THE CALIBRATION OF DIFFUSION MEMBRANES AND THE CALCULATION OF MOLECULAR VOLUMES FROM DIFFUSION COEFFICIENTS

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INTRODUCTION

In the membrane method for measuring diffusion coefficients (Northrop and Anson (1928–29)) diffusion takes place through a porous disc. Under these conditions convection currents which usually make diffusion measurements difficult are avoided, the time required for a measurement is greatly shortened, and it becomes possible to remove the diffused substance for estimation. Under suitable conditions the rate of diffusion of one substance is independent of other substances present. To calculate the diffusion coefficient it is necessary to know only the per cent, not the absolute amount, of the original material which passes through the disc in a given time. The method can be applied, therefore, even to impure biological substances which can be estimated only by activity measurements. Since the dimensions of the pores are unknown, the membrane is calibrated by measuring the rate of diffusion through it of a substance of known diffusion coefficient.

The first part of this paper describes the calibration of diffusion membranes with sodium chloride, potassium chloride, and hydrochloric acid and summarizes the evidence that the membrane method yields correct diffusion coefficients. The second part of this paper states the assumptions made in calculating molecular volumes from diffusion coefficients by Einstein's law, states the consequences if these assumptions are not valid, and discusses the possible reasons why molecular volumes of various proteins calculated from their diffusion coefficients are higher than the molecular volumes calculated from osmotic pressures and sedimentation data. The third part

outlines the uses to which diffusion measurements can be put despite the limitations in the application of Einstein's law.

Our original paper was primarily concerned with the technical problems involved in diffusion measurements and a detailed discussion of the limitations and possibilities in the use of the method was omitted. This omission gave rise, on the part of some wishing to use the method for biological materials, to a certain amount of confusion which we hope will now be avoided.

1. The Validity of the Membrane Method for Measuring Diffusion Coefficients

Before discussing the validity of calculations made from diffusion rates measured by the membrane method we shall first state the evidence that the diffusion data themselves are correct. First, it has been shown by special experiments that there is no important disturbance from convection currents, that there is adequate stirring of the solutions above and below the membrane, and that the results are independent of the material, structure and, within certain limits, of the dimensions of the membrane (Northrop and Anson (1928–29)). Secondly, the assumption that there is a linear concentration gradient across the membrane, while not strictly correct, does not lead to any significant error (Barnes (1934)). Lastly, the diffusion coefficients of various salts and non-electrolytes and of hemoglobin are the same, within the experimental error, whether measured by the membrane method or by the classical method which does not involve the use of a membrane. Thus, the measurements given in this paper show that the membrane and the classical methods give the same results for the effect of temperature on the rate of diffusion of sodium chloride and for the ratio of the diffusion coefficients of sodium and potassium chlorides. Similar data can be found in the paper of McBain and Liu (1931). The value of Northrop and Anson (1928–29) for the diffusion coefficient of 2 per cent hemoglobin at 5°C. is 0.034 cm.^{2}/day (assuming the diffusion coefficient of 0.1 N hydrochloric acid at 5°C. to be 1.45 cm.^{2}/day). The value of Tiselius and Gross (1934) for 1 per cent hemoglobin at 20°C. obtained by the classical method is 0.0542 cm.^{2}/day. Extrapolated to 5°C. by Einstein's equation it is 0.0332 cm.^{2}/day. Lamm and Polson (1936) have recently obtained a value

for the diffusion coefficient of hemoglobin which is 9 per cent higher than that obtained by Tiselius and Gross. As yet there has not been a sufficiently detailed study of the diffusion of any protein by both the classical and the membrane methods to prove that the two methods when applied to proteins yield exactly the same results. In particular, the comparison of the two methods has not been made in any case in which the diffusion coefficient is known to be independent of concentration. Most of the values of diffusion coefficients of proteins obtained by the membrane method, furthermore, were obtained from experiments incidental to other work, so that each value is based on only a small number of measurements.

The Calibration of Diffusion Membranes

Sodium Chloride.—Sodium chloride is a suitable substance for the standardization of diffusion membranes because its diffusion coefficient is not sensitive to concentration. As measured by the membrane method at 5°C. the diffusion coefficients of 0.2 N, 1.0 N, and 2.0 N sodium chloride are the same within the experimental error of 2 per cent. In practice 2.0 N sodium chloride is used for calibration because the greater the amount of diffused sodium chloride available for estimation the easier and more accurate is the titration with silver nitrate. When the diffusion coefficient is sensitive to concentration the results obtained by different methods are not comparable because the changes of concentration involved in the different procedures are different. The differences in concentration changes are more serious the more concentrated the solution. Very few of the diffusion coefficients in the literature are true diffusion coefficients, *i.e.* diffusion coefficients which really apply to the concentrations to which they are supposed to apply and are not averages of the diffusion coefficients corresponding to various concentrations.

