[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE APPLICATION OF THE OIL TURBINE TYPE OF ULTRACENTRIFUGE TO THE STUDY OF THE STABILITY REGION OF CARBON MONOXIDE-HEMOGLOBIN

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Depending on the experimental conditions, there are two principles by which molecular weights may be determined by means of the ultracentrifuge: the establishment of a sedimentation equilibrium between centrifuging and diffusion, and the measurement of the sedimentation velocity.[2] The first requires only a moderate centrifugal force of about 5000 times that of gravity but a relatively long time, generally two days. Unstable substances, for example, acid hemoglobin near the region of hematin formation, may be altered appreciably during this length of time. The second principle, on the other hand, requires for molecules of the order of magnitude of the proteins, centrifugal forces of at least 70,000 times that of gravity, introducing certain experimental difficulties, but enabling measurements to be made in as short a period as two hours of centrifuging, if necessary.

Although the sedimentation equilibrium method is sounder thermodynamically, the other method has certain advantages which justified the construction of an oil turbine type of ultracentrifuge capable of running at a speed of 42,000 r.p.m. and giving an effect 104,000 times that of gravity. In addition to the much smaller chance for decomposition of an unstable substance in the short time of centrifuging, it is possible to obtain more exact information when a mixture of molecules is present in a solution, whereas in the sedimentation equilibrium method only the resultant effect of centrifuging and diffusion is obtained; by using higher speed we determine the diffusion and the velocity of sedimentation separately. Under these conditions it is possible to detect anomalies in the diffusion produced by labile gel formation or hydration that might otherwise cancel out or be disguised.

Previous communications[3] have given results obtained through the use of the sedimentation equilibrium method on hemoglobin and egg albumin. The present paper will give a short description of the high-speed oil turbine type of ultracentrifuge and determinations on the range of P_H over which hemoglobin can exist in its normal form.

[1] Fellow of the International Education Board.

[2] The Svedberg, *Kolloid-Z.*, **36**, 57, 60 (1925).

[3] (a) Svedberg and Fåhraeus, THIS JOURNAL, **48**, 430 (1926); (b) Svedberg and Nichols, *ibid.*, **48**, 308 (1926).

Theory of the Sedimentation Velocity Method

After the very short initial period the centrifugal force per mole, $M(1-\rho V)\omega^2 x$, becomes equal but is of opposite sign to the frictional force, $f(\mathrm{d}x/\mathrm{d}t)$, where for dilute solutions $f = (RT/D)$. These quantities have their usual significance; M, the molecular weight; ρ, the density of the solution; V, the partial specific volume; ω, the angular velocity; x, the distance from the axis of rotation of the centrifuge; f, the frictional coefficient; t, the time; T, the absolute temperature; and D, the diffusion constant.

The expression for the molecular weight is, then, for small x-intervals,

$$M = \frac{RT}{D(1 - V\rho)} \cdot \frac{\frac{\mathrm{d}x}{\mathrm{d}t}}{\omega^2 x} = \frac{RTs}{D(1 - V\rho)} \qquad (1)$$

where $(1/\omega^2 x)\cdot(\mathrm{d}x/\mathrm{d}t) = s$ is the specific sedimentation velocity, a characteristic constant for every molecular species at a given temperature and for a given solvent. For large x-intervals it is necessary, of course, to use the integrated form of equation (1):

$$M = \frac{RT \ln \left(\frac{x_2}{x_1}\right)}{D\,(1 - V\rho)\omega^2(t_2 - t_1)} \qquad (2)$$

With a sufficiently strong centrifugal field of force it would be possible to measure directly the movement of the boundary of the dissolved substance with time, but generally sufficient diffusion occurs so that the boundary becomes too blurred to read off the x-values accurately. The boundary will become especially diffuse if there are several molecular species present of not very different weights. However, if sedimentation occurs rapidly enough so that pure solvent appears at the inner end of the solution column in a time short compared to the total time of centrifuging and if there is still a region of solution where there is no change of concentration with distance, then the true position of the boundary can easily be located. The theory of diffusion tells us that the place where the boundary would have been if there had been no diffusion is the surface where the concentration is half that of the original concentration of the solution. After a certain time of centrifuging, the constant part does not correspond to the original concentration in a sectorial cell, due partly to the fact that the molecules are thrown out radially and partly to the increased acceleration with distance. The gradual decrease in concentration with time can be calculated from the relation[4]

$$c_t = c_0 \left(\frac{x_0}{x_t}\right)^2, \qquad (3)$$

c_0 being the original concentration and c_t the concentration at time t.

The diffusion constant can be determined independently of the centri-

[4] Svedberg and Rinde, THIS JOURNAL, **46**, 2684 (1924).

fuging, if desired, but it is more reliable to calculate it under the same conditions from the same observations used to determine the sedimentation velocity. When pure solvent is obtained above the solution in a time short with respect to the total time, then the diffusion constant may be conveniently determined from the simple diffusion equations

$$\left.\begin{aligned} c_z &= \frac{c_0}{2}\left(1 - \frac{2}{\sqrt{\pi}}\int_0^y \epsilon^{-y^2}\,dy\right) \\ y &= \frac{z}{\sqrt{4\,Dt}} \end{aligned}\right\} \qquad (4)$$

in which c_z is the concentration at the distance z from the meniscus after the time t; c_0, the concentration of the unchanged part of the solution; and $\frac{2}{\sqrt{\pi}}\int_0^y e^{-y^2}\,dy$, the probability integral.

