A METHOD FOR THE DETERMINATION OF DIFFUSION CONSTANTS AND THE CALCULATION OF THE RADIUS AND WEIGHT OF THE HEMOGLOBIN MOLECULE.

By JOHN H. NORTHROP AND M. L. ANSON.

(From the Laboratories of The Rockefeller Institute for Medical Research,
Princeton, N. J.)

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The diffusion coefficient of a substance in solution is a useful constant since from it may be calculated, by Einstein's equation (1), the radius and weight of the particle. It is particularly useful in the case of substances which cannot be obtained in pure solution and which are frequently of interest in biological work. In such cases the diffusion coefficient is the only practical means of determining the molecular size or weight. The classical method for determining the diffusion coefficient is so beset with experimental difficulties and, in the case of slow moving substances, requires such a long time that it is useless for most biological material.

It occurred to the writers that if the diffusion could be made to occur across a thin porous plate the process would be greatly accelerated owing to the high concentration gradient, and at the same time the elaborate precautions to prevent convection currents, etc. inherent to the classical method would be eliminated (2). Search of the literature revealed that several attempts (3-6) had already been made in this direction, but none were practical for biological material owing to the unsuitable nature of the membranes used. It was found, after a number of trials, that rapid and reproducible results could be obtained with thin membranes made either of alundum or glass powder.

Theory of the Method.—The diffusion coefficient is defined as the quantity of material that will diffuse across a plane of unit dimensions in unit time under unit concentration gradient, or

$$(1) \quad D = \frac{dQ}{A \, dt \, \frac{dc}{dx}}$$

where D is the diffusion coefficient, dQ is the quantity which passes across the plane of area A in time dt under a concentration gradient of dc/dx . There are a number of solutions of this differential equation depending on the conditions of the experiment (*cf.* Mellor (7)). The present case is in many respects the simplest. Suppose a solution of concentration C_1 is separated from a more dilute solution of concentration C_2 by a porous membrane through the pores of which the solute can diffuse. Both solutions are stirred so that diffusion occurs only across the membrane. The solute will diffuse from C_1 to C_2 and if the volume of these solutions is relatively large and the experiment carried on for a short time the concentrations will remain practically constant.* Let the effective area of the pores be A and the effective

* The equation for diffusion, under the above conditions, for the general case in which the concentration cannot be considered constant during the duration of the experiment, may be derived as follows:

Let

S = total solute at beginning of experiment

v_1 = volume concentrated solution

v_2 = volume dilute solution

Q = quantity solute in dilute solution

$= c_2 v_2$

$S - Q = c_1 v_1$

From equation (1)

$$\frac{dQ}{dt} = \frac{DA}{h} (c_1 - c_2)$$

substituting for c_1 and c_2

$$= \frac{DA}{h} \left(\frac{S - Q}{v_1} - \frac{Q}{v_2} \right)$$

or

$$= \frac{DA}{h v_1 v_2} (v_2 S - (v_2 + v_1) Q)$$

Integrating, substituting $K = \frac{h}{A}$

$$D = \frac{2.3 K v_1 v_2}{(v_1 + v_2) t} \log \frac{v_2 S - (v_2 + v_1) Q_0}{v_2 S - (v_2 + v_1) Q}$$

where $Q = Q_0$ when $t = 0$.

length (the distance through which the solute diffuses) be h . The concentration gradient will then be constant and equal to $\frac{C_1 - C_2}{h}$ and the quantity Q diffusing in time t will be simply

$$(2) \quad Q = D A t \frac{C_1 - C_2}{h}$$

or

$$(3) \quad D = \frac{h Q}{A t (C_1 - C_2)}$$

If the experiment is so arranged that the dilute solution is originally pure solvent, C_2 is zero and the equation is still further simplified to

$$(4) \quad D = \frac{h Q}{A t C_1}$$

Dimensions of D.—Since concentration may be expressed as quantity per unit of volume, the units used to measure the quantity cancel out provided the same unit is used to express the concentration as is used to measure the quantity diffusing. D therefore reduces to area over time, or if time is expressed in days and length and volume in centimeters, to cm.^2 per day. This may be seen from the following equation