The rate of diffusion of 2 N sodium chloride has been measured by the membrane method at 5°, 10°, 18°, 20°, and 25°C. See Table I. The data fit within 2 per cent the straight line equation

$$\frac{D_{t_2} - D_{t_1}}{t_2 - t_1} = 0.0275 \text{ cm.}^2\text{/day per degree} \qquad (1)$$

t = °C.

Taking D to be 0.72 cm.^{2}/day for $t_2 = 5$ (Oholm (1905)) equation (1) becomes

$$D = 0.588 + 0.0263\, t \tag{2}$$

On this basis $D_{18} = 1.06$ cm.^{2}/day, which agrees within the experimental error with the results of Oholm (1905) and Clack (1917, 1921, 1924). See Table I. It must not be assumed that the equation holds for temperatures lower than 5°C. or higher than 25°C. The data fit the Nernst (1888) equation.[1]

The diffusion cells used in most of the measurements given in this paper were of about 50 ml. capacity. 50 ml. of water was in outside vessels similar to the one pictured in the paper of Scherp (1932–33). The membranes were of Jena sintered glass,[2] porosity G 4, and about 4 cm. in diameter and 1.5 mm. in thickness. At 25°C. about 1 per cent of the sodium chloride diffused through the membranes in an hour.

As an indicator for the titration of sodium chloride with silver nitrate, 4 drops of 5 per cent potassium chromate are added to 50 ml. of solution and the titration

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[1] The temperature coefficient, α, of diffusion is usually defined as

$$\frac{D_{t_2} - D_{t_1}}{D_{t_2}} \cdot \frac{1}{t_2 - t_1} = \alpha \qquad t_2 > t_1$$

The value of α as given by this equation is not the same for different temperature ranges unless the calculations are made from a constant D_{t_2} as a base. If D_{t_1} is taken to be constant, the equation becomes identical with equation (1).

[2] Diffusion cells with fused in membranes can be obtained from Schott and Company, Jena, Germany (American agents, Fish-Schurman Corporation, New York City).

In the catalogue of the Jena Glass Company our diffusion apparatus is pictured in the form used by McBain. This differs in two unessential details from the form now used by us. First, the membrane is several times thicker than ours. The thicker membrane may be an advantage for very accurate measurements of the rate of diffusion of small molecules. When the rate of diffusion of slowly diffusing biological molecules is being measured thinner membranes which permit more rapid diffusion are more desirable. Secondly, we have the stop-cock immediately above the wide part of the diffusion cell, whereas McBain has it higher on the tubing attached to the wide part, as in our original apparatus. The purpose in placing the stop-cock lower is to avoid a dead space in which convection currents may not produce adequate stirring. The manufacturers will make diffusion cells of any desired design and dimensions.

is carried to a definite brown. Since the greater the amount of silver chloride in suspension the more silver nitrate is needed to turn the indicator brown, known amounts of sodium chloride are titrated to obtain a calibration curve.

Potassium Chloride.—McBain and his associates have used our membrane method extensively. They calibrate their membranes with 0.1 N potassium chloride at 20°C. taking for the diffusion coeffi-

TABLE I

Diffusion Coefficients at Various Temperatures — Cm.²/Day

	5°	10°	12°	16°	18°	20°	25°
2 N sodium chloride	0.720	0.848			1.04	1.12	1.27
	(0.720)	(0.851)			(1.06)	(1.11)	(1.25)
1 N sodium chloride (Oholm)	0.720				1.06		
2 N sodium chloride (Scheffer)	0.72						
1 N sodium chloride (Clack)					1.04		
					1.07		
0.5 N potassium chloride							1.56
0.5 N potassium chloride (McBain and Dawson)							1.57
1 N hydrochloric acid	1.60	1.85	(1.96)	2.16	(2.27)		2.65
	(1.59)	(1.85)		(2.17)			(2.64)
1 N hydrochloric acid (Oholm)			1.96				
0.4 — 0.5 N hydrochloric acid (Scheffer)	1.55	1.78					
0.1 N hydrochloric acid	1.45	1.68	(1.77)	1.94			2.38
	(1.45)	(1.68)		(1.96)			(2.37)
0.1 N hydrochloric acid (Oholm)			1.98	2.13			

The values are diffusion coefficients expressed in cm.²/day.

The values in parenthesis are calculated from the equations given in the text.

