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MOLECULAR WEIGHT DETERMINATIONS OF BIOLOGICAL SUBSTANCES BY MEANS OF DIFFUSION MEASUREMENTS

BY

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The methods generally applied in chemistry to the determination of the molecular weight of any substance are based on the supposition that the substance in question is available in a pure state. In biochemistry, however, where we often work with very small quantities or with substances which cannot—or can only with difficulties—be obtained in a pure state, it would often be desirable to perform a molecular weight determination without isolating the substance. In the case of high-molecular substances, obtainable in rather concentrated and pure solutions, the ultracentrifugation may offer satisfactory information. In all other cases, we are forced to use diffusion (or dialysis) as a basis for the determination of the molecular weight.

This method has been applied in a great number of older and more recent investigations. However, the results have not always been satisfactory and the applicability of the method was, therefore, considered doubtful. It is the aim of the present work to give a theoretical and an experimental contribution to the elucidation of this problem.

Theory.

Definition of the Diffusion Coefficient.

We consider the diffusion of a substance in dilute solution in a cylinder where the concentration c of the respective substance is constant within an arbitrary plane perpendicular to the axis of the cylinder, however, varying with the situation x of the plane. In this case, FICK's second law holds, viz.

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial x^2}, \qquad (1)$$

where D is the diffusion coefficient and t the time. On the supposition that the gas-law is valid for the substance, D is independ-

ent of c. Equation (1) may then be integrated, assuming the cylinder to be infinitely long, and at the initial state ($t = 0$) putting $c = 0$ for $x > 0$; moreover, for $x = 0$ c be constant $= c_s$. Hereby, we arrive at

$$\frac{c}{c_s} = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{x}{2\sqrt{Dt}}} e^{-y^2} dy. \tag{2}$$

This equation gives the dependence of the concentration on the path of diffusion after the lapse of the time t, if the diffusion coefficient is D, and if the diffusion occurs in an infinitely long cylinder, where at the height $x = 0$ we have the constant concentration c_s of the diffusing substance, while above this height the concentration at the start is equal to 0.

(2) generally forms the basis for the experimental determination of D, since $\frac{c}{c_s}$, x, and t can be measured.

Since FICK's second law and, thus, equation (2) have been derived without paying regard to the shape of the diffusing molecule, we must be able to determine D for all molecules—even non-spherical ones—and, therefore, FICK's law involves a definition of the diffusion coefficient of a dissolved substance obeying the gas law.

RICHARD GANS (1928) has subjected the problem of the diffusion of non-spherical particles to a thorough mathematical treatment. By means of rather complicated calculations, he arrived at a formula for the interdependence between c, x, and t, which is valid under the same conditions as (2) and comprises magnitudes which are dependent on the shape of the molecule. On the basis of these calculations, GANS has given some tables from which it should be possible—when starting from measurements of the relation between c and x—to calculate not only the magnitude of the diffusing molecules but, moreover, a figure which indicates their deviation from the spherical shape.

By differentiation, GANS' formula leads to an equation which is in contradistinction to FICK's second law.

The definition of the diffusion coefficient has, furthermore, been treated by KUUSINEN (1935).

The Relation between Diffusion Coefficient and Molecular Weight.

Large, Spherical Molecules.

If we suppose the validity of the gas law for the diffusing substance it is possible, by means of STOKE's law, to derive the following formula which holds for large, spherical molecules:

$$D = \frac{kT}{6\pi\eta r}, \tag{3}$$

where k is BOLTZMANN's constant, T the absolute temperature, η the viscosity of the medium, and r the radius of the molecules. This formula, the EINSTEIN-STOKE equation, has been used by NORDLUND (1914) in order to determine AVOGADRO's figure. The value obtained was in good agreement with the values found in other ways, thus formula (3) must be regarded as verified experimentally.

If m denotes the mass and d the specific gravity of a spherical molecule, we have

$$r = \sqrt[3]{\frac{3m}{4\pi d}}, \tag{4}$$

putting

$$M = m \cdot N,$$

where N is AVOGADRO's figure, and inserting (4) into (3) we get

$$D\sqrt[3]{\frac{M}{d}} = \frac{kT\sqrt[3]{\frac{4}{3}\pi N}}{6\pi\eta}. \tag{5}$$

At constant temperature and viscosity, the right side of this equation is constant. For water at 10 °C. we get ($k = 1.374 \cdot 10^{-16}$, $N = 6.06 \cdot 10^{23}$, $\eta = 0.013$, abs. u.)

$$D_{10}\sqrt[3]{\frac{M}{d}} = 1.86, \tag{6}$$

where D is calculated in cm²/day.

This equation may serve the determination of M from measurements of D, provided that the specific gravity of the molecules is known and all conditions for the validity of the equation are fulfilled.

If we have to do with an unknown substance, the specific gravity must be estimated: in the case of water-soluble organic substances of a high molecular weight, the value should be put to somewhat higher than 1, for proteins for example around 1.3. The relative error of the estimated specific gravity will give rise to a correspondingly great relative error of the value of M—as it appears from the formula. However, it will be seen from the following that this error generally will be smaller than the uncertainty for other reasons encumbering molecular weight determinations of this kind.

