where $[\eta]$ is the intrinsic viscosity and ν_1 is the viscosity increment of the protein. But if we retain the molecular interaction terms in (40) we obtain the new limiting expression

$$\lim_{\phi \to 0} \frac{(\eta/\eta_0) - 1}{\phi} \equiv 100[\eta] \Big/ \left(\bar{V}_p + \frac{H}{d_0} \right) = \nu_1 + f_1 \quad (42)$$

Equation 42 shows that the measured value of $100[\eta]/[\bar{V}_p + (H/d_0)]$ is in general greater than the viscosity increment ν_1 by an amount equal to the coefficient f_1 in (40). The usual heuristic explanation that the effect of molecular interactions on $[\eta]$ disappears at infinite dilution is based on the implicit assumption that the contribution due to these interactions involves only terms of the type $f_n\phi^n$ with $n > 1$. There is no definite experimental support to this assumption. Indeed, for solutions of simple electrolytes we should even include an interaction term of the form $f_{1/2}\phi^{1/2}$ according to Falkenhagen.[17]

For protein solutions, $(\eta/\eta_0 - 1)/\phi$ approaches a constant value as $\phi \to 0$, consequently we have $f_{1/2} = 0$. But there seems to be no reason for us to neglect the term $f_1\phi$.

The evaluation of the coefficients f_1, f_2, etc., would require a theoretical treatment of molecular interaction in protein solutions, but the simple formal relationship considered above should suffice to show the heuristic nature of the usual interpretation of intrinsic viscosity.

While for macromolecules of a high degree of asymmetry we may expect that f_1 is indeed negligible as compared to ν_1, for molecules with low axial ratios the existing structural information obtained from viscosity data may need considerable revision in view of (42). Furthermore (42) also suggests a new experimental approach for studying molecular interactions in protein solutions.

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(17) See, for example, Harned and Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 1950, p. 67.

[CONTRIBUTION NO. 1138 FROM THE DEPARTMENT OF CHEMISTRY OF YALE UNIVERSITY AND THE LABORATORY OF CELLULAR PHYSIOLOGY OF NATIONAL HEART INSTITUTE]

The Self-diffusion Coefficients of Water and Ovalbumin in Aqueous Ovalbumin Solutions at 10°

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The self-diffusion coefficients of water and ovalbumin in aqueous ovalbumin solutions at 10° have been determined with H_2O^{18} and C^{14}-labeled ovalbumin, respectively, as the tracers.

One of the problems that interests and puzzles many experimenters engaged in sedimentation and ordinary diffusion studies on proteins is the quantitative relationship between the mobility of the protein molecules and the measured viscosity of the solution. Theoretical investigation of this problem is complicated by the inhomogeneity of the liquid phase in the direction of sedimentation or diffusion. For self-diffusion, however, there is neither net back-flow of the solvent nor variation in the activity coefficients of the components along the diffusion path. Consequently the situation becomes much simpler theoretically, and one may expect to get a better understanding of the problem by careful examination of the self-diffusion coefficients of proteins in solution. Unfortunately, no such data exist in the literature. In the present work the self-diffusion coefficients of water and ovalbumin in aqueous ovalbumin solutions at 10° have been determined. The results of similar measurements on several other protein solutions will be reported in later communications.

Experimental

Diffusion Measurements.—The improved capillary method[1] was used in the present work. The rate of stirring in the diffusion bath was so adjusted that $2\Delta l/l$ is negligible as compared to other experimental error. Consequently Δl could be neglected and the simple relationship

$$\frac{c_{av}}{c_0} = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2\eta + 1)^2} \exp\left[-(2n + 1)^2\pi^2 Dt/4l^2\right] \quad (1)$$

could be used to compute the self-diffusion coefficient D from the measured values of t, l and c_{av}/c_0. In the present work the self-diffusion coefficients of both water and ovalbumin in aqueous ovalbumin solutions at 10.00 ± 0.01° were determined as a function of protein concentration. In the measurements on the self-diffusion of water, capillaries of about 0.002 cm.² in cross-sectional area and with length between 2 and 3 cm. were used. The diffusion time t was between 1 and 1.5 days. Under these conditions Dt/l^2 was almost always greater than 0.2 so that it is sufficiently accurate to omit all terms after the first on the right-hand side of equation 1. Thus we have

$$\frac{Dt}{l^2} = \frac{4}{\pi^2} \ln\left(\frac{8}{\pi^2} \times \frac{c_0}{c_{av}}\right) \quad (2)$$

