# MOLECULAR WEIGHT DETERMINATIONS WITH A MAGNETICALLY SUPPORTED ULTRACENTRIFUGE[1]

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A magnetically suspended ultracentrifuge has been used to determine the molecular weights and related properties of various substances. Techniques are described which make this apparatus particularly suitable for equilibrium studies of macromolecules in the $10^5$ to $10^7$ molecular weight range.



The equilibrium method is generally considered to be the most reliable of the several centrifuge techniques for molecular weight determinations and related problems because it is based upon rigorous equilibrium thermodynamic theory.[2] In fact, in the case of large macromolecules of unknown shapes, particularly with synthetic polymers, the equilibrium method is probably the only centrifuge technique which yields direct meaningful results. The principal practical disadvantage has been the long centrifuging time required for equilibrium to take place. However, in recent years this problem essentially has been solved through the use of one or a combination of three techniques for effectively reducing the equilibrium time.

The first is the use of short ultracentrifuge cell columns as discussed by Van Holde and Baldwin[3] and illustrated by Yphantis[4] and others. In general the time required to reach equilibrium is proportional to the length of the column squared. Secondly, a considerable decrease in the time required to reach equilibrium is found both experimentally[5,6] and theoretically[7] in the case where the rotor speed is very slowly decreasing. Thirdly, the introduction of a predetermined step function reduction in angular speed was found to reduce greatly the centrifuging time without sacrifice of accuracy.[8]

## Experimental

An improved magnetically suspended ultracentrifuge has been described in detail elsewhere,[5,6] so only its essential characteristics need be mentioned here. The rotor is magnetically suspended in a vacuum and "coasts" freely during the sedimentation experiments. It loses less than one part in $10^8$ of its speed per second when the pressure surrounding the rotor is between $10^{-6}$ and $10^{-7}$ mm. For rotor speeds of 100 r.p.s. as used in some equilibrium centrifugation, this amounts to less than $^1/_{10}$ r.p.s. per day. The rotor may be accelerated or decelerated during an experiment by a detachable high speed electric motor. The rotor is free of all hunting, and its speed is measured routinely to better than one part in $10^5$. The rotor temperature is constant and known to at least one part in $10^4$. The centrifuge may be used either for sedimentation equilibrium or velocity of sedimentation experiments, although its principal use has been for the former. The maximum speed is set only by the strength of the rotor and the minimum can be made as small as desired, since stability is independent of the speed.

One type of ultracentrifuge cell used is shown in Fig. 1. It contains two sector-shaped compartments, has a depth of 1 cm., and is 2.7 cm. in diameter. Circular shaped cells have also been used in this Laboratory as well as circular and sector-shaped multichannel cells which permit multiple determinations during the course of one experiment. The one-piece cells are made of either anodized duralumin or Kel-F. It is to be noted that pressure is applied to the quartz or glass windows normally by five pressure screws, which eliminates distortion of the windows often found when they are tightened using screw caps. Although it is much easier to construct circular or rectangular rather than sector-shaped compartments and the equilibrium concentration distribution is theoretically independent of the shape of the cell, the sector-shaped compartments have been found to be much preferable. This probably is due to convection currents set up in the non-sector-shaped cells which are easily observed with the associated optical system.

The sedimentation in the cell is measured by a Jamin type interferometer which is used with parallel light as shown in Fig. 2. Two light sources at equal optical distances from the lens $L_1$ can be alternated during an experiment by placing the mirror $M_w$ into or out of the beam. One is a 1500-watt projection lamp which serves as a white light source and the other a 1200-watt mercury arc lamp which is used with a filter as a monochromatic light source. The lens $L_1$ focuses the light on the "chopper" slit K′ mounted in the rotor with its length along the radius. The light is then focused by the lens $L_2$ on a small hole $S_1$. This provides a small point source. The lens $L_3$ renders the light parallel which is reflected from $M_4$, limited by the slit $S_2$ before incidence upon the first Jamin plate $I_1$. The Jamin plate splits the light into two parallel displaced beams. Each of the beams passes through a sector compartment of a compensating ultracentrifuge cell $K_1$, then through a compartment of an identical cell $K_2$ mounted in the rotor, and impinges on a second Jamin plate $I_2$ where the two beams are reunited. $I_1$ and $I_2$ are matched glass interferometer plates 30% silvered on the front and 100% silvered on the back. The light is then focused by $L_4$ on spectroscopic plates which are sensitive in the spectral region used. Each of the two identical cells, $K_1$ and $K_2$, contains two sector shaped vacuum tight compartments side by side, one of which contains the solvent and the other the solution. The cells are so placed that the light beam which traverses the compartment in $K_1$ containing the solvent passes through the compartment in $K_2$ containing the solution and *vice-versa*.

