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Interactions of Phosphatidylcholine Vesicles with 2-*p*-Toluidinylnaphthalene-6-sulfonate†

Ching-hsien Huang* and James P. Charlton

ABSTRACT: The interaction of 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) with phosphatidylcholine vesicle, a closed shell-like sphere with a continuous bilayer surrounding a volume of solvent, was studied by the gel filtration method. Thermodynamic parameters were estimated from the temperature dependence of the apparent intrinsic association constant from 5 to 40°. In the presence of spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl the binding affinity of TNS to phosphatidylcholine vesicle was decreased slightly, whereas the maximum number of equivalent binding sites was unaffected. These data suggest that TNS molecules are most likely located in a geometrically restricted region near the interface between the hydrocarbon core and the polar head region of the lipid bilayer and that the phosphatidylcholine vesicle–TNS binding is of the "nonclassical" hydrophobic type. Sedimentation velocity analysis revealed that the sedimentation coefficient of the phosphatidylcholine vesicle–TNS complex increased linearly as a function of $\bar{r}$, the ratio of moles of bound TNS to moles of phosphatidylcholine, over the TNS concentration range studied. These results provide evidence to argue against gross conformational change of the vesicle due to TNS binding.

The fluorescent compounds ANS[1] and TNS have been widely used as hydrophobic probes to investigate the conformational changes in proteins (Edelman and McClure, 1968; Stryer, 1968) and more recently the structural changes in biological membranes (Tasaki *et al.*, 1968; Azzi *et al.*, 1969). In view of the fact that phospholipids are major components of most biomembranes, and that the hydrophobic core of the phospholipid bilayer seems likely to be the binding site for these hydrophobic probes, a study of the phospholipid–probe interaction can provide information of value for the interpretation of data obtained from probe studies on biological membranes.

This communication describes studies of the binding of TNS to phosphatidylcholine vesicles using the gel filtration method of Hummel and Dreyer (1962). The stoichiometry and apparent intrinsic association constant of the phosphatidylcholine vesicle–TNS complex formation were measured at various temperatures, and were also determined in the presence of the electron spin-label Tempo. These binding results were further correlated with hydrodynamic studies of the phosphatidylcholine vesicle–TNS complex in an attempt to reach a consistent picture of the structure of the complex. The advantage of using the spherical phosphatidylcholine vesicle as a membrane model system stems from its well-characterized properties (Huang, 1969; Huang *et al.*, 1970; Huang and Charlton, 1971).

Methods

Materials. The phosphatidylcholine was isolated from hen egg yolk by the column chromatographic method (Huang, 1969). The purity of the preparation was checked by thin-layer chromatography (Skipski *et al.*, 1964). Tempo was prepared according to the method of Rozantzev and Neiman (1964). TNS and Tris were supplied by Sigma Chemical Co., St. Louis, Mo. TNS solutions of desired concentration were prepared in 0.1 M KCl–0.01 M Tris, adjusted to pH 8.0.

Preparation of Phosphatidylcholine Vesicles. The procedure for the preparation of phosphatidylcholine vesicles was a modification of that described previously (Huang, 1969). Lyophilized phosphatidylcholine (300–400 mg) was suspended in 8 ml of buffered 0.1 M KCl solution (0.1 M KCl in 0.01 M Tris buffer) at pH 8.0. The suspension was ultrasonically irradiated (20 kHz) under nitrogen at 2° for 2.5 hr and then centrifuged at 105,000*g*, at 4°, for 60 min. The resulting supernatant was first concentrated to about 2 ml in a Sartorius collodion bag and then was subjected to upward-flow gel filtration, at 4°, on a column (25 × 50 cm) of Sepharose 4B which had been previously equilibrated with the same buffered KCl solution. The elution pattern of the phosphatidylcholine dispersion consists of two distinct peaks (fractions I and II). Those portions of fraction II which show a linear relation between absorbance at 300 nm and lