formation and breakdown of the ternary complex so that reciprocal plots of velocity versus either substrate concentration are linear even if quasi-equilibrium is not obtained provided an inhibitor possessing not competitive character is not present.

Zewe and Fromm (1962) have reported a study of the lactate dehydrogenase reaction. Examination of the reciprocal plots reported by these workers shows that they are slightly nonlinear, even in the absence of added inhibitor. This result is suggested by equation 5 when a two substrate enzyme system adds the substrates randomly or even preferentially provided quasi-equilibrium is not obtained. The data of Zewe and Fromm also show that this non-linearity in the reciprocal plots is greatly exaggerated when certain inhibitory products are added.

Fromm and associates suggest that the reduced or oxidized coenzymes form abortive ternary complexes with the fully reduced or fully oxidized enzyme substrate complexes. These inhibitory products do not form complexes only with the free enzyme then but also interact with the complex between the second substrate and the enzyme. Thus, these inhibitors would be expected to have not-competitive character. This, in turn, would be expected to induce a non-linearity in the reciprocal plots or, if the plots were already non-linear, to aggravate that non-linearity. The data reported by Zewe and Fromm (1962) clearly show that increasing concentrations of these inhibitory products cause increasing curvature in the reciprocal plots. This confirms the prediction that the deviations increase as the inhibitor concentration increases and also as the ratio of inhibitor concentration to inhibitor dissociation constant increases.

References

Botts, J., and Morales, M. F. (1953), *Trans. Faraday Soc. 49*, 696.

Dixon M., and Webb, E. (1958), Enzymes, New York, Academic Press, Inc.

Edelhoch, H., and Coleman, J. (1955), *J. Biol. Chem. 219*, 351.

Fromm, H. J., and Nelson, R. D. (1962), *J. Biol. Chem. 237*, 215.

Kielley, W. W., and Kielley, R. K. (1953), *J. Biol. Chem. 209*, 213.

Morales, M. F. (1955), *J. Am. Chem. Soc. 77*, 4169.

Nordlie, R. C., and Fromm, H. J. (1959), *J. Biol. Chem. 234*, 2523.

Zewe, V., and Fromm, H. J. (1962), *J. Biol. Chem. 237*, 1668.

Diffusion Measurements in Agar Gel

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An improved method for the employment of agar gel as a medium for diffusion measurements and a mathematical treatment of the data for the calculation of diffusion coefficients are described. Diffusion is allowed to proceed under controlled conditions into a column of agar gel held in a cell made of a 50-ml hypodermic syringe that has had the needle end of the barrel cut off. By means of a micrometer the gel is pushed from the cell, sectioned with a fine wire, and assayed. The use of arithmetic probability paper affords a convenient means of plotting the data, making necessary correction for the presence of the gel, and calculating diffusion coefficients. Advantages include the possibility of using mixtures and very dilute solutions. Coefficients obtained on some salts, sugars, amino acids, and proteins are in agreement with those obtained by free diffusion.

The employment of gels as media for the study of diffusion under circumstances where concentrations are very low and where mixed solutes are present appears to have good possibilities. The rigidity of the gel enables sharp boundaries to be produced and controls to a great extent the errors resulting from thermal or mechanical mixing. Gels can be sectioned accurately, and precise analyses for several substances can be made on each section. In contrast, other methods employed for studies on mixed solutes, such as diffusion through a porous disk (Northrop and Anson, 1929), or free diffusion with various means of layer analysis (Bourdillon, 1941; Cohen and Bruins, 1924; Polson, 1944), either do not allow a study of concentration patterns or are subject to the usual difficulties in forming boundaries and preventing mixing. The use of gels, however, is not without some complications. The particles that make up the structure occupy space and thereby reduce the solvent volume within unit volume of gel. The particles also get in the path of diffusing solute molecules and thereby increase

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the distance they must travel. In addition the, diffusing solute might react with the gel.

Stiles (1923), Stiles and Adair (1921), Mann (1924), and Fricke (1925) found that the diffusion coefficients of salts in 2% agar gel are 5 to 10% lower than those obtained by free diffusion in water. Coefficients of salts in 10% gelatin were as much as 25% lower. Friedman (1930) has attributed this lowering to obstructive effects of the gel, small pore size, and increased viscosity. Diffusion of proteins into agar gels, as measured by Oudin (1946), Ouchterlony (1949), and Becker (1961), are 15 to 20% lower than in free diffusion. Felicetta *et al.* (1949) have employed light absorption to study the diffusion of some substances into agar gel. Geddes and Pontius (1960) have reviewed and described the various techniques employed in diffusion studies.

The work described in this paper is limited to agar gel because it has been found to be generally more satisfactory than others, particularly for biological substances at or near the physiologic *p*H range. An improved procedure for the rapid handling and sectioning of agar gels is presented. Furthermore, a new approach has been developed to the analyses of data from diffusion in gels.

Materials and Methods

The diffusion cell for holding the gel is made of a 50-ml hypodermic syringe (Fig. 1) with the needle end of the barrel cut off squarely so that the plunger can slide through the cut-off end. The ground glass surface inside the barrel was found to make an ideal binder to hold the gel tightly and rigidly. The close-fitting plunger provides a means for loosening the gel from the barrel and pushing it out for sectioning. The micrometer apparatus for holding the diffusion cell and accurately sectioning the gel is illustrated in Figure 1 and is similar in basic principle to that described by Fricke (1925). A fine stainless steel wire (about 0.003 in. dia.) is used for sectioning the gels.

Commercial bacteriologic agar, Difco No. B140 or 014-01, was used for making the gels. Analyses for chloride salts were made by titration with silver nitrate using potassium dichromate as the indicator with the section of gel broken up and suspended in a small volume of water. Urea, amino acids, and proteins were assayed directly on a section of gel by a micro-Kjeldahl procedure (Johnson, 1941). Bioassays for botulinum toxin and mussel poison were carried out with mice on an extract of a section of gel (Schantz *et al.*, 1957; Schantz and Spero, 1957). Sugars were determined on extracts of gel sections by reduction methods. Sucrose and raffinose were hydrolyzed with invertase (Nutritional Biochemicals Corporation).

Data were plotted on normal arithmetic probability paper, *e.g.* K and E 358-23 or Codex Book Co. No. 3227. Because it is possible to use only one half of conventional paper for a plot and because it is often advantageous to plot C'/C_0' for some biologically active substances beyond the lowest values given, a modified paper was constructed (FD Form 6-134), as partially illustrated in Figure 2.

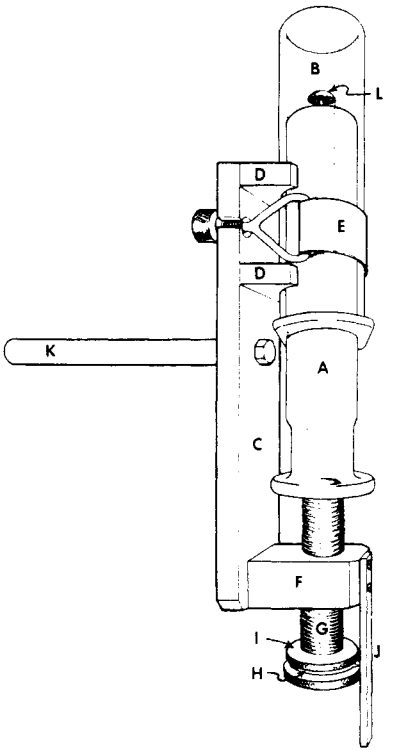

FIG. 1.—The diffusion apparatus. A is the plunger and B the barrel of a 50-ml hypodermic syringe with the needle end cut off squarely. C is an aluminum or brass plate, 7 × 2 × 1/2 in. D and D are "V" blocks to hold the syringe. E is a metal strap to hold the syringe tightly into the "V" blocks. F is a brass block, 1-1/2 × 2 × 3/4 in., to hold the micrometer screw rigidly. G is a micrometer screw, 3-3/4 in. long and 3/4 in. in diameter, with a thread pitch of 1 mm. H is an index ring which is free to rotate and is tightened by the threaded ring, I. J is an index bar. K is a rod to attach the apparatus to a ring stand. L is a collar button or similar object cemented to the end of the plunger to help hold the gel in place.

Experimental Procedure

Preparation of Agar Gel.—A 2-g sample of commercial agar is prepared by washing 4 or 5 times with cold water by decantation and bringing to a boil in the appropriate amount of water to make 100 ml of solution. Because of moisture in the agar and because some of it is washed away, the actual amount of agar in this solution is about 1.5% and is sufficient for a good gel. After cooling to about 60° the agar solution is skimmed and poured into the syringe so that it comes slightly over the cut-off end but does not run over. To hold the syringe in place for filling with gel, a rubber band is placed around the plunger in such

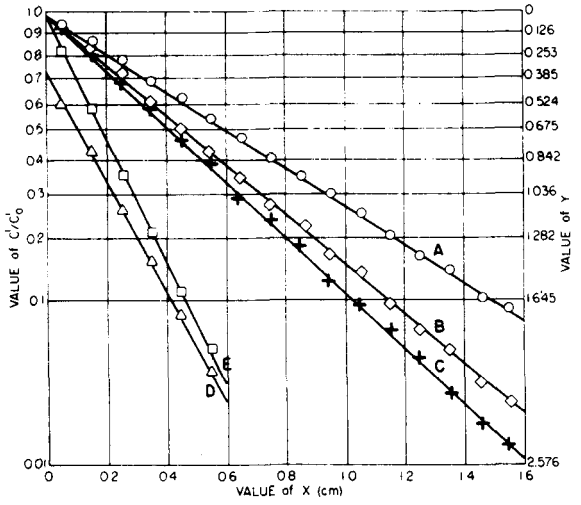

FIG. 2.—Diffusion patterns of various substances in agar gel. Details of experiments are listed in Table I. A, Urea diffusing for 10 hours at 20°; B, NaCl for 5 hours at 20°; C, glucose for 10 hours at 20°; D, C'/C_0 for bovine serum albumin for 36 hours at 4°; E, corrected and plotted data (C'/C_0') for serum albumin at 4°. For publication the intermediate ordinates and abscissas were removed along with the lower portion of the figure (FD Form 6-134) from $C'/C_0' = 0.01$ to 0.0001.

a position that a cup 5 to 6 cm deep will remain in the barrel when the syringe stands on the plunger end. When the gel has hardened, the open end of the syringe with the gel exposed is placed in solvent to prevent drying. It can be stored several days if kept in a cool place. The solvent can be water or buffer solutions from *p*H 4 to 10 without affecting the quality of the gel for diffusion work. In alkaline or acidic buffers it is best to prepare a more concentrated agar solution and mix with concentrated buffer to obtain the desired ionic strength just before the gel forms.

Various types of commercial agar have been used with success. However, some of the new and more purified agars have a tendency to crack soon after hardening in the diffusion cell. This difficulty can be prevented by dipping the barrel into hot 0.5% agar to a depth of about 5 cm, swinging or wiping out the excess gel, and allowing the film to dry.

Diffusion Procedure. The solution and diffusion cell containing the gel are brought to thermal equilibrium in a constant-temperature room (± 0.5°) or water bath. The portion of gel extending beyond the barrel is carefully sliced off. If the gel is pulled away from the wall of the cell, erratic results might be obtained because of the solution containing the solute creeping into the capillary space. The difficulty is prevented by dipping the freshly cut gel into the solvent before starting the diffusion. The gel is then placed in contact with the solution so that it just touches. Care should be taken that no air bubbles are trapped beneath. The solution is well stirred throughout the diffusion period. The volume of solution in these experiments was such that the concentration did not decrease more than 2 to 3% during the diffusion period and was considered to be constant.

Sectioning of Gel.—When the diffusion is terminated, the excess liquid on the gel surface is blotted with filter paper and the rubber band around the plunger is removed. The gel is loosened in the cell by careful pushing up on the plunger. The cell is then placed in the holder in a vertical position for sectioning the gel and the screw adjusted against the plunger so that the gel is even with the end of the barrel. This is the zero point, and the index pointer on the screw is set at the index bar. Each complete turn of the screw pushes the gel out of the barrel 1 mm, and sections are cut with the fine wire pulled across the end of the barrel. The section is removed with tweezers having flattened tips.

The greatest error in thickness occurs in the first section because a visual estimate must be made for the zero point. It was found, by weighing individual gel sections 1 mm in thickness, that with some practice the first section can be cut to ± 10% of the mean weight of the sections, while subsequent sections can be cut within ±3%.

Chemical or Biological Assay.—In many cases assays for the solute can be made directly on the individual sections of gel. The solute can also be extracted from the section of gel or suspended in water to a definite volume and allowed to come to equilibrium with the solvent. The latter method appears to be most satisfactory because it eliminates complications in the analysis caused by the gel. An average section of 1.5% agar gel 1 mm in thickness (0.6 ml) was found to contain about 5 μg of nitrogen. Water or buffered solvents, *p*H 4 to 10, were found to extract sufficient carbohydrate material from the gel to give a weak anthrone color test, but these extracts showed no evidence of anything that interfered with any other colorimetric tests or chemical assays or resulted in any toxic symptoms in mice when injected intraperitoneally or intravenously.

Determination of Free Volume in Gel.—It has been shown on theoretical grounds by Lauffer (1961) that the calculation of diffusion coefficients from analyses of gel sections makes it necessary to distinguish between solute concentration in the gel expressed as amount per total volume, C', and amount per free volume, C. It also was shown that

$$C' = C(1 - \phi) \qquad (1)$$

where $(1-\phi)$ is the fraction of the total volume in the gel free to dissolve solute. In the simplest case, ϕ would be the volume fraction actually occupied by the gel substance, but it is possible that ϕ could be larger than this because of hydration or because of exclusion of solute particles from the neighborhood of gel particles for mechanical, electrostatic, or any other reason. The

excluded volume fraction, ϕ, is important also because it is involved in a correction for obstruction of diffusion. It was shown (Lauffer, 1961) that

$$D' = D/[1 + (\alpha - 1)\phi] \quad (2)$$

where D' is the coefficient of diffusion in the gel; D, that in the absence of gel; and α is a coefficient having a value for 5/3 for randomly oriented rods.[1] Under some circumstances, ϕ can be measured by freezing point depression when salt is dissolved in gel. The value of ϕ for a 1.5% agar gel as determined from the freezing point of gels containing salt was found to be about 0.05.

There are circumstances under which a procedure like this might not give the value of ϕ actually applicable to the case of a large charged particle such as a protein molecule. A more direct method of evaluating ϕ is inherent in the diffusion data themselves and is accomplished by plotting the data on arithmetic probability paper. When gel is analyzed for solute concentration, one obtains C', and when the solution bathing the gel is analyzed, one measures C_0. The values of C'/C_0 are plotted against x, the distance in the gel, on probability paper. If the sections are sufficiently thin, 1 mm, the average concentration is practically equal to the concentration at the midpoint. Thus, plotting points of sections cut 1 mm thick would be at 0.05 cm for the first section, 0.15 cm for the second section, and so on. When C'/C_0 is plotted against x on probability paper (Fig. 2) the data will not extrapolate to 1.0 where x = 0 but to a lower value, usually about 0.95 for sugars and salts and between 0.7 and 0.8 for proteins. This intercept can be taken as a direct measure of (1-ϕ) because the most reasonable explanation for C' values being too low in all gel sections, even the first, is that the volume fraction ϕ is not free to dissolve solute. When the value of (1-ϕ) is thus determined, it can be used with equation (1) to convert the values of C'/C_0 to C/C_0 or the equivalent C'/C_0', shown to be the concentration function best adapted to interpreting diffusion in gels (Lauffer, 1961).

Calculation of Diffusion Coefficients.—The values of C'/C_0' are next plotted against x. The slope, y/x, of this plot (Fig. 2) is used in equation (3)[2] to calculate the diffusion coefficient in gel, D',

$$D' = \frac{1}{(y/x)^2 2t} \quad (3)$$

where x is the distance in the gel in cm, t is the time in seconds, and y is a function of the concentration in the relationship shown in equation (4),

$$C'/C_0' = 1 - \frac{2}{\sqrt{2\pi}} \int_0^y e^{-y^2/2} dy \quad (4)$$

and its value can be obtained from probability tables (Hodgman, 1960). For convenience, the values of y are given in the right column of the modified probability paper (Fig. 2).

The value of D, the diffusion coefficient of the solute in absence of gel, is calculated from equation

$$D = D'(1 + 2\phi/3) \quad (5)$$

(5).

Results and Discussion

The data given in Table I indicate that diffusion in an agar gel is similar to free diffusion in water if the volume excluded from solute by the gel and the obstructive effect of the gel are accounted for. Salts, sugars, amino acids, and other low-molecular-weight substances gave corrected values for their diffusion coefficients in line with published values for free diffusion. The same was found true for proteins under conditions where interaction with the gel did not occur. Three crystalline proteins investigated in this study—bovine serum albumin, hemoglobin, and botulinum toxin—precipitated at or just within the surface of the gel when attempts were made to carry out diffusion measurements in systems of water and gel (pH about 6) or 0.05 M acetate and gel at pH 4

[1] Equation (2) reduces for small values of ϕ to the definition of D' contained in equation (2) of Lauffer (1961). Some of the values of ϕ encountered in the present study are too large to permit the use of this approximation. Equation (2) can be derived from equation (13) of Fricke (1924), who considered the electrical conductivity of a suspension containing spheroids. For a suspension of randomly oriented extremely anisometric, nonconducting prolate ellipsoids of revolution, Fricke's equation reduces to $k' = k(1 - \phi)/[1 + (\alpha - 1)\phi]$, where α has a value of 5/3 and k' and k are the specific conductivity of the suspension and of the suspending medium, respectively. Diffusion of solute through a plug of gel bathed on both sides by solutions at constant concentrations, one high and one low, is an exact analog. One can write Fick's first law: $dS = -D[(1 - \phi)/[1 + (\alpha - 1)\phi]]Q(dc/dx)dt$, where dS is the amount of solute transported across the plug of cross section Q in time dt when the concentration gradient, dc/dx, is the difference between the concentrations, expressed in amount per unit volume of solution, of the two solutions divided by the thickness of the plug. When concentrations are expressed in terms of amount per total volume of gel, C', one applies equation (1) and obtains: $dS = -[D/[1 + (\alpha - 1)\phi]]Q(dc'/dx)dt$ or $dS = -D'Q(dc'/dx)dt$, where $D' = D/[1 + (\alpha - 1)\phi]$. Expressing concentration in terms of C' is a necessary preliminary for obtaining Fick's second law for obstructed diffusion in a form analogous to that for unobstructed diffusion.

[2] If the gel sections are 0.1 cm or less and the slope of the plotted data is about 3 or less, the error involved in the calculation of D' in equation (3) is less than 0.5% and insignificant. If the diffusion time is short and the sections are greater than 0.1 cm, a significant error may occur and can be corrected by multiplying the value obtained for D' by $[1 - p^2/48D't]^2$, as explained by Lauffer (1961), where p is the thickness of the gel section, t is the time in seconds, and D' is taken as the uncorrected value obtained by equation (3). Then D' (corrected) = D' (uncorrected) $[1 - p^2/48\ D'$ (uncorrected) $t]^2$.

TABLE I
DIFFUSION COEFFICIENTS (D) DETERMINED IN AGAR GEL

Substance	Conc. of Solute Beneath Gel (mg/ml)	Gel Slicing[a] Agar ($D \times 10^5$)	Literature[a] Value ($D \times 10^5$)
Sodium chloride	11.7	1.39[f]	1.39[h]
	1.0	1.38	
Potassium chloride	10	1.64	1.67[h]
Glucose	18	0.59[f]	0.60[h]
	1.8	0.60	
Sucrose	34	0.42	0.42[h]
Raffinose	54	0.34	0.36[h]
Urea	1.0	1.20	1.18[h]
Mixture of urea and glucose			
Urea	6.0	1.24	
Glucose	18.0	0.58	
Shellfish poison (purified)[b]	1.0	0.48	0.49[i]
Shellfish poison (crude)[b]		0.47	
Bovine serum albumin[c]	1.25	0.060[g]	0.060[j]
Hemoglobin[c]	1.25	0.061[g]	0.062[j]
Glycine[c]	1.0	0.95	0.95[k]
Botulinum toxin[d]	0.1	0.075[g]	0.018[l]
Tobacco mosaic virus protein[e]	0.5	0.090[g]	0.030[m]

[a] All values at 20° in water unless otherwise stated. [b] Poison from California mussels (*Mylitus californianus*) conc. 5000 mouse units per ml. [c] In 0.05 M phosphate buffer at *p*H 6.8. [d] In 0.05 M phosphate and 0.1% gelatin buffer at *p*H 6.8. [e] In carbonate buffer at *p*H 10.2. [f] Standard deviation about ±5% based on 12 observations. The pooled standard deviation for all substances also was about ±5% based on at least 3 observations on each substance. [g] Values determined at 4° and calculated to 20°. [h] International Critical Tables (1929), vol. 5, New York, McGraw-Hill Book Co., p. 63. [i] Schantz *et al.* (1957). [j] Neurath and Bailey (1953). [k] Cohn and Edsall (1943). [l] Putnam *et al.* (1946, 1948); Wagman and Bateman (1951). [m] Schramm and Zillig (1955); Anderer (1959); Ansevin and Lauffer (1959).

However, in systems of gel and 0.05 M acetate buffer at *p*H 5.0, or 0.1 M phosphate buffers at *p*H6.0, 6.8, and 7.3, these proteins did not interact wtih the gel and diffusion proceeded in the expected manner. The precipitation in water at about *p*H 6 and in acetate gel at *p*H 4.0 appeared to be an aggregation of some type caused by an interaction of the gel and protein and not a denaturation. When the precipitated botulinum toxin was redissolved by raising the *p*H, the original toxicity was found to be present.

The method of correcting for the volume within the gel excluded to the diffusing solute is justified as follows. It might be assumed that some hydration of the agar takes place, thus increasing the effective volume of the gel, but additional factors may come into play. The gel possesses a negative charge, and because of this charge may combine with a molecule of solute possessing a net positive charge and exclude or repulse a molecule of solute possessing a net negative charge. The results of these experiments appear to fit this situation quite closely. Neutral molecules or those possessing no great amount of net positive or negative charges, such as salts and sugars, gave small values for ϕ (equation 1) of 0.03 to 0.05, indicating that the volume excluded to the diffusing solute must be 3 to 5%. A determination of the amount of hydration existing in the gel would be very difficult, but the volume of dried agar particles will increase two- to three-fold when placed in cold water. If it can be assumed that this increase in volume is similar to hydration in a gel, then the volume excluded to the solute by a 1.5% agar gel would be 3 to 4.5% and in line with values obtained by plotting and from freezing points of gels containing salt. Proteins on the other hand show an abnormally high value for ϕ, *i.e.*, about 0.25. One explanation of this high value could be supported on the basis that if the radius of the protein molecule is high compared to the radius of a gel particle, the centers of the protein molecules will be excluded from a volume large compared to that actually occupied by the gel particles. A more plausible explanation, however, is the possibility of electrostatic repulsion between like charged particles resulting in a smaller amount of solvent available to the diffusing solute. Lauffer and Bendet (1954) showed that such considerations could explain some abnormal hydration values calculated for viruses from studies involving sedimentation in bovine serum albumin solutions.

When C'/C_0 is plotted against x and the intercept with the ordinate, $x = 0$, is at a value greater than 1.0, the interpretation is that an interaction between gel and solute has taken place and the diffusion will not proceed normally in the gel. An intercept of less than 1.0 is interpreted as the volume excluded to the diffusing solute, and the results of diffusion measurements made in agar gel support these interpretations. If the column of agar were like a pack of tubes with the walls in line with the direction of diffusion, the above correction would be sufficient. It is more likely, however, that the fibers making up the gel structure are randomly distributed, making it necessary for the solute molecules to detour around a certain number of the fibers. The concentration therefore at distance x in the gel is less than would be encountered in the absence of gel. It has been pointed out by Lauffer (1961) that this factor can be corrected on the basis of the value of ϕ as shown in equation (5). This obstructive effect does not change the shape of the diffusion curve but does increase the slope. The correction factor in equation (5) has been found to be from about 1.03 for salts, sugars, and other low-molecular-weight compounds up to 1.2 for some proteins.

Linearity of the corrected and plotted data

should indicate that the diffusion is obeying Fick's law and that no appreciable interaction between the solute and gel took place and that there was no association or dissociation of the solute during the diffusion. Nonlinearity means that these reactions might be occurring or that the solute under test was not a single molecular species. However, the extent of this heterogeneity can be detected only when two or more substances responding to the same assay possess diffusion coefficients which differ by a factor of about two or more. For example, when glucose was mixed with raffinose ($D = 0.6 \times 10^{-5}$ and 0.36×10^{-5} $cm^2\ sec^{-1}$) in equal amounts and analyses were made for total sugar throughout the gel, nonlinearity could be detected in the plotted data, but when glucose and sucrose ($D = 0.6$ and 0.46) were mixed, nonlinearity could not be detected.

Results obtained with sodium chloride showed that the calculation of the diffusion coefficient of this salt between 4° and 20° from values obtained at 20° or 4° were in accordance with the equation, $D_2 = D_1 T_2\eta_1/T_1\eta_2$, generally used for the conversion of coefficients from one temperature to another in free diffusion.