The optical system is adjusted, while the rotor is at rest, using the monochromatic light source. The fringes are accurately aligned parallel to the radius of the cell and made very broad. White light is then substituted for the monochromatic light and the Jamin plates are further adjusted to give a single broad white light fringe which covers the entire field of view on the plate P. This is possible because the optical paths of the two beams between $I_1$ and $I_2$ are equal. The centrifuge is then started and the concentration gradient produced in the compartment of $K_2$ containing the solution will give rise to fringes which lie along lines of constant radial distance if the adjustment of $I_1$ and $I_2$ is done properly. These fringes and the cell $K_2$ are in accurate focus on the photographic plate P; *i.e.*, there is a point to point correspondence between cell and fringes which immediately shows any convection in the cell if it exists. The change in con-

(1) Supported by N. I. H. Grant and Navy Bureau of Weapons Grant.

(2) (a) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, New York, N. Y., 1940; (b) H. K. Schachman, "Ultracentrifugation in Biochemistry," Academic Press, New York, N. Y., 1959.

(3) K. E. Van Holde and R. L. Baldwin, *J. Phys. Chem.*, **62**, 734 (1958).

(4) D. A. Yphantis, *Ann. N. Y. Acad. Sci.*, **88**, 586 (1960).

(5) J. W. Beams, R. D. Boyle, and P. E. Hexner, *J. Polymer Sci.*, **57**, 161 (1962).

(6) J. W. Beams, R. D. Boyle, and P. E. Hexner, *Rev. Sci. Instr.*, **32**, 645 (1961).

(7) W. J. Archbald, private communication.

(8) P. E. Hexner, L. E. Radford, and J. W. Beams, *Proc. Natl. Acad. Sci. U. S.*, **47**, 1848 (1961).

centration $\Delta c$ in the cell is given by the relation $\Delta c = (\lambda/KL)\Delta n$ where $K = \Delta u/\Delta c$, $L$ is the thickness of the cell, $\lambda$ is the wave length of the light, $\Delta u$ the change in refractive index of the solution under test, and $\Delta n$ is the number of fringes produced on the plate. Also, $\lambda\,\Delta n = L\,\Delta u$. $K$ is measured outside the centrifuge by a differential refractometer or a Michelson type interferometer. The position of the fringes on the plate P are measured by a comparator and a special microphotometer. From these measurements of the positions of the fringes produced by monochromatic light, the change in concentration between any two points in the cell is determined. The position of the central white light fringe gives the position in the cell where the concentration is the same as the initial uniform concentration $c_0$ in the cell before the centrifuging is started. Consequently, with the known value of $c_0$, the concentration $c_r$ at every point in the cell can be determined. Also, it should be noted that with this optical system little or no extrapolation to the ends of the cell is required.

When equilibrium is obtained in the centrifuge cell $K_2$, for dilute solutions, the molecular weight $M$ is given by the relation

$$M = \frac{2RT \ln (c_2 f_2/c_1 f_1)}{4\pi^2 N^2 (1 - \bar{v}\rho)(r_2^2 - r_1^2)} \qquad (1)$$

where $c_1$ and $c_2$ are the concentrations at the radial distances $r_1$ and $r_2$ in the ultracentrifuge cell, $T$ is the absolute temperature, $R$ is the gas constant, $f_1$ and $f_2$ are activity coefficients, $\rho$ is the density of the solution, $\bar{v}$ is the partial specific volume, and $N$ is the number of r.p.s. For an ideal monodisperse dilute solution eq. 1 becomes

$$c_r = \frac{c_0 AM(b^2 - a^2) \exp\langle -AM(b^2 - r^2)\rangle}{1 - \exp\langle -AM(b^2 - a^2)\rangle} \qquad (2)$$

where $A = (1 - \bar{V}\rho)\,4\pi^2 N^2/2RT$, $b$ is the peripheral radius of the cell, and $a$ is the radius of the top or meniscus in the cell. Also

