coemergent with authentic malonamide. The hydrolysis is complete and no other peaks are visible. A blank without the hemoprotein does not reduce the bromide.

With horse heart myoglobin the identical procedure can be employed except that the purchased material (Mann Biochemicals) is treated first with potassium ferricyanide and passed through a short G-75 Sephadex column in buffer before commencing with its reduction.

Kinetics. Two milliliters of 5×10^{-6} *M* iron(II) hemoprotein was prepared directly in 1-cm spectrophotometric cells in the fashion described for reaction. The same buffer and salts were employed. The cell was fitted with a tight serum cap and thoroughly flushed with argon *via* hypodermic needles. The requisite amount of a fresh dithionite solution (10 μl) was injected to generate the iron(II) complex. In all cases reduction was complete in 15 min or less. At time 0, 10–20 μl of a stock freshly prepared and argon purged $2.5–6.25 \times 10^{-3}$ *M* bromomalononitrile solution was injected. The solution was tipped once and placed in the spectrophotometer cavity. The decrease in the Fe(II) soret band was plotted by the recorder. A reference cell contained salts and buffer. All operations were conducted at ambient temperature (18°).

Other Reactive Substrates. A 5×10^{-6} *M* solution of hemoglobin was oxidized by the addition of 1–2 μl of a saturated buffer solution of the following substrates: iodoacetic acid, 2,3-dibromosuccinic acid, α-bromobutyrolactone, allyl bromide, allyl chloride, and 2,3-dibromopropanol. The halides are grouped in a decreasing order of reactivity. However, all of these reactions caused some denaturation of hemoglobin. A turbidity of the solution and an incompleteness of reaction were typical of this phenomenon.

Diffusion Studies on Phosphatidylcholine Vesicles

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Abstract: Diffusion measurements of homogeneous phosphatidylcholine vesicles in 0.1 *M* KCl–0.01 *M* Tris–11.5% D_2O, pH 8.0, have been carried out at 20° in the ultracentrifuge. Since the apparent specific volume of phosphatidylcholine vesicles is nearly equal to the reciprocal of the density of this medium, no appreciable sedimentation occurs during the high speed experiment. Consequently, the measurement of diffusion coefficients of the lipid system in the ultracentrifuge at high speed is virtually identical with that of the free diffusion experiments performed in the stationary diffusion apparatus. Procedures are described in detail for calculating the diffusion coefficient from Rayleigh interference data. The averaged diffusion coefficient is $2.03 \pm 0.04 \times 10^{-7}$ cm^2 sec^{-1}.

Reports on determinations of the translational diffusion coefficients of proteins and viruses in solutions have been well documented in the literature.[1–3] Since the determination of diffusion coefficients from data obtained *via* the optical systems built into the analytical ultracentrifuge is relatively simple and straightforward to perform in comparison with *free diffusion* experiments of stationary diffusion apparatus, the ultracentrifuge has frequently been employed for diffusion studies. A general account on the evaluation of diffusion coefficients of both native and denatured proteins from sedimentation boundary curves obtained at various speeds with a synthetic boundary cell has been extensively discussed by Kawahara.[4] In the case of a pure lipid system, however, no detailed reports on the measurements of diffusion coefficients have yet appeared in the literature.

Based on the theoretical works of Fujita,[5–7] the diffusion coefficient, D, of macromolecules can be related to the maximum height–area ratio (H/A) of the sedimenting boundary obtained with a synthetic boundary cell and the schlieren optical system according to the following equation[4]

$$(A/H)^2 = 4\pi Dt(1 - z)(1 + s\omega^2 t) \qquad (1)$$

with $z = 2r_0\omega^2 s^0 kC_0(H/A)t$ and $0 < z < 0.16$. Here t is the time, ω the angular velocity of the ultracentrifuge rotor, r_0 the radial position of the initial synthetic boundary, C_0 the initial solute concentration, and s the sedimentation coefficient which varies with concentration, C, obeying the linear relation: $s = s^0(1 - kC)$, where s^0 is the extrapolated value of s at infinite dilution and k is a constant.

It is obvious from eq 1 that the values of z and $s\omega^2 t$ may become negligibly small compared with unity if the experiment is performed at low speed. At the rotational speed of 12,590 rpm, for example, the values of z are all smaller than 0.02 and the values of $s\omega^2 t$ are less than 0.001 for native proteins such as aldolase, bovine serum albumin, hemoglobin, myoglobin, and ribonuclease.[4] The diffusion coefficient can, therefore, be calculated from the experimental data obtained at low speed by the familiar simple equation for the *free diffusion* method[1] which can be easily derived from eq 1 as follows

$$D = \frac{1}{4\pi t}(A/H)^2 \qquad (2)$$

Alternatively, eq 1 can also reduce to eq 2 if the value of s for a given system is so small that the values of both z and $s\omega^2 t$ are much less than unity. Under the condition $s \simeq 0$, the diffusion experiment carried out in the ultracentrifuge is equivalent to that performed at low speed. Consequently, the measurement of diffusion coefficients is virtually identical with that of the *free diffusion* experiment.

