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A Method for Calculating Differential Diffusion Coefficients in Two Component Systems: Application to Glycine-Water and Bovine Mercaptalbumin-Buffer Systems¹

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A procedure, based on the Boltzmann method, is described for calculating the concentration dependence of the diffusion coefficient in two component systems from Rayleigh Diffusiometer data. Application of the method is made to two systems at 20°: glycine-water and bovine mercaptalbumin-buffer (sodium acetate:acetic acid, $pH = 4.75$, $\Gamma/2 = 0.05$). Differential diffusion coefficients and the magnitude of the concentration dependence for the glycine system are in good agreement with existing literature values. The concentration dependence of D in the bovine mercaptalbumin system is in satisfactory agreement with the value obtained from a series of differential experiments in which the mean concentration of the boundary varied from 0.04 to 1.1 g./dl. protein. Certain modifications of the diffusion interferometer and of the technique of boundary formation are described.

In free diffusion work on two-component systems, in which the concentration in the boundary region is recorded refractometrically, it has long been evident that the diffusion process rarely gives rise to a boundary region which may be characterized by a Gaussian probability function. This lack of ideality may be attributed to two causes; (1) a non-linear relation between refractive index and concentration, and (2) a dependence of the diffusion coefficient on concentration.

The functional dependence of refractive index on concentration can be determined by independent experiment. In many systems a linear relation has been shown to hold up to relatively large solute concentrations. For example, the protein systems thus far studied show a linear dependence of refractive index on concentration up to protein concentrations in excess of 10 g./dl.^{3,4} On the other hand, there are often substantial deviations from linearity in electrolyte⁵ and dipolar ion⁶ systems. In systems of this type, if the refractive index-concentration function is known, then the primary refractive index data obtained for the diffusion boundary can always be reduced to concentration data.

It is customary in the investigation of concentration dependence of the diffusion coefficient to perform a series of experiments at different mean solute concentrations. If the concentration difference in each experiment is kept small, differential diffusion coefficients corresponding to each mean concentration may then be calculated to establish the degree of concentration dependence. This procedure is time-consuming and in the case of protein work may require more material than is available. Also, if the Rayleigh Diffusiometer is used, this procedure is less than satisfactory since it is evident that the necessary information for calculating the concentration dependence is present in the data of any individual experiment.

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(3) G. E. Perlmann and L. G. Longworth, *J. Am. Chem. Soc.*, **70**, 2719 (1948).

(4) R. Barer and S. Tkaczyk, *Nature*, **173**, 821 (1954).

(5) L. J. Gosting, *J. Am. Chem. Soc.*, **72**, 4418 (1950).

(6) M. S. Lyons and J. V. Thomas, *ibid.*, **72**, 4506 (1950).

Apart from the theoretical interest attached to the concentration dependence of the diffusion coefficient, it is of importance in the study of proteins and other macromolecules to have a diffusion coefficient at zero concentration which may be used with other data to give information about the size and shape of the kinetic unit of the solute.

Creeth⁷ has described an elegant method for calculating the concentration dependence of the diffusion coefficient in two component systems using the high precision data obtained with the Rayleigh Diffusiometer. Creeth's method is based on a series solution to the differential equation of diffusion obtained when a cubic relation between diffusion coefficient and concentration is assumed.

It is the purpose of this paper to describe a different procedure for calculating the concentration dependence from Rayleigh diffusion data. This method, unlike that of Creeth, requires no *a priori* assumption regarding the nature of the dependence of the diffusion coefficient on concentration. Application of this method is made in studying the concentration dependence of the glycine-water system and of the system of bovine mercaptalbumin at pH 4.74, in sodium acetate-acetic acid buffer, $\Gamma/2 = 0.05$.

Theory.—Fick's Second Law⁸ for the case of one-dimensional free diffusion in a two-component system, may be written

$$\frac{\partial C}{\partial t} = \frac{\partial}{\partial x} \left(D_c \frac{\partial C}{\partial x} \right) \quad (1)$$

where C is concentration, t is time, x is the vertical position coordinate in the diffusion cell and D_c is the diffusion coefficient at concentration C . It is convenient to arrange experimental conditions such that at $t = 0$, for

$$x > 0, C = C_2 \quad x < 0, C = C_1 < C_2$$

In addition, the length of the diffusion cell is such that throughout the course of the experiment the composition of the solution does not change at the top and bottom of the cell.

If the diffusion coefficient is a constant independent of concentration, the well-known solution involving the probability integral

$$\frac{2(C - \bar{C})}{\Delta C} = \frac{2}{\sqrt{\pi}} \int_0^{x/\sqrt{4Dt}} e^{-\alpha^2} d\alpha \quad (2)$$

(7) J. M. Creeth, *ibid.*, **77**, 6428 (1955).

(8) A. Fick, *Ann. Physik, u. Chem.*, **94**, 59 (1855).

is obtained^{9,10} where $C_2 - C_1 = \Delta C$ and $(C_1 + C_2)/2 = \bar{C}$.

However, for the general case, in which the diffusion coefficient is an unspecified function of the concentration, no simple solution is possible. One method of approach to the problem, exemplified by Creeth's procedure, is to assume a function describing the concentration dependence of the diffusion coefficient and then solve the resulting differential equation. With certain concentration dependence functions either exact solutions or a series solution have been obtained.^{7,11-15} Gosting has discussed these solutions in some detail.¹⁶

A second way of attacking the problem, which involves no assumption about the form of the concentration dependence, was first proposed by Boltzmann.¹⁷ Boltzmann pointed out that if equation 1 adequately describes the diffusion process for the system under investigation, then a new variable $z = x/\sqrt{t}$ may be introduced.

When the substitution of z is made, equation 1 becomes

$$-\frac{z}{2} \frac{dC}{dz} = \frac{d}{dz} \left(D_0 \frac{dC}{dz} \right) \quad (3)$$

which may be integrated to give

$$D_0 = -\frac{1}{2} \frac{dz}{dC} \int_{C_1}^C z dC \quad (4)$$

subject to the restriction, imposed by the conservation of mass

$$\int_{C_1}^{C_2} z dC = 0 \quad (5)$$

It is evident that this condition defines $x = 0$. For any time, t , equation 4 becomes

$$D_0 t = -\frac{1}{2} \frac{dx}{dC} \int_{C_1}^C x dC \quad (6)$$

The solution in this form may be used with concentration and vertical cell coordinate data to calculate D_0 as a function of C . That the condition C be a function of x/\sqrt{t} is obeyed may be verified experimentally. Actually, even in the cases of large concentration dependence, this condition appears to be satisfied.¹⁸⁻²¹

In order to calculate D_0 using equation 6, it is necessary to perform an integration and a differentiation by numerical or graphical methods. In

(9) J. W. Williams and L. C. Cady, *Chem. Revs.*, **14**, 171 (1934).