As it appears from the derivation of equation (6), it is a condition for its validity 1) that the gas laws can be applied to the dissolved substance; this is the case with a dilute solution of an uncharged substance, and 2) that we have to do with large, spherical molecules. Such a molecule will be surrounded by a layer of water which follows the motions of the molecule so that the friction determining the resistance to the motions of the molecule occurs between the interior layer and the surrounding layer of water further outside. The latter will follow the motions to a smaller extent with increasing distance from the molecule.

If D depends on M, as expressed by equation (6), the weight of the water layer bound to the molecule must be small as compared with the weight of the molecule. In order that the molecule can be regarded as "large", we must, therefore, require at least that the weight of a monomolecular layer of water on the surface of the molecule must be small as compared with the weight of the molecule. For example, we may consider a substance with the molecular weight 100000 and the molecular specific gravity 1.3. We can then easily calculate the weight of a monomolecular water layer, surrounding a gram molecule of this substance, to be 20000 g. or a fifth of the weight of the molecules. In other words, not even protein molecules can be considered to be "large".

Furthermore, the demand of a spherical shape of large molecules will be fulfilled in rare cases only and it is, therefore,

obvious that the application of equation (6) as an exact formula for the determination of M must be confined to extremely rare cases.

The procedure was used in a number of earlier and more recent works, for example, by EDMAN (1945) for the determination of the molecular weight of hypertensin.

Large, Non-Spherical Molecules.

We shall assume that non-spherical molecules with some approximation can be regarded as rotation ellipsoids.

On the basis of relations stated by GANS (1928), HERZOG, ILLIG and KUDAR (1933) derived the formulae given below, where b is the axis of rotation, and a the other axis of the ellipsoid.

For a flat ellipsoid, we have

$$D = \frac{kT}{6\pi\eta r}\left[\sqrt[3]{\frac{b}{a}}\,\frac{\arcsin\sqrt{1-\frac{b^2}{a^2}}}{\sqrt{1-\frac{b^2}{a^2}}}\right] \tag{7}$$

and for an elongated rotation ellipsoid we get

$$D = \frac{kT}{6\pi\eta r}\left[\frac{1}{2}\,\frac{\sqrt[3]{\frac{a^2}{b^2}}}{\sqrt{1-\frac{a^2}{b^2}}}\cdot ln\,\frac{1+\sqrt{1-\frac{a^2}{b^2}}}{1-\sqrt{1-\frac{a^2}{b^2}}}\right], \tag{8}$$

where r is the radius of a sphere with the same volume as the ellipsoid.

These formulae are analogous to formula (3). The magnitudes in the brackets can be considered correction factors by means of which we correct for the deviation from the spherical shape. It holds for both correction factors that they have a limit value 1 for $\frac{a}{b} \to 1$ and a limit value 0 for $\frac{a}{b} \to 0$. For all intermediate values of $\frac{a}{b}$ the correction factors are between 0 and 1.

Since the shape of the molecule of an unknown biological

substance, the molecular weight of which we wish to determine, is unknown, the correction factors cannot be taken into account. In this case, the molecular weight determined by formula (6) must thus be a maximum value indicating the highest possible value of the molecular weight. If the molecule is spherical, this maximum value is identical with the molecular weight, while in all other cases the molecular weight must be smaller, and the smaller the greater the deviation from the spherical shape.

Polson (1936) described a method for the determination of the molecular weight of proteins from measurements of the diffusion constant and the viscosity, since a/b can be calculated from the viscosity, and r and hence M from formula (8), if we suppose the shape of the molecule to be an elongated rotation ellipsoid. This method must be assumed to be applicable to biological substances even if they are not available in pure solutions, however, if it may be considered granted that the solution does not contain other substances contributing to the viscosity value.

Small, Spherical Molecules.

In contrast to the conditions prevailing for large molecules, it is impossible in the case of small molecules to derive an exact formula for the interdependence between D and M, since the molecular-kinetic conditions in liquids are but scarcely known.

Herzog, Illig and Kudar (1933) have derived the following formula on the basis of kinetic considerations, viz.

$$D = \frac{kT}{4\pi\eta r}. \tag{9}$$

The only difference between this formula (9) and the formula (3) is the factor 4 in the denominator of (9) instead of the factor 6 in formula (3). This is due to the fact that the derivation of (9) assumes completely smooth molecules so that motion occurs between the molecule and the immediately surrounding water layer; in the derivation of formula (3) it is assumed for large molecules that the water layer in direct touch with the molecule surface moves together with the latter; in this case, motion takes place between the water molecules in the neighbourhood of the

large molecules and the surrounding molecules (HERZOG and KUDAR 1933).

For not completely smooth molecules, the coefficients in the denominator of the corresponding formulae must be between 4 and 6. Thus, the equations (3) and (9) represent extreme cases. As regards such substances which do not obey any of these formulae, it is impossible on this basis to give any precise description of the relation between M and D, except that the equations (3) and (9) give the limits of the existing possibilities.

A corresponding equation which is said to hold for all molecule sizes was given by SUTHERLAND (1905):

$$D = \frac{kT}{6\pi\eta r} \cdot \frac{1 + \frac{3\eta}{\beta r}}{1 + \frac{2\eta}{\beta r}} \qquad (10)$$

where β is the friction coefficient on the boundary surface between the molecule and the closely surrounding water molecules. For $\beta = \text{infin.}$ and $\beta = 0$, we arrive at (9) and (3), respectively, from the equation (10). Since β can scarcely be a definite function of M, it is clear from equation (10) that there does not exist a definite relation between D and M, not even in the case of spherical molecules.