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(2) H. Svensson and T. E. Thompson, "Analytical Methods of Protein Chemistry," Vol. III, P. Alexander and R. J. Block, Ed., Pergamon, New York, N. Y., 1961, p 58.
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(5) H. Fugita, *J. Chem. Phys.*, **24**, 1084 (1956).
(6) H. Fugita, *ibid.*, **31**, 5 (1959).
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When the boundary with a negligible value of s is recorded by the Rayleigh interference optical system, the vertical reflections of a single fringe give a direct plot of the concentration of the sedimenting macromolecule, C, against position, r, in the synthetic boundary cell. If the initial synthetic boundary is formed at position r_i from the center of rotation of the centrifuge rotor, and the initial concentration is C_0, the following diffusion equation can be derived from Fick's second law, $\partial C/\partial t = D\partial^2 C/\partial r^2$, by the integral transform method[8]

$$C = \frac{C_0}{2}\left[1 + \frac{2}{\sqrt{\pi}}\int_0^{\frac{r-r_i}{\sqrt{4Dt}}} \exp(-\alpha^2)\mathrm{d}\alpha\right] \quad (3)$$

for the boundary conditions $t = 0$, $C = 0$, $-\infty < r < r_i$; $C = C_0$, $r_i < r < +\infty$, where α is a dummy variable of integration. Equation 3 may be rewritten as

$$(2C/C_0 - 1) = erf\left[\frac{r - r_i}{\sqrt{4Dt}}\right] \quad (4a)$$

or

$$D = \frac{1}{2t}\left[\frac{r - r_i}{\sqrt{2}erf^{-1}(2C/C_0 - 1)}\right]^2 \quad (4b)$$

where erf is the Gauss error integral or error function

$$erf(\xi) = \frac{2}{\sqrt{\pi}}\int_0^{\xi} \exp(-\alpha^2)\mathrm{d}\alpha$$

and erf^{-1}, the inverse error function, is given by $\mathrm{erf}^{-1}(\sigma) = \xi$ where $\sigma = erf(\xi)$.

In eq 4, C_0 is the initial concentration which is directly proportional to the vertical position coordinate in the plateau region of a fringe near the bottom of the synthetic boundary cell and D is the diffusion coefficient corresponding to the vertical position of C at radial position r from the center of rotation and time t from the start of the experiment (Figure 1). It should be noted that only the concentration ratio, C/C_0, is expressed in eq 4; the absolute concentration is thus not important in diffusion measurements. Since the error function is a standard program for most digital computers, the diffusion coefficient can be readily calculated with the aid of a computer, according to eq 4, from experimental data obtained with interference optics.

In the present paper, the analysis of the sedimentation boundary curves obtained with either schlieren or interference optics has been adapted for the measurement of diffusion coefficients of monodispersed homogeneous phosphatidylcholine vesicles. Since the specific volume of phosphatidylcholine vesicles is very close to unity, it has been demonstrated earlier that the density of the supporting medium corresponding to zero redistribution of phospholipids during sedimentation, or the isodensity point, can be obtained by adding a small amount of D_2O into the phosphatidylcholine vesicles solution.[9] The diffusion experiments reported in this article were performed under the condition that the sedimentation coefficients of phosphatidylcholine vesicles were made nearly zero ($<0.1S$) by adding sufficient D_2O into the supporting aqueous medium, so that the simple mathematical analysis, eq 2 and 4, of the diffusion process for lipids obtained. We believe that

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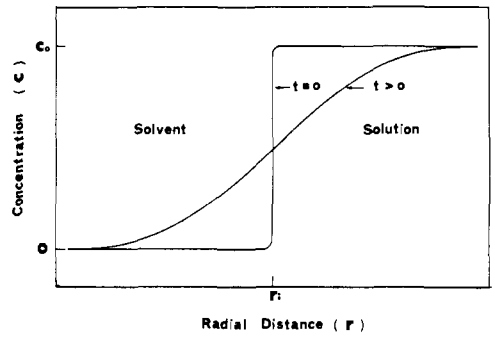

Figure 1. A schematic representation of the spreading of a diffusion boundary with zero sedimentation in the synthetic boundary cell.

phosphatidylcholine vesicles could serve as a model and that the method for diffusion measurements described here is also applicable to other pure phospholipid systems in aqueous solution.

Experimental Section

Materials. Phosphatidylcholine was isolated from hen egg yolk by the silicic acid column chromatographic method.[10] Deuterium oxide (90 mol % D_2O, lot 4566) was supplied by Bio-Rad, Richmond, Calif., tris(hydroxymethyl)aminomethane from Sigma, St. Louis, Mo., Sepharose 4B from Pharmacia Fine Chemicals, Piscataway, N. J., and silicic acid from Clarkson Chemical, Williamsport, Pa. All other chemicals were of reagent grade. Water was doubly glass-distilled.

