THE MAGNETICALLY SUPPORTED EQUILIBRIUM ULTRACENTRIFUGE*

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THE ultracentrifuge methods for measuring molecular weights originally were introduced by Svedberg and his students over thirty years ago, and have been used extensively by them in their classical experiments on the characterization of proteins (1). At the present time, as a result of the development of good high speed ultracentrifuges, some of which are commercially available, the methods are widely used in studies especially of high molecular weight compounds both in this country and abroad. In addition to the determination of molecular weights, the ultracentrifuge is of great use in the purification of many different substances (1, 2). It is fortunate that the centrifugal fields necessary to purify a substance or to measure its molecular weight are far too small to deactivate appreciably even the most unstable substances so far studied.

Two principal methods of determining molecular weights by the ultracentrifuge method are in use (1). The first known as the velocity of sedimentation method requires a relatively high centrifugal field and a centrifuging time of a few hours, while the second, known as the equilibrium method, requires a much smaller centrifugal field but a centrifuging time of several days, or even weeks. In the past, the velocity of sedimentation method has been more widely employed where the molecular weights are large enough to give an appreciable rate of sedimentation. The principal reason for the limited use of the equilibrium method in the past has been the great difficulty of maintaining the rotor speed and rotor temperature of the ultracentrifuge sufficiently constant for the long times required for equilibrium to take place. On the other hand the equilibrium method is more reliable because it is based directly upon thermodynamic theory instead of Stoke's Law, as is the case with the velocity of sedimentation method. In addition, it gives absolute values of the molecular weights directly. Furthermore, with the same rotor speed, the equilibrium method may be used for much smaller molecular mass measurements and does not require a knowledge of the diffusion constants.

The purpose of this paper is to describe some improvements in the equilibrium ultracentrifuge technique which we have under way at the University of Virginia (3, 4, 5). If M_e is the molecular weight of a mono-disperse substance, then by the equilibrium method

$$M_e = \frac{2RT \log_e c_1/c_2}{4\pi^2 N^2 (1 - Vd)(r_1^2 - r_2^2)}$$

where c_1 and c_2 are the concentrations at the radial distances r_1 and r_2 respectively, V is the partial specific volume, d is the density of the solution, N the number of revolutions per second of the rotor and T is the absolute temperature. The above relation holds strictly only for an ideal dilute incompressible solution so that when the solution is non-ideal and not dilute, it must be corrected (1). Professor John W. Williams and his students (5) especially have shown how this may be done. However, in all cases the three quantities to be measured while the centrifuge is spinning are the rotor speed, rotor temperature, and the concentration of the solution at the various radial distances in the cell. The other factors such as V and d are determined from measurements made outside the centrifuge cell.

Figure 1 is a schematic diagram of the apparatus. The rotor is freely suspended inside a non-magnetic vacuum chamber by means of the axial magnetic field of the solenoid. The rotor is accelerated to operating speed by means of an air turbine situated below the vacuum chamber. The turbine is connected with the rotor by a small-diameter flexible steel shaft which passes through vacuum tight low vapor pressure oil glands. When the desired operating speed is reached, the shaft is disengaged from the rotor by means of an air piston arrangement located below the turbine and the rotor coasts freely during the course of the sedimentation measurements. Figure 2 shows the drive system in detail. It might be noted that when the shaft is pulled out of the rotor the air