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(11) J. Wagner, *J. Chem. Phys.*, **18**, 1227 (1950).

(12) R. H. Stokes, *Trans. Faraday Soc.*, **48**, 887 (1952).

(13) J. Gillis and O. Kedem, *J. Polymer Sci.*, **11**, 545 (1953).

(14) H. Fujita, *J. Colloid Sci.*, **9**, 269 (1954).

(15) L. J. Gosting, H. Fujita, *J. Am. Chem. Soc.*, **79**, 1359 (1957).

(16) L. J. Gosting, *Advances in Protein Chem.*, **11**, 429 (1956).

(17) L. Boltzmann, *Wied. Ann.*, **53**, 959 (1894).

(18) F. N. Rhines and R. F. Mehl, *Trans. Am. Inst. Mining Met. Engrs.*, **128**, 185 (1938).

(19) B. Gerlach, *Ann. Physik*, **10**, 437 (1931).

(20) E. Munter, *ibid.*, **11**, 558 (1931).

(21) The substitution of a new variable z for x/\sqrt{t} is valid provided equation 1 adequately describes the diffusion process in the system under investigation. This equation is limited in applications to systems of two components and in addition, to systems which show no volume change on mixing. Over the small concentration differences customarily employed in diffusion studies, the volume change on mixing in most systems is very small and is generally assumed to be zero. The fact that for proteins the partial specific volume is independent of concentration indicates that the volume change on mixing can be expected to be zero in protein systems.

general the use of such procedures results in a loss of precision, and usually, unless the concentration dependence is extreme, the loss of precision may be so great as to make the final result of little value.

Beckmann and Rosenberg,²² using a modification of equation 6 and normalized data obtained with the Schlieren method, devised a procedure which minimizes the graphical integration errors. This procedure is based upon a difference function calculated from a comparison of the normalized data with a standard probability curve. A graphical integration then is performed on the difference function and the results are combined with tabular values of the probability integral to give D_0 as a function of $2C/\Delta C$ and $D_{2,0}$ as calculated from the zeroth and second moments of the gradient curve.

The method described in this paper is similar in principle to that of Beckmann and Rosenberg.

The Modified Boltzmann Method.—As primary data, the Rayleigh²³⁻²⁵ system gives a record of refractive index increments of the solution in the diffusion boundary region as a function of a photographic plate coordinate, X . The refractive index increments are recorded as interference fringes. If the refractive index of the solution is a linear function of the solute concentration, that is dn/dC is a constant, then the total fringe number, J_m , will be proportional to ΔC . Consequently, if the fringes are numbered consecutively, starting with the fringe corresponding to C_1 as zero, then a given fringe number at a specified level in the boundary region will be proportional to the concentration in excess of C_1 . Thus, if C' is the concentration in excess of C_1 , $H(\theta)$ may be written

$$H(\theta) = 2(C - \bar{C})/\Delta C = (2C' - \Delta C)/\Delta C = (2j - J_m)/J_m = (2j/J_m) - 1 \quad (7)$$

If dn/dC is not a constant, the first two equalities of equation 7 are still true, but the final relationship becomes more complex, and this situation can be handled by defining H as a function $H(\theta^\ddagger)$, as described by Creeth.⁷ Under these conditions, we have²⁶

$$H(\theta^\ddagger) = 2(C - \bar{C})/\Delta C = (2C' - \Delta C)/\Delta C = (2j/J_m)[1 + \beta(J_m - j) + \dots] - 1 \quad (7a)$$

where β is a small correction term as described in the footnote. Value of θ (or θ^\ddagger) are then determined by inverse interpolation of tables²⁷ of the probability function $H(\theta)$ (or $H(\theta^\ddagger)$).

The vertical plate coordinate X may be normalized in the following manner. An arbitrary zero for X is established for the value of X corresponding to $J_m/2$. This will be referred to as X_0 . For

(22) C. O. Beckmann and J. L. Rosenberg, *Ann. N. Y. Acad. Sci.*, **46**, 329 (1945).

(23) J. St. L. Philpot and G. H. Cook, *Research (London)*, **1**, 234 (1948).

(24) H. Svensson, *Acta Chem. Scand.*, **3**, 1170 (1949).

(25) L. G. Longworth, *Rev. Sci. Instr.*, **21**, 524 (1950).

(26) Here the refractive index is expressed as

$n - n_0 = R_0 C + R_1 a_1' C^2 + \dots = j\lambda/a$, and $\beta = a_1' \lambda/a R_0$. Here λ is the wave length (in cm.) of the light used in the interferometer, and a is the thickness of the diffusion column in the direction of the light path. In these experiments, λ was 5461×10^{-10} cm., and a was 2.50 cm. $R_1 = (\partial n/\partial C)_{C=0}$, and $a_1' = (\partial^2 n/\partial C^2)_{C=0}/2R_0$.

(27) "Tables of the Error Function and Its Derivative," National Bureau of Standards Applied Mathematics Series #41, U. S. Government Printing Office, Washington 25, D. C., 1954.

each fringe a new coördinate X' may be established by

$$X' = X - X_0 \tag{8}$$

Each X' of the plate measurement is related to a conjugate x' in the diffusion cell by

$$x' = X'/G_v \tag{9}$$

where G_v is the vertical magnification factor of the optical system.²⁸

Values of X' are normalized by multiplication with an arbitrary factor m , to give $\Psi = mX'$. The normalizing factor, m , is selected such that over the extent of the boundary, $(\Psi - \theta)$ is as small as possible. This variable Ψ can be related to the variable z introduced into equations 3-6 by means of the equation

$$\Psi - \Psi_0 = (x' - x'')/(2\sqrt{D^*t}) = z/(2\sqrt{D^*t}) = (X' - X'')/(2G_v\sqrt{D^*t}) = m(X' - X'') \tag{10}$$