The membranes were calibrated with 2 N sodium chloride, taking $D_6° = 0.72$ from Oholm.

cient the accurately determined value of Cohen and Bruins (1924), 1.249 cm.²/day. The diffused salt is estimated with an interferometer. 0.1 N potassium chloride has two disadvantages as a substance for calibration. First, the accurate estimation of very small amounts of potassium chloride is, in the absence of suitable optical equipment, not convenient. Secondly, according to McBain and Dawson (1935) the rate of diffusion of dilute potassium chloride is not independent of the concentration so that the results obtained by the membrane method

are not strictly comparable to those of Cohen and Bruins. The possible error here is only a few per cent.

Using membranes calibrated with 0.1 N potassium chloride McBain and Dawson (1935) found the diffusion coefficient of 0.5 N potassium chloride at 25°C. to be 1.57 cm.²/day. Using membranes calibrated with 2 N sodium chloride, we find the diffusion coefficient of 0.5 N potassium chloride to be 1.56 cm.²/day.

Hydrochloric acid.—The rate of diffusion of 0.1 N and of 1.0 N hydrochloric acid through membranes calibrated with 2 N sodium chloride has been measured at 5°, 10°, 16°, and 25°C. The results (Table I) within 1 per cent are expressed by the equations

$$D_{0.1N\ HCl} = 1.22 + 0.046\,t$$
$$D_{1.0N\ HCl} = 1.33 + 0.0525\,t$$

Our results with 1 N hydrochloric acid agree with Oholm's. Our results with 0.1 N hydrochloric acid do not. Oholm's value for the diffusion coefficient of 0.1 N hydrochloric acid is 11 per cent higher than ours at 12°C., 9 per cent higher at 16°C.

Scheffer measured the diffusion coefficient of 0.5 N hydrochloric acid at 5°C. and 10°C. His values are between our values for 0.1 N hydrochloric acid and 1 N hydrochloric acid. See Table I.

It should be pointed out that if the diffusion coefficient of hydrochloric acid is dependent on the concentration, then the results obtained by Oholm's method and the membrane method are not strictly comparable. The deviation from the true diffusion coefficient is more serious when Oholm's method is used. Furthermore, Oholm regarded his experiments with hydrochloric acid as less accurate than those with sodium chloride. In his more concentrated acid solutions visible precipitates were formed by the reaction of the acid with the mercury supporting the solutions.

Correction of Old Calibration.—The diffusion coefficient of 0.1 N hydrochloric acid at 5°C., as measured with a membrane calibrated sodium chloride, is 1.45 cm.²/day. In our original use of the membrane method we calibrated our membranes with 0.1 N hydrochloric acid at 5°C. and took the diffusion coefficient as 1.85 cm.²/day. This value was obtained by extrapolation from Oholm's data. Zeile (1933) pointed out that we made a numerical error in calculation and that

the effect of temperature on the rate of diffusion of hydrochloric acid is greater than that given by Oholm. We have now found, in addition, a discrepancy between Oholm's absolute values and the absolute values obtained by the membrane method with membranes calibrated with sodium chloride. Since from the data in the literature, one cannot be certain what the absolute diffusion coefficient of hydrochloric acid is at any given temperature or what the effects of temperature and concentration are on the diffusion coefficient, it is clear that hydrochloric acid is not at present a suitable fundamental standard for the calibration of diffusion membranes.

The Assumptions Involved in the Calculation of Molecular Weights from Diffusion Coefficients

Although the measurement of rates of diffusion by means of the membrane method is simple, the calculation of molecular weights from diffusion constants involves many uncertainties. These uncertainties have nothing to do with the validity of the diffusion data themselves. They are, for the most part, independent of the technique used in making the diffusion measurements. According to Einstein (1908) the diffusion coefficient D is related to the friction coefficient F as follows

$$D = \frac{RT}{N} \cdot \frac{1}{F} \tag{1}$$

This equation states that the rate of diffusion of a substance from one part of a solution to another is directly proportional to the difference in osmotic pressure due to the difference in concentrations of the substance in the two parts of the solution, and inversely proportional to the coefficient of friction, which is the force needed to produce unit rate of motion. The assumptions made are: (1) that D is a true diffusion constant independent of the concentration of the diffusing substance; (2) that van't Hoff's osmotic pressure law is obeyed, which again means that D is independent of the concentration of the diffusing substance; and (3) that D is not influenced by other diffusing substances. The diffusion of protein from hydrochloric acid solution into water, for instance, is much faster than the diffusion of isoelectric protein into water. The small negatively charged chloride ions diffuse

rapidly and drag with them the large protein ions of opposite charge from which they cannot be separated. This accelerating effect can be abolished by salt just as Donnan effects can be abolished in osmotic pressure experiments. If diffusion experiments are carried out with dilute solutions containing salt, and if D is independent of the concentration of the diffusing substance and of the salt, then the assumptions made in deriving equation (1) may be considered as experimentally justified and F may safely be calculated from D.

Einstein takes F to be related to the radius of the diffusing particle r and the viscosity of the solvent, η, by Stokes' law

$$F = 6\pi r\eta \tag{2}$$

Combining equations (1) and (2) one obtains

$$D = \frac{RT}{N} \cdot \frac{1}{6\pi r\eta} \tag{3}$$

which is Einstein's law. The assumptions involved in Stokes' law are that the diffusing particles are large and few in number in comparison with the molecules of the solvent and that they are spherical.

If the diffusing particles are not spherical the calculated radius is too great. It is impossible to find out from diffusion experiments alone whether the diffusing particles are spherical or how great an error is made in making the assumption that they are spherical. Theoretical calculations have been made for particles of certain definite shapes (Svedberg (1928)). For instance, the coefficient of friction of an ellipsoid whose long axis is ten times its short axis is about 20 per cent greater than the coefficient of friction of a sphere of the same volume.

Finally, the friction which, other things being equal, determines the rate of diffusion is the friction between the whole kinetic unit and the solvent. If the diffusing molecule is hydrated then the radius given by Einstein's law is the radius of the hydrated molecule and the molecular volume calculated from the radius is the volume of the hydrated molecule. In contrast, osmotic pressure measurements yield information about the volume of the unhydrated molecule. There is no way of telling from diffusion experiments alone whether a molecule is hydrated or not, or to what extent it is hydrated.

If the diffusion has been accelerated by ionic effects then the calculated radius is too low. This acceleration is easily abolished by salt. If, on the other hand, any of the other assumptions made in arriving at Einstein's law is not justified in any particular case, then the calculated radius is too high.

We have seen that some of Einstein's assumptions, in particular those involved in the equation relating D to F, can be tested experimentally by diffusion measurements. Others cannot. Even if all the possible tests have been carried out, the molecular weight of the unhydrated molecule calculated from D may be higher than that obtained from osmotic pressure data because the molecule is either hydrated or non-spherical or both.

Comparison of Molecular Volumes Calculated from Osmotic Pressures, Sedimentation Data, and Diffusion Coefficients

Table II shows that the volumes of various protein molecules calculated from osmotic pressure and sedimentation data agree but are lower than the volumes calculated from diffusion coefficients. The volumes calculated from osmotic pressure and sedimentation data do not include any water of hydration. The calculations do not involve any assumptions about the shapes of the molecules. The volumes calculated from diffusion data, as we have seen, do include water of hydration and are based on all the assumptions involved in Einstein's law. There are three possible reasons why the volumes calculated from diffusion coefficients are higher than those from osmotic pressure.

1. The diffusion coefficients are too low. We have already presented the evidence for the correctness of the diffusion coefficients.

2. The molecules are hydrated and the difference between the volume calculated by the other two methods represents water of hydration. This hypothetical water of hydration, however, is higher than the hydration of these proteins calculated from viscosity data by the empirical equation of Kunitz (1925–26). This disagreement is not conclusive proof that hydration is not responsible for the high volumes calculated from diffusion coefficients because the application of Kunitz's equation may not be valid.

In a previous paper (Kunitz, Anson, and Northrop (1933–34)) the

TABLE II

Molecular Volumes

	Calculated from osmotic pressures	Calculated from sedimentation data	Calculated from diffusion coefficients
Hemoglobin	50,200 (Adair (1925))	52,000 (Lamm and Polson (1936))	104,500* (Northrop and Anson (1928–29))
Pepsin	26,300 (Northrop (1929–30))	26,000 (Philpot and Eriksson-Quensel (1933))	56,000* (Northrop (1929–30))
Trypsin	27,500 (Kunitz and Northrop (1934–35))		92,400* (Scherp (1932–33))
Chymo-trypsinogen	17,100 (Kunitz and Northrop (1934–35))		56,100† (Kunitz and Northrop (1934–35))
Chymo-trypsin	30,700 (Kunitz and Northrop (1934–35))		56,100† (Kunitz and Northrop (1934–35))

* Membrane calibrated with 0.1 N hydrochloric acid — $D_5° = 1.45$ cm.^{2}/day.

† Membrane calibrated with 0.23 N sodium chloride — $D_5° = 0.72$ cm.^{2}/day.

hydration calculated from viscosity data was found to agree with the difference between the volumes calculated from diffusion coefficient and osmotic pressure. This result must be rejected, however, because the diffusion coefficients used were based on a wrong value for the diffusion coefficient of 0.1 N hydrochloric acid at 5°C.