Small, Non-Spherical Molecules.

Apparently, the problem of diffusion of small, non-spherical molecules has never been treated theoretically. However, it can scarcely be quite incorrect to assume that, for these molecules, a deviation from the spherical shape causes similar changes of D as it is the case for large molecules. In other words, we consider the formulae (7) and (8) to be valid for smaller molecules if the factor 6 in the denominator is replaced by a figure between 6 and 4.

The Charge of the Molecules.

The considerations outlined above are based on the supposition that the diffusing molecules are uncharged. An unknown

biological substance will probably be present in solution as charged molecules, i. e. either as low-molecular ions or as high-molecular colloidal particles. Therefore, the problem of the influence of charge upon the diffusion coefficient is of decisive significance for our present question. Most investigators who applied diffusion measurements to molecular weight determinations tacitly assumed that charged and uncharged molecules of the same molecular weight diffuse equally rapidly. However, even other views can be found in the literature. For example, HEVESY (1913) stated that the diffusion constants of ions depend only on the charge and not on the molecular weight. According to HEVESY, all monovalent ions at 10 °C. have a diffusion constant of 1.08, and the diffusion constant of all divalent ions is about half that value, i. e. c. 0.54. However, JANDER and WINKEL (1930) found this view hardly correct.

The difference between the diffusion of an uncharged substance and the same substance carrying charge can be ascribed to three different circumstances, viz.

1) Diffusion of a charged particle must always be accompanied by diffusion of one or a number of other charged particles in such a way that the total transport of electricity becomes zero.

2) With increasing salt concentration, the inter-ionic forces will cause a decreasing diffusion rate (DEBYE and HÜCKEL 1923).

3) Charge causes hydration of the molecule, whereby the molecular weight increases. Hence, the diffusion velocity will be smaller than corresponding to the molecular weight of the non-hydrated substance.

The influence of the circumstances mentioned under 1) might be eliminated by adding to the solution so much of a neutral salt that the electric conductivity is great as compared with the conductivity due to the ion whose diffusion has to be determined. The resistance to diffusion owing to the transport of electricity must be small as compared with the resistance owing to the viscosity of the medium. Thus, the determination must be carried out on a rather dilute solution of the respective substance in a relatively concentrated solution of a neutral salt.

In a solution of this type, the ions of the substance to be measured follow the gas laws, and this is a necessary condition —as mentioned previously—for the validity of equation (2).

If we assume the same relation between M and D both for charged and uncharged molecules, the determination of the molecular weight will be encumbered with errors for the reasons mentioned under 2) and 3). Presumably, these errors increase with increasing salt concentration, with increasing charge, and with decreasing molecular weight. The magnitude of the errors, however, cannot be calculated theoretically. We have, therefore, subjected the problem to an experimental investigation and measured the diffusion coefficients of primary and secondary phosphate ions and, moreover, of primary and secondary pyrophosphate ions in 1 M potassium chloride.

If, generally, the charge of a molecule causes a considerable reduction of the diffusion coefficient, we must expect to find a great difference in the diffusion coefficients of ions of the same molecular weight, but of different charge, especially if the charge of the ions is great relative to the molecular weights, as it is the case here, and moreover if the measurements are performed in so strong a salt solution.

As regards the greater details of the experimental procedures, the reader is referred to the experimental section. Here, we give only the average values found for the diffusion coefficients at 10 °C., measured in cm^2/day.

primary phosphate ion	0.495
secondary " "	0.468
secondary pyrophosphate ion	0.371
tertiary " "	0.356

The values indicate that, in the present cases, the charge is only of minor influence upon the diffusion coefficients. The errors involved for other reasons in a molecular weight determination of this type are considerably greater. Whether the same will be true for other substances can obviously not be decided on the basis of so few experimental data. But probability considerations are in favour of this view, since deviations must be expected to be especially great in these experiments, as already outlined above.

For all molecules regardless of their size, shape, or charge it will be possible to determine a maximum value of M from the equation

$$M_{max} = \left(\frac{2.79}{D_{10}}\right)^3 d. \qquad (11)$$

The real molecular weight will be smaller than (or, in the limit case, equal to) this maximum value, mainly for two reasons: the friction against the surrounding water molecules and the deviation from the spherical shape. The first mentioned error can amount at the most to a factor $(6/4)^3 = 3.4$ and the second, for molecules with a molecular weight below c. 1000, will be of a similar magnitude. Thus, we may expect that the maximum molecular weights obtained in this manner will be up to ten times higher than the true values.

For larger molecules, the first mentioned source of error can be partly eliminated, while the possibility for errors of the last mentioned type will be considerably greater.

Empirical Equations.

SUTHERLAND (1905) arrived at the following empirical equation which he found to be in agreement with the experimentally found D-values of substances with a molecular weight between 2 and 500.

$$D\sqrt[3]{\frac{M}{d}} = b + \frac{k}{\sqrt[3]{\left(\frac{M}{d}\right)^2}} \qquad (12)$$

where b and k are constants at constant temperature and for one and the same medium. In the case of water at 10 °C., SUTHERLAND's constants can be calculated to be $b = 1.5(7)$ and $k = 16.(4)$ if D is calculated in cm²/day. Sutherland: $b = \frac{21}{10^6}$, $k = \frac{220}{10^6}$, D in $\frac{cm^2}{sec}$ for water at 16 °C.