If a sufficiently high field of force is not available, then the more involved expression[5] must be used

$$\frac{c_x}{c_0} = -\frac{B\sqrt{t}}{\sqrt{D\pi}}\,e^{-\frac{(Bt-x)^2}{4Dt}} + \frac{1}{2}\left[1 - \Theta\left(\frac{Bt-x}{\sqrt{4\,Dt}}\right)\right] + \frac{1}{2}\,e^{\frac{Bx}{D}} \left[1 + \frac{B}{D}(Bt+x)\right]\left[1 - \Theta\left(\frac{Bt+x}{\sqrt{4\,Dt}}\right)\right] \qquad (5)$$

where B is the sedimentation velocity and $\Theta\left(\frac{Bt-x}{\sqrt{4\,Dt}}\right)$ and $\Theta\left(\frac{Bt+x}{\sqrt{4\,Dt}}\right)$ are probability integrals with the arguments indicated. Since this equation was derived for a gravitational field, it should be used only for very small x-intervals in a centrifugal field. Furthermore, there must be a region of constant concentration so that the liquid may be regarded as unlimited in the direction of the periphery.

With a centrifugal field 70,000 times that of gravity it is permissible to use the simple equations (4) after four hours of centrifuging for a substance of molecular weight 16,700 and after two hours for a weight of 33,400.

Apparatus and Method

The apparatus developed[6] may be conveniently divided into the centrifuge proper,[7] the oil circulation system, the vacuum and hydrogen system, the optical system and the stroboscopic tachometer.

The most important conditions which the centrifuge had to fulfil were: a centrifugal force in the cells of 80,000–100,000 times the force of gravity,

[5] Mason and Weaver, *Phys. Rev.* [2] **23**, 424 (1924).

[6] For a complete description of the apparatus see Svedberg and Lysholm, *Nova Acta Reg. Soc. Scient. Upsaliensis*, Vol. ex. ord., ed. **1927**.

[7] The centrifuge proper was built in the workshops of Ljungström Steam Turbine Company through the courtesy of Mr. F. Ljungström, who suggested the use of high pressure oil turbines for driving the centrifuge. Most of the other parts of the apparatus were made in the workshops of this Laboratory. Mr. A. Lysholm, chief engineer, should be credited as the designer of the centrifuge.

temperature in the cells not higher than 35°, amplitude of vibration small enough so that sharp photographs could be taken during centrifuging at high speed, optical parts inside the centrifuge remaining free from oil dust. Fig. 1 shows the rotor, cylindrical in shape, 150 mm. in diameter, and 60

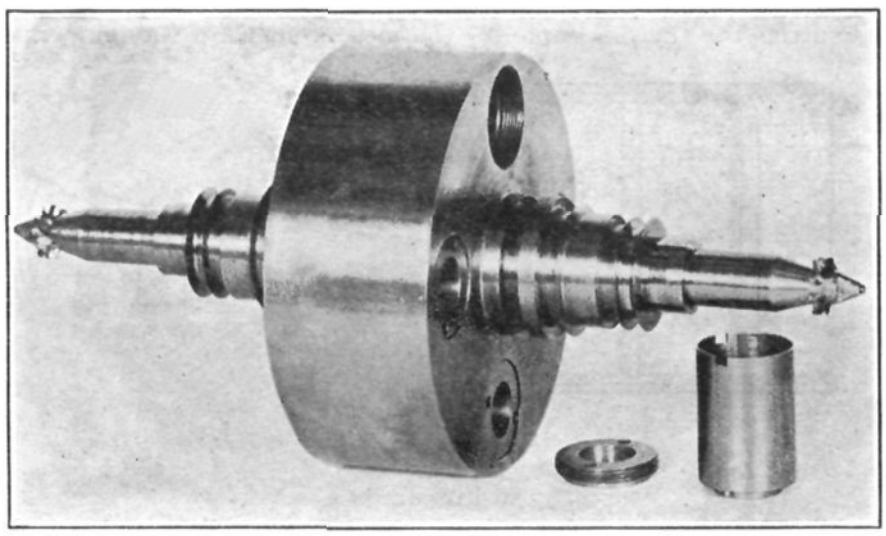

Fig. 1.

mm. thick. It is made of chrome nickel steel and has openings for four cells. It is driven by high pressure oil impinging against two eight-bladed oil turbines, one at each end of the shaft. The turbines receive 240

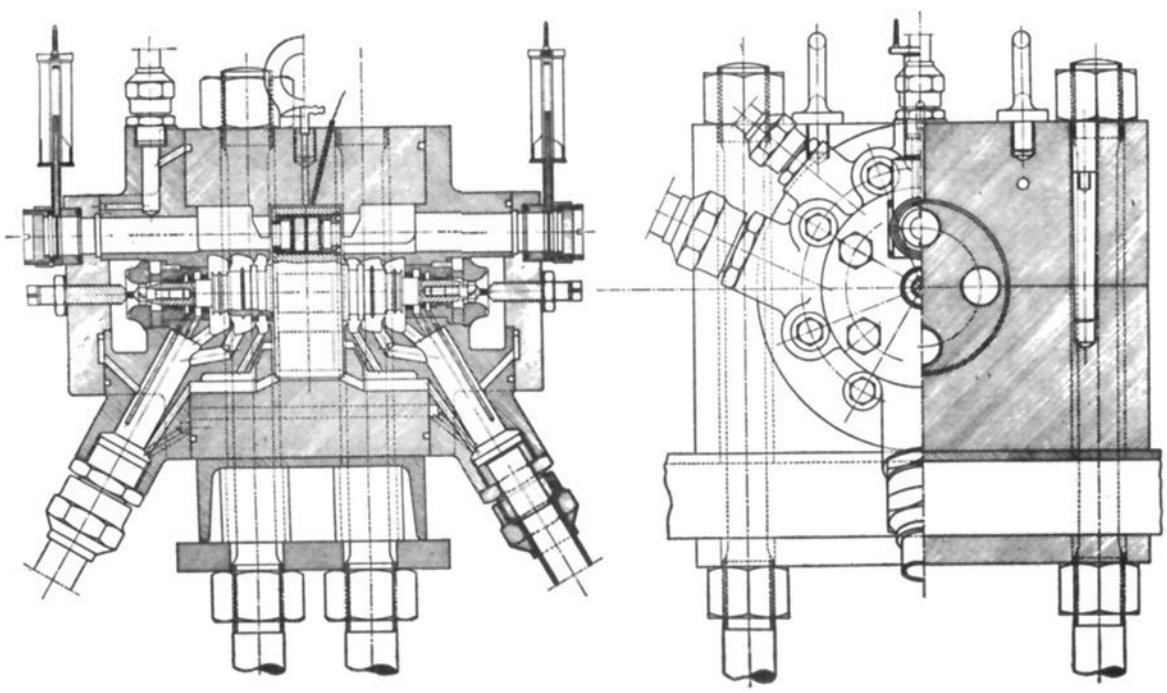

Fig. 2.

liters of oil per minute, and at the same time 7 liters of oil per minute lubricate and cool the bearings. The rotor revolves in hydrogen gas at a reduced pressure to avoid undesirable heat production in the rotor chamber.

The oil from the bearings is prevented from entering the rotor chamber by an elaborate system of oil rings and oil deflectors.

The centrifuge is shown diagrammatically in Fig. 2. To the left is an axial section, and to the right a section at right angles to the axis of rotation. The chief parts are the rotor, top and bottom casings, and the two end brackets. The top casing has the hydrogen inlet and carries the thermocouple for the measurement of the temperature of the

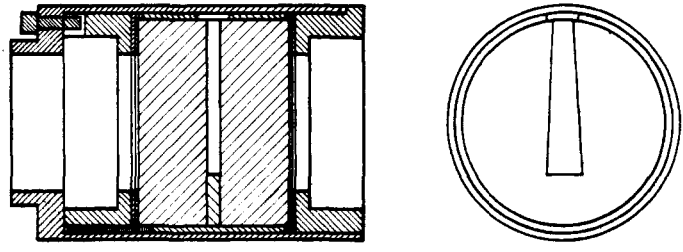

Fig. 3.

interior of the centrifuge. The end brackets contain the turbine chambers with inlets and outlets for the oil to the turbines and also outlets for the oil that has been pressed through the bearings. The main bearings and the plates for the pivot thrust bearings of the shaft are here also. Thermocouples are inserted into the bearings to ascertain their temperatures. An observation channel 34 mm. in diameter is bored through the end brackets and top casing and closed by round glass or quartz windows 10 mm. thick, protected from oil dust by special electromagnetically operated shutters.

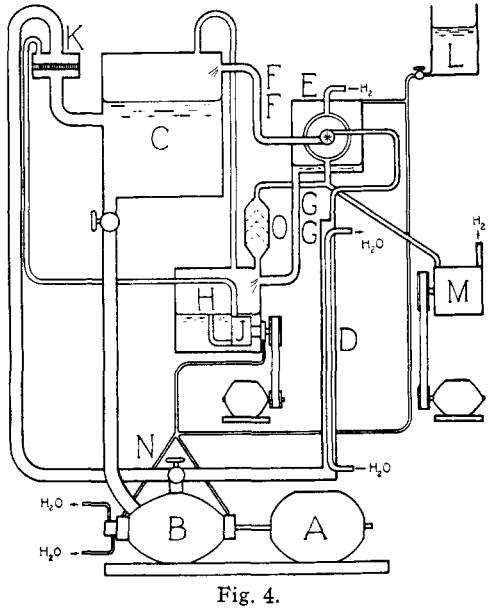

Fig. 4.

The cells for the solution to be studied are made as follows. Two round glass or quartz plates 30 mm. in diameter and 10 mm. thick are cemented with a de Khotinsky cement having a high softening temperature to a third plate 30 mm. in diameter and 1.90 mm. thick. The latter has a sectorial aperture of 5°. The cell is cemented to a steel collar (a brass collar becomes deformed in time) having an opening coincident with the opening of the sectorial cell. This collar containing the cell is then put into a steel shell (shown in Fig. 1) and supported at both ends by a system of sectorial disks and diaphragms (Fig. 3) so that actually a 3° sectorial cell is used. The steel shell bearing the cell is held in place in the rotor by means of a guard ring.

Fig. 4 shows the oil circulation system. The centrifugal pump B, driven by the

20 H. P. three-phase motor A, sucks the oil[8] from the container C and forces it through the oil cooler D to the centrifuge E. From the turbine the oil passes through the pipes FF (only one is shown in the diagram) and back to the container. The oil pumped through the bearings drains down to the lower container H through the pipes GG. A small pump J then raises it again to the upper container C, forcing it through the filter K on its way. Before starting a run the whole oil flow is directed through the filter by means of the three-way valve N in order to remove suspended material which would wear away the bearings too rapidly. M is the vacuum pump, L the container for the oil employed for keeping the various joints vacuum-tight and O is a trap for oil droplets. Speed regulation of the centrifuge is obtained through changing the oil pressure by varying the speed of the main oil pump motor. In Fig. 5 is given the relation between oil pressure and speed of the centrifuge.

In hydrogen as well as in air at atmospheric pressure the rotor warmed up more than 10° above room temperature in a few minutes. Even a 0.5 mm. air vacuum obtained by the oil vacuum pump (M, Fig. 4) did not prevent injurious heating because the heat generated in the residual air cannot be conducted away rapidly enough. However, the heat conductivity of hydrogen is high and independent of pressure down to about 20 mm. of Hg, below which the conductivity decreases.[9] But heat production decreases continuously with decreasing pressure and would become negligible at very small hydrogen pressures (as indicated by the dotted line, Fig. 6) if its dissipation were not radically hindered by the rapid decrease in heat conductivity of air below a pressure of 1 mm. of Hg and of hydrogen below 20 mm. of Hg. Therefore there must be an optimum pressure of hydrogen which will give the smallest temperature difference. The most favorable hydrogen pressure was found to be 12–15 mm. of Hg, as indicated by Fig. 6, where the ordinates give the temperature differences found to exist between the rotor and the casing of the centrifuge for different pressures of hydrogen. Therefore, hydrogen is let in from a container of compressed gas at a rate sufficient to maintain a pressure of 15 mm. of Hg while the vacuum pump keeps the system air-free during the centrifuging.

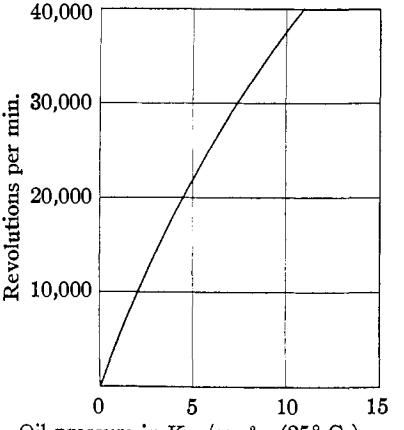

Fig. 5.

Resistance thermometers are used to ascertain the temperatures of the incoming and outgoing water of the oil cooler, the incoming and outgoing oil, the oil container and the cold junction of the thermocouples. Thermo-

[8] Gargoyle Velocite Oil E is most suitable because of its low vapor pressure and low viscosity.

[9] Soddy and Berry, *Proc. Roy. Soc.* (*London*), **84A**, 582 (1911).

couples give the temperature of the main bearings and the top casing of the centrifuge. To determine the difference in temperature between the rotor and the top casing a substance or mixture of known melting point was put into the cell and subjected to the same experimental conditions as in the experiment. Azoxybenzol and piperonal mixtures give a temperature region in the vicinity of 30°. If a thin layer of substance is used the pressure effect on the melting point is negligible. The temperature difference was found to be 1.4°, ordinarily, with a hydrogen pressure of 12–15 mm. of Hg, so the temperature of the cell is then determined by adding this value to the reading of the top casing thermocouple.

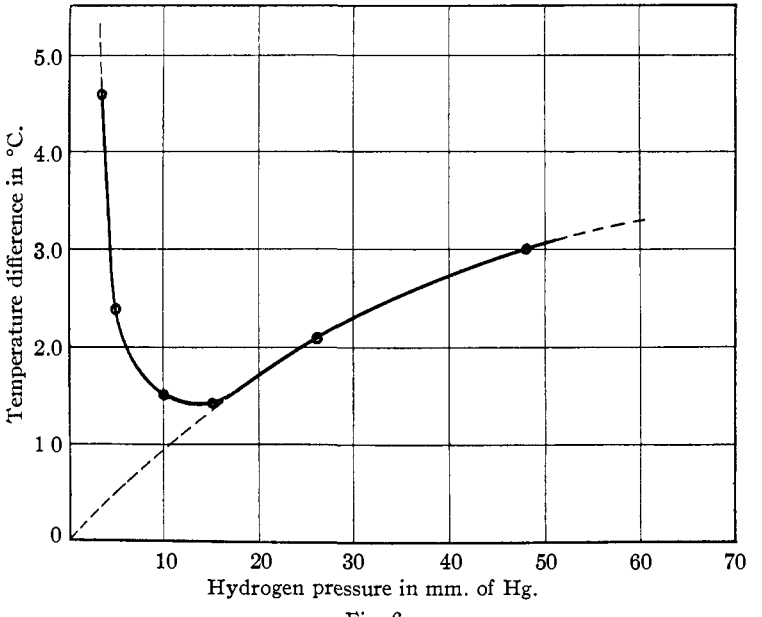

Fig. 6.

The optical system is shown in Fig. 7. Parallel light is produced by focusing the image of the incandescent ball of the fixed-focus 100 c. p. "Pointolite" lamp B on the diaphragm D by means of a Dallmeyer lens C_1 of aperture f:1.9 and focal length 7.5 cm. The light from the diaphragm is then made parallel by means of a Zeiss-Tessar lens C_2, of aperture f: 4.5 and focal length 15 cm. E_1 and E_2 are a water filter and a light filter, respectively. For the visible and the long-waved ultraviolet region of the spectrum a special Zeiss anastigmatic lens H of aperture f:12.5 and focal length 99 cm. was used. G is the camera.

Since the rotor should run free of any gearing arrangement, the speed of the centrifuge is determined by a stroboscopic tachometer. At the back of the camera a motor J is mounted (Fig. 7) carrying a perforated disk K. The stroboscope motor is connected to a small generator M, which acts on a hot-wire ammeter calibrated to read revolutions per minute directly. To measure the speed, the rotating cell is observed through the holes of the revolving disk of the stroboscope and the speed of the motor adjusted by

means of the resistance L until the cell appears to come to a stop, at which time the speed is read off from N.

The rotor must be balanced to within a few centigrams, otherwise injurious vibration will occur. A balancing machine similar to the Lawaczeck-Heymann apparatus[10] is quite suitable. The rotor is supported by two horizontal V-bearings lined with Babbitt metal. The bearings are fixed on top of two stiff vertical steel springs, one of which carries a small mirror for reflecting the image of a straight filament lamp onto a scale. By means of a small motor the rotor is given a speed corresponding to a vibration period above the resonance value of the springs. The rotor is then allowed to run free, and as its speed decreases through the resonance point the amplitude of vibration is read off on the scale. By systematic trial of small lead pieces put into the steel shells the unbalance can be reduced to a few milligrams.

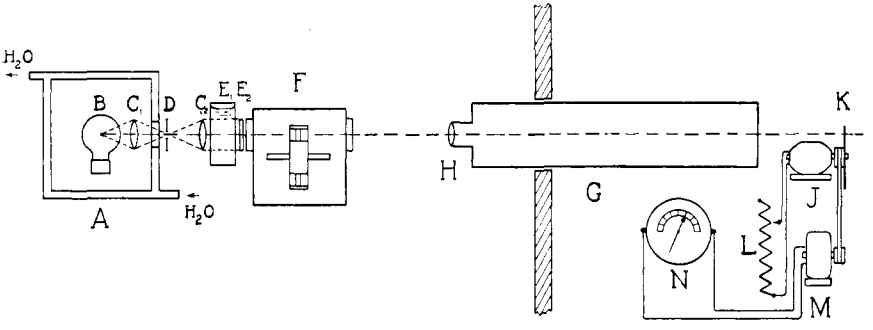

Fig. 7.