Equation 2 was used to compute all the self-diffusion coefficients of water from the experimental data. For measurements on the self-diffusion of ovalbumin, capillaries with the same cross-sectional area as those described above but with length between 0.7 and 0.9 cm. were used. The use of these short capillaries for the protein is necessary because of the small self-diffusion coefficient of ovalbumin and the ease with which ovalbumin denatures. By using these extremely short capillaries it was found possible to complete the diffusion measurements in from six to 14 days without excessive amounts of denaturation. Despite these long diffusion times, the value of Dt/l^2 for the protein was often much less than 0.2 so that it was necessary to evaluate D from the experimental data by means of equation 1. This

(1) J. H. Wang, C. V. Robinson and I. S. Edelman, THIS JOURNAL, 75, 466 (1953).

was most conveniently done by plotting c_{av}/c_o as a function of Dt/l^2 as given by equation 1, and then reading off the desired value of Dt/l^2 corresponding to the measured value of c_{av}/c_o from such a theoretical curve.[2]

Preparation of Protein Solutions.—The ovalbumin used in this work was prepared from 100 dozen fresh chicken eggs according to the procedure of Kekwick and Cannan.[3] After separation from other proteins, the ovalbumin was recrystallized three times from sodium sulfate solution at *p*H 4.74. The final crystalline product was centrifuged and kept in the form of thick paste under saturated sodium sulfate solution at 1°. The salt-free ovalbumin solutions for diffusion measurements were prepared by dialyzing the protein paste against frequently renewed distilled water at 1°. Part of the dialyzed ovalbumin solution was further concentrated by evaporation through cellulose membrane at 1°. The *p*H of these ovalbumin solutions remained constant at 4.74 before, during and immediately after the diffusion measurements.

H_2O^{18} was used as tracer to determine the self-diffusion coefficients of water in protein solutions. This was supplied by Stuart Oxygen Company, San Francisco, Calif., and obtained in allocation from the Isotopes Division, U.S. Atomic Energy Commission. Solutions of ovalbumin in tagged water with protein concentration precisely equal to that of the bath solution were prepared by the following procedure. Small aliquot parts (about 0.5 ml. each) of the bath protein solutions were placed in small cellulose bags and rapidly weighed on a semi-micro balance. These small bagfuls of protein solution were then dialyzed in two steps to reach H_2O^{18}-exchange equilibrium with some O^{18}-enriched water. These dialyzed solutions have in general lower protein concentrations than before because of osmosis. Consequently these bagfuls of H_2O^{18}-enriched protein solutions were concentrated by low temperature evaporation through the cellulose membrane until the weight of each bag and the solution in it was equal to or a few milligrams less than the weight before dialysis. At this point the solution in each bag was rapidly mixed and used to fill the capillaries for diffusion measurements.

C^{14}-Labeled ovalbumin was used as tracer for ovalbumin. This was prepared by incubation *in vitro* of hen oviduct minces in the presence of $C^{14}O_2$ and isolated and purified according to a procedure described earlier.[4] This C^{14}-labeled ovalbumin was stored in 70% ammonium sulfate solution at *p*H 4.7. An aliquot part of this radioactive ovalbumin stock solution was taken before each measurement and dialyzed in distilled water at 1° until practically salt-free. Estimated amount of inert protein solution was then mixed with this radioactive protein solution to make the protein concentration of the mixture somewhat less than that of the corresponding bath solution to be studied. The resulting solution was then filled in a loosely tied cellulose bag (loosely tied to eliminate hydrostatic pressure) and dialyzed for two days in the bath solution to be studied until osmotic equilibrium was reached. The bag was then taken out of the bath, the radioactive protein solution inside was taken out and transferred into diffusion capillaries by means of a fine pipet.

Determination of Protein Concentrations.—The concentrations of ovalbumin solutions were determined by three independent methods, *viz.*, the micro-Kjeldahl method of nitrogen analysis (assuming that ovalbumin contains 15.76% of N), the density increment method, and the dry weight method. In the last mentioned method, weighed amounts of protein solutions were carefully evaporated to dryness on stainless steel planchets of known weight under an infrared lamp. These dry samples were then baked in an oven at 105° until constant weight. The concentration of each protein solution determined by these three methods agreed within 1%. The agreement not only confirmed the reliability of each of these methods for determining protein concentration but also indicated that the amount of salt present in each of these protein solutions was negligible.