$$\int_a^b c_r r\,dr = c_0 \int_a^b r\,dr$$

Consequently, a plot of ln $c$ *vs.* $r^2$ gives a straight line for an ideal monodisperse solution at equilibrium. In general, if the curve is concave upward, polydispersity is indicated, and if concave downward, the solution is probably non-ideal. From the slope of this line the value of the molecular weight can be determined. At the radial distance where $c_r = c_0$, eq. 2 may be written

$$1 - \exp\langle -AM(b^2 - a^2)\rangle = AM(b^2 - a^2) \exp\langle -AM(b^2 - r^2)\rangle$$

and the molecular weight is obtained[9] by solving for $M$. This, of course, does not require waiting for equilibrium to be established and can be carried out in a short centrifuging time. The position of the "hinge point" can be precisely determined from two photographs, one with white light and one with monochromatic light.[5,6] Also, it might be noted that this "hinge point" can be determined by photographs taken with two monochromatic, widely separated wave lengths such as 5460.74 and 4358.35 Å of the mercury arcs, or the ruby Laser light 6943 Å. and either of the mercury lines. Incidentally, the pulsed Laser may be used without the chopper slit.

The above measurements give $c_r$ as a function of $r$. From these values $\partial c/\partial r$ can be derived. On the other hand, it sometimes is desirable to measure $\partial c/\partial r$ directly. This also can be carried out with the interferometer shown in Fig. 2. It will be observed that the light beam is incident on the first Jamin mirror $I_1$ at approximately 45°. This is done to get a proper separation of the two beams which pass through the two compartments in $K_1$ and $K_2$. On the other hand, if the light is incident normally on $I_1$ then the beams are not separated. Consequently, the beams may be made to have any separation from zero to at least the distance between the centers of the two cell compartments by varying the angle of incidence on $I_1$ and $I_2$ accordingly. If now the distance between the beams is reduced and the plates $I_1$ and $I_2$ oriented so that the two beams are separated in a direction parallel to the radius rather than perpendicular to the radius as in Fig. 2, then one sees in the field of view images of the two compartments of $K_2$. With proper adjustment, fringes will appear in the image of the compartment containing the solution if a concentration gradient exists. The number and position of these fringes make it possible to determine $\partial c/\partial r$ directly. No fringes should appear in the image of the compartment containing the solvent unless distortions in the windows occur so that this image serves as a control.

(9) W. J. Archibald, *J. Phys. Chem.*, **51**, 1602 (1947).

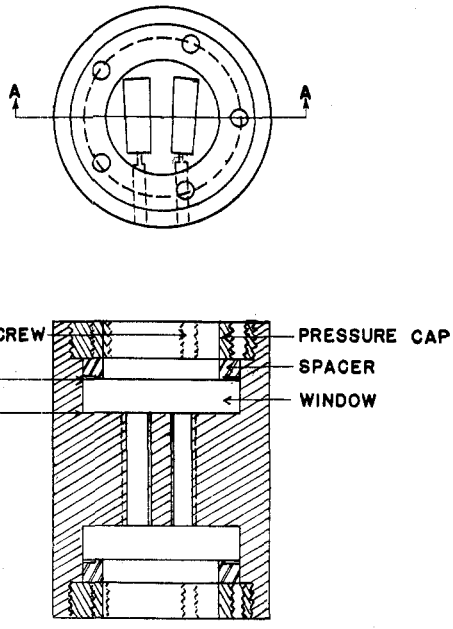


Fig. 1.—Sector-shaped cell.

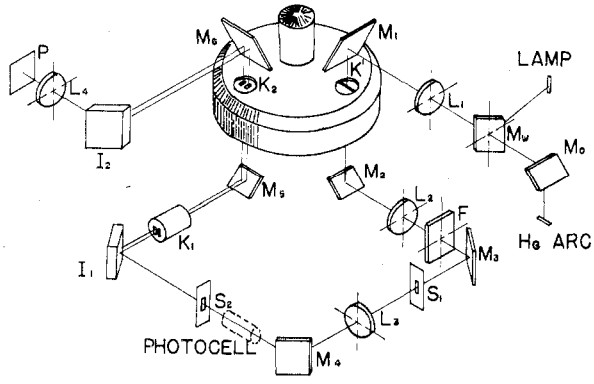


Fig. 2.—Optical method of measuring sedimentation.