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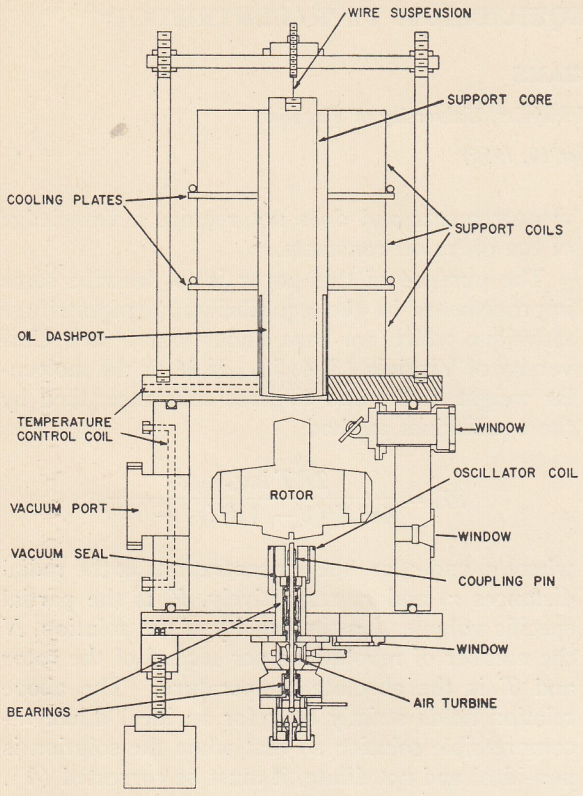

FIG. 1. Diagram of ultracentrifuge.

turbine is stopped and the shaft clamped down in such a way that the base of the coupling pin completely seals the vacuum chamber.

Two types of rotors for equilibrium measurements are in use. The first which weighs about 10 lbs. is made of duralumin with a steel core while the second which weighs about 30 lbs. was machined from a single piece of high-strength alloy steel. Each rotor has four 2.4 cm. channels drilled parallel to the axis of rotation with their centers 65 mm. from the axis. These channels carry the centrifuge cells and counterbalances. The maximum diameter of each rotor is 9.4 cm. and the moments of inertia are such as to insure stable rotation about the vertical axis shown in figure 1. The cylindrical brass vacuum chamber is 22.2 cm. high, 23 cm. i.d. with a wall thickness of 1.76 cm. The top and bottom plates of the vacuum chamber are made of brass and are 30 cm. in diameter and 1.9 cm. thick. Neoprene O rings provide the vacuum seal between the end plates and the cylinder. Channels which carry a liquid at constant temperature are provided in both the cylinder and the end plates. The temperature of the chamber is held constant to about 0.03° C. by circulating water from a thermostat bath flowing through the cooling channels. A radial slit is cut in the upper plate as indicated by the shading in figure 1, and then filled with a low vapor pressure thermoplastic or a wax. The purpose of this slit is to suppress possible eddy currents in the upper plate which might interfere with the support. Four glass windows 4.15 cm. in diameter and 1.02 cm. thick of good optical quality provide two different light paths through the rotor. The vacuum chamber is evacuated through a 7.6 cm. metal tube by means of an oil diffusion pump and a "Megavac" fore pump. The pressure in the chamber is determined by an ionization gauge.

The solenoid or support coil which suspends the rotor magnetically is situated above the vacuum chamber and consists of 36,000 turns of No. 22 insulated copper wire. The core of the solenoid is a soft iron rod 2.4 cm. in diameter and 30 cm.

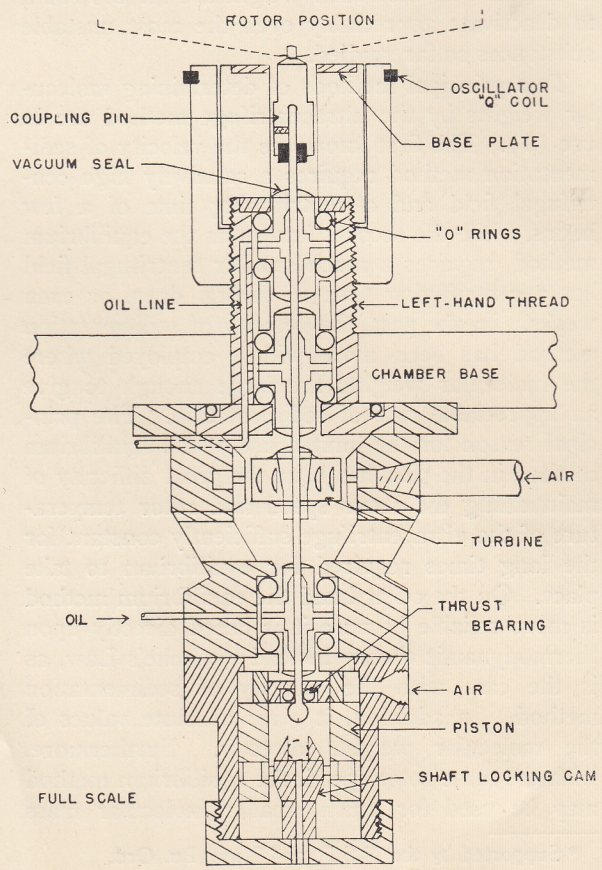

FIG. 2. Air drive system.

