figure 2 is an example of the simultaneous sedimentation of two protein components and shows the correlation existing between photographs taken of the same material at the same time by the refractive-index and the absorption methods.

For most cases the refractive-index method is preferable to the absorption method, particularly in the study of mixtures and where it is desired to determine the absolute or relative concentrations of several components. There is a considerably greater proportional variation in the absorption coefficients of proteins in the ultraviolet region, for example, than there is in their specific refractive indices in the visible region. As a matter of fact, good approximations of the concentrations of protein components of unknown optical properties can nearly

always be made with the refractive-index method. It is also an advantage to be able to observe the sedimentation on the screen with visible light during sedimentation. Furthermore, it is more expensive and less convenient to work with ultraviolet light, which requires quartz optical parts.

A good refractive-index system is subject to fewer and less serious errors than is an absorption system. The pictures shown in figure 1 represent an unusually sharp boundary of a fairly concentrated, slowly diffusing material. Measurements can be made directly from the photographs. In actual practice, however,

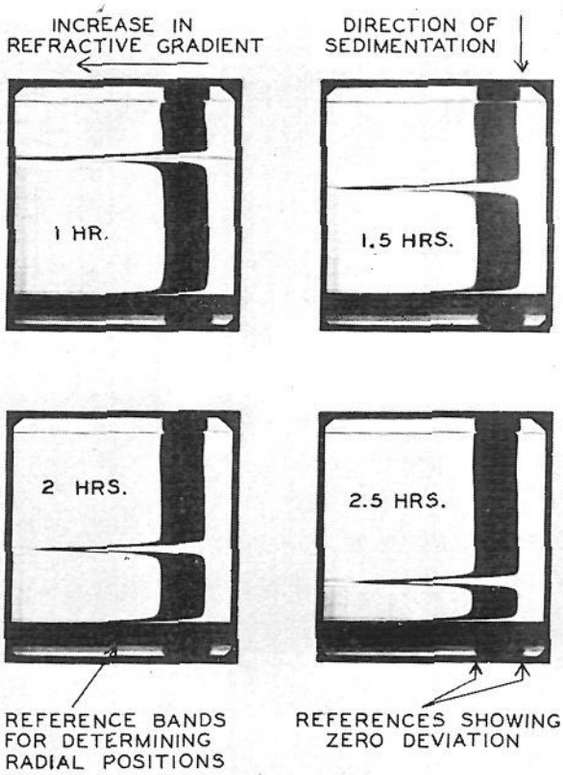

FIG. 4

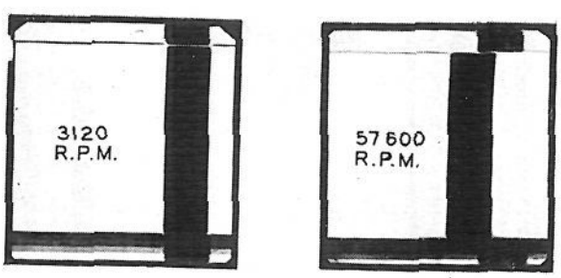

FIG. 5

FIG. 4. Pictures taken during the experiment illustrated in figure 1 and showing sedimentation in the same hemocyanin solution, as recorded by the refractive-index method.

FIG. 5. Photographs illustrating the gradients of refractive index introduced in a water-filled ultracentrifugal cell at high speed by hydrostatic compression and distortion of the cell.

boundaries of smaller, more common proteins are more diffuse, such as those shown in figure 2. To analyze absorption photographs, it is then necessary to employ a microphotometer which will measure the photographic density of the plate at all levels in the cell picture. Results obtained must then be translated into measurements of concentration. The analysis is subject to all the usual photographic errors, as has been pointed out by Pedersen (1). The apparent position of a diffuse boundary and the apparent concentration distribution throughout the cell can be greatly altered by photographic overexposure or underexposure and also by the use of a highly absorbing preparation in the cell. It is imperative to use monochromatic light of high purity and to illuminate the cell uniformly.

In some cases, however, the absorption method is to be preferred. For example, Pickels and Bauer (14) found that the virus of yellow fever in infected monkey serum showed a high differential absorption in the upper ultraviolet region, and they were able to study, by absorption, sedimentation boundaries which could not be detected with the refractive-index system. Since the precision of the refractive method depends on the magnitude of the concentration gradients present rather than on the concentration itself, the absorption method is sometimes more applicable with unusually ill defined boundaries or with very inhomogeneous preparations.

VI. EXPERIMENTAL CONSIDERATIONS

As Svedberg (13) has indicated, it is necessary that the sedimenting material be not disturbed by convection currents in the solution. In our work it has been found very important to have the cell aligned correctly within the rotor to prevent sedimentation of particles against the side walls. When even slight departures are made from this rule, photographs are obtained with a monodisperse substance which falsely give the impression of varying amounts of inhomogeneous material sedimenting below the main boundary.

The principal potential sources of convection currents are temperature gradients which must exist within the cell to insure the passage of heat from the sections of the rotor exposed to the greatest proportion of frictional resistance to those exposed to smaller amounts. The convection action is enormously magnified by high centrifugal force and can interfere seriously with the sedimentation of slowly moving particles. The gradients are quite small in most modern ultracentrifuges, having been estimated at about 5×10^{-4} °C. per centimeter for a rotor of the vacuum-type ultracentrifuge operating at 60,000 R.P.M. in rarified air at a pressure of 5×10^{-7} atm. However, gradients should not be discounted when the rotor is spinning under less favorable circumstances, for instance, in hydrogen gas at a pressure of 0.025 atm. Theoretically a density gradient due to temperature differences would then be of such a magnitude that a concentration gradient of protein amounting to 10^{-3} per cent per centimeter would be required to neutralize it. Wherever concentration gradients are very small, which is the case above and below boundaries of monodisperse proteins, for example, convection can take place, and it should be expected that the behavior of a diffuse boundary of a material in an unusually low concentration might easily be influenced.

It is a common impression that the radial density gradient introduced into a liquid by the application of centrifugal force is a marked stabilizing influence against convective disturbances. In a filled ultracentrifuge cell revolving at 57,600 R.P.M., for example, the outer layer of liquid is actually at least 1 per cent more dense than the inner layer. The magnitude of the concentration gradient is readily visualized by reference to the illustrations in figure 5. Only a small proportion of the refractive-index gradient is due to distortions of the cell. The maximum temperature gradient through the cell, for instance, could never cause a density gradient of the order cited, and it might at first thought seem that the

compressed fluid would tend to remain in the lower part of the cell and thus prevent any stirring which might otherwise be caused through the action of weak opposing gradients due to thermal influences. The fallacy of this viewpoint can be realized by considering the energy changes in the system when it is disturbed while free from temperature gradients. If the state of the system is the most stable one, i.e., the one of lowest potential energy, then any interchange of fluid at different heights by some outside mechanism should increase the potential energy of the system with respect to the axis of rotation. Actually, if the top and bottom layers of fluid are interchanged, their respective densities reverse immediately in accordance with their new locations and the potential energy of the system is just the same as before. The result would be quite different if a cold (dense) layer of water at the bottom of a cell were exchanged with a warm (less dense) top layer. In this case the density of neither layer would be affected immediately by the transition, the potential energy would be increased, and the system would try to seek its original condition. The fundamental difference is that while the compressional density is a direct function of the depth below the fluid surface, the thermal density is not. Therefore, no account has to be taken of the compressional effects in considering the disturbances caused by temperature gradients in the ultracentrifuge.

It is, of course, important to know accurately the temperature of the solution while it is revolving in the ultracentrifuge in order to compare its viscosity with that of the standard medium. Adequate methods for making such measurements are in use with both the oil-driven (1) and the air-driven (9) machines. Runs are usually made at temperatures within a few degrees Centigrade of room temperature. For the investigation of proteins which exhibit excessive instability under such conditions, Rothen has surrounded his air-driven ultracentrifuge rotor with stationary refrigerated metal surfaces which permit him to maintain the material under study near 0°C. (16). Hydrogen at a reduced pressure of about 0.1 mm. of mercury furnishes the thermal conduction. For reasons of stability, as well as to avoid convective disturbances, it is often advisable to perform long diffusion experiments at about +4°C. In view of the theoretical importance of making sedimentation and diffusion measurements at the same temperature, Rothen's arrangement promises to widen the scope of studies suited to the Svedberg equation.

In the earlier experiments of Svedberg (4), determinations of the diffusion constant were attempted upon the material under study while it was sedimenting in the ultracentrifuge. An argument in favor of the procedure was that the environmental conditions were identical for both sedimentation and diffusion measurements. In later years it was found that the diffusion values obtained in this manner were not very reliable. The probable reason for the discrepancy between diffusion measurements made in the centrifuge and in a diffusion apparatus can be understood when one considers that a small inhomogeneity in the sedimentation rate of a group of particles can produce a comparatively large effect as far as the sharpness of a sedimentation boundary is concerned. For example, small amounts of aggregated or denatured material settling at slightly faster rates than

the primary particles could produce such an effect. Small amounts of convection below or above the boundary could definitely change its form and apparent diffusion rate without appreciably altering the sedimentation rate. The potentialities of this mechanism must be considered when low concentrations of material are used. It will be recalled in this connection that the Svedberg equations apply strictly only for very dilute solutions in which no interaction between particles can be assumed. It has been the practice of Svedberg and his associates to make determinations of the sedimentation rate on a series of progressively lower concentrations of the solute under investigation. The results are extrapolated to zero concentration, and the respective sedimentation rate obtained is assigned as the true sedimentation constant of the material. Often there have been unexpectedly large differences between the rates for different dilutions (1). Most of the variations have been explained on the basis of a dissociation phenomenon. It is quite possible, however, that some of the discrepancies at very low dilutions were caused at least partly by convection.

Even with a convection-free, monodisperse system there is still another factor which affects the sharpness and apparent diffusion rate of a sedimenting boundary. Its influence increases with the concentration. There is through the boundary a relatively rapid shift in the solute concentration, so that the sedimenting particles at the trailing edge of the boundary are in a very dilute concentration compared to those on the leading edge. The more widely separated particles tend to sediment slightly faster, since the effective viscosity of the medium increases to some extent with increasing concentration of the sedimenting component, possibly because some interaction phenomenon exists between crowded particles, or perhaps because the effective buoyancy force is a function of concentration, or possibly because the backward streaming of the displaced medium becomes an important factor at higher concentrations. In any event, the boundary tends to resharpen itself continually in opposition to the tendency of the solute to diffuse. The effect is well illustrated by the photographs of figures 1 and 4. They show boundaries which with time spread only a small proportion of the amount to be expected from measured values of the diffusion constant. The contrast with the appearance of the more dilute boundaries in figure 2 is at once evident.

In our laboratory, tests are being made of a method by which it is hoped that reliable diffusion measurements can be made in the ultracentrifuge under certain conditions. It would offer great advantages in the study of mixtures the individual components of which cannot conveniently be isolated in pure form for diffusion measurements. With the vacuum-type ultracentrifuge it has been found possible to decelerate the rotor to very low speeds without disturbing boundaries partially sedimented rapidly at high speed. The boundaries are then allowed to diffuse, and diffusion constants are determined from measurements taken at the low speed. With insignificant centrifugal force, the migration of the boundary becomes negligible and the principal objections to the determination of diffusion constants in the ultracentrifuge cell are avoided. Minor corrections for the sectoral shape of the cell can be applied.

VII. CONCLUSION

The development of the ultracentrifugal method by Svedberg and his collaborators and their application of the technique to the investigation of molecular weights have become accepted as outstanding scientific achievements. The theoretical validity of the method is well established, and the principal sources of error in the earlier experimental procedures appear to have been realized and corrected. The field of research has become even more promising with time, and at present the scope of application is still being widened by further refinements and by the introduction of new related techniques.

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