## Results and Discussion

The method of shortening the time required to attain sedimentation equilibrium by a predetermined reduction in angular speed early in an experiment has proven to be most useful in recent experiments with very large macromolecules. As reported previously,[8] a ribonuclease molecular weight determination can be reduced with no apparent loss of precision from 14 to approximately 2 hr. with a 3 mm. cell. The real value of the method becomes more apparent with two specific examples.

An 18S protein, extracted from green leaves of bean plants, having a molecular weight of ap-

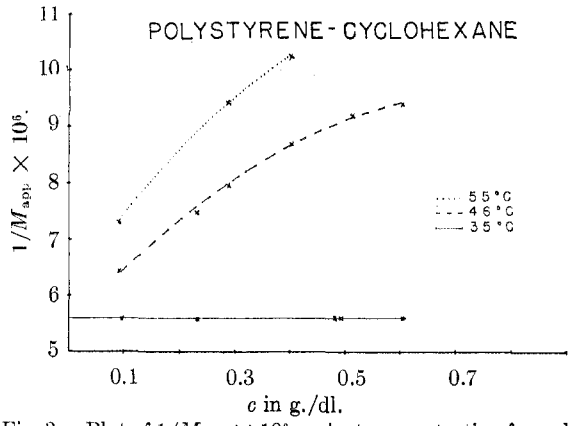


Fig. 3.—Plot of $1/M_{app} \times 10^6$ against concentration for polystyrene ($M_w$ = 180,000) in cyclohexane at 35, 46, and 55°.

proximately $2.75 \times 10^5$, requires a sedimentation equilibrium time on the order of 5 days with a 3 mm. cell, at maximum permissible speeds. Even though the magnetically suspended ultracentrifuge is well suited for experiments of this duration, the sample both dissociates and aggregates after prolonged exposure at temperatures of interest. Due to the polydispersity of the sample, the Archibald approach to equilibrium method[9] yields inconclusive results. By reducing the rotor speed from 100 to 80 r.p.s., equilibrium is achieved in 10 hr.

A sample of polystyrene with a weight average molecular weight of $3.5 \times 10^6$ in cyclohexane at 35° requires 14 days to reach equilibrium in a 3 mm. cell at the maximum permissible speed of 28 r.p.s. Again, the magnetically suspended ultracentrifuge is quite capable of operating for this duration, but a 14 day experiment is at best inconvenient. Utilizing the "cut back" method from 28 to 20 r.p.s., excellent results were obtained in approximately two days. This sample also illustrates the stability of the rotor suspended in a vacuum of $10^{-6}$ Torr by the magnetic field. No hunting of the rotor was observed at these speeds and excellent photographs were obtained at 20 r.p.s., a speed at which stable operation of a mechanically linked rotor is very difficult.

A well fractionated, essentially monodisperse sample of polystyrene, kindly furnished by Dr. D. McIntyre of the National Bureau of Standards, has been used extensively with this apparatus to determine the accuracy of the "cut back" method in the $10^5$ molecular weight range and to study the behavior of polymers in solutions at temperatures other than theta.[10] Table I shows the results obtained at 35°, the theta temperature in cyclohexane, the first experiment listed being a "true" equilibrium run of 36 hr. duration. The other results were obtained using the "cut back" method in approximately 5 hr. Figure 3 shows results obtained at 46 and 55°.

The purpose of the temperature variation experiments is to determine whether it is possible to find the true molecular weight of a polymer by extrapolation to infinite dilution of data obtained

(10) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

TABLE I

| Concn., g./dl. | $M_w$ |
|---|---|
| 0.4805 | 179,000 |
| .0903 | 180,500 |
| .2312 | 180,100 |
| .4935 | 179,200 |
| .6110 | 179,500 |

at temperatures other than theta.[11,12] Figure 3 indicates that the answer to the question depends, at least in the case of a relatively low molecular weight and essentially monodisperse polymer, on the deviation from theta temperature and on the accuracy desired. Data with this sample in benzene at room temperatures confirm the above conclusions. Experiments on a higher molecular weight polymer with a greater $M_z/M_w$ ratio are now being conducted.