For high molecular weights, we obtain from this

$$D_{10}\sqrt[3]{\frac{M}{d}} = 1.5(7).$$

In the case of lower molecular weight, this magnitude assumes higher values, in qualitative agreement with the considerations mentioned above in connection with equation (9). For molecules where $M/d < 50$, the equation (12) leads to values of D higher than those determined from (9). In practice, however, this is of little interest if we have to do with biological substances.

In the biological literature we often meet with the simple relation

$$D\sqrt{M} = \text{const.}, \tag{13}$$

which was proposed for the first time by RIECKE (1890) on an empirical basis. ØHOLM (1910) stated that the constant assumed the value c. 7 at 20 °C. Furthermore, the value of the constant depends somewhat on the group of substances to which the diffusing substance belongs. However, when comparing related substances, the constant is said to be independent of M within wide limits (JANDER and WINKEL 1930). Equation (13) holds if the mean free path of the molecules is independent of the molecular weight.

Experimental Methods.

In the course of time, a great number of different methods has been described for the experimental determination of D. In principle, we can differentiate between two groups of methods, viz. those where diffusion occurs through a membrane (dialysis) and others where diffusion takes place freely in the liquid.

Dialysis has been treated thoroughly by BRINTZINGER (1940) and by JANDER and SPANDAU (1941). In practice, these methods have considerable advantages as compared with those based upon diffusion in liquids, because errors due to convection are excluded. Since, on the other hand, the theory of diffusion in such gels as used for the membrane is but poorly known, and in certain cases deviates from the theory of diffusion in liquids, a closer investigation of these phenomena will be necessary in order that the dialysis methods may reach the same extent of applicability as the free diffusion. The same holds presumably for the methods where the diffusion medium is an agar gel.

Diffusion measurements in agar were carried out by Poul Larsen (1944) with extremely small quantities of a plant growth substance. The method outlined by Northrop and Anson (1929) based upon diffusion through a porous glass plate theoretically belongs to those methods which apply free diffusion; but, in contrast to these methods, it necessitates calibration with a substance of a known diffusion coefficient.

The main interest is directed towards methods which are based upon free diffusion in liquids, chiefly in view of their simplicity in theoretical respect. Even these methods may be subdivided in two groups dependent on whether the concentration of the diffusing substance is determined optically or analytically. If we have to do with biological substances in impure solutions, the analytical method will generally be preferable and, therefore, this method only will be treated in the following. The "analysis" may, for example, be a biological test based upon the effect of the respective substance on the organism. Frequently, this is the only way out.

In practice, the determinations were performed in the following way.

A vertical cylinder contains in its lower part the solution of the substance and, placed over the solution, the pure solvent. The two liquids should not mix because of currents, which claims special precautions and often will be impossible if the difference in specific gravity is too small. This difference will be small because the concentration of the diffusing substance should not be high. For this reason, 1 per cent glucose was added to the lower liquid; this facilitates considerably the formation of a sharp boundary layer. We can hardly assume that this addition of glucose should have any significant influence upon *D*. The cylinder must stand at constant temperature under vibration-free conditions. Since by diffusion the specific gravity decreases gradually upward, an addition of glucose will reduce the risk of currents due to vibration or temperature fluctuations.

Now, the diffusion process begins, the concentration of the diffusing substance increases in the layers above the boundary plane and it decreases below the boundary. So long as the concentrations at the uppermost and the lowest part of the cylinder have not suffered any significant changes, the cylinder may be

regarded as infinitely long in both directions. In this case, it is easy to show that equation (2) describes the dependence of the concentration upon the height above the boundary layer at the time t, if the initial concentration in the lower layer is $2\,c_s$. After the lapse of some time, however, the changes in concentration will even reach the ends of the cylinder and, then, equation (2) no longer holds. In the upper part of the cylinder this disturbing effect can easily be avoided if only the cylinder is sufficiently high. Thereby, the concentration of the diffusing substance in the upper part of the cylinder remains small as compared with the concentration in the highest layer taken into account for the calculation. As regards the conditions prevailing in the lower part of the cylinder, we can keep the concentration constant by using a sufficiently high layer of the lower solution. Since it will frequently be of importance, however, to perform the measurement with as little of the substance as possible, it is of special interest to study the course of the diffusion process applying a layer of the lower liquid of a finite height a.

We shall consider an infinitely high cylinder containing between the height $x = 0$ and the height $x = -2\,a$ a solution of the diffusing substance and, above and below this, the pure solvent. If we disregard the gravity, it is clear that diffusion—owing to the symmetry around the plane $x = -a$ —will occur according to the same law as in a cylinder which is limited in the direction downward containing a layer of the solution of the height a. After the lapse of the time t, the concentration at the height x (> 0) will be c'_x. If we imagine the lowest phase of the solvent to be replaced by the solution, we would obtain the concentration c_x which is greater than c'_x, because c_x includes the substance which diffused from the solution below the height $-2\,a$. If we denote this difference as c_{x+2a} we get