Here we have introduced the new constants

$$\Psi_0 = \frac{1}{2} \int_{-1}^{+1} (\Psi - \theta) dH \tag{11}$$

$$x'' = 2\Psi_0\sqrt{D^*t} \tag{11a}$$

and

$$X'' = 2G_v\Psi_0\sqrt{D^*t} = \Psi_0/m \tag{11b}$$

Use of these constants in equation 10 will give values of x or z which will satisfy equation 5 and are therefore the correct values for use in equation 4. It is evident that

$$m = 1/(2G_v\sqrt{D^*t}) \tag{12}$$

and that the choice of m is in effect a choice of D^* . Equation 12 is used to calculate D^* . If the units of X' and t are cm. and sec., respectively, then D^* will have the usual units of cm.²/sec. This value of D^* is of course an arbitrary choice, and it can only be related to true diffusion coefficient values by an application of equation 4. Also, it is necessary to correct the measured time, t_m , by the use of a zero time correction, Δt , obtained by making D_c a linear function of $1/t_m$. The values of t in the equations above are thus equal to $t_m + \Delta t = t_{corr}$.

We find that equation 10 is true within the experimental accuracy of our measurements in the case of both glycine and bovine mercaptalbumin. That is, $(X' - X'')$ is a linear function of $1/\sqrt{t_{corr}}$ within the limit of precision of our data, and hence $(\Psi - \Psi_0)$ is independent of t within the limit of precision, justifying our use of the variable z in the integration of equation 3.

Values of the variable $(\Psi - \Psi_0)$ are nearly equal to the variable θ (or θ^\pm) defined by equation 7 (or 7a) if a good choice of m has been made. It is then useful to define a new variable

$$\phi = \Psi - \Psi_0 - \theta \tag{13}$$

From equations 10 and 13 we then obtain

$$z = 2\sqrt{D^*}(\Psi - \Psi_0) = 2\sqrt{D^*}(\theta + \phi) \tag{14}$$

We also can obtain $dH(\theta)/dC$ (or $dH(\theta^\pm)/dC$)

(28) The choice of $j = J_m/2$ instead of $C = \bar{C}$ to determine x may seem somewhat arbitrary and undesirable if dn/dC is not constant. However since the value of x must be that value which will allow

$$\int_{c_1}^{c_2} x dC \approx 0$$

and hence will be neither that corresponding to $J_m/2$ or \bar{C} , the choice of $J_m/2$ seems preferable as its use is operationally much simpler.

from equation 7 or 7a

$$dH(\theta)/dC = 2/\Delta C \tag{15}$$

It is now easy to calculate dz/dC and $\int_{c_1}^c z dC$ for use in equation 4

$$\frac{dz}{dC} = \frac{dz}{dH} \cdot \frac{dH}{dC} = \frac{4\sqrt{D^*}}{\Delta C} \left[\frac{d\theta}{dH} + \frac{d\phi}{dH} \right] \tag{16}$$

$$\int_{c_1}^c z dC = \int_{-1}^H z \frac{dC}{dH} dH = \Delta C \sqrt{D^*} \left[\int_{-1}^H \theta dH + \int_{-1}^H \phi dH \right] \tag{17}$$

But

$$d\theta/dH \equiv 1/H' \text{ and } \int_{-1}^1 \theta dH = -H'/2$$

Therefore

$$D_c = -\frac{1}{2} \frac{dz}{dC} \int_{c_1}^c z dC = D^* \left[\frac{1}{H'} + \frac{d\phi}{dH} \right] \left[H' - 2 \int_{-1}^H \phi dH \right] \tag{18}$$

$$D_c = D^* \left[1 + H' \frac{d\phi}{dH} - \frac{2}{H'} \int_{-1}^H \phi dH - 2 \frac{d\phi}{dH} \int_{-1}^H \phi dH \right] \tag{19}$$

A plot of $(\Psi - \theta) = (\phi + \Psi_0)$ vs. H can easily give us $\frac{d\phi}{dH}$ and

$$\int_{-1}^H \phi dH = \int_{-1}^H (\Psi - \theta) dH - \Psi_0(H + 1) \tag{20}$$

by graphical differentiation and integration of the smoothed curve. H' and H are both functions of θ , or θ^\pm , and can be found in suitable tables.²⁷

The order of the terms in the right-hand side of equation 20 is such that each term contributes less to D_c/D^* than the term to the left of it. If a proper choice of D^* is made, even in cases of rather large concentration dependence, the maximum contribution to D_c/D^* made by the second, $H' \frac{d\phi}{dH}$, usually can be kept to within ± 0.10 . Since it is not difficult to measure the slope, $d\phi/dH$, graphically with an accuracy of a few per cent. and since the contribution of the third term is about one-tenth of the second term (the fourth term is then usually negligible), it is possible to achieve a precision of a few tenths of a per cent. in the calculation of D_c/D^* .²⁹

(29) If the solute is not homogeneous and if there are no interactions between flows of the solute species, then the average diffusion coefficient, \bar{D}_0 , calculated by the modified Boltzmann method, will have the form

$$\bar{D}_0 = \sum_i D_{ci} \frac{dC_i}{dz} / \sum_i \frac{dC_i}{dz} \tag{A}$$

If in addition, each solute diffuses ideally, equation A may be written

$$\bar{D}_0 = \frac{\sum_i \sqrt{D_i} \Delta C_i e^{-z^2/4D_i}}{\sum_i \frac{\Delta C_i}{\sqrt{D_i}} e^{-z^2/4D_i}} \tag{B}$$

where ΔC_i is the concentration difference across the boundary of species i . Thus

$$\Delta C = \sum_i \Delta C_i$$

At $z = 0$, equation B reduces to

$$\bar{D}_0 = \left(\sum_i \sqrt{D_i} \cdot \Delta C_i \right) / \sum_i \left(\Delta C_i / \sqrt{D_i} \right) \tag{C}$$

This type of average lies between the height-area and weight averages. Gralen³⁰ has shown that the weight average is always larger than the height-area average.

(30) N. Gralen, *Kolloid Z.*, **95**, 188 (1941).

Experimental

The apparatus used in this research is a convergent light double beam interferometer of the Rayleigh type described by Svensson²⁴ and Longworth.²⁵ Following Svensson,²⁴ a transmission grating with vertically oriented rulings is used as the coherent light source for the interferometer. Light of wave length 5461 Å. is isolated from an H-4 General Electric mercury arc by a combination of Corning filters 3484, 4303 and 5120.