3. Einstein's equation is not valid.

(*a*). The relation between the coefficients of diffusion and friction

$$D = \frac{RT}{NF}$$

is not correct when applied to proteins. Lamm and Polson (1936), however, have shown that the molecular weight calculated from sedimentation equilibrium agrees with the molecular weight calculated from sedimentation velocity using the coefficient of friction obtained from the diffusion coefficient. This agreement would not exist were not the coefficient of friction calculated from the diffusion coefficient correct.

(*b*). Stokes' law

$$F = 6\pi r\eta$$

is not valid when applied to protein molecules. As we have already pointed out, if the molecule is not spherical the radius and hence the volume calculated from the diffusion coefficient by Einstein's law which includes Stokes' law is too high.

Polson (1936) has assumed that the molecular weights calculated from diffusion coefficients are higher than those calculated from sedimentation data because the molecules are ellipsoids and not spheres, and that corrections for the non-sphericity of the molecules can be calculated from viscosity data, using the equations of Kuhn and Arrhenius. When such corrections were made he found that the molecular weights of a number of proteins calculated from diffusion and viscosity data were about 70 per cent of those calculated from sedimentation data.

(*c*). Einstein assumes that the viscosity, η, in Stokes' equation is the viscosity of the solvent and that the viscosities of the solvent and the solution are the same. We have used the viscosity of the solvent in calculating the molecular volume from the diffusion coefficient but

the viscosities of the solutions were, in general, higher than the viscosities of the solvents. The volumes calculated from diffusion coefficients, however, would still be high even if the viscosities of the solutions were used in the calculations.

In general, it may be said that the molecular volumes calculated from diffusion coefficients are higher than those calculated from osmotic pressure and sedimentation data, but that it cannot be decided whether these high values are due to hydration or non-spherical shape or both because there is no reliable information at present about the hydration and shapes of protein molecules.

The Kinds of Information Which Can Be Obtained from Diffusion Measurements

Since Einstein's law involves assumptions which in practice may not be valid, there is some doubt as to the significance of the molecular volumes calculated from diffusion coefficients. Useful information, however, can be obtained from the diffusion measurements despite this limitation.

In the first place, by assuming Einstein's law to be correct one can obtain a rough notion of the maximum molecular size from a knowledge of the diffusion coefficient. The membrane method, in fact, was originally devised to obtain rough information about molecular size of biologically active substances when the ordinary methods of measuring molecular size cannot be applied at all. By means of the membrane method one can measure the diffusion coefficient of, for instance, bacteriophage whose presence is known only by its biological activity and which is available only in low, unknown absolute concentration in impure solution (Hetler and Bronfenbrenner (1930–31)).

In the second place, by making only assumptions which can be tested experimentally, one can calculate the coefficient of friction, F, from the diffusion coefficient, D. Knowing F one can calculate the molecular weight of a particle from the rate at which it moves in a gravitational field, as has been done by Svedberg, or the charge on a particle from the rate at which it moves in an electrical field, or in general, add to the information which can be obtained from a knowledge of the rate at which a particle moves under the influence of a given force.

In the third place, several different kinds of useful information can be obtained from suitable diffusion experiments without any calculation of friction coefficient or molecular size, without any assumptions whatever about the shape of the molecule or its degree of hydration. First, diffusion experiments can, under suitable conditions, give some information about the constitution of a substance. If the rate of diffusion of a substance in the absence of salt is not affected by acid or base, then the substance is a non-electrolyte. If the rate of diffusion is at a minimum at a given pH and is increased by both acid and base, then the substance is amphoteric. Secondly, diffusion experiments can be used to test the homogeneity with respect to particle size of the diffusing substance. If the first part of the substance which has diffused through the membrane is removed and the rate at which this diffusate diffuses through a membrane is measured in a separate experiment, and if the diffusion coefficient of the first diffusate is the same as that of the original substance, then the original substance is homogeneous. Thirdly, when an active material has been supposedly isolated diffusion experiments can be used to test whether the substance isolated is actually the active substance. If the active substance and the isolated substance are of similar size and constitution, then the rate of diffusion under all circumstances, even in the absence of salt and in the presence of acid or base, should be the same whether the amount diffused is measured by activity measurements or by direct estimation of the substance, for instance, by nitrogen determination.

Finally, by means of diffusion substances which diffuse at different rates can be separated. Large differences in rate of diffusion may exist between two substances of similar size if one substance is ionized and the other is not.

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