$$c'_x = c_x - c_{x+2a}. \tag{14}$$

c_x and c_{x+2a} satisfy the formula (2). For a given substance and a given time of experiment it would, thus, be simple to find the error of D at a given height of the solution if we apply formula (2) to the calculation and if we, thus, disregard the term $-c_{x+2a}$. Reversely, we may find out which height we need in order to

arrive at a given accuracy in the determination of D. If we wish to work with small quantities of the substance, the formulae (2) and (14) can be used for the calculation, the latter formula being written as follows

$$c_x = c'_x + c'_{x+2\alpha}, \tag{15}$$

where $c_{x+2\alpha}$ is replaced by $c'_{x+2\alpha}$, the measured concentration at the height $x + 2\alpha$, which generally will be permissible because $c_{x+2\alpha}$ often will be rather small as compared with c_x. If this is not sufficiently accurate, we must use some further terms of the infinite series

$$c_x = c'_x + c'_{x+2\alpha} + c'_{x+4\alpha} + c'_{x+6\alpha} + \cdots\cdots c'_{x+2n\alpha} + \cdots\cdots \tag{16}$$

The desired number of terms on the right side is accessible for measurements if only the cylinder is sufficiently high. Then, D is found from c_x by means of formula (2). However, this procedure is based on the supposition that the bottom of the apparatus is horizontal, which is not the case in the apparatus used by us. For this reason, we have ascertained in each case that the height of the solution in the cylindrical part of the apparatus was so great that the measured concentrations directly satisfied equation (2).

The duration of the experiment depends, apart from certain regards paid to the dimensions of the apparatus as treated above, on the lability of the substance and, finally, on two more points concerning the analytical determination of the concentration, viz. the relative accuracy of the determination and the least concentration relative to c_s at which the determination can be performed.

The curves of Fig. 1, illustrating the relation between distance and concentration show, how the mentioned factors determine the duration of the experiment and the accuracy of the determination. It is of special significance for the applicability of the method to biologically active substances that we can reach a satisfactory accuracy of the determination of D although the measurement of the concentration is encumbered with a considerable relative uncertainty, if only the substance to be measured is available in a concentration which is high as compared with the lowest pos-

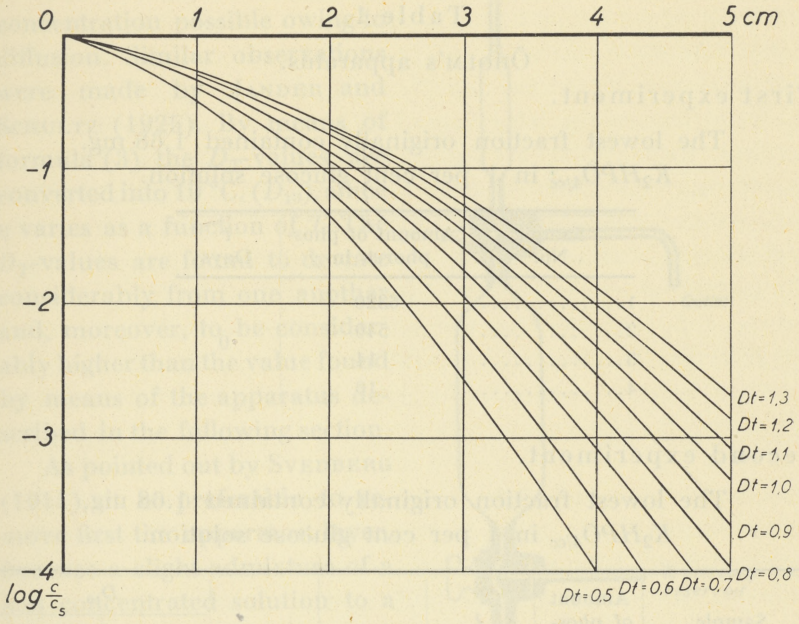

Fig. 1. The interdependence between the distance from boundary and $\log \frac{c}{c_s}$, for different values of Dt.

sible concentration for the analytical determination of the substance. This supposition will frequently be fulfilled.

When the time of diffusion is elapsed the experiment is interrupted by taking samples from the solution at different heights. This may be done in three different manners:

Cohen and Bruins (1923), and later Theorell (1934), separated the cylinder mechanically in numerous sections which were then emptied individually. From a theoretical point of view this is the most correct method, however, it necessitates a rather complicated apparatus.

Less satisfactory is Öholm's (1905) procedure, where the whole quantity of the liquid is removed in portions through the bottom of the cylinder in such a way that the layers with the highest concentration are removed first. The portions containing lower concentrations of the substance will thereby be mixed with small quantities of a more concentrated solution, and this may be the cause of rather significant errors, as it is illustrated by the course of the curve corresponding to equation (2). The experiment

Table 1.

Öholm's apparatus.

First experiment.

The lowest fraction originally contained 1.68 mg. K_2HPO_4/cc in 1 per cent glucose solution.

Sample No.	Amount of phosphorus in γ	t Days
1.....	6320	0
2.....	540	
3.....	14	
4.....	10	

Second experiment.

The lowest fraction originally contained 1.68 mg. K_2HPO_4/cc in 1 per cent glucose solution.

Sample No.	Amount of phosphorus in γ	t Days	D_T	T °C.	D_{10}	
					Öholm's apparatus	The authors' experiments
1.....	3360	0.187	0.97	13.2	0.74	0.475
2.....	2040		..		..	
3.....	480		0.63		0.58	
4.....	72		0.65		0.60	

represented in Table 1 shows clearly that such errors actually occur and that they play a very significant part.