$$(5) \quad D = \frac{h \text{ cm.}}{A \text{ cm.}^2} \frac{Q \text{ units}}{t \text{ day} \frac{Q_1 \text{ units}}{\text{cm.}^3}} = \frac{h Q \text{ cm.}^2}{A Q_1 t \text{ day}}$$

in which Q_1 is the number of units per cc. of the concentrated solution. If the amount contained in 1 cc. of the concentrated solution is taken as the unit of quantity, *i.e.* if $Q_1 = 1$, and the amount diffused is expressed in this unit (*i.e.* as the number of cc. of the concentrated solution containing the quantity diffused), the equation may be still further simplified and written

$$(6) \quad D = \frac{h Q_{\text{cc.}} \text{ cm.}^2}{A t \text{ day}} = \frac{K Q_{\text{cc.}} \text{ cm.}^2}{t \text{ day}}$$

where $Q_{\text{cc.}}$ is the number of cc. of the concentrated solution that contains the amount of substance diffused. For instance, if it were found in an

experiment with HCl that 10 cc. of 0.10 acid had diffused and the concentrated solution were 0.10 M acid then Q_{∞} would be 10. If the concentrated solution had been 1.0 M, Q_{∞} would be 1.0, etc. Any comparative unit of quantity may therefore be used to express the quantity diffused provided the concentration is expressed in the same units and this is an advantage in dealing with biological material.

It is evident that in order to obtain the diffusion coefficient in absolute units it is necessary to know the dimensions of the membrane through which the diffusion occurs. In the case of porous membranes this value cannot be measured directly since the effective radius and the arrangement of the pores is not known. For any one membrane, however, the effective thickness and area may be assumed constant and therefore h/A is constant and may be called K , the membrane constant. In order to obtain this value it is necessary to standardize the apparatus against some solution the diffusion constant of which is known, just as is the case with a conductivity cell. K , the cell constant, may then be found from the equation

$$(7) \quad K = \frac{D t}{Q_{\infty}}$$

When this constant has been determined for a particular membrane it may then be used to determine the diffusion coefficient for unknown substances, provided of course that the effective pore area is the same for the standard and for the unknown.

Construction of the Apparatus.—It follows from the preceding considerations that the following conditions must be fulfilled by the apparatus.

1. The concentration of the two solutions on opposite sides of the membrane must be kept constant, by stirring or otherwise, so that diffusion occurs only across the membrane.
2. The quantity allowed to diffuse must be small enough so that the difference in concentration between the two sides may be considered constant during the experiment.
3. The membrane must be thick enough so that the liquid in the pores is not disturbed by the stirring of the solutions. On the other hand, the thinner it is the more rapidly the experiment can be completed.
4. The pores must be small enough to prevent convection currents in the liquid held in them and large enough to allow free diffusion of the particles (or molecules) of the solute.

5. The membrane must be level in order to prevent flow from one solution to the other.

It was found after a number of trials that porous glass or alundum discs gave satisfactory results.

The Membranes.—Filter discs made by pressing together a uniform powder of Jena glass are obtainable from the Jenaer Glaswerk, Schott and Gen., Jena, Germany—American agent J. E. Bieber, 1123 Broadway, New York. Discs of the porosity now called No. 4, but formerly designated as <7, were used. According to the manufacturers the pore diameter is 5 to 10 microns or thousandths of a millimeter. The radius of the hemoglobin molecule is about 2.7×10^{-3} microns. The discs are cut down to approximately the desired diameter with an ordinary hack saw and rounded off exactly on a carborundum wheel. Then the pores of the membrane are filled with melted rosin by gentle suction applied through the stop-cock of the glass vessel to which the disc is to be attached. This is to prevent the clogging of the pores with the carborundum powder with which the disc is next ground down to about 0.5 mm. thickness. At the beginning the grinding is done on a carborundum stone, and finished on a flat piece of glass. The rosin is removed with a suitable solvent such as chloroform.

The glass discs are usually sold already fused on to vessels of various shapes, one of them very like the one used in these experiments. It is better, however, to start with the loose discs, since as a result of the fusion the effective area of the disc is lessened.