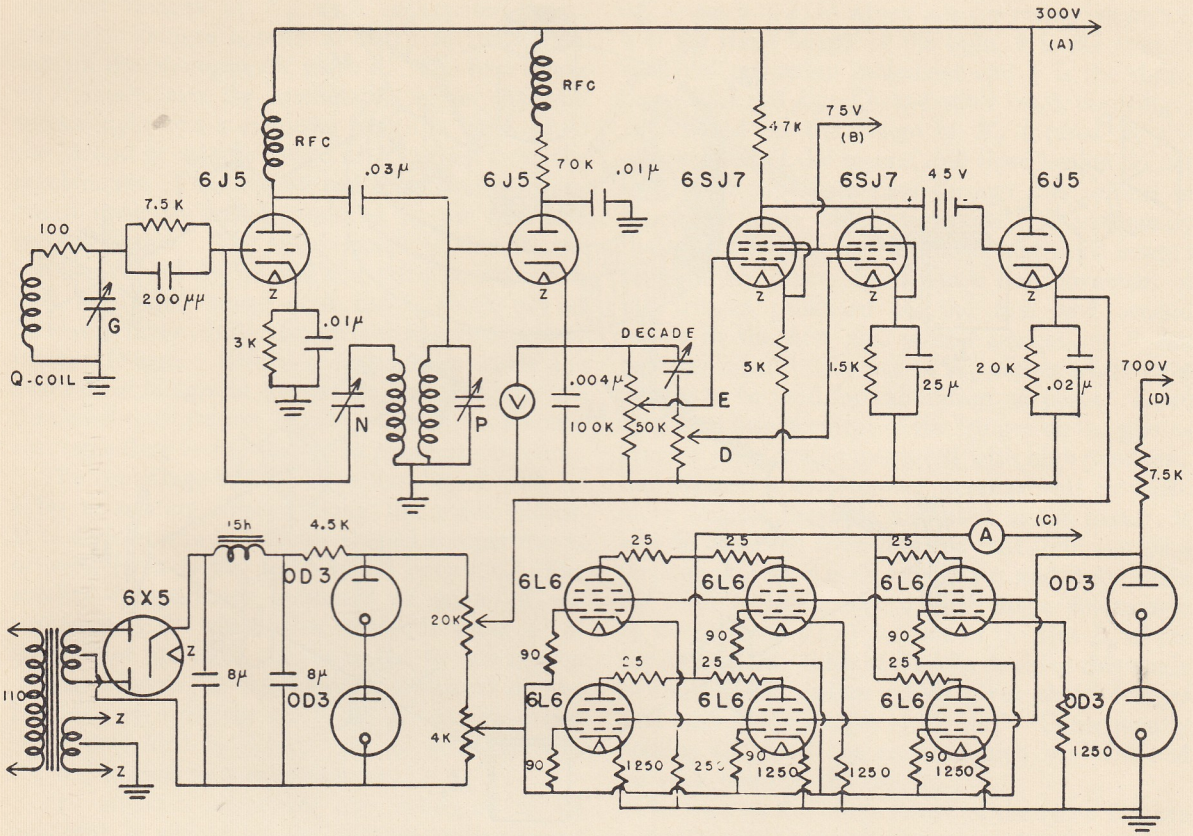

FIG. 3. Magnetic suspension circuit.

long. It is suspended by a 0.0623 cm. piano wire. The lower end of the core is immersed in an oil (No. 30 motor oil) dash pot and is about 0.15 cm. above the top of the vacuum chamber. The purpose of this pendulum-like core is to damp out any horizontal motion of the rotor that might develop.

The servo circuit which regulates the current in the support solenoid is shown in figure 3. The principle of this circuit has been described in detail previously (3, 4) so only a very brief outline of its function will be given here. A small sensing or Q-coil situated below the rotor is in the grid circuit of a tuned-grid tuned-plate oscillator which has a frequency of several megacycles per second. If the rotor moves downward, the impedance of the sensing coil is changed in such a way as to increase the current through the solenoid which in turn raises the rotor. If the rotor moves upward the signal from the sensing coil reduces the current in the solenoid and the rotor moves back down. By inserting so called "anti hunt" into the servo circuit the rotor is automatically held in a given vertical position without any motion which can be observed by a microscope focused on the rotor surface. Since the rotor is suspended freely inside a vacuum chamber by an axial magnetic field the rotor friction should be very small. In practice, the 30 lb. rotor when coasting freely at 300 rps with the vacuum chamber pressure below 10^{-6} mm. of Hg loses the order of one rps in three days. The rotor speed is determined by a photomultiplier tube placed between M_2 and I_2 in figure 4, in a light beam which is interrupted once each revolution of the rotor. The signal from the photomultiplier is amplified and compared on an oscilloscope screen with an amplified signal from the National Bureau of Standards radio station WWV or a signal generator calibrated and continuously monitored by WWV. The rotor speed is adjusted to be approximately equal to one of the broadcast frequencies of WWV or one of its multiples or submultiples. This gives a precision in the rotor speed of at least one part

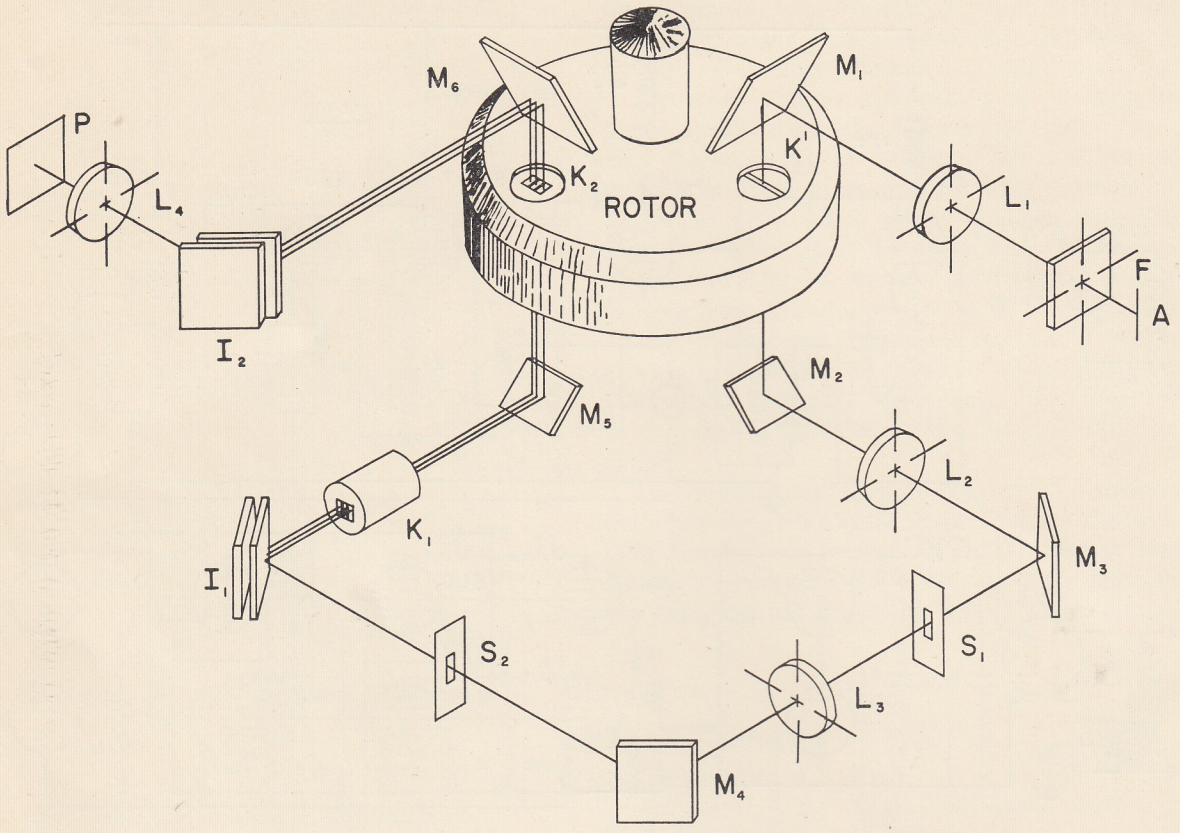

FIG. 4. Interferometer and light chopper.

in 10^6. The high precision in the rotor speed together with the fact that the rotor temperature can be held constant at a known temperature to the order of one part in 10^4 makes it highly desirable to increase the reliability with which the concentration in the centrifuge cell as a function of the rotor radius can be measured.

The concentration of the sedimenting substance in the centrifuge cell is usually determined by measuring the index of refraction of the solution while the centrifuge is spinning. The relation between the concentration and the refractive index n is easily determined outside the centrifuge with high precision by standard methods. Figure 4 shows a combination light chopper and Jamin type interferometer used for determining the refractive index in the centrifuge cell at various rotor radii. Light from a high intensity mercury arc not shown is focused on the slit A. It is then made monochromatic by the filter F and brought to focus on the slit K^1 by the lens L_1. K^1 is a radial slit in the centrifuge rotor. The light is next focused by L_2 on S_1 and made parallel by the lens L_3. S_2 is a diaphragm which limits the width of the beam to prevent overlapping in the centrifuge cells K_1 and K_2. The interferometer plates I_1 are properly silvered to split the incident light beam into three beams of very roughly the same intensity which pass on through K_1 and K_2. The interferometer plates I_2 recombine the three beams and the lens L_4 brings the cell K_2 to focus on the photographic plate P. I_1 and I_2 are each made of a pair of optically flat glass plates with their silvered surfaces facing each other. The front plate is silvered to give from 25 to 35 per cent reflection while the rear plate is full silvered. If each pair of interferometer plates is accurately parallel the interferometer fringes will be at infinity. However, if one of the plates in I_2 is rotated or inclined with respect to the other by the proper small angle, the fringes appear in sharp focus on the photographic plate superposed on the images of the centrifuge cell K_2. Since there are three beams passing through K_2, at least three

separate images of K_2 with superposed interferometer fringes appear in sharp focus side by side on the photographic plate P. The first image on P arises from the combination of the first and second light beams counting from the right, while the second or middle image of K_2 arises from the combination of the second and third beams superposed upon an image produced by the first and second beams. The third image is due to the combination of the second and third beams. The first and third image each has a single set of intense fringes while the second has two sets of intense fringes. Before sedimentation starts the two sets of fringes in the middle image usually are displaced with respect to each other by an amount corresponding to the change in phase at reflection.

The centrifuge cell K_2 is sector shaped with its sides accurately aligned along the rotor radius. It is divided into two sector shaped compartments or cells by a liquid tight radial partition. The smaller compartment subtends an angle of approximately 2° while other subtends about 4° at the axis of the rotor. K_2 is sealed by one crystal quartz window on the top and a second on the bottom. Its construction is similar to one previously described in detail.[4] K_1 is identical with K_2 and is used as a compensator.

The solution containing the substance to be analyzed is placed in the smaller compartment while the solvent is placed in the larger compartment of K_2 to exactly the same radial depth. K_1 is then filled in the same way except that the solvent fills the smaller and the solution the larger compartment. The cell K_2 is next sealed and precisely aligned in the centrifuge rotor. The vacuum chamber is evacuated to at least 10^{-5} mm. Hg pressure and the rotor with the drive shaft attached is magnetically supported. The rotor is turned to the position shown in figure 4 by rotating the air turbine by hand. Next the interferometer is adjusted so that the first beam on the right passes through the compartment in K_2 containing the solution while the second and third beams pass side by side through the larger compartment in K_2 containing the solvent. K_1 is placed in the beam so that the first beam passes through the small compartment containing the solvent and the second and third pass through the larger compartment containing the solution. As sedimentation takes place in the solution, the fringes formed by the first and second beams shift their position on the photographic plate P. The change in concentration ΔC due to a shift of one fringe is $\Delta C = \Delta n/k = \lambda/kh$ where n is the refractive index, λ is the wave length of the light, k is the specific index of refraction increment and h is the thickness of the cell K_2. On the other hand, the fringes superposed on the image of K_2 formed by the combination of the second and third beams do not shift since they both pass through the solvent in K_2 which does not sedimentate. Consequently the sets of fringes in the second and third images serve as references from which the movement of the fringes produced by the first and second beams in the first and second images on P can be determined. The use of K_1 allows white light fringes to be used for calibration purposes. Since a relative displacement of the fringes on the photographic plate can be measured with high precision, the present interferometer arrangement is much more reliable than the one used in the past.[4] It will be observed from figure 4 that light can pass through K_2 once each revolution and only while the rotor turns through a small angle determined by the properties and position of the lens L_2 of the slit K^1 and the width of S_1. In practice this angle is about 0.003 radian which gives sharp fringes over each of the images of K_2 on P. Consequently precise values of the concentration are obtained for each point in the cell K_2.

As soon as the temperature of the rotor reaches a steady value the interferometer fringes are adjusted so that they are parallel or preferably perpendicular to the radius of the rotor and photographed both in monochromatic and in white light. The centrifuge is next accelerated to operating speed as quickly as possible and the drive shaft disconnected. This allows the centrifuge rotor to "coast" freely during the sedimentation and completely eliminates the troublesome problem of rotor "hunting." The fringes are again photographed both in white and in monochromatic light at regular intervals until the observed concentration gradient in K_2 stops changing, i.e. until equilibrium is effectively established.

Figure 5 shows the distribution of concentration at different times during the sedimentation of human albumin and figure 6 shows the log C at radius X plotted versus $X^2 - X_n^2$, where X_n is the peripheral radius of the centrifuge cell, for sucrose dissolved in water after 58 hours of centrifuging. According to theory this should be a straight line, the slope of which gives the molecular weight if the sedimenting substance is mono-disperse. The value obtained for the molecular weight of sucrose was 343 as compared with the known value of

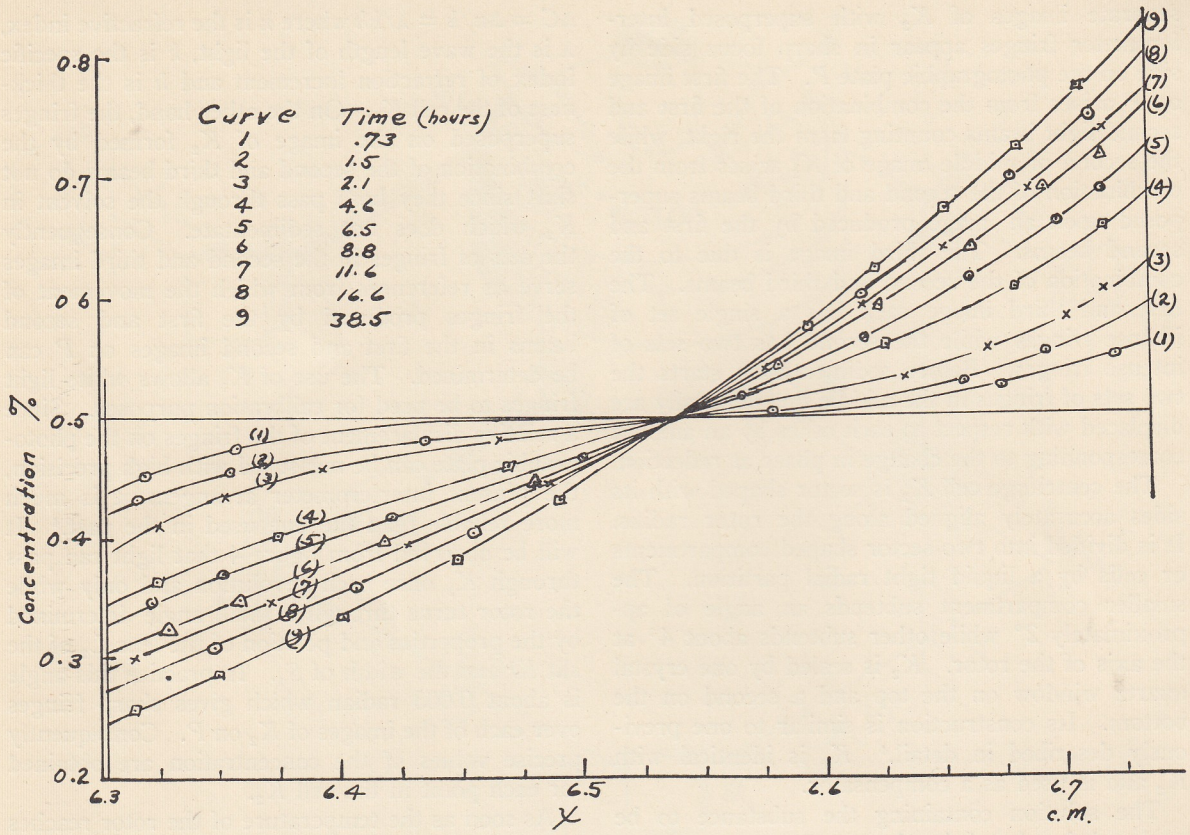

FIG. 5. Distribution of concentration during sedimentation of albumin.

342.3. The overall precision of the measurements made while the rotor is spinning gives a value of the molecular weights to about three significant figures over the molecular weight range above 100.

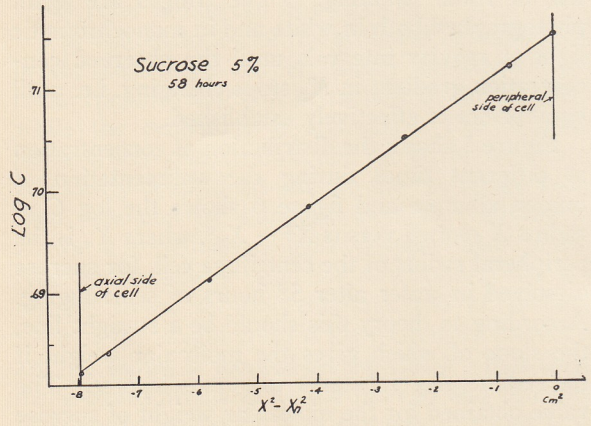

FIG. 6. Log C versus $X^2 - X_n^2$ for sucrose after 58 hours.

It was soon found that, in practice, equilibrium was established in the centrifuge cell much quicker than should be expected from the well-known formula which applies when the rotor speed is constant (1). In the above experiments since the rotor is "coasting" its speed is decreasing very slightly and this allows equilibrium to be established in a much shorter time. Archibald (7) has shown theoretically that if $1/N(dN/dt)/4\pi^2N^2S << 1$, where S is the sedimentation constant and N the rotor speed, equilibrium will remain once it is established in the cell. As a result a considerable amount of centrifuging time may be saved by adjusting the air pressure around the rotor to a value that will introduce just the proper deceleration to give a minimum time for the establishment of equilibrium. In all cases these decelerations are so small, i.e. the order of a few rev. per sec. per day, that the rotor temperature remains sensibly constant. In this way the equilibrium times in some cases may be reduced from

the order of weeks to days. In addition to the measurement of molecular weights the equilibrium ultracentrifuge also may be applied to the determination of molecular weight distributions.

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