The modified 11 ml. quartz electrophoresis center section with window extension, described by Longworth,²⁵ has been used in all of this work. A cell holder, designed so that the top and center sections of the cell assembly are immovably held but permitting the bottom section to be moved aside to disconnect the two arms of the cell, is secured to a mounting frame which can be positioned reproducibly on the optical track of the apparatus. A double slit system with reference slits similar to that described by Creeth⁷ is mounted rigidly on the cell holder.

In order to make the fringe correction described below, a fiducial point on the axis of the reference fringe system is necessary. A one mil phosphor-bronze wire is soldered across the upper end of the reference slits. The image of this wire serves as the fiducial mark.

All starting boundaries were formed by siphoning³³ using a syringe with a beveled tip.³⁴ At the conclusion of siphoning, the bottom section was moved aside to disconnect the two arms of the cell.

During siphoning, just before the siphon was closed and withdrawn, a picture of the fringe pattern was taken. This picture was used to determine the fractional fringe number. Throughout the course of an experiment eight to ten exposures were taken at roughly equal time intervals. In all of this work Kodak Metallographic 4 by 5 inch plates were used.

The experimental relationship between fringe number, j , and the vertical plate coordinate, X , was obtained with the use of a Mann one-coordinate precision screw comparator, equipped with a monocular microscope containing a micrometer ocular. This ocular was set to allow measurements of small displacement in the direction perpendicular to the screw axis. The microcomparator illumination system employed point-source, divergent, monochromatic light to increase the precision of measurement and has been described elsewhere.³⁵

The vertical magnification factor, G_v , of the apparatus was determined as follows: A transparent ruled scale was fixed in the diffusion cell holder so that the plane of the horizontal scale lines coincided with the center plane of the diffusion cell. The glass slab of the scale (equivalent in thickness to the front window of the diffusion cell) was between the scale lines and the photographic plate. A photograph of the glass scale in position in the cell holder was then taken and the positions of 60 scale lines (1 mm. intervals) were measured on both the actual scale and the photographic image, using the precision comparator. Scale line differences were then calculated over 2 cm. (20 line) intervals beginning with the first line and continuing through to the last (making 40 such intervals) on both scale and image. The ratios of these scale intervals as measured on the plate to those as measured on the scale were then averaged to give a value of $G_v = 1.07834$ with a standard deviation of 0.00015. With the scale at our disposal, it was important to eliminate inherent scale errors by correlating the image and scale intervals. Deviations in G_v as a function of position were random, showing that there was no observable curvature of the field in the optical system from plate to the center plane of the cell.

The temperature of the diffusion bath³⁶ was maintained to $\pm 0.005^\circ$ during all diffusion experiments. However, the bath was not always adjusted to exactly 20.00° in all ex-

periments. The observed diffusion constants were corrected to this temperature by the equation $D_{20} = D_T(1 - 0.0280 \Delta T)$. This temperature correction never amounted to more than 0.5%.

Since all of the components of the apparatus are not optically perfect, the fringes formed in the absence of a concentration gradient are not perfectly straight. It is very important to determine the true shape of these fringes and to eliminate this factor as a source of error. To do this the cell was filled with water and the fringe pattern photographed. This plate was aligned on the comparator stage so that the fringes were parallel to the comparator screw. The alignment was made using only the ends of the reference fringe system. Using the image of the fiducial wire as a reference zero, the plate was translated along the fringe system. At 0.1 mm. intervals the displacement of the center of a selected fringe from the microscope cross-hairs was recorded by means of a micrometer ocular. The record of the fringe displacements as a function of the distance from the fiducial image gave the required deviations of the base line.

Corrections for the base line deviations were applied in the following manner: The record of the fringe pattern for a given time was aligned on the comparator stage using only the ends reference fringe system. Measurements of the positions of the centers of all of the diagonal fringes were made with the fiducial image as a zero reference. Using the base line data, a correction in the ocular setting for each fringe was determined. The positions of the fringe centers were then remeasured, each with the appropriately set ocular correction. Thus for each exposure a table of fringe number *vs.* corrected comparator reading, X , was compiled. It proved very convenient to make the correction in this manner which permitted the fringe number to be retained as an integer.

Materials and Solutions.—The bovine mercaptalbumin—BMA—used in this work was prepared by modification of the method of Dintzis.³⁷ The BMA was three times recrystallized as the mercury dimer, the mercury removed by treatment with thioglycolate and then all salt removed by passage over a mixed bed ion exchange column. The BMA solution was then brought to the point of incipient precipitation by slow addition of an equal volume of cold ethanol, 30% (v./v.) keeping the temperature near freezing, and allowed to crystallize at -5° . The crystals were centrifuged off, the resulting paste slurred with three volumes of distilled water and lyophilized. The preparation was stored as the dry powder, isoionic, salt free, at -5° . All BMA work reported in this paper was done in sodium acetate: acetic acid buffer, ionic strength 0.05, and pH 4.75 (measured at 25°).

Sodium acetate (Mallinckrodt) and glacial acetic acid (du Pont) used to prepare this buffer were reagent grade as supplied by the manufacturer. Glycine, supplied by Dow, was once recrystallized from water.

The BMA solutions for use in the diffusion experiments were made up to approximately the desired concentration and dialyzed against the buffer for 15 hr. at 20.0° . Since J_m is proportional to ΔC , the correct value of C_2 and \bar{C} in the experiments in which $C_1 = 0$ was calculated from a value of $C = 0.01138 j$. For the three experiments in which $C_1 \neq 0$, the values of \bar{C} were calculated from values of C_1 and C_2 determined spectrophotometrically at 280 $m\mu$. An extinction coefficient, $E_{1\%}^{1\text{cm}}$, was determined on the basis of dry weight at 75° *in vacuo* to be 6.55.

Viscosity measurements were made with a Cannon-Fenske type viscometer at 20.00° . The kinetic energy correction was found to be negligible. Solutions for the viscosity work were made up by weight dilution from a 10 g./dl. stock solution of BMA. The concentration of the stock solution was determined by dry weight as above. The density of the sodium acetate:acetic acid buffer was determined pycnometrically and found to be 1.0000 g./ml. at 20.00° . Densities and concentrations of the dilution series were calculated using the known concentration of the stock solution, the weight dilution data, the density of the buffer and the partial specific volume of bovine albumin, \bar{V}_{20} ($= 0.734_3$), as given by MacInnes, *et al.*,³⁸ and confirmed by Charlwood.³⁹

(31) H. Svensson, *Acta Chem. Scand.*, **5**, 1301 (1951).

(32) L. G. Longworth, *J. Am. Chem. Soc.*, **74**, 4155 (1952).

(33) D. S. Kahn and A. K. Polson, *J. Phys. and Colloid Chem.*, **61**, 816 (1947).

(34) L. G. Longworth, *J. Am. Chem. Soc.*, **75**, 5705 (1953).

(35) J. L. Oncley, M. Ludwig and T. E. Thompson, *Rev. Sci. Instr.*, **29**, 985 (1958).

(36) T. E. Thompson and H. Svensson, "Analytical Methods of Protein Chemistry," edited by P. Alexander and R. J. Block, Vol. III, Chapter 3, Pergamon Press, New York, N. Y., 1961; see especially pp. 106-108.

(37) H. M. Dintzis, Ph.D. Thesis, Harvard University (1952).

(38) M. O. Dayhoff, G. E. Perlmann and D. A. MacInnes, *J. Am. Chem. Soc.*, **74**, 2515 (1952).

(39) P. A. Charlwood, *ibid.*, **79**, 776 (1957).

Results

The primary interest in this investigation is centered on the application of the modified Boltzmann method to the BMA system. However, in order to obtain a more critical evaluation of the success with which this method may be applied, a study of the glycine-water system is included.

Glycine.—Aqueous glycine was chosen as a system which may be characterized as strictly two component and for which a considerable body of data is available in the literature.

Concentration dependence of the diffusion coefficient was calculated by the procedure described in this paper for a glycine solution for which $\Delta C = 0.9860$ g./dl., $\bar{C} = 0.4930$ g./dl. and $J_m = 83.175$. The zero time correction, $\Delta t = 18$ seconds, was calculated by plotting D_c vs. $1/t$. These data were fitted by the least squares method.

TABLE I
FRINGE DATA FOR DIFFUSION OF GLYCINE
 $\Delta C = 0.9860$ g./dl., $\bar{C} = 0.4930$ g./dl., $J_m = 83.175$, $t + \Delta t = 14,718$ sec., $T = 20.00^\circ$

(1)	(2) $H(\theta^\pm)$	(3) X' (cm.)	(4) ψ^a	(5) θ^\pm	(6) $\psi - \theta^\pm$
2	-0.9521	-1.1164	-1.4055	-1.3986	-0.0069
4	-.9041	-.9367	-1.1793	-1.1774	-.0019
6	-.8561	-.8212	-1.0339	-1.0335	-.0004
8	-.8082	-.7329	-0.9231	-0.9229	.0002
10	-.7602	-.6601	-.8310	-.8312	.0002
12	-.7122	-.5977	-.7525	-.7516	-.0009
14	-.6642	-.5397	-.6795	-.6806	.0011
16	-.6162	-.4882	-.6146	-.6159	.0013
18	-.5682	-.4407	-.5548	-.5559	.0011
20	-.5202	-.3963	-.4987	-.4997	.0008
22	-.4722	-.3530	-.4444	-.4464	.0020
24	-.4242	-.3128	-.3939	-.3956	.0017
26	-.3761	-.2743	-.3453	-.3468	.0015
28	-.3281	-.2368	-.2981	-.2995	.0014
30	-.2801	-.2004	-.2523	-.2535	.0012
32	-.2320	-.1645	-.2071	-.2086	.0015
34	-.1839	-.1295	-.1630	-.1645	.0015
36	-.1359	-.0948	-.1194	-.1210	.0016
38	-.0878	-.0608	-.0765	-.0780	.0015
40	-.0397	-.0268	-.0337	-.0352	.0015
42	.0084	.0070	.0088	.0074	.0014
44	.0565	.0411	.0517	.0501	.0016
46	.1046	.0746	.0939	.0930	.0009
48	.1527	.1089	.1371	.1362	.0009
50	.2008	.1435	.1807	.1799	.0008
52	.2489	.1785	.2247	.2243	.0004
54	.2971	.2147	.2703	.2697	.0006
56	.3452	.2513	.3164	.3162	.0002
58	.3934	.2891	.3640	.3641	.0001
60	.4415	.3285	.4136	.4137	-.0001
62	.4897	.3695	.4652	.4655	-.0003
64	.5378	.4122	.5190	.5199	-.0009
66	.5860	.4581	.5767	.5776	-.0009
68	.6342	.5067	.6379	.6395	-.0016
70	.6824	.5597	.7047	.7066	-.0019
72	.7306	.6183	.7784	.7809	-.0025
74	.7788	.6849	.8623	.8650	-.0027
76	.8270	.7635	.9612	.9635	-.0023
78	.8752	.8595	1.0821	1.0854	-.0033
80	.9234	.9889	1.2450	1.2522	-.0072
82	.9717	1.2193	1.5351	1.5504	-.0153

^a $\psi = 1.2590 X'$. This corresponds to $D^* = 9.2157 \times 10^{-6}$ cm.²/sec. for $G_v = 1.07834 \pm 0.00015$ (equation 18).

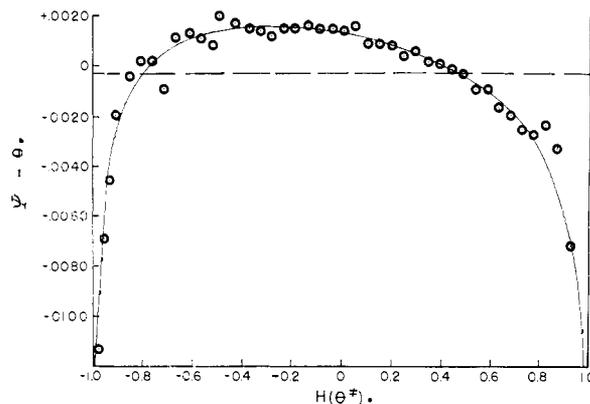


Fig. 1.—Difference plot based on the data from Table I. The $(\psi - \theta)$ values for $j = 1$ and $j = 3$ are also included, while the value for $j = 82$ is off the scale used here. ψ_0 was found to equal -0.00035 .

From the data of Lyons and Thomas⁶ it is evident that the refractive index increment cannot be expected to be constant for this system. As a consequence the reduced fringe number, $H(\theta^\pm)$ must be calculated from equation 7a. While values for a_1' are lacking for this system at 20° , it seemed that only a negligible error would be incurred if it was assumed that a_1' is the same at 20° as the 25° value given by Lyons and Thomas.⁶ The value of R_0 was estimated using this value of a_1' , and the values of J_m and ΔC with equation 43, reference 7. This gave $\beta = -3.75 \times 10^{-3}$.

Table I illustrates a convenient manner in which the data may be arranged for the calculation of the difference function for each time. In this example the corrected time is 14,187 seconds. Column 1 gives the fringe number, and column 2 gives $H(\theta^\pm)$. In column 3 are listed the differences between the comparator readings for each fringe and the comparator reading calculated for the fringe $J_m/2$. Column 4 gives the normalized values of column 3 entries, and column 5 gives the values of θ^\pm . In column 6 the differences between corresponding values in columns 4 and 5 are listed.

Figure 1 is the difference plot based on the data of Table I. The dashed horizontal line in this figure marks the zero of the ϕ axis after adjustment to the condition

$$\int_{-1}^1 \phi dH(\theta^\pm) = 0$$

Table II illustrates the way in which the data derived from the plot of Fig. 1 may be arranged for the calculation of D_c at nineteen equally spaced intervals of $H(\theta^\pm)$. Column 1 gives $H(\theta^\pm)$, column 2 the tabular value $H'(\theta^\pm)$, column 3 the slope of the difference plot, $d\phi/dH(\theta^\pm)$, measured at corresponding $H(\theta^\pm)$, values and column 4 the values of

$$2 \int_{-1}^{H(\theta^\pm)} \phi dH(\theta^\pm)$$

Column 5 gives the product of the entry in column 2 times the corresponding entry in column 3. Column 6 gives the quotient of the entry in column 4 divided by the corresponding entry in column 2. Values of D_c/D^* are listed in column 7 and in column 8, values of D_c .

TABLE II
BOLTZMANN ANALYSIS OF FRINGE DATA FOR THE DIFFUSION OF GLYCINE
Glycine, $C = 0.9860$ g./dl., $J_M = 83.175$, $\bar{C} = 0.4930$ g./dl.
 $t + \Delta t = 14,718$ sec., $T = 20.00^\circ$

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
H	H'	$\frac{\partial\phi}{\partial H}$	$2 \int_{-1}^H \phi dH$	$H \frac{\partial\phi}{\partial H}$	$\frac{2}{H^2} \int_{-1}^H \phi dH$	$\frac{D_c}{D^*}$	$D_c \times 10^6$	$D_c \times 10^6$	$\sigma \times 10^6$
							cm. ² sec. ⁻¹		
-0.9	0.29177	0.0276	-0.0011	0.0081	-0.0038	1.0119	9.325	9.293	± 0.026
-.8	.49638	.0137	-.0012	.0068	-.0024	1.0092	9.300	9.277	.020
-.6	.79184	.0037	-.0008	.0029	-.0010	1.0039	9.252	9.246	.013
-.4	.98341	.0013	0	.0013	0	1.0013	9.228	9.216	.011
-.2	1.09273	-.0002	0.0009	-.0002	0.0008	0.9990	9.206	9.199	.008
0	1.12838	-.0019	.0017	-.0021	.0015	.9964	9.182	9.178	.004
0.2	1.09273	-.0030	.0023	-.0033	.0021	.9946	9.166	9.160	.005
.4	0.98341	-.0048	.0028	-.0047	.0028	.9925	9.147	9.138	.010
.6	.79184	-.0071	.0028	-.0056	.0035	.9909	9.132	9.121	.013
.8	.49638	-.0170	.0022	-.0084	.0044	.9842	9.098	9.096	.004
.9	.29177	-.0315	.0016	-.0092	.0055	.9853	9.080	9.083	.013

^a Diffusion constant calculated at $t + \Delta t = 14718$ sec. ^b Mean and standard deviation of the diffusion constant at the tabulated H from measurements at $t + \Delta t = 7398, 11238, 14718$ and 18558 sec.

D_c values were calculated for three other times: 123, 187 and 309 minutes, corrected for Δt and the four values at each concentration were averaged. These average values of D_c are listed in column 9 of Table II. The standard deviation σ of the sample of D_c at any C , as obtained at different times is listed in column 10 and was estimated from the range.⁴⁰ The average D_0 values so obtained were then fitted to a linear equation C by the method of least squares. Expressing this result in the form

$$D_0 = D_c(1 - kC) \quad (21)$$

where D_0 is D_c at $C = 0$, gives $D_0 = 9.288 \times 10^{-6}$ cm.²/sec. at 20.00° and $k = 0.0243$ dl./g. The error associated with k is less than $\pm 5\%$. At $\bar{C} = 0.4930$ g./dl., $D_c = (9.178 \pm 0.005) \times 10^{-6}$ cm.²/sec. at 20.00° .

It seemed of interest to calculate the concentration dependence of D from the data of this experiment by Creeth's method.⁷ For this calculation the same four exposures and the same comparator measurements (from the even numbered fringes) were used for the Boltzmann calculation. The result of this calculation, expressed in the form of equation 21 is: $D_c = 9.284(1 - 0.0231C) \times 10^{-6}$ cm.²/sec. D at $\bar{C} = 0.4930$ g./dl. was found to be 9.178×10^{-6} cm.²/sec., the same result as that obtained with the Boltzmann calculation.

These values of k may be compared with $k = 0.0241$ as determined by Lyons and Thomas at 25° from a series of six differential experiments covering the concentration interval from 0.25 to 4.2 g./dl. Comparison with the value of Lyons and Thomas is justifiable on the grounds that the variation of k with temperature, as estimated from the data of these authors at 25° and 1° , is very much less than the accuracy of $\pm 5\%$ assigned to the Boltzmann result. Creeth⁷ has determined $k = 0.0251$ at 25° for glycine with $\bar{C} = 0.6101$ g./dl., $\Delta C = 1.2202$ g./dl.