Preparation of Phosphatidylcholine Vesicles. Lyophilized phosphatidylcholine (300–400 mg) was suspended in 8 ml of buffer solution at pH 8.0. The suspension was ultrasonically irradiated (20 kHz) for 2.0–2.5 hr under nitrogen at 2°. Titanium fragments released from the sonication probe (Branson Sonifier, Model 125, Danbury, Conn.) and any undispersed phospholipid were removed by centrifugation at 105,000g for 60 min. The resulting supernatant was first concentrated by ultrafiltration under nitrogen pressure in a 10-ml magnetically stirred Diaflo apparatus (Amicon Model 12 cell, Amicon Co., Lexington, Mass.) using a Diaflow Membrane UM-2 to about 2 ml, and then was subjected to upward flow gel filtration, at 4°, on a column of Sepahrose 4B (2.5 × 50 cm). Prior to use, the column was washed with 250 ml of $10^{-5}M$ pentachlorophenol to kill any bacterial contaminants, and then eluted with the buffer solution overnight to remove the germicide and to equilibrate the Sepharose 4B with the buffer. After the concentrated vesicle dispersion was introduced into the column bed, it was followed by the same buffer solution. The elution diagram of the phosphatidylcholine dispersion consists of two distinct fractions (I and II). Those portions of fraction II which show a linear relation between absorbance at 300 nm and lipid phosphorus content and that from which the linear regression line passes through the origin were collected and used for subsequent studies. The detailed experimental procedures for ultrasonic disruption, gel filtration, and collection of the homogeneous vesicle solution in those portions of fraction II have been published elsewhere.[11] Phosphatidylcholine concentrations in the eluted fractions were expressed in terms of lipid phosphorus (P_i) as determined colorimetrically according to Gomori.[12] A solution of potassium chloride (0.1 M) and tris(hydroxymethyl)aminomethane–HCl (0.01 M), adjusted to pH 8.0, was used as the buffer solution throughout this work.

The vesicle solutions with various densities of the supporting media were prepared by mixing an aliquot of the homogeneous phosphatidylcholine vesicle solution, collected from some selected portions of the Sepharose 4B column effluent as described above, with an appropriate amount of the stock buffered D_2O solution (60% D_2O in 0.1 M KCl–0.01 M Tris–HCl aqueous solvent). Buffered D_2O supporting media of different densities without the

(10) C. Huang, *Biochemistry*, **8**, 344 (1969).
(11) C. Huang and J. P. Charlton, *Biochem. Biophys. Res. Commun.*, **46**, 1660 (1972).
(12) G. Gomori, *J. Lab. Clin. Med.*, **27**, 955 (1942).

Table I. Summary of Sedimentation Studies on Phosphatidylcholine Vesicles Suspended in Various Percentages of D_2O in 0.1 *M* KCl–0.01 *M* Tris Buffer at 20°

Physical parameter	% D_2O[a] in buffer solution			
	0	5	10	15
η_r	1.0010 ± 0.0011	1.0027 ± 0.0006	1.0046 ± 0.0010	1.0150 ± 0.0006
s^0	1.84 ± 0.09	1.24 ± 0.06	0.32 ± 0.04	−0.53 ± 0.03
$\eta_r s^0$	1.84 ± 0.09	1.24 ± 0.06	0.32 ± 0.04	−0.55 ± 0.03
ρ(g ml^{-1})	1.00340 ± 0.00003	1.00837 ± 0.00003	1.01357 ± 0.00003	1.01817 ± 0.00003
$S^\circ_{20,w}$[b]	2.63 ± 0.23	3.03 ± 0.15	2.96 ± 0.36	3.46 ± 0.18

[a] Deuterium oxide with a purity of ~90 mol % D_2O was obtained from Bio-Rad (lot no. 4366). [b] Values of $S^\circ_{20,w}$ in Svedberg unit were calculated according to the equation $S^\circ_{20,w} = s^\circ\eta_r(1 - \phi'\rho_{H_2O})/(1 - \phi'\rho_{salt-D_2O-H_2O})$, where ϕ' is the reciprocal of ρ^0 ($\rho^0 = 1/\phi' = 1.0154$) and equals 0.9848 ± 0.0007 ml/g.

phosphatidylcholine vesicles were similarly prepared by mixing the buffer solution with a calculated volume of the stock buffered D_2O solution, and were expressed as per cent of D_2O in the buffer solution.

Viscosity and Density Measurements. A Cannon-Ubbelhode semimicrodilution viscometer with a shear rate of about 1500 sec^{-1} and a water flow time of about 287 sec was used in all the viscosity measurements on the different suspending media. No kinetic energy correction was necessary. The viscosity of the medium relative to that of water, η_r, was calculated using the relation $\eta_r = (td/t_\omega d_\omega)$, where t is the flow time and d the density of the medium. The subscript, ω, represents the measurements with redistilled water. Flow times determined with an electric timer were the result of a minimum of five experiments. The density measurements of each suspending medium were determined in triplicate with a 5-ml calibrated pycnometer. Weighings were made with a Mettler H30T analytical balance to an accuracy of ±0.01 mg. Both the viscosity and density measurements were carried out at 20° in a thermostated bath with a temperature control of ±0.005°.

Determination of the Isodensity Point of Phosphatidylcholine Vesicles in Buffered D_2O Supporting Media. The sedimentation velocity method was employed to determine the isodensity point of phosphatidylcholine vesicles suspended in buffered D_2O solution.[9] The sedimentation coefficients, s, of the vesicles suspended in media of various densities were measured using a Beckman-Spinco Model E analytical ultracentrifuge equipped with schlieren optical system. The ultracentrifuge experiments were performed at 20° as controlled by the RTIC unit. The An-D rotor, operated at 42,040 rpm, was employed; a double-sector, capillary-type, synthetic boundary cell with 12-mm optical path was used for most experiments. The relative amounts of vesicle solution and its corresponding supporting medium introduced separately into the two sectors of the centerpiece were so chosen as to allow the formation of the boundary near the center of the cell.