The general procedure for carrying out an experiment is as follows. One cell is filled with the solution to be studied and another with a standard for comparison of light intensities (potassium chromate is satisfactory for the whole range blue-short ultraviolet of the spectrum). Then vacuum oil is added to form a layer 2 mm. thick on top of the solutions to prevent boiling caused by the hydrogen vacuum in the rotor chamber and to prevent the rise of convection currents. At a speed of 10,000 r.p.m. the 2 mm. of oil in the cells exerts a pressure of one atmosphere; therefore, since the vacuum pump is not started until an oil pressure of 3 kg./cm.^{2} is reached, corresponding to a speed of 14,000 r.p.m., the solution is protected the whole time.

The cells in their steel shells are inserted into the rotor and the contact surfaces of the top casing are rubbed with wool fat to make the centrifuge vacuum-tight. Then the lid is put on and screwed tight and the windows with their electromagnetic shutters are put in place at the ends of the observation channel of the centrifuge.

[10] Heymann, *Elektrotech. Z.*, **40**, 235 (1919).

After removing air bubbles from the centrifugal oil pump the big motor is gradually set in motion and the vacuum pump started when a pressure of 3 kg./cm.^{2} is reached. The pump for the lower oil container is also started at this time. As soon as an air vacuum of 5 mm. of Hg is reached, hydrogen is admitted to the rotor chamber and the flow regulated at 1.0–1.5 kg./cm.^{2} pressure to give a total pressure of 15 mm. of Hg in the centrifuge. The oil pressure is now increased until the desired speed is attained, regulating at the same time the water to the oil cooler to keep the oil entering the turbines at a temperature of 25°.

During the centrifuging, photographs of double natural size are taken at 30-minute intervals. After three hours a substance of molecular weight approximately 68,000 has centrifuged so completely at a speed of 40,000 r.p.m. that there is no longer a region of constant concentration in a column of solution 1.2 cm. in length. After stopping the centrifuge, a series of different concentrations of the same solution are pipetted into the cell and photographed on the same plate, the centrifuge being run at a low speed for this purpose. The plate after development is microphotometered and treated as previously described in the sedimentation equilibrium determinations.

Temperature and the speed of the rotor are measured from time to time during centrifuging, partial specific volume is determined pycnometrically and the diffusion constant either independently or from the photographs of the solution during centrifuging. It is better, however, to determine the diffusion constant from the centrifuging because its value may be decreased by the pressure developed at the high centrifugal forces used (about 10 atmospheres per millimeter of solution at a speed of 42,000 r.p.m.).

Experimental Part

Adair[11] found by osmotic measurements that hemoglobin, over a considerable range of hydrogen-ion concentrations (P_H 6.8–8.3), gives a normal osmotic pressure corresponding to a molecular weight of about 68,000, after correcting for the pressure of diffusible ions. However, the ultracentrifuge offers a means of determining the molecular weight directly, eliminating any correction for the presence of diffusible ions, provided a sufficient concentration of electrolyte is present to repress the Donnan effect.[12] A series of determinations was therefore made to verify and extend Adair's results.

Carbon monoxide-hemoglobin was prepared in the following manner. Horse blood was defibrinated by shaking for fifteen minutes with Jena glass pearls, saturated with carbon monoxide, and cooled to 0°. The corpuscles were separated from the serum by centrifuging and then washed five times with 1% sodium chloride. The concentrated mass of corpuscles was then dialyzed at 0° to hemolyze and yield crystals and a

[11] Adair, *Skand. Arch. Physiol.*, **49**, 76 (1926).

[12] Tiselius, *Z. physik. Chem.*, **124**, 458 (1926).

saturated stock solution. Since the stroma cannot be centrifuged off from a concentrated solution of hemoglobin, they were removed by a method suggested by A. Tiselius of this Laboratory and based on an observation by Heidelberger.[13] According to the latter, a toluene layer over a solution of hemoglobin containing stroma soon acquires some swelled stroma due to the action of the toluene on the cholesterol of the stroma. The contents of the bags were shaken with toluene in a shaking machine for one hour at 0° and then centrifuged, yielding a thick jelly of swelled stroma which could be skimmed off. Complete removal of the stroma was effected in two shakings with toluene. Then an equal quantity of 0.2 *M* disodium phosphate was added to complete the solution of the crystals and sufficient molar potassium dihydrogen phosphate added to bring the hemoglobin to its isoelectric point, P_H 6.7, following a procedure similar to that of E. J. Cohn.

The solution at this point still contains globulins, so further purification is necessary. Saturated ammonium sulfate solution was added slowly until crystals of hemoglobin just started to form, as indicated by the streaming effect of the small plate-like crystals in motion. This occurs when the solution becomes about 15% saturated with ammonium sulfate. Crystallization was allowed to continue for three days and then the crystals were centrifuged off and dialyzed to remove the ammonium sulfate, yielding an 11% solution of pure hemoglobin with a mass of crystals at the bottom of the bags. The solution was resaturated with carbon monoxide and used exclusively for the centrifuging. The crystallization from 15% saturated ammonium sulfate solution is not complete, so after the first crop of crystals is removed more ammonium sulfate may be added to the mother liquor to cause the remaining hemoglobin to crystallize. This crop may be somewhat contaminated, so it should be dissolved again and recrystallized.

The partial specific volume of the hemoglobin was determined at 30° over the range of hydrogen-ion concentrations studied and found to increase gradually from 0.7545 at a P_H of 6.0 to a low maximum, 0.759, in the neighborhood of a P_H of 9 for dilute solutions.

All of the centrifuging experiments were performed on carbon monoxide-hemoglobin solutions of 0.95–1.0 g. per 100 cc. Parallel light with a Lifa blue filter was employed throughout. The determinations therefore refer to the light absorption of the hemoglobin absorption band at 410 mμ. The usual time of exposure for Hauff Ultra Rapid plates was 20–40 seconds, and the plates were ordinarily developed three minutes in Ilford metol developer or two minutes in Eclipse metol-hydroquinone developer.