No recent data are available in the literature for the diffusion coefficient of aqueous glycine solutions at 20° . However the values determined in this

work may be referred from 20 to 25° with negligible error by the usual relation

$$D_{25} = D_{20} \frac{\eta_{20}}{\eta_{25}} \frac{298.16}{293.16}$$

For the purpose of comparison, a 20° value of D_c at $C = 0.6101$ g./dl. from this work becomes 1.0490×10^{-5} cm.²/sec. at 25° . At this temperature and concentration a value of 1.0479×10^{-5} cm.²/sec. may be calculated from the data of Lyons and Thomas.⁶ At the same temperature and concentration, the value obtained from the data of Dunlop⁴¹ is 1.0446×10^{-5} cm.²/sec., and the values given by Creeth⁷ are 1.0451×10^{-5} cm.²/sec. (Rayleigh) and 1.0458×10^{-5} cm.²/sec. (Gouy).

Table III gives the fractional difference in parts per thousand between each of the D_c values obtained by the Boltzmann calculations and the corresponding value of D_c using equation 21 and the recorded least squares values of D_0 and k . It can easily be seen that the Boltzmann calculation gives results accurate to about 1 or 2 parts per thousand, except at the two extremes, where the errors become somewhat larger but never as great as 5 parts per thousand. This accuracy is quite equivalent to that obtained using the Creeth method or using Longworth's³⁰ method for calculating differential diffusion coefficients.

It is interesting to note that in this experiment virtually no concentration dependence was observed in the data at times of 10, 20 and 30 minutes, even though the concentration dependence is large enough so that it should be measurable at these short times. A similar anomaly also was observed in all of the experiments on BMA discussed below. Creeth⁷ has reported a similar observation and has discussed imperfections in the cylinder lens and second order optical effects as possible causes for it. In addition to these causes it is quite probable that the lack of skewness in early pictures is at least in part due to an anomaly in the shape of the starting boundary. Observations in this Laboratory indicate that the starting boundary formed by siphoning with a single centrally placed needle is not

(40) E. B. Wilson, "An Introduction to Scientific Research," McGraw-Hill Book Co., New York, N. Y., 1952, page 244.

(41) P. J. Dunlop, cited in ref. 7.

planar⁴² and that the time which must elapse before the expected skewness is observed is correlated with the magnitude of the time correction. (The larger the time correction, the longer the time interval before skewness is observed.)

TABLE III

ERROR ANALYSIS FOR BOLTZMANN CALCULATION OF DIFFUSION COEFFICIENT OF GLYCINE

<i>t</i> (sec.)	7398 [<i>D_c</i> - <i>D₀</i> (1 - <i>kC</i>)] × 1000/ <i>D_c</i> ^a	11238	14718	18558	Average
-0.9	2.3	-0.9	4.9	-0.5	1.4
-.8	-1.2	0	3.4	1.4	0.9
-.6	-1.8	-0.3	0.5	1.0	-0.1
-.4	-2.0	-1.6	.3	-0.4	-1.0
-.2	-1.5	-0.9	.3	.2	-0.4
0	-0.8	.1	.1	-.5	-.3
0.2	.2	-.2	.8	-.4	.1
.4	0	.4	1.1	-1.1	.1
.6	0.3	1.6	1.8	-1.1	.6
.8	.3	0.8	0.5	-0.2	.3
.9	1.2	1.1	-0.2	-0.5	.1

^a *D₀* = 9.288 × 10⁻⁶ cm.²/sec., and *k* = 0.0243 dl./g. at 20.00°.

Bovine Mercaptalbumin.—A series of seven differential diffusion experiments were carried out covering the range of concentration from zero to 1.5 g./dl. Differential diffusion coefficients were calculated for each of these runs by the method described by Longworth.³² The zero time correction was estimated as described above. The results of these experiments are summarized in Table IV. Run 222 was carried out on the same preparation as the other six runs, but after the BMA, salt free and isoionic, had been stored as a dry powder at -5° for about a year. Apparently storage under these conditions for this period produced no change in the albumin which could be detected by diffusion experiment. Equation 21 was fitted to these differential diffusion data, using the method of least squares.

TABLE IV

DIFFERENTIAL DIFFUSION COEFFICIENTS FOR BOVINE MERCAPTALBUMIN IN SODIUM ACETATE-ACETIC ACID

BUFFER, pH = 4.75, Γ/2 = 0.05, T = 20.00°

Run no.	<i>C</i> , g./dl.	<i>J_M</i>	Δ <i>t</i> , sec.	<i>D_c</i> × 10 ⁷ , cm. ² /sec.	σ × 10 ⁷ , cm. ² /sec.
207	0.0461	8.098	40	5.763	±0.030
206	.1174	20.618	43	5.745	.008
202	.2544	44.670	514	5.721	.002
203	.4780	83.941	555	5.685	.004
205	.6500	40.085	195	5.647	.004
222	1.0140	83.232	371	5.565	.003
204	1.1540	38.090	115	5.545	.003

The data of experiments 203 and 222 which cover the concentration range of zero to 0.9 and 0.5 to 1.5 g./dl., respectively, were analyzed for concentration dependence by the modified Boltzmann method. The results of these calculations are summarized in Table V. In this table the values of [*D_c* - *D₀*(1 - *kC*)] - *x* 1000/*D_c* are given as a function of concentration, *C*, and time, *t*. The values of *D₀* and *k* used for this calculation were those from the least square calculation of the dif-

(42) These observations are reported in some detail in the Ph.D. thesis, ref. 1.

ferential experiments; *D₀* = 5.774 × 10⁻⁷ cm.²/sec., and *k* = 0.0344 dl./g. The Boltzmann results show a considerable tendency toward higher values near *H* = 1, but even here the error is usually less than 0.5%. In dilute solutions, the errors in the Boltzmann calculation are no larger than those in the differential diffusion experiments—indeed, they are considerably smaller in these studies.

The concentration dependence of the diffusion coefficient also was estimated from the data of run 203 by Creeth's method.⁷ All values of *D₀* and *k* are summarized in Table VI. The deviations in the Boltzmann calculation and the Creeth calculation were found to be of the same order of magnitude. The agreement among the various values of *D₀* and *k* is good. However, in all cases the value of *k* determined by either the Boltzmann or Creeth method for a single experiment is less than the value of *k* determined from the six differential diffusion experiments.