In experiment 1, tapping was carried out immediately after filling, secondary potassium phosphate being used as the diffusing substance. According to the diffusion theory we should expect the total quantity of phosphate in fraction 1 and nothing in any other fraction. However, the table shows that especially fraction 2 contains considerable quantities of phosphate.

In the second experiment, the time of diffusion was 0.187 day. When applying Kawalki's table, improved by Jander and Schultz (1925), and inserting the quantities of phosphate found, we arrive at values for D_T given in the fourth column of the table. The concentration found in fraction 2 is higher than the highest

concentration possible owing to diffusion. Similar observations were made by JANDER and SCHULTZ (1925). By means of formula (3) the D_T-values are converted into 10 °C. (D_{10}), since η varies as a function of T. The D_T-values are found to deviate considerably from one another and, moreover, to be considerably higher than the value found by means of the apparatus described in the following section.

As pointed out by SVEDBERG (1911), it is preferable to remove first the uppermost layer, because a slight admixture of a less concentrated solution to a more concentrated one does not give rise to great errors. This kind of tapping is most easily performed by replacing the liquid in the cylinder by mercury which is introduced from below, while the solution flows through a tube inserted at the uppermost part of the cylinder. The principle of the apparatus applied by the present authors appears from Fig. 2.

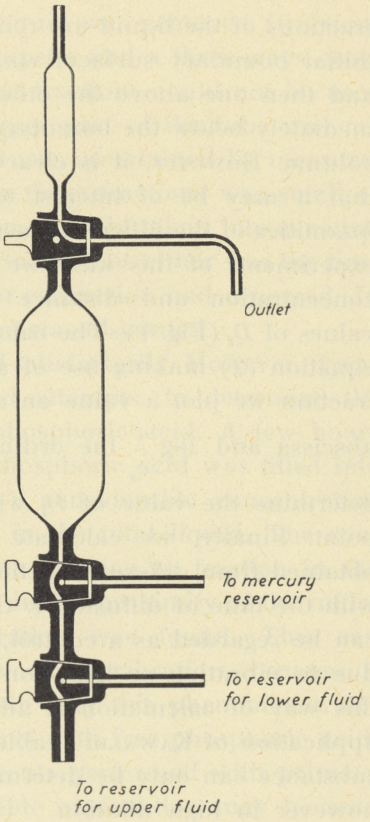

Fig. 2. The apparatus employed.

The distance of the single fractions from the initial boundary layer is calculated from the cross section area and the volume of the cylinder and from the volumes of the fractions and the lowest solution.

Calculation of *D* from the Results of the Measurements.

KAWALKI (1894) has given a table for the calculation of D which was extended later by JANDER and SCHULTZ (1925) and which is frequently used in connection with ÖHOLM's apparatus. The application of the table is based on the supposition that three

fractions of the liquid are removed which are situated above the initial boundary surface, viz. immediately above the boundary and then one above the other and, moreover, one fraction immediately below the boundary. All fractions must have the same volume. However, it is clear from the preceding considerations that it may be of interest to apply other positions and other quantities of the different fractions. For the calculation of D from experiments of this kind we have plotted the relation between concentration and distance from the boundary for different values of D_t (Fig. 1). The calculation of these curves is based on equation (2) making use of a table of error integrals. For each fraction we plot a value on a graph like Fig. 1, where x is the abscissa and $\log \frac{c}{c_s}$ the ordinate. By a simple interpolation we determine the value of D_t which corresponds to the respective point. Finally, we calculate the mean of the different values obtained from different fractions, and D is calculated by division with the time of diffusion t. Good agreement of the values of D_t can be regarded as a control, indicating the absence of currents due to vibration or heat convection. In biological experiments, this way of calculation is advantageous—as compared with an application of KAWALKI's table—i. e. in cases where the diffusing substance can only be determined with a low relative accuracy, however in high dilution.

Experimental.

The measurements were performed by means of the apparatus designed in Fig. 2, which was placed in a well-insulated cellar laboratory situated below earth level. The room temperature varied somewhat with the season, as it appears from Table 2, but the fluctuations during the day were very small.

After filling with the different solutions, the apparatus was left standing for c. one hour in order to secure temperature equilibrium. Subsequently, the experiment was started by opening the lower stop-cock so much that the lower liquid flew very slowly into the cylinder (c. 10 ml. per hour). The start of the experiment was reckoned from the moment when this cock was opened. At the conclusion of the experiment a number of equally

great volumes (1.489 ml.) of the liquid was removed (fractions Nos. 1, 2, 3, . . .) by means of a pipette and a three-ways cock. The concentrations of the three first fractions could not be used for the calculation because the upper part of the tube was not cylindrical (cf. Fig. 2). However, it was ascertained by measurements whether the concentration in these fractions was small as compared with the highest fraction actually taken into account. If this was not the case, it was concluded that either was the time of experiment not sufficiently long or convection had occurred. In both cases, the respective experiment was discarded.