Three different buffers were used: Walpole's acetic acid-sodium acetate for solutions more acid than a P_H of 5.5, Sørensen's primary-secondary phosphate mixtures for the range P_H 5.8–7.7, and Ringer's secondary sodium phosphate-sodium hydroxide mixtures for solutions more alkaline than P_H 7.7.

Table I is typical of the results obtained for the sedimentation velocity from centrifuging. It refers to a solution of carbon monoxide-hemoglobin at a P_H of 7.56 in primary-secondary phosphate buffer 0.0191 *M* with respect to phosphate. Fig. 8 gives a reproduction of the photographic record of the centrifuging, showing the conditions of the solution 0.5 hr., 1 hr., etc., up to 3 hrs. after the start of the run. Immediately above the sedimentation record is the potassium chromate standard for adjusting the light intensity to the same value throughout. The top row is the scale of concentrations.

From the variation of concentration with distance from the center of rotation after 1, 1.5, 2, 2.5 and 3 hours the diffusion constant was cal-

[13] Heidelberger, *J. Biol. Chem.*, **53**, 32 (1922).

TABLE I

EXPERIMENT 56. SPECIFIC SEDIMENTATION VELOCITY OF CARBON MONOXIDE-HEMOGLOBIN, P_H = 7.56

Concentration 0.99 g. per 100 cc.; primary-secondary phosphate buffer, 0.0191 M phosphate; V = 0.7575 at 30°; ρ = 1.0005; T = 303.1°; length of column = 1.17 cm.; thickness of column = 0.20; Hauff Ultra Rapid plates, Lifa blue filter; exposure time = 30 sec.; Ilford metol developer, $2^3/_4$ min.

Time interval, hours	Δx per 0.5 hr.	x med., cm.	Speed, r.p.m.	Centrif. force, $\omega^2 x$	Spec. sediment. velocity, s in cm./sec.
0.5–1.0	0.080	4.911	38,700	8.066×10^7	5.46×10^{-13}
1.0–1.5	.080	4.990	38,820	8.246×10^7	5.36×10^{-13}
1.5–2.0	.085	5.072	38,750	8.353×10^7	5.58×10^{-13}
2.0–2.5	.086	5.158	38,700	8.471×10^7	5.48×10^{-13}
2.5–3.0	.087	5.244	38,720	8.622×10^7	5.44×10^{-13}
					Av. 5.46×10^{-13}

culated, giving a mean value of 0.0736 cm.²/day at 30°. Using equation (1), the above data then give a mean molecular weight of 66,800.

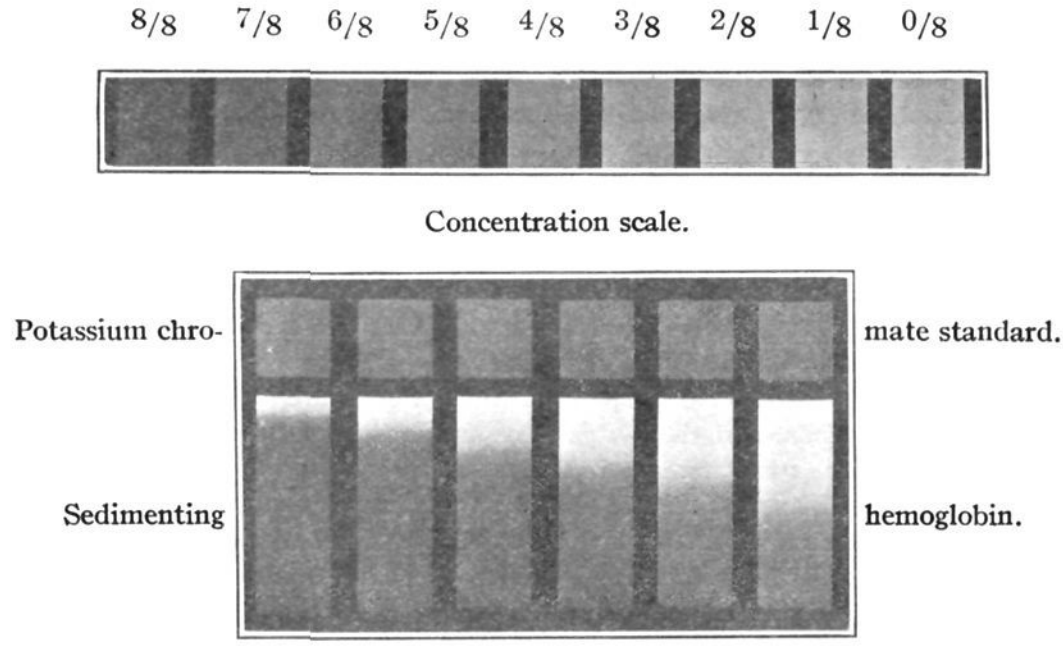

Fig. 8.

Fig. 9 gives the variation of concentration with distance after 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours of centrifuging of the same hemoglobin solution. These curves have been corrected by means of formula (3) for the gradual decrease in concentration of the solution with time due to the sector-shaped cell and the increase in acceleration with distance from the center of rotation. The dotted curves for 2 and 3 hours represent the theoretical diffusion curves of a substance of only one molecular species if subjected to the same experimental conditions. The agreement is evidently extremely

good, and since the small deviations at the ends of the curves cancel one another in the curves for 2 and 3 hours they are probably purely accidental.