Viscosity measurements were carried out for this system in the concentration interval from zero to 8 g./dl. The results of the viscosity measurements at 20.00° may be expressed conveniently

$$1/\eta_0 = 1 - 0.0398 C - 0.00014 C^2$$

This type of plot gives $[\eta] = 0.0398$. An intrinsic viscosity $[\eta] = 0.0398$ also was found for this system by plotting $Q = (1/C) \ln (\eta/\eta_0)$ vs. *C* and determining $[\eta]$ as the extrapolated value of *Q* at *C* = 0.⁴³ The value of $[\eta]$ may be compared with $[\eta] = 0.037$ determined in KCl at 25° by Tanford and Buzzell⁴⁴ for crystalline Armour bovine serum albumin. Tanford and Buzzell note that $[\eta]$ for this system is virtually independent of ionic strength for the interval 0.01 < Γ/2 < 0.50 and independent of pH in the interval from 4.3 to 7.3. In view of this fact a comparison may also be made with the value of $[\eta] = 0.038$, calculated from the data of Yang and Foster by Tanford and Buzzell,⁴⁴ for bovine serum albumin in KCl, Γ/2 = 0.10 at 25°. The data of several authors summarized by Tanford and Buzzell indicate that if indeed any dependence of $[\eta]$ on temperature exists, it is probably such that $[\eta]$ increases slightly with decreasing temperature.

Discussion

Application of the method presented in this paper to the glycine data is quite satisfactory. Both the value of *k* and the differential diffusion coefficient at *C* agree well with the literature values. However, in the case of BMA, while the agreement is good between the value of *k* obtained by the series of differential experiments and *k*'s obtained by the Boltzmann method and Creeth's method, it is by no means excellent.

Ultracentrifuge studies of this protein preparation in several different buffers indicate that there is about 4% of a faster sedimenting component present. Consequently, this preparation will be a two-component system only as a first approxima-

(43) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Colloid Chem.*, **51**, 184 (1947).

(44) C. Tanford and J. G. Buzzell, *J. Phys. Chem.*, **60**, 225 (1956).

TABLE V
ERROR ANALYSIS FOR BOLTZMANN CALCULATION OF DIFFUSION COEFFICIENT OF BOVINE MERCAPTALBUMIN
In sodium acetate-acetic acid buffer, $pH = 4.75$, $\Gamma/2 = 0.050$, $T = 20.00^\circ$

Time (sec.) H	$C_2 = 0.956$ g./dl.; $C_1 = 0$				$C_2 = 1.511$ g./dl.; $C_1 = 0.517$ g./dl.			
	183,835	205,995	241,995	Average	149,471	160,511	180,911	Average
-0.9	-1.8	-2.6	2.1	-0.2	-2.3	-0.5	4.9	0.5
-.8	-1.1	-1.9	0.5	-.9	-2.3	.9	5.1	1.2
-.6	-0.5	-0.9	.4	-.4	-1.2	.7	1.6	0.4
-.4	0	-.4	.9	.4	-2.0	-.9	-1.2	-1.4
-.2	-0.9	-.4	.2	-.2	-1.8	-1.4	-0.4	-1.3
0	-.5	.7	1.2	.5	-1.8	-0.5	-1.3	-1.3
0.2	.5	1.4	2.6	1.6	0	1.1	-0.4	0.2
.4	2.5	3.2	3.0	2.8	2.4	2.9	2.4	2.5
.6	2.6	4.6	3.0	3.4	4.9	5.8	4.0	4.9
.8	3.7	5.1	4.6	4.4	6.7	8.4	4.5	6.5
.9	2.8	5.7	2.8	3.7	5.8	9.1	5.8	6.9

^a $D_0 = 5.774 \times 10^{-7}$ cm.²/sec., and $k = 0.0344$ dl./g. $C = C_1 + (C_2 - C_1)(H + 1)/2$.

TABLE VI
CONCENTRATION DEPENDENCE OF THE DIFFUSION COEFFICIENT OF BOVINE MERCAPTALBUMIN IN SODIUM ACETATE ACETIC ACID BUFFER, $pH = 4.74$, $\Gamma/2 = 0.05$, $T = 20.00^\circ$

	Buffer as solvent $D_0 \times 10^7$, cm. ² /sec.	Water as solvent $D_0^w \times 10^7$, cm. ² /sec.	k , dl./g.
Differential diffusion experiments	5.772	5.905	0.0344
All Boltzmann results (Runs 203 and 222)	5.768	5.901	.0315
Boltzmann calculation (Run 203)	5.764	5.896	.0287
Boltzmann calculation (Run 222)	5.742	5.874	.0280
Creeth calculation (Run 203)	5.761	5.893	.0274

tion.⁴⁵ It might be expected that this paucidispersity will be reflected in the concentration dependence determinations on single experiments.

It is evident from equation A, footnote 24, that \bar{D}_c will be a function of the concentration over the extent of the boundary, even if the individual species exhibit no concentration dependence. At the ends of the boundary, that is, at $H(\theta) = -1$ and $+1$, the value of \bar{D}_c will be equal to the value of the largest diffusion constant in the collection D_i , at the concentrations corresponding to these values of $H(\theta)$. As a result, the plot of \bar{D}_c vs. C will in general be concave. However, the detailed shape of this curve is difficult to define

(45) Recently Creeth and Costing have developed a method based on Rayleigh fringe data for evaluating solute heterogeneity: J. M. Creeth and L. J. Gosting, *J. Phys. Chem.*, **62**, 58 (1958); J. M. Creeth, *ibid.*, **62**, 66 (1958).

in terms of the concentration dependences and concentrations of the various solute species. Caution must be applied in interpreting results obtained by the Boltzmann method on systems which are not strictly two component. Consequently, the concentration dependence of BMA obtained by means of the series of differential experiments must be considered more reliable than that calculated from the data of single experiments. Nevertheless, the results obtained for BMA in single experiments indicate that values of the diffusion coefficient at zero concentration may be obtained with good precision by the Boltzmann method.

In addition to the problem of heterogeneity of the solute, there is also the possibility of complications arising from the multi-component nature of the solvent buffer. At present it is impossible to predict what effects may be attributable to a complex solvent. It seems quite reasonable to expect, however, that such effects will be minimal, if not absent altogether, when the protein is isoelectric. For this reason the work on BMA reported in this paper was confined to conditions under which the albumin is isoelectric. In addition, from the light scattering work of Kay⁴⁶ the interaction constant, $\partial \ln \gamma / \partial C$, is also zero under these conditions.

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(46) C. M. Kay, Ph.D. Thesis, Harvard University, 1956.