Generally, the apparatus worked satisfactorily. However, it was found impossible, despite repeated attempts, to determine the diffusion coefficients of the free phosphoric acid. A few hours after the lower liquid containing phosphoric acid was filled into the lower part of the apparatus, a considerable concentration of phosphoric acid was measured in the total liquid. The concentration was found constant from fraction to fraction in the upper 4 cm. of the container, while in the proximity of the initial boundary an increasing concentration was found. This can hardly be due to heat convection or to vibration, because it would then be impossible to explain why this phenomenon occurred in each of the six experiments with free phosphoric acid, while it was absent in six experiments performed with primary and secondary phosphate. A possible explanation may be seen in the assumption that the phosphoric acid might migrate along the boundary glass-liquid. Similar movements of substances along boundary surfaces are known between air and solid substances. For example, it is well known that water can creep along a glass surface against the direction of gravity.

The phosphates were determined colorimetrically by means of molybdic acid, and the pyrophosphates were measured in the same way after hydrolysis (E. Jacobsen 1933).

Methyl red was determined colorimetrically in acid medium.

Histamin was determined by its effect on an isolated intestine of the guinea pig (Ahlmark 1944).

The pH-values required in these experiments—in order that only the given ions of phosphate and histamine to be measured should be present in the solution—were determined by electrometric titration in 1 *M* potassium chloride. As an upper liquid

Tabl

Compounds	*M* Mol. Wt.	pH	Fraction No.	log c/c_S
Prim. phosphate $H_2PO_4^-$	97	4,5	4	−2.89
			5	−2.47
			6	−2.09
			7	−1.66
Prim. phosphate H_2PO^{4-}	97	4,5	6	−2.80
			7	−2.35
Sec. phosphate HPO_4^{--}	96	8,5	5	−2.68
			6	−2.22
			7	−1.87
			8	−1.46
Sec. phosphate HPO_4^{--}	96	8,5	5	−2.55
			6	−2.21
			7	−1.85
Sec. phosphate HPO_4^{--}	96	8.5	5	−2.55
			6	−2.17
			7	−1.81
Sec. pyrophosphate $H_2P_2O_7^{--}$	176	3.9	4	−2.56
			5	−2.22
			6	−1.90
Tert. pyrophosphate $HP_2O_7^{---}$	175	6.7	5	−2.34
			6	−2.08
			7	−1.73
Methyl red $C_{15}H_{14}O_2N_3^-$	268	$p_H > 8$	6	−3.11
			7	−2.55
			8	−2.11
			9	−1.56
			10	−1.06
Methyl red $C_{15}H_{11}O_2N_3^-$	268	$p_H > 8$	6	−3.00
			7	−2.47
			8	−1.99
			9	−1.56
Histamine $C_5H_{10}N_3^+$	112	..	4	−3.01
			5	−2.59
			6	−2.21
			7	−1.91
			8	−1.57

All experiments are performed in 1 *M* potassium chloride.

2.

x Distance cm.	$D_T \cdot t$	$D_T \cdot t$ Mean	t Days	D_T	$T\,°C$	D_{10}
4.82	1.20					
4.41	1.15	1.19	1.88	0.635	18.3	0.492
4.00	1.17					
3.58	1.27					
4.00	0.81	0.805	1.25	0.642	18.6	0.497
3.58	0.80					
4.41	1.04					
4.00	1.08	1.08	1.88	0.577	17.0	0.466
3.58	1.08					
3.17	1.12					
4.41	1.11					
4.00	1.09	1.10	1.90	0.579	16.4	0.475
3.58	1.09					
4.41	1.11					
4.00	1.05	1.11	1.97	0.564	16.5	0.463
3.58	1.05					
4.82	1.30					
4.41	1.29	1.29	2.86	0.452	16.5	0.371
4.00	1.29					
4.41	1.23					
4.00	1.17	1.19	2.75	0.434	16.3	0.356
3.58	1.17					
4.12	0.76					
3.70	0.78					
3.30	0.73	0.754	1.81	0.418	19.0	0.319
2.72	0.76					
2.08	0.74					
4.21	0.83					
3.76	0.84	0.84	1.92	0.435	18.3	0.337
3.32	0.84					
2.86	0.84					
4.82	1.08					
4.41	1.08					
4.00	1.08	1.06	1.78	0.600	14.7	0.531
3.58	1.05					
3.17	1.02					

Tabl

Compounds	Ref.	M Mol. Wt.	D_T	T °C	D_{10}
Sec. phosphate ..... HPO_4^{--}	..	96	..	..	0.468
Prim. phosphate ..... $H_2PO_4^-$	..	97	..	..	0.495
Histamine..... $C_5H_{10}N_3^+$	..	112	..	..	0.531
Tert. pyrophosphate ..... $HP_2O_7^{---}$	..	175	..	..	0.356
Sec. pyrophosphate ..... $H_2P_2O_7^{--}$	..	176	..	..	0.371
Methyl red..... $C_{15}H_{14}O_2N_3^-$	..	268	..	..	0.328
Saccharose	Lamm 1928	342	0.399	20	0.295
Colloidal gold	Svedberg 1909	6 100	0.27	11.7	0.26
Lactoglobulin.....	Polson 1936	40 000	0.0626	20	0.0464
Ovalbumin.....	Polson 1936	40 500	0.0670	20	0.0496
CO-hemoglobin	Svedberg and Nichols 1927	67 000	0.071	30	0.041
Edestin.....	Lamm 1929	228 000	0.0445	20	0.0329
Amandin.....	Polson 1936	330 000	0.0313	20	0.0232
Thyroglobulin	Polson 1936	676 000	0.0233	20	0.0172
Octopus hemocyanin	Polson 1936	2 780 000	0.0142	20	0.0104