Analysis of H_2O^{18} Diffusion Samples.—The c_{av}^- and c_o^- samples for the water diffusion measurements were sealed in tiny glass tubes and stored in a refrigerator at below freezing temperature until analyzed. This was necessary because although there should be no detectable amount of O^{18}-exchange between water and protein under the present experimental conditions, complication may arise should the protein decompose due to some bacterial action. Before the mass-spectrometric analysis of each sample the solution (usually less than 0.005 ml. in volume) was taken out by means of a dry micro-pipet and transferred to a 4-ml. gas sample bulb prefilled with dry nitrogen. The nitrogen was subsequently separated from the frozen solution by evacuating the gas sample bulb when immersed in a Dry Ice–acetone bath. Water in the sample was then vaporized, and aliquot parts of the water vapor from the same sample were passed successively through a Consolidated-401 mass spectrometer for isotope analysis. The $[O^{18}]/([O^{18}] + [O^{17}] + [O^{16}])$ ratio in each sample was determined directly from the ratio of intensities of the peaks due to singly charged ions of mass 18 and 20, respectively. Prior to the analyses of the diffusion samples, the mass spectrometer was first calibrated by means of a series of water samples of known isotope ratios. The calibration curve so obtained was then used in turn to correlate the observed ratio of intensities to the atom per cent. of O^{18}. Memory effects of the mass spectrometer were eliminated by preflushing the mass spectrometer three times with aliquot parts of the sample vapor. After the preflushing, four or five aliquot parts of the sample were passed successively through the instrument and had their O^{18}-content determined. By controlling the sample pressure, ionizing current and scanning speed, etc., it was found possible to make the result reproducible within 0.5%. Under these operating conditions, there was no detectable variation in the measured O^{18}-content of the successive aliquot parts of each sample that could be attributed to fractionation effects. The c_o^- and c_{av}^- samples of each batch were analyzed on the same day. The O^{18}-content of distilled water at Yale was also determined from time to time. This remained fairly constant at about 0.202 atom per cent. of O^{18}. This value was subtracted from the measured O content of all the diffusion samples so that all the c_o's and c_{av}'s were expressed in atom per cent. excess of O^{18}. The over-all accuracy in the final values of c_o/c_{av} was about one per cent.

Analysis of Radioactive Ovalbumin Diffusion Samples.—Accurately weighed amounts of radioactive protein solutions were evaporated slowly to dryness on copper disks under an infrared lamp, and kept in a desiccator until moisture-free before counting. A heavily shielded windowless flow counter was used to count all the samples. The weights of solution taken for evaporation in preparing the c_o-samples were so adjusted that each c_o-sample contained about the same amount of dry protein (in the form of a thin protein layer of definite area) as the corresponding c_{av}-sample. Since the computed value of D depends only on the measured value of the ratio c_{av}/c_o, possible errors due to self-absorption of β-radiation by the solid samples were automatically eliminated. The sampling and washing procedure to prepare the c_{av}^- samples was identical with that described earlier.[5]

Results and Discussion

The measured self-diffusion coefficients of water and ovalbumin, respectively, in aqueous isoelectric ovalbumin solutions at 10.0° are summarized in Table I. Each value listed in Table I is the average result of 3 to 6 measurements. The limiting self-diffusion coefficient of ovalbumin in infinitely dilute aqueous solution listed in Table I was computed from Kegeles and Gutter's sedimentation data[6] converted to 10°. The molecular weight of ovalbumin was taken as 44,000 in computing this limiting value for the diffusion coefficient of ovalbumin. In general the self-diffusion coefficients of water listed in Table I are from 3 to 6 times more accurate than those for ovalbumin. Chief sources of error in the measurement of the self-diffusion of ovalbumin are short capillary length, slight denaturation of the protein during the long diffusion period, large statistical fluctuations in counting

(2) J. H. Wang, THIS JOURNAL, **73**, 510 (1951).
(3) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 232 (1936).
(4) D. Steinberg and C. B. Anfinsen, *J. Biol. Chem.*, **199**, 25 (1952).
(5) J. H. Wang, THIS JOURNAL, **74**, 1182, 6317 (1952).
(6) G. Kegeles and F. J. Gutter, *ibid.*, **73**, 3770 (1951).

very weak samples of radioactive protein, etc. The uncertainty in the computed limiting diffusion coefficient of ovalbumin due to both the uncertainty in the accepted molecular weight of ovalbumin and the experimental errors in sedimentation measurements is about 5%. However, extrapolation of ordinary diffusion data for ovalbumin leads to much the same result,[7] indicating that this computed limiting value cannot be too far off.