Table II gives the results obtained over the P_H range 5.4 to 10.2. In many cases the values of the diffusion constant and sedimentation velocity were determined for each experiment from two plates taken with different times of exposure. The acetate buffer used for the P_H 5.4 was 0.091 M with respect to acetate. The double phosphate buffer for the two experiments at P_H 6.0 and for the two at P_H 7.56 was 0.0191 M, but for P_H

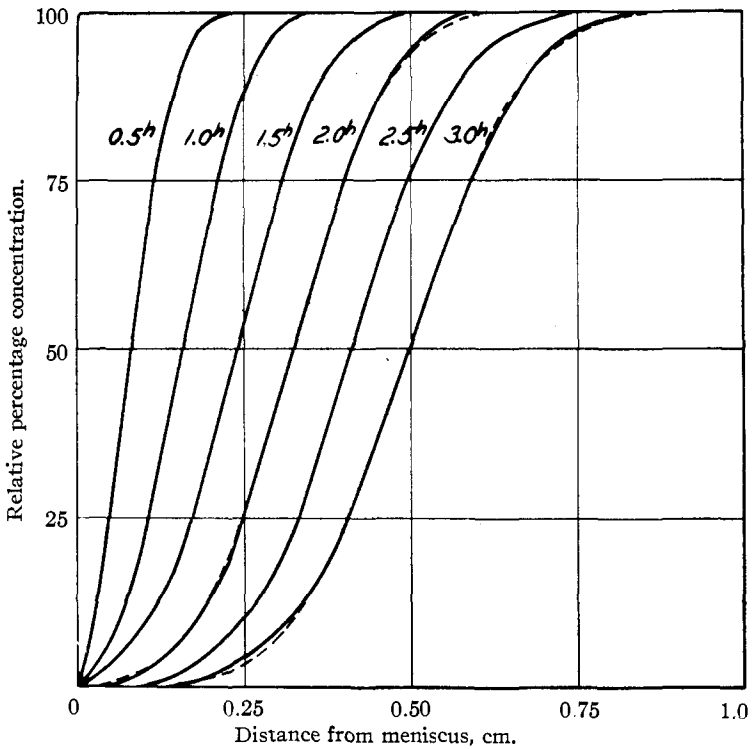

Fig. 9.

6.5 it was 0.06 M with respect to phosphate. The secondary sodium phosphate-sodium hydroxide buffer for the two experiments at P_H 9.05 was 0.0191 M, but for P_H 10.2 it was 0.091 M with respect to phosphate. The length of the column of solution was 11–12 mm. for all runs except No. 54 and D, for which it was 13.7 and 15.5 mm., respectively. Experiment D refers to a dialyzed solution, salt-free.

It will be seen from the table that carbon monoxide-hemoglobin exists as molecules of weight 68,000 over the whole range of P_H 6.0 to 9.05, but that below P_H 6.0 and above P_H 9.05 some change has occurred, as indicated by the low sedimentation velocity and high relative diffusion

TABLE II

INFLUENCE OF P_H ON THE DIFFUSION CONSTANT, SEDIMENTATION VELOCITY AND MOLECULAR WEIGHT OF CARBON MONOXIDE-HEMOGLOBIN

Expt.	P_H	Speed, r.p.m.	Mean centrifugal force	Partial specific volume, V	D cm.²/day	M	Spec. sediment. velocity, s in cm./sec.
66	5.40	41,800	9.90×10^7	0.7545	0.085	....	4.58×10^{-13}
58	6.0	38,700	8.32×10^7		.073	66,700	5.49×10^{-13}
				0.7545	.069	70,110	5.47×10^{-13}
59	6.0	38,500	8.32×10^7		.070	70,650	5.48×10^{-13}
54	6.5	38,900	8.09×10^7				
				0.755			
D	6.6	39,300	7.95×10^7		.070	68,340	5.41×10^{-13}
56	7.56	38,700	8.35×10^7		.074	66,800	5.46×10^{-13}
				0.7575			
57	7.56	38,900	8.45×10^7		.071	68,650	5.43×10^{-13}
61	9.05	41,500	9.72×10^7		.0655	69,210	5.00×10^{-13}
				0.759			
68	9.05	42,400	10.29×10^7		.0636	70,500	4.93×10^{-13}
67	10.2	41,200	9.59×10^7	0.7575	.070	....	4.48×10^{-13}

constant. The diffusion constant for P_H 10.2 has a fairly low absolute value on account of the hydration of the molecule which will be mentioned below. This change may be only apparent and in reality be caused by a small Donnan effect still unrepressed, or it may be due to a splitting up of the molecules. Sufficient data have not been accumulated as yet to decide between these possibilities because qualitatively these two phenomena act similarly on the diffusion constant and the sedimentation velocity of an ionized particle.

Experiments 61 and 68 very clearly show a hydration effect. For both runs a low sedimentation velocity and an equally low diffusion constant were obtained, giving an average of 0.91 of the normal values $D = 0.071$ cm.²/day and $s = 5.46 \times 10^{-13}$ cm./sec. Table III shows in more detail the effect of hydration on the centrifuging.

TABLE III

EXPERIMENT 61. CARBON MONOXIDE-HEMOGLOBIN AT P_H 9.05

Concentration, 0.99 g. per 100 cc.; secondary phosphate-sodium hydroxide buffer, 0.0191 M phosphate; $V = 0.759$ at 30°; $\rho = 1.0007$; $T = 303.1°$; length of column = 1.17 cm.; exposure time, 20 sec.; 3 min. development in half-strength Eclipse metol-hydroquinone developer.

Time int., hours	Δx per 0.5 hr.	x med. in cm.	Speed, r.p.m.	Centrif. force, $\omega^2 x$	D cm.²/day	M	s, cm./sec.
1.0–1.5	0.086	4.9950	41,230	9.31×10^7	0.0654	68,430	4.94×10^{-13}
1.5–2.0	.090	5.0830	41,570	9.65×10^7	.0649	69,300	4.97×10^{-13}
2.0–2.5	.091	5.1735	41,400	9.72×10^7	.0639	70,400	4.96×10^{-13}
2.5–3.0	.094	5.2660	41,530	9.96×10^7	.0669	67,550	4.99×10^{-13}
2.5–3.03	.102	5.2700	41,530	9.97×10^7	.0635	70,900	5.06×10^{-13}
				Av.	.0649	Av.	4.98×10^{-13}

The diffusion constant and the specific sedimentation velocity should be reduced in exactly the same ratio if hydration is present. This can easily be shown from the following considerations.