Since the vesicles were homogeneous with respect to size, and the schlieren patterns were highly symmetrical,[9,10] s was calculated from the least-squares slope of a plot of t *vs.* log r_H, where r_H is the radial distance from the center of rotation of the centrifuge rotor to the point of the maximum ordinate on the schlieren peak at time, t. The sedimentation coefficients of the vesicles suspended in each percentage of buffered D_2O solution were first measured as a function of phospholipid concentrations, and were then extrapolated linearly by a least-squares treatment of the experimental data to obtain s^0, the value of sedimentation coefficient at infinite dilution. The individual value of s^0 obtained in a given buffered D_2O solution was multiplied by the relative viscosity, η_r, of the buffered D_2O suspending medium, and all the values of $\eta_r s^0$ were plotted against the density of the suspending buffered D_2O solution. At the zero value of $\eta_r s^0$, the density of the supporting D_2O buffer solution, corresponding to the reciprocal of the specific volume of the phosphatidylcholine vesicles, is defined as the isodensity point.[9] Phosphatidylcholine vesicles suspended in 11.5% D_2O buffer solution were used to determine the diffusion coefficients. Since the density of the supporting D_2O buffer was only slightly less than the isodensity point (see Results), the sedimentation coefficients of the vesicles suspended in 11.5% D_2O buffer were all less than 0.1*S* over the phospholipid concentration range studied in diffusion experiments.

Diffusion Experiments. A Beckman-Spinco Model E analytical ultracentrifuge equipped with both schlieren and Rayleigh interference optical systems was used in these experiments. The temperature of the centrifuge rotor (An-D) was maintained at 20° by the RTIC unit during all diffusion experiments. Double-sector, capillary-type, synthetic boundary cells equipped with sapphire windows and an interference mask were used for most of the diffusion experiments.

Phosphatidylcholine vesicle solutions were mixed volumetrically with the stock D_2O buffers to reach a final D_2O concentration of 11.5%. The phospholipid concentration of the final solution was calculated from the known dilution factor. The vesicle solution (0.12 ml) with 11.5% D_2O was introduced in the right-hand compartment of the synthetic boundary centerpiece (as viewed from the screw ring end), and 0.43 ml of solvent (11% of D_2O in 0.1 *M* KCl–0.01 *M* Tris buffer) was placed in the other compartment. The very small difference in D_2O concentration (0.5%) between the right and left compartment was aimed at the anticonvection stabilization of the diffusion boundary during the layering. The refractive increment of heavy water ($dn/dC_{D_2O} = -0.0046$ ml/ml of D_2O[13]) perturbed at the boundary due to the small concentration difference in D_2O between the layered solvent and the supporting medium was negligible as demonstrated by the blank experiment using a regular double-sector centerpiece.

The schlieren patterns were recorded on Kodak metallographic plates. The peaks were enlarged (~25 times) on the tracing papers; the peak areas were determined by cutting out the tracings and weighing the papers on an analytical balance. The maximum height measurements were also made from the same tracings.

The Rayleigh interference patterns were photographed on Kodak spectrographic plates, emulsion type II-G. The plates were measured with a two-coordinate microcomparator (Nikon shadowgraph, Model 6). Readings of the vertical displacement along a single fringe followed the procedures suggested for the high-speed sedimentation equilibrium method.[14] In general, a minimal 50 readings were taken for a central white fringe. The total number of fringes across the boundary was counted. The fractional fringes counted from patterns taken at different time intervals for a given experiment were checked, and no significant deviations were observed indicating that no appreciable sedimentation occurred during the experiment. Diffusion coefficients were calculated according to eq 4. Computations and figures were carried out on a CDC 6400 computer (Control Data Corp.) equipped with a Calcomp plotter.

Results and Discussion

The results of the sedimentation velocity studies on phosphatidylcholine vesicles suspended in various proportions of D_2O in buffer solutions and the density and viscosity data of the various suspending media are summarized in Table I.

Figure 2 shows the plot of the viscosity-corrected sedimentation coefficient, $\eta_r s^0$, of phosphatidylcholine vesicles *vs.* the density of the supporting medium, the D_2O–buffer solution. The value of ρ^0, the isodensity point of D_2O–buffer corresponding to zero redistribution of phosphatidylcholine vesicles, can be obtained from the point where the least-squares line for the plot intersects with the density axis. This isodensity point, $\rho^0 = 1.0154 \pm 0.0007$ g/ml, can be calculated from the known densities of various percentages of D_2O in the buffer (Table I) to be the density of 11.88% D_2O in 0.1 *M* KCl–0.01 *M* Tris buffer.

(13) J. Strassburger and K. E. Reinert, *Biopolymers*, **10**, 263 (1971).
(14) D. A. Yphantis, *Biochemistry*, **3**, 297 (1964).