The membranes are attached to the glass vessel with de Khotinsky cement applied to the bottom of the vessel. Then the edges of the discs which may stick out slightly here and there are carefully ground down even with the vessel and covered with cement. Leaks are tested for by blowing through the stop-cock while the membrane is under water.

Alundum filter discs, porosity RA 225, are made by the Norton Company, Worcester, Massachusetts. The disc is ground down to the desired diameter on a wheel and to the desired thickness by being rubbed against another disc. At the finish a fresh flat disc should be used. After the powder produced in the grinding is washed out the disc is attached.

Some batches of alundum discs contain air spaces large enough to cause small holes when the discs are ground thin. Such discs cannot be used.

Assembly of the Apparatus.—The apparatus is assembled as shown in Fig. 1. The cell contains about 20 cc. and the outside solution is 10 to 50 cc. depending on the rate of diffusion, the time of the experiment, and the analytical method used. The cell is filled by gentle suction through the membrane and rinsed with water. It is then clamped in position and leveled by means of a small level or by placing a dish of mercury just below it. The membrane is made parallel to the mercury by adjusting the universal clamp. The mercury is then removed and replaced with sufficient pure solvent to cover the membrane. The time is noted and the diffusion allowed to continue until the desired amount has passed through

the membrane. The outside solution is then pipetted off and analyzed and replaced by a new portion. This process is continued until the quantity passing through the membrane is constant for equal time intervals, *i.e.*, the system is in the steady state. This constant figure is then used. If more than a few per cent of the solute diffuses out it is necessary to correct the concentration of the inside solution by this amount. The amount diffusing during any one determination should be not more than 2 or 3 per cent of the total in the concentrated solution, and under these conditions a sufficiently accurate correction is obtained by con-

sidering the concentration as the average value for the duration of the experiment. For instance, if it is found that 4 per cent of the solute has dialyzed out during the experiment, then the concentration of the inside solution has dropped from 100 to 96 during the experiment. For purposes of calculation it is considered to have remained constant at 98 per cent.

The arrangement shown in which the heavier solution is above obviates the necessity of stirring since, as the solute diffuses through, a solution is formed near the membrane which is heavier than the pure solvent and so sinks to the bottom. This process could be clearly observed in the experiments with hemoglobin. As the solution in contact with the upper side of the membrane becomes more dilute it is replaced by the heavier concentrated solution above it. There are also slight convection currents due to temperature effects and vibrations from the stirrer of the water bath which aid this process. The fact that even with HCl the quantity diffusing remains constant for constant time intervals shows that the two solutions must remain nearly homogeneous,

FIG. 1. Apparatus for determination of diffusion coefficients.

i.e., that diffusion occurs only across the membrane as is assumed in the calculation. Any flow across the membrane would result in variable results in different experiments and would cause an increase in the relative amount of solution passing the membrane with more concentrated solutions.

Evidently the change in concentration due to the diffusion would be less if the volumes were larger. However, a larger volume of the cell increases the error caused by temperature fluctuations and a large volume of outside solution renders

the analysis more difficult. The apparatus is placed in a water bath at 5°C. since the coefficient of expansion of water is near the minimum at this temperature and also since biological material is more stable at this temperature. The temperature regulation was obtained by placing an ordinary electrically regulated glass water bath in a refrigerating room which had a temperature of about 3° to 4°C.

Calibration of the Apparatus.—It was stated above that since the actual dimensions of the membrane are not known it is necessary to standardize the apparatus by means of a solution whose diffusion coefficient is known. HCl, lactose, and several salts were used for this purpose and it was found that the same cell constant was obtained for any particular membrane with all substances. The results with HCl will be given as an example.

The results of the experiment are shown in Table I. It was found that the constant for the cell was 0.148. The physical significance of this figure, as may be seen from equation (6), is evidently the ratio of effective thickness to pore area of this membrane.

TABLE I.
Diffusion of HCl through Alundum Membrane 1, 5°C.