It will be observed from Table I that the diffusion coefficients of tobacco mosaic virus protein and botulinum toxin are several times greater than the values obtained by other investigators. These differences are explained on the basis that the proteins are probably dissociated into smaller units under the conditions prevailing in these experiments (Table I).

Assays for biologically active substances usually entail a considerable margin of variability. However, the slope of the best straight line through the points plotted on probability paper should represent an acceptable average for the calculation of the diffusion coefficient. If the deviations from the mean value for the assay of a biological substance in a series of sections of gel are randomly distributed, the standard deviation of the diffusion coefficients should not be greater than that obtained for substances where the assay is more precise. The theoretical reasons for this are that the fractional error of y (equation 4) is of necessity much less than that of C'/C_0' and that the error of the slope of a line fitted to sixteen points is much less than that of the points themselves. Bioassays employing mice for shellfish poison have yielded diffusion coefficients within a standard deviation of $\pm 5\%$, a value in line with that obtained for substances such as sodium chloride and glucose.

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References

Anderer, F. A. (1959), *Z. Naturforsch. 14b*, 24.
Ansevin, A. T., and Lauffer, M. A. (1959), *Nature 183*, 1601.
Becker, E. L. (1961), *Arch. Biochem. Biophys. 93*, 617, 631.
Bourdillon, J. (1941), *J. Gen. Physiol. 24*, 459.
Cohen, E., and Bruins, H. R. (1924), *Z. physik. Chem. 113*, 157.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids, and Peptides, New York, Reinhold Publishing Corporation, p. 412.
Felicetta, V. F., Markham, A. E., Peniston, Q. P., and McCarthy, J. L. (1949), *J. Am. Chem. Soc. 71*, 2879.
Fricke, H. (1924), *Phys. Rev. 24*, 575.
Fricke, R. (1925), *Z. Electrochem. 31*, 430.
Friedman, L. (1930), *J. Am. Chem. Soc. 52*, 1311.
Geddes, A. L., and Pontius, R. B. (1960), Physical Methods of Organic Chemistry, vol. 1, Weissberger, A., editor, New York, Interscience Publishers, p. 895.
Hodgman, C. D. (1960), Handbook of Chemistry and Physics, ed. 42, Cleveland, Chemical Rubber Publishing Co., p. 211.
Johnson, M. J. (1941), *J. Biol. Chem. 137*, 575.
Lauffer, M. A. (1961), *Biophysical J. 1*, 205.
Lauffer, M. A., and Bendet, I. J. (1954), *Advances in Virus Research, vol. II*, New York, Academic Press, p. 241.
Mann, C. E. T. (1924), *Proc. Roy. Soc. London A. 105*, 270.
Neurath, H., and Bailey, K. (1953), The Proteins, vol. 1, pt. B, New York, Academic Press, Inc., p. 636.
Northrop, J. H., and Anson, M. L. (1929), *J. Gen. Physiol. 12*, 543.
Oudin, J. (1946), *Compt. rend. Acad. sc.* (Paris) *222*, 115.
Ouchterlony, O. (1949), *Arkiv Kemi, Mineral. Geol. 26B, 14*, 1.
Polson, A. (1944), *Nature 154*, 823; (1947), *Onderstepoort J. Vet. Sci. Animal Ind. 22*, 41.
Putnam, F. W., Lamanna, C., and Sharp, D. G. (1946), *J. Biol. Chem. 165*, 735; (1948), *ibid. 176*, 401.
Schantz, E. J., Mold, J. D., Stanger, D. W., Shavel, J., Riel, F. J., Bowden, J. P., Lynch, J. M., Wyler, R. S., Riegel, B., and Sommer, H. (1957), *J. Am. Chem. Soc. 79*, 5235.
Schantz, E. J., and Spero, L. (1957), *J. Am. Chem. Soc. 79*, 1623.
Schramm, G., and Zillig, M. (1955), *Z. Naturforsch. 10b*, 493.
Stiles, W. (1921), *Biochem. J. 15*, 629; (1923), *Proc. Roy. Soc. London A. 103*, 260.
Stiles, W., and Adair, G. S. (1921), *Biochem. J. 15*, 620.
Wagman, J., and Bateman, J. B. (1951), *Arch. Biochem. Biophys. 31*, 424.