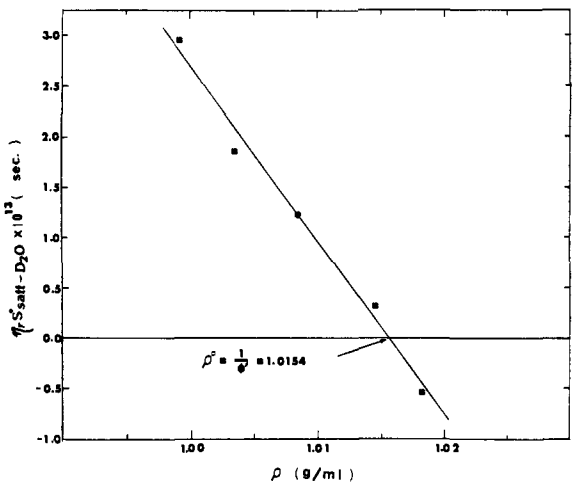

Figure 2. Plot of $\eta_i s^0$ *vs.* ρ for phosphatidylcholine vesicles suspended in KCl–Tris–D_2O–H_2O solvent, μ = 0.11, at pH 8.0. The straight line was calculated by least-squares fit of all the experimental data and the value of ρ^0 was obtained from the point of intersection of the least-squares line with the density axis.

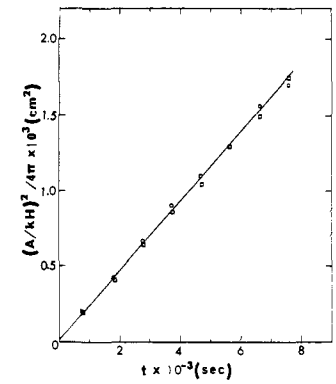

Figure 3. Plot of $(A/KH)^2/4\pi$ *vs.* t for phosphatidylcholine vesicles, P_i = 11.6 mM, in 0.1 M KCl–0.01 M Tris–11.5% D_2O suspending medium. A is the peak area of the schlieren pattern photographed at time t; H is the maximum height of the same pattern and K is the magnification factor in the radial direction. The open squares represent experimental data obtained at 42,040 rpm and the open circles represent experimental data obtained at 12,590 rpm. The apparent diffusion coefficient calculated from the slope of the least-squares line is 1.99 ± 0.03 × 10^{-7} cm^2 sec^{-1}.

Figure 3 shows two sets of diffusion data obtained with the schlieren optical system. The lipid phosphorus was 11.56 μmol/ml for both experiments. As is evident from Figure 3, the linear least-squares line which best fits one set of the data obtained at 42,040 rpm is indistinguishable from that of the other at 12,590 rpm. The identical slope of the plots indicates that the values of diffusion coefficients determined near the isodensity point are independent of the rotor speed. It is well known in practice that the rotor becomes unstable as the speed is lowered beyond a certain limit. The usual method of diffusion at low speed may, therefore, be ill adapted to accurate measurements because of the essentially uncontrollable hazard of convection. Because it is independent of the rotor speeds, the present method of diffusion measurements can avoid this difficulty by performing the experiments at higher speed. Most experiments reported in this communication were carried out at 42,040 rpm.

At 42,040 rpm, the sedimentation coefficients of the phosphatidylcholine vesicles in 11.5% of D_2O–buffer covering the range of concentration of P_i from 0 to 17.7 μmol/ml (or 0–1.36% by phospholipid weight) were all smaller than 0.1S. The value of k in eq 1 was 0.018 dl/g as obtained from the least-squares calculation of s *vs.* C. It can be estimated easily that the value of $s\omega^2 t$ in eq 1 is smaller than 2 × 10^{-3} and that the value of z is less than 3 × 10^{-5} for an average diffusion run of 3 hr; hence, both eq 2 and eq 4 derived under the assumption that the boundary does not move appreciably are valid for the calculation of diffusion coefficients of the system under study.

Table II illustrates a convenient manner in which the data read off a central white fringe of a Rayleigh interference pattern may be arranged for the calculation of D, according to eq 4, at time t. In this example, the time is 6941 sec from the start of the centrifuge run, without taking the zero time correction into account. Column 1 gives the radial distances in centimeters from the center of rotation to the measured points as calculated from microcomparator readings in horizontal directions. Column 2 gives the direct microcomparator readings along the vertical direction. Column 3 gives

Table II. Fringe Data for Diffusion of Phosphatidylcholine Vesicles[a]

r, cm	Y, cm	$\frac{C}{C_0} = \frac{Y}{Y_0}$	$(2)^{1/2}erf^{-1}\left(\frac{2C}{C_0} - 1\right)$	$D \times 10^7$, cm^2 sec^{-1}
6.6114	0.0587	0.1794	−0.9176	2.0669
6.6138	0.0614	0.1877	−0.8865	2.0076
6.6161	0.0662	0.2023	−0.8333	2.0495
6.6185	0.0692	0.2115	−0.8012	1.9889
6.6208	0.0729	0.2228	−0.7626	1.9574
6.6232	0.0766	0.2341	−0.7253	1.9156
6.6255	0.0812	0.2482	−0.6801	1.9136
6.6279	0.0857	0.2619	−0.6373	1.8969
6.6302	0.0912	0.2787	−0.5865	1.9291
6.6326	0.0960	0.2934	−0.5434	1.9132
6.6349	0.1010	0.3087	−0.4995	1.8997
6.6373	0.1057	0.3230	−0.4591	1.8556
6.6396	0.1107	0.3383	−0.4170	1.8186
6.6420	0.1164	0.3557	−0.3697	1.8238
6.6443	0.1215	0.3713	−0.3283	1.7657
6.6467	0.1315	0.4019	−0.2484	2.2572
6.6490	0.1372	0.4193	−0.2037	2.3181
6.6514	0.1425	0.4355	−0.1623	2.3182
6.6537	0.1486	0.4542	−0.1151	2.5534
6.6561	0.1541	0.4710	−0.0728	2.7551
6.6584	0.1605	0.4905	−0.0238	5.8957
6.6608	0.1654	0.5055	0.0138	0.1434
6.6631	0.1708	0.5220	0.0552	1.5289
6.6655	0.1770	0.5410	0.1029	1.6305
6.6678	0.1817	0.5553	0.1391	1.9531
6.6702	0.1877	0.5737	0.1857	1.9222
6.6725	0.1925	0.5883	0.2232	2.0631
6.6749	0.1993	0.6091	0.2769	1.9200
6.6772	0.2037	0.6226	0.3121	2.0488
6.6796	0.2093	0.6397	0.3576	2.0316
6.6819	0.2149	0.6568	0.4036	2.0148
6.6843	0.2198	0.6718	0.4446	2.0453
6.6866	0.2249	0.6873	0.4881	2.0504
6.6890	0.2298	0.7023	0.5309	2.0599
6.6913	0.2356	0.7200	0.5829	2.0037
6.6937	0.2403	0.7344	0.6262	2.0116
6.6960	0.2457	0.7509	0.6773	1.9724
6.6984	0.2501	0.7644	0.7205	1.9820
6.7007	0.2544	0.7775	0.7636	1.9906
6.7031	0.2590	0.7916	0.8116	1.9745

[a] Y_0 = 0.3272 cm, t = 6941 sec, $r_{C=C_0/2}$ = 6.6606 cm, and P_i = 4.7 μmol ml^{-1}.

the normalized values of the column 2 entries, *i.e.*, the concentration ratios. Column 4 presents the calcu-

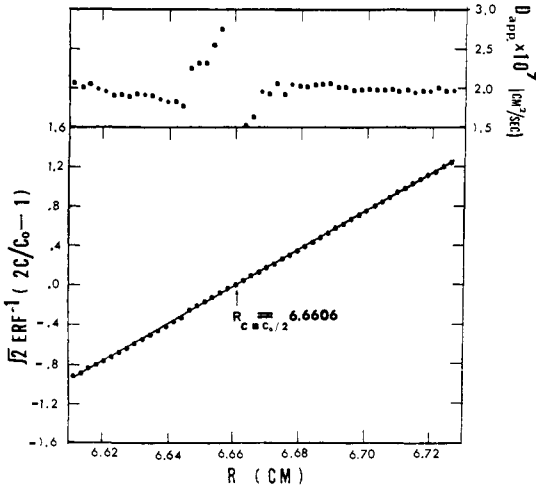

Figure 4. Determination of the averaged apparent diffusion coefficient from the data points across the sedimentation boundary in the synthetic boundary cell. Plot of $(2)^{1/2}erf^{-1}(2C/C_0 - 1)$, obtained at a single time as in Table II, as a function of radial distance. The individual apparent diffusion coefficient at each radial position is also given.

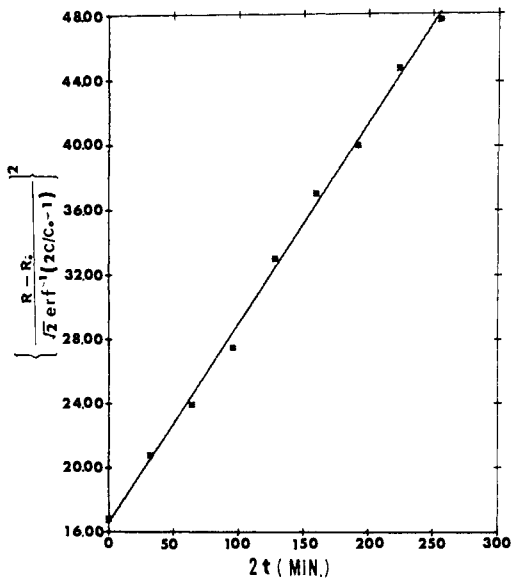

Figure 5. Plot of $[(r - r_i)/(2)^{1/2}erf^{-1}(2C/C_0 - 1)]^2$ as a function of time. The diffusion coefficient is the slope of the least-squares line. The concentration of phosphatidylcholine vesicles used in this experiment was 4.7 mM lipid phosphorus.

lated values of $(2)^{1/2}erf^{-1}(2C/C_0 - 1)$, and column 5 gives the apparent diffusion coefficients. Based on these data, a straight line was obtained for the plot of $(2)^{1/2}erf^{-1}(2C/C_0 - 1)$ against r, Figure 4, indicating the Gaussian character of the boundary curve. The apparent diffusion coefficients calculated from data points across the boundary are also presented in the same figure. A few values of D near the center of the boundary curve can be found which deviate visibly from a smooth line through all the other values. These deviations are expected, because the term $(2C/C_0 - 1)$ becomes extremely sensitive to the accuracy of C since $2C$ is approximately equal to C_0 as the data points approach the center of the boundary curve. An averaged value of 1.98×10^{-7} cm^2 sec^{-1} can be calculated as the averaged apparent diffusion coefficient of the vesicle by

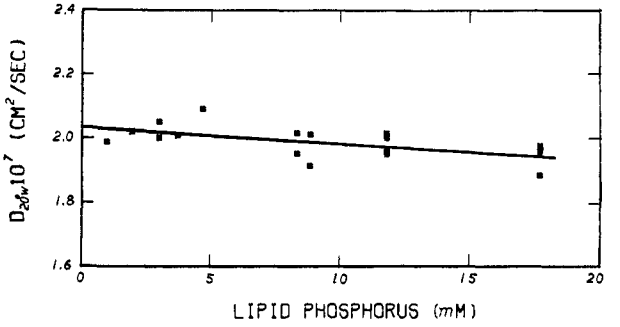

Figure 6. Plot of the diffusion coefficient, $D_{20,w}$, of phosphatidylcholine vesicles *vs.* concentration.

taking account of all the data points. This is obtained by multiplying the value of $1/2t$ by the square of the inverse slope of the least-squares line best fitted to all data points across the boundary for the plot of $(2)^{1/2}$. $erf^{-1}(2C/C_0 - 1)$ against r as shown in Figure 4. It should, however, be noted at this point that the method of calculations described here is similar, in principle, to the probability paper method reported by Mommaerts and Aldrich,[15] and Markham and Reichmann cited in Markham.[3] As we have already emphasized earlier, in the method described here one takes a minimum of 50 microcomparator readings with great care along a single fringe and utilizes a computer program to calculate the value of D at each radial position as well as the value of an averaged D obtained by a least-squares fit of all the data points. It is, therefore, reasonable to conclude that the method reported here is more accurate, although more tedious, than the probability paper method in which the value of D is essentially determined at two points (16 and 84%) on the arithmetical probability paper.

Since the interference patterns are always recorded as a function of t, the value of D can thus be calculated from the slope of a plot of

$$\left[\frac{r - r_i}{(2)^{1/2}erf^{-1}(2C/C_0 - 1)}\right]^2$$

the square of the inverse slope of the linear least-squares line shown in Figure 4, against $2t$. The diffusion coefficient obtained by this plot is more reliable than the ones obtained from a single fringe at a fixed t presented above (Table II), because the value of D calculated from the t function is a real averaged diffusion coefficient since the zero time correction can easily be shown to be unnecessary. An average value of D obtained from a representative plot, Figure 5, was 2.07×10^{-7} cm^2 sec^{-1}.

The concentration dependence of diffusion coefficients is shown in Figure 6. Below the concentration of 5.0 μmol ml^{-1}, values of D were calculated from data obtained with Rayleigh interference optics using the method illustrated in Figure 5. The schlieren optical system was employed for higher concentrations. All values of D were reduced to standard values in water at 20°, $D_{20,w}$. As can be seen in Figure 6, diffusion coefficients are slightly concentration dependent with the data best fitted by least-squares to the expression $D_{20,w} = D^\circ_{20,w}(1 - 0.0024P_l)$, where $D^\circ_{20,w}$, the diffusion

(15) W. F. H. M. Mommaerts and B. B. Aldrich, *Biochim. Biophys. Acta*, **28**, 627 (1958).

coefficient extrapolated to infinite dilution, was found to be 2.03 ± 0.04 × 10^{-7} cm^2 sec^{-1}. The Stokes' radius of the phosphatidylcholine vesicle can be calculated, based on the Stokes–Einstein relation ($a = RT/N6\pi\eta D$), to be 105 ± 4 Å. This result is not inconsistent either with our earlier preliminary observation (a = 114 ± 3 Å)[10] or with the recent conclusion of others (120 ± 4 Å).[16]

The effective specific volume, ϕ', of phosphatidylcholine vesicles in 0.1 *M* KCl–0.01 *M* Tris can be calculated from the reciprocal of the isodensity point (Figure 2) as 0.9848 ± 0.0007 ml g^{-1}. Using this value, together with the new value of the diffusion coefficient and the reported value of the sedimentation coefficient ($S^\circ_{20,w}$ = 2.63*S*) determined in 0.1 *M* KCl–0.01 *M* Tris (Table I), the vesicle weight of phosphatidylcholine in this medium can be calculated from the relation $M = RTS/(1 - \phi'\rho)D$ to be 1.86 ± 0.18 × 10^6 daltons. In this calculation, D_2O–H_2O is considered to be a one-component system in which preferential binding of the different forms of water to phosphatidylcholine vesicles has been neglected.[9] This value of vesicle weight is fully consistent with our earlier reported value (2.06 ± 0.05 × 10^6 daltons)[10] obtained in 0.1 *M* NaCl–0.01 *M* Tris.

(16) S. M. Johnson, A. D. Bangham, M. W. Hill, and E. D. Korn, *ibid.*, **233**, 820 (1971).