3.

d	Einstein-Stoke's equation				Sutherland's equation		Riecke's equation	
	M_{exp} from equation (6)	$\frac{M_{exp}}{M}$	M_{max} from equation (11)	$\frac{M_{max}}{M}$	M_{exp} from equation (12)	$\frac{M_{exp}}{M}$	M_{exp} from equation (13) $k = 5{,}00$	$\frac{M_{exp}}{M}$
1.88	118	1.2	398	4.1	200	2.1	114	1.19
1.88	101	1.0	341	3.5	190	2.0	102	1.04
1.1	48	0.43	162	1.4	105	0.9	89	0.79
1.88	270	1.5	910	5.2	340	1.9	197	1.13
1.88	233	1.3	785	4.5	330	1.9	182	1.03
1.20	212	0.8	715	2.7	270	1.0	232	0.87
1.59	400	1.2	1 350	4.0	450	1.3	287	0.84
19.3	7 000	1.1	23 600	3.8	7 300	1.2	370	0.06
1.35	87 000	2.2	290 000	7.2	51 000	1.3	12 000	0.30
1.35	71 000	1.8	240 000	6.0	42 000	1.0	10 000	0.25
1.35	125 000	1.9	420 000	6.3	75 000	1.1	15 000	0.22
1.35	240 000	1.1	810 000	3.5	140 000	0.6	18 000	0.08
1.35	700 000	2.1	2 400 000	7.3	410 000	1.2	46 000	0.14
1.35	1 700 000	2.5	5 700 000	8.4	1 000 000	1.5	85 000	0.13
1.35	7 700 000	2.8	26 000 000	9.4	4 500 000	1.6	230 000	0.08

we always used an 1 M potassium chloride to which a dilute buffer was added with the pH of the lower liquid. Moreover, potassium chloride was added to the lower liquid until the total salt content was 1 M and glucose to 1 %.

The experimental results are given in Table 2.

Discussion.

Table 3 comprises the results of our own experiments and a number of measurements performed by different authors according to methods which may be assumed to lead to reliable results. The diffusion constants are converted into 10 °C. by means of formula (6), using for η the viscosity of water, which is dependent on T. Most of the molecular densities given in the table refer to pure substances. In the case of proteins, we used the estimated value 1.35. All specific gravities must be looked upon with some reservation.

From the D_{10}-values we calculated the values of M in different ways, already discussed in the theoretical section; finally, the table contains the ratio between the values found in this way and the molecular weights calculated from the formulae.

In agreement with the theory, it is found that the maximum values of M determined from equation (11) all are higher than the true values and, moreover, that the deviations from the true values are of the order of magnitude which was to be expected theoretically.

Einstein-Stoke's equation leads to values which—for low molecular weights—can be both higher and lower than the true values, while in the case of large molecules, the values become too high. This is presumably due to the fact that these molecules are not spherical.

Using Sutherland's empirical equation (12), the values for the low molecular weights turn out to be too high, while the high molecular weights are more correct than those determined from Einstein-Stoke's formula. It appears, however, from the theoretical considerations that, for large molecules, Sutherland's equation leads to a lower constant value of $D\sqrt[3]{\frac{M}{d}}$ than would be obtained from Stoke's law. Whether this leads to values

which agree better with the true values than do those determined by EINSTEIN-STOKE's law, will depend on the shape of the molecules. Since we cannot imagine that the shape will show any definite relation to the molecular weight, generally, the better agreement of SUTHERLAND's equation with the figures found here is presumably due to a mere chance.

RIECKE's equation ($D\sqrt{M} = k$) leads to much too low values in the case of high molecular weights. For low molecular weights, however, the agreement with the true values is rather good. This fits very well for the observation made by STUMPF (1945) who found this equation applicable to molecular weights between 32 and 500 while, in the case of higher molecular weights, he found a better agreement with EINSTEIN-STOKE's equation. In our experiments at 10 °C. k has a value around 5. As it was to be expected, this formula cannot be applied to the gold sol studied by SVEDBERG (1909), owing to the extremely high specific gravity of these "molecules".

Conclusion.

The order of magnitude of the molecular weight of a substance dissolved in water can be calculated from its diffusion constant by means of EINSTEIN-STOKE's equation. The inaccuracy of the method is about equally great for all sizes of molecules above 100. For very high molecular weights the values found must be considered maximum values.

From HERZOG, ILLIG and KUDAR's formula we can calculate maximum values which are independent of the size and the shape of the molecules to be studied. RIECKE's equation seems to lead to rather good values for the weight of small molecules.

Summary.

The relation between diffusion coefficient and molecular weight is treated theoretically for large and small molecules and due regard is paid to the significance of their deviation from the spherical shape.

The influence of the charge of the molecules is treated theoretically and experimentally.

The principle for the measurement of the diffusion coefficient is described and the sources of error involved in the generally applied methods are discussed.

There is given a simple method for the graphic calculation of D from the measuring results which, especially in the case of biological measurements, has certain advantages to the hitherto used tables.

Some values of the diffusion coefficient of known substances are measured experimentally. By means of these values and others taken from the literature the different relations between molecular weight and diffusion coefficient are checked and discussed.

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