If we assume the water molecules attached to the hemoglobin to have their normal density, the centrifugal force on the hydrated particle will remain unchanged but the opposing viscous force will be increased. Calling the radius of the hemoglobin molecule r and that of the hydrated molecule R_1, then

$$\frac{4}{3}\pi r^3 \ (\rho_{\mathrm{Hb}} - \rho_d) \ \omega^2 x = 6\pi\eta R_1 \frac{\mathrm{d}x}{\mathrm{d}t}$$

where $(\rho_{\mathrm{Hb}} - \rho_d)$ is the difference in density of the hemoglobin molecule from that of the dispersion medium. Rearranging

$$\frac{2}{9}\frac{(\rho_{\mathrm{Hb}} - \rho_d)}{\eta} \cdot \frac{r^3}{R_1} = \frac{1}{\omega^2 x} \cdot \frac{\mathrm{d}x}{\mathrm{d}t} = S_{\mathrm{obs.}}$$

$$s_{\mathrm{obs.}} = \frac{r}{R_1} \cdot s$$

where $s_{\mathrm{obs.}}$ and s are, respectively, the observed and the normal specific sedimentation velocities.

Likewise $D_{\mathrm{obs.}} = (RT/6\pi\eta R_1) = (r/R_1) \cdot D$, where $D_{\mathrm{obs.}}$ and D are the observed and the normal diffusion constants, respectively. Thus, both s and D should be reduced by the same ratio, (r/R_1), if hydration has taken place; (r/R_1) was found to be 0.91 at a P_{H} of 9.05.

Now the number of water molecules held by a hemoglobin molecule can be calculated. Taking the density of a normal hemoglobin molecule as 1.33, the volume of a particle of molecular weight 66,800 is 82.9×10^{-21} cc. The volume of the hydrated particle is then 110.0×10^{-21} cc. and the volume of the water attached is 27.1×10^{-21} cc. The molecular volume of water at 30° is about 18.1 cc.; therefore the *free* volume of one molecule is 2.99×10^{-23} cc. The number of water molecules held by a hemoglobin molecule is then $27.1 \times 10^{-21} \div 2.99 \times 10^{-23} = 907$.

It is interesting to compare this value with the minimum number required to form a monomolecular layer on the hemoglobin molecule. The total surface of the Hb molecule considered as a sphere is 91.95×10^{-14} sq. cm. The cross section of a water molecule taken as a cube is 9.64×10^{-16} sq. cm. Therefore the minimum number that can be packed on the surface of a hemoglobin molecule is 954.

If the water molecules are considered as spheres the effective surface to be covered is that of a sphere of radius equal to the sum of the radii of the Hb and of the water molecules and is 105.7×10^{-14}. Assuming "cubical" packing the minimum possible number of water molecules is 713, but with hexagonal packing the number becomes 823. Hexagonal packing is the most probable mode of packing and since 823 is the *minimum* number possible, it is permissible to conclude that at a P_{H} of 9.05 there is a monomolecular layer of water surrounding the Hb molecules. A similar effect

of lesser magnitude was observed at a P_H of 7.73, using a low concentration of the secondary phosphate-sodium hydroxide buffer. But both solutions were poorly buffered, because the addition of the 1% protein changed the original P_H of the buffer by more than one P_H unit; therefore, since at P_H 7.56 a solution well buffered with primary-secondary buffer showed no hydration, this hydration effect is perhaps repressed if the solution is properly buffered. When more data have been collected this point can be decided.

The condition of the hemoglobin at the extremes of P_H and a fuller discussion of the phenomena occurring will be taken up in a later paper.

The rather high expenses connected with the construction of this centrifuge have been defrayed by grants from the foundation "Therese och Johan Anderssons Minne" and from the Nobel Fund for Chemistry.

Summary

1. An oil turbine type of ultracentrifuge has been described capable of running at a speed of 42,000 r.p.m. and yielding a centrifugal force 104,000 times that of gravity.

2. Determinations of the influence of P_H on the diffusion constant, molecular weight and specific sedimentation velocity of carbon monoxide-hemoglobin are reported over a P_H range 5.4–10.2. The diffusion constant and the specific sedimentation velocity are normal, respectively, 0.071 cm.^{2}/day and 5.46×10^{-13} cm./sec. at 30° over the range of P_H 6.0–7.56, and the molecular weight is normal, 68,000, at least from a P_H of 6.0 to 9.05.

3. At a P_H of 9.05, in the neighborhood of a maximum in the partial specific volume curve, the Hb molecule appears to hold a monomolecular layer of water at its surface.

UPSALA, SWEDEN

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF ILLINOIS]

VARIOUS ω-CYCLOHEXYLALKYL ALKYL ACETIC ACIDS AND THEIR ACTION TOWARD B. LEPRAE. VIII[1]

BY ROGER ADAMS, W. M. STANLEY, S. G. FORD AND W. R. PETERSON[2]

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In an earlier paper the ω-cyclohexyl derivatives of various normal aliphatic acids containing from one to thirteen carbon atoms in the side chain were described and their bactericidal character toward *B. Leprae*

[1] For previous articles in this field see (a) Shriner and Adams, THIS JOURNAL, **47**, 2727 (1925); (b) Noller with Adams, *ibid.*, **48**, 1074 (1926); (c) **48**, 1080 (1926); (d) Hiers with Adams, *ibid.*, **48**, 1089 (1926); (e) Van Dyke and Adams, *ibid.*, **48**, 2393 (1926); (f) Sacks with Adams, *ibid.*, **48**, 2395 (1926); (g) Hiers with Adams, *ibid.*, **48**, 2385 (1926).

[2] This communication is an abstract of the theses submitted by W. M. Stanley, S. G. Ford and W. R. Peterson in partial fulfilment of the requirements for the degree of Master of Science in Chemistry at the University of Illinois.