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Communications to the Editor

Absolute Configuration of Natural (+)-Abscisic Acid

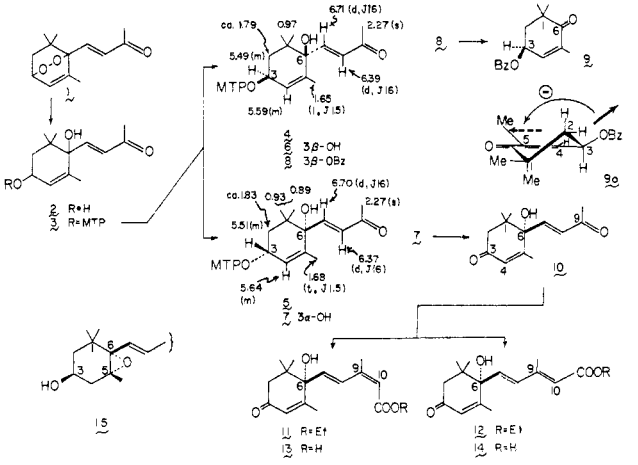
Sir:

The absolute configuration proposed[1] for the plant growth regulator (+)-abscisic acid (ABA)[2] (6β-OH in **13**) and optical data[2,3] appeared to be in conflict with the predicted chiral interaction between its two chromophores. In fact, it was reported that either the configuration of (+)-ABA or violaxanthin required revision;[4] on the other hand, two recent publications[5,6] implied revision of the (+)-ABA configuration.

The evidence presented below establishes the absolute configuration of natural (+)-ABA to be **13**[7] and violaxanthin to be **15** (only the pertinent portion is shown). We also describe preparation of ABA enantiomers employing high speed liquid chromatography (lc) of (+)-α-methoxy-α-trifluoromethylphenyl acetates (MTP esters)[8] **3** for resolution.

Hydrogenation of peroxide **1**[9] (Lindlar's catalyst) gave in quantitative yield the *dl-cis*-diol **2**,[10] which was converted into the diastereomeric MTP esters **3** (96%) with (+)-MTP acetyl chloride (see Scheme I).[8] The

Scheme I



crude mixture **3** (300 mg) was successfully separated into 120 mg each of diastereomers **4** and **5** by four recycles through a lc column[11] (3 × 3 ft) of Porasil T with 1% *i*-PrOH in hexane.

The less polar ester **4** was hydrolyzed to the diol **6** in 86% yield with 5% KOH in H_2O–MeOH (1:3, v/v) at room temperature. Oxidation of benzoate **8** with the same amount of $KMnO_4$ for 1 hr in acetone–piperidine (2:1, v/v) gave 6% of the enone benzoate **9** (or **9a**) [mass spectrum 258.1255 (calcd for $C_{16}H_{18}O_3$, 258.1256); ir ($CHCl_3$), 1714, 1677 cm^{-1}; nmr ($CDCl_3$) δ 1.25 (s, 6 H, 1-Me), 1.86 (t, 3 H, J = 1.5 Hz, 5-Me), 2.10 (d of d, 1 H, J = 9, 13 Hz, 2β-H), 2.31 (d of d of d, 1 H, J = 1.5, 6, 13 Hz, 2α-H), 5.89 (m, 1 H, $W_{1/2}$ = 17 Hz, 3-H), and 6.65 ppm (sextet, 1 H, J = 1.5 Hz, 4-H)], and 41% of

(1) J. W. Cornforth, W. Draber, B. V. Milborrow, and G. Ryback, *Chem. Commun.*, 114 (1967).
(2) P. F. Wareing and G. Ryback, *Endeavour*, **29**, 84 (1970).
(3) B. V. Milborrow, *Planta*, **76**, 93 (1967).
(4) R. S. Burden and H. F. Taylor, *Tetrahedron Lett.*, 4071 (1970).
(5) S. Isoe, S. Be Hyeon, S. Katsumura, and T. Sakan, *ibid.*, 2517 (1972).
(6) T. Oritani and K. Yamashita, *ibid.*, 2521 (1972).
(7) According to the presently accepted nomenclature for assigning *R* and *S* configurations in carotenoids, the revised configuration at C-6 of (+)-ABA **13** is *S*. See: R. S. Cahn, C. K. Ingold, and V. Prelog, *Angew. Chem., Int. Ed. Engl.*, **5**, 385 (1966).
(8) A recently developed reagent for judging optical purity of secondary alcohols by nmr: J. A. Dale, D. L. Dull, and H. S. Mosher, *J. Org. Chem.*, **34**, 2543 (1969). The MTP ester was used for separation of diastereomers by Dr. D. A. Schooley in connection with insect juvenile hormone studies [*cf.* K. Nakanishi, D. A. Schooley, M. Koreeda, and J. Dillon, *Chem. Commun.*, 1235 (1971)].
(9) M. Mousseron-Canet, J. C. Mani, J. P. Dalle, and J. L. Olive, *Bull. Soc. Chim. Fr.*, 3874 (1966).
(10) Spectroscopic data of all compounds prepared were in agreement with their structures.
(11) A Waters ALC-100 high-speed liquid chromatograph was employed.