Comparative measurements on the above sample have been made,[13] using an L.K.B. Produkter ultracentrifuge with a Lamm scale optical system at the National Bureau of Standards, and a Spinco Model E ultracentrifuge with a Raleigh interferometric system at the Department of Biochemistry of the University of Virginia School of Medicine. The results are very encouraging although the quantitative agreement of the molecular weights is not exact. The dispersion of the numerical results with the magnetically suspended ultracentrifuge is much smaller than with the other instruments. This is probably due to the difficulty of measuring the concentration gradient near the ends of the cell with the Lamm scale optical system in the case of the L.K.B. centrifuge, and to variations in speed and relative instability of the rotor at low speeds with the particular Model E used. Only constant angular speed equilibrium runs were performed with the L.K.B. The "cut back" procedure was attempted with the Model E and, although relatively successful at higher speeds with samples such as ribonuclease, did not significantly reduce the equilibrium time with this sample due to stirring during "cut back" at low speeds.

The precision of the magnetically suspended ultracentrifuge has been coupled with a Burroughs 205 digital computer to gain valuable information on the molecular weight distribution in polydisperse samples. This essentially is a self-consistent guessing procedure made feasible by the rapidity of computer calculations. In a system of $n$ components, the concentration in the cell at some point $r$ from the center of rotation at time $t$ is the sum of the concentrations of the $n$ components. Assuming the same buoyancy and specific refractive increments for all components, this can be written

$$c(r,t) = \sum_{i=1}^{n} c_i(r,t) = \sum_{i=1}^{n} A_i e^{\lambda_i r^2} + \sum_{i=1}^{n} F_i(r,t)$$

where $F_i(r,t) = 0$ at $t = \infty$.

(11) L. Mandelkern, L. C. Williams, and S. G. Weissberg, *J. Phys. Chem.*, **61**, 271 (1957).

(12) H. Fujita, A. M. Linklater, and J. W. Williams, *J. Am. Chem. Soc.*, **82**, 379 (1960).

(13) P. E. Hexner, H. G. Kim, F. N. Weber, D. McIntyre, L. C. Williams, R. F. Bunting, and D. W. Kupke, in press.

Since the only unknowns in the above equations are $c_{0i}$ and $M_i$, both contained in $A_i$, $\lambda_i$, and $F_i$, educated guesses for the unknowns are self-consistently programmed into the computer until the results agree with the experimental data. If an adequate supply of sample is available, a series of equilibrium runs is made to ensure uniqueness and to preclude the use of the time dependent term in the above equation. Excellent results have been obtained with this method on known mixtures and with proteins which have a tendency to aggregate.

In the case where $n$ is very large, the above method is not practical and the problem reduces to solving an integral equation. A formal solution has been found, but practicability awaits a sample with a known distribution.

The magnetically suspended ultracentrifuge has yielded excellent results with low molecular weight compounds such as sucrose, raffinose, ribonuclease, insulin, lysozyme, and others.[5,6,14] Its greatest potential, however, seems to be in the field of large macromolecules of unknown shapes due to the extreme stability of the rotor at very low speeds and the inherent advantages of the associated optical system.

(14) R. D. Boyle and P. E. Hexner, *Science*, **134**, 339 (1961).

**Acknowledgments.**—The authors are greatly indebted to H. G. Kim (American Machine and Foundry Fellow) and F. N. Weber, who participated in most of the experiments, and to R. F. Bunting, D. W. Kupke, and D. McIntyre for valuable advice. F. Linke, P. Sommer, and their co-workers constructed most of the apparatus.

## DISCUSSION

L. GROPPER (Beckman Instruments, Inc.)—At low speed when the cell is tilted to offset gravity in the downward direction, are the optics tilted also?

P. E. HEXNER.—The mirrors directly below and above the cell are tilted proportionately to ensure that the light passes through the cell normal to the direction of the resultant forces.

R. TRAUTMAN (Plum Island Animal Disease Laboratory, USDA).—Is it better to tilt the cell or solve the equations for equilibrium in a gravitational and centrifugal field?

P. E. HEXNER.—The purpose of tilting the cell is to prevent convection due to the non-radiality of the resultant forces on the particles. The equations must be solved in two dimensions in this case.

NORMAN G. ANDERSON (Oak Ridge National Laboratory).—You are very close to the point where the centrifuge can be eliminated and gravity itself used to produce the gradient.

P. E. HEXNER.—Since this is a very interesting and stimulating field we certainly hope that the centrifuge will not be eliminated. For large enough particles gravity can, of course, be used to produce a concentration gradient.