TABLE I

THE SELF-DIFFUSON COEFFICIENTS OF WATER AND OVALBUMIN IN AQUEOUS OVALBUMIN SOLUTIONS AT 10.0° WITH *p*H 4.76

Concn. of ovalbumin (% by wt. of dry protein)	$D_{H_2O} \times 10^5$, cm.²/sec.	$D_{protein} \times 10^7$ cm.²/sec.
0	1.675 ± 0.016	(5.80)[6]
10.6	1.38 ± .03	3.32 ± 0.10
19.0	1.13 ± .015	1.62 ± .07
24.4	0.979 ± .009	0.87 ± .05
24.5	0.978 ± .006	

The self-diffusion coefficients listed in Table I are compared with viscosity data[8] in Table II.

TABLE II

COMPARISON OF THE SELF-DIFFUSION COEFFICIENTS WITH VISCOSITY DATA OF AQUEOUS OVALBUMIN SOLUTIONS AT 10.0°

Concn. of ovalbumin, % by wt.	η/η_0	$D_p(\eta/\eta_0) \times 10^7$, cm.²/sec.	$D_{H_2O}(\eta/\eta_0) \times 10^5$, cm.²/sec.
0	1	(5.80)[6]	1.675
10.6	1.61	5.34	2.22
19.0	3.4	5.51	3.84
24.4	6.7	5.83	6.56

(7) J. T. Edsall, Chap. 7, "The Proteins" (Edited by H. Neurath and K. Bailey), Vol. 1, Part B, Academic Press, Inc., New York, N. Y., 1953.

(8) H. Chick and E. Lubrzynska, *Biochem. J.*, **8**, 59 (1914).

Because of the low specific activity of the radioactive ovalbumin, the short capillaries and the ease with which ovalbumin denatures at 10°, the experimental uncertainties in the above D_p values may be as high as 5%. It is hoped that these values can be much improved in the near future by using labeled proteins of higher specific activity, *e.g.*, radioactive iodo-proteins, etc. Values of the relative viscosities in Table II were computed from the data of Chick and Lubrzynska[8] by interpolation. In spite of the tentative nature of these values, Table II shows that the product $D_p(\eta/\eta_0)$ remains fairly constant when η/η_0 varies by a factor of 6.7, indicating that the self-diffusion coefficient of ovalbumin is approximately proportional to the fluidity of the solutions. This observation may also be taken as a suggestion that no appreciable amount of permanent aggregates of ovalbumin molecules exists even in fairly concentrated solutions, because if these aggregates do exist in considerable amount it is very unlikely that they will affect D_p and η/η_0 to exactly the same extent so that their product remains constant.

On the other hand, values in Tables I and II indicate that the relationship between the self-diffusion coefficient of water and the viscosity of ovalbumin solution is more complicated. A quantitative treatment of the concentration dependence of the self-diffusion coefficient of water in protein solutions is given in a separate communication.[9]

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(9) J. H. Wang, THIS JOURNAL, **76**, 4755 (1954).

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[CONTRIBUTION FROM DEPARTMENT OF CHEMISTRY, BOSTON UNIVERSITY]

Electrochemical Behavior of Cation Exchange Membranes[1,2] in Liquid Ammonia

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Cationic ion-exchange membrane electrodes are used successfully in liquid ammonia, alcohol and water solutions to measure junction potentials. These measurements may be then utilized in standard electrochemical equations to calculate cationic concentrations. In addition such membrane electrodes are shown to be valuable as a means of determining the ratios of activity coefficients of salts in the above mentioned media.

The electrochemical properties and uses of ion-exchange membranes depend on the fact that they form a special type of junction between two solutions. When two solutions containing different concentrations of the same electrolyte are in contact, a potential will be set up at the junction which is caused by the difference in speed of migration of the various ions. The potential may be expressed by the well-known equation

$$E = (2t_+ - 1)RT/F \ln a_1/a_2 \qquad (1)$$

or

$$E = \frac{t_+ - t_-}{t_+ + t_-} RT/F \ln a_1/a_2$$

in which $t_+ + t_- = 1$. E is, therefore, a function of the transference numbers of the anions and cations.

If the two solutions are separated by a cation-exchange membrane, the migration of the negative ions can be restricted. Membranes made of ion-exchange material act as ion sieves. An ideal membrane made of cation-exchange material would allow only cations to pass through, *i.e.*, $t_+ = 1$ and $t_- = 0$. Equation 1 than reduces to

$$E = RT/F \ln a_1/a_2 \qquad (2)$$

It has been demonstrated experimentally that an

(1) From Ph.D. Thesis of Martha J. Bergin, May, 1952, presented in part at the 122nd A.C.S. Meeting, Atlantic City, N. J., Sept., 1952.

(2) Supported in part by a Grant in Aid of the Research Corporation, New York, N. Y.

(3) Sylvania Electric Products, Inc., Salem, Mass.