Solution	Time <i>t</i>	Equivalent cc. 0.1 N HCl diffused	Average
0.1 N HCl	<i>days</i>	<i>Q_{cc.}</i>	0.261
	0.0208	0.262	
	.0208	.258	
	.0208	.262	

$$K = \frac{Dt}{Q_{cc.}} = \frac{1.85 \times 0.0208}{0.261} = 0.148$$

D = diffusion coefficient 0.1 N HCl at 5°, extrapolated from Ohölm's data = 1.85 cm.² per day.

It is now possible to determine the diffusion coefficient of another substance by means of this membrane. Carbon monoxide hemoglobin was chosen for this purpose since the molecular weight of hemoglobin is known from osmotic pressure measurements and the weight calculated from the diffusion experiment may therefore be checked against this value.

Preparation of Hemoglobin.—Whipped beef blood is saturated with CO, cooled, and washed three times with cold isotonic salt solution which is likewise saturated with CO. An equal volume of water is added to the corpuscles. The laked corpuscles are shaken with a quarter their volume of toluene (Heidelberger (10)) and the mixture allowed to stand in the cold overnight. The toluene and cell debris are removed by centrifuging. By dilution with 1/20 M phosphate or borax buffers the hemoglobin is brought to the desired concentration and hydrogen ion concentration. It is filtered through fine filter paper and saturated with CO.

During the experiments the hemoglobin is allowed to diffuse into buffer solution saturated with CO.

The carbon monoxide hemoglobin which has diffused through the membrane is estimated colorimetrically.

Carbon monoxide hemoglobin was used since it is the most stable form of hemoglobin.

TABLE II.
Determination of Diffusion Coefficient of Hemoglobin, 5°C.

Membrane	Hemoglobin solution	Time	Hemo- globin diffused as cc. con- centrated solution	K for membrane	D hemoglobin cm. ² /day
		<i>days</i>	<i>Q_{cc.}</i>		
Alundum 1	1 per cent pH 6.8	0.75	0.202	0.150	0.0405
		.92	.264		.0430
		2.06	.558		.0407
	2.5 per cent pH 6.8				.0430
					.0412
					.0428
Alundum 2	2.5 per cent COHb pH 6.8			.168	.0401
	2.5 per cent COHb M/20 borax pH 9.3				.0397
Glass 1	2.5 per cent COHb pH 6.8			.100	.0434
Glass 2	2.5 per cent COHb pH 6.8 M/20 phosphate			.0833	.0456
Average.....					0.0420 ± 0.0005

Diffusion Coefficient of Hemoglobin.—The results of several diffusion experiments are given in Table II. The table gives the amount of hemoglobin found to have diffused through various membranes after various time intervals and from different hemoglobin solutions. This quantity has been expressed as the number of cc. of the concentrated solution (used for the experiment), which contained the same amount

of hemoglobin. The diffusion coefficient of hemoglobin may therefore be calculated from these figures by means of equation (8).

$$(8) \quad D = \frac{K Q_{cc}}{t}$$

The values obtained in this way are given in the last column of the table. The average value is 0.0420 ± 0.0005 cm.² per day.

Calculation of the Radius and Molecular Weight of the Molecule from the Diffusion Coefficient.—According to Einstein the diffusion coefficient is related to the radius of the molecule by the following equation

$$(9) \quad D = \frac{R T}{N} \cdot \frac{1}{6 \pi r \eta}$$

$$R = 8.3 \times 10^7 \text{ erg} \cdot \text{deg.}^{-1} \cdot \text{mole}^{-1}$$

$$N = 6.06 \times 10^{23} \text{ mole}^{-1}$$

$$T = 278 \text{ deg.} \text{ — for } 5^\circ\text{C.}$$

$$r = \text{radius of particle including water of hydration, in cm.}$$

$$\eta = \text{viscosity of water at } 5^\circ\text{C.}$$

$$= 0.01519 \text{ gm. cm.}^{-1} \text{ sec.}^{-1}$$

$$= 0.01519 \text{ erg} \cdot \text{sec. cm.}^{-3}$$

$$D = \frac{1.33 \times 10^{-13}}{r} \frac{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.}^3}{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.} \cdot \text{sec.}}$$

$$= \frac{1.33 \times 10^{-13} \text{ cm.}^2}{r \text{ sec.}}$$

or for H₂O at 5°C.

$$(10) \quad = \frac{1.148 \times 10^{-8} \text{ cm.}^2}{r \text{ day}}$$

therefore

$$(11) \quad r = \frac{1.148 \times 10^{-8}}{D}$$

$$= 2.73 \times 10^{-7} \text{ cm.}$$

The molecular weight of the particle may be calculated from the radius by the following equation provided the specific gravity is known.

$$\begin{aligned}
 (12) \quad m &= \frac{4}{3} \pi r^3 g N \\
 g &= \text{specific gravity} = \text{gm. cm.}^{-3} \\
 &= 1.33 \\
 &= \frac{4}{3} \times 3.14 \times 6.06 \times 10^{23} g r^3 \\
 &= 25.4 \times 10^{23} \cdot g \cdot r^3 \\
 &= 25.4 \times 10^{23} \times 1.33 \times 20.2 \times 10^{-21} \\
 &= 68,500 \pm 1,000
 \end{aligned}$$

The specific gravity is assumed to be the same as that of solid hemoglobin and the calculation gives a molecular weight of $68,500 \pm 1,000$ which agrees within the experimental error with that of 67,000 found by Adair (8) by osmotic pressure measurements, and of 68,000 found by Svedberg (11) from experiments on the rate of sedimentation.

Einstein's equation assumes that the solute molecules are large compared to the solvent molecules and therefore could not be used for substances of small molecular weight. It was found by Euler (9), however, that for such molecules the diffusion coefficient is inversely proportional to the square root of the molecular weight, *i.e.*

$$(13) \quad \frac{D_1}{D_2} = \sqrt{\frac{m_2}{m_1}}$$

In such cases the molecular weight could be obtained by simple proportion from the diffusion constant of some known substance of about the same molecular weight.

Sources of Error in the Method and Assumptions Underlying the Theory.—The effect of temperature, leveling, and change of concentration during the experiment have already been mentioned. It is conceivable that the substance might stick to the membrane and so fill up the pores, or that some of the pores would not allow the unknown solution to pass while the solution used as a standard could pass. For this reason it is advisable to use different types of membrane since it is

unlikely that the effect would be the same in membranes made of different material. Another method of testing for this error is to determine the effect of filtering the solution through the membrane. Any appreciable adsorption would be shown by a change in concentration on filtering.

Einstein's equation for calculating the radius and molecular weight from the diffusion coefficient is based on the following assumptions. The diffusing particles (molecules) are few and large compared to those of the solvent. They are spherical and are impelled by a force equal to the osmotic pressure as given by Van't Hoff's law against a resistance as given by Stokes' law. The work of Perrin and others has shown that Einstein's equation is valid for a number of colloidal solutions. The specific gravity of the particle, however, is more uncertain since it might very well consist partially of water and so be lighter than the dry solid in bulk.

In the case of ionized substances another complication is introduced since the diffusion rate of one ion is influenced by the accompanying ion. Most biological substances, however, are weak electrolytes so that the quantity present in the form of ions is usually small. It is conceivable in the case of such things as enzymes that several different size molecules might be determined simultaneously by the method of analysis used. In this case no constant diffusion coefficient would be obtained since the smaller molecules would diffuse first. If the proportion or the relative number and sizes of the different molecules were known this effect could be corrected for, at least in theory. Practically, however, decreasing coefficients found on succeeding diffusions would indicate a mixture of substances but could hardly be analyzed further in a quantitative way. The problem is evidently the same as the separation of gases by diffusion.

Finally, it may be said that the molecular weight obtained in this way is that defined by the properties of the solution and used by the physicist and physical chemist.

SUMMARY.

A method is described for determining the diffusion coefficient of solutes by determining the rate of passage of the solute through a thin porous membrane between two solutions of different concentration.

The method has been used to determine the diffusion coefficient of carbon monoxide hemoglobin. This was found to be 0.0420 ± 0.0005 cm.² per day at 5°C.

The molecular weight of carbon monoxide hemoglobin calculated by means of Einstein's equation from this quantity is $68,600 \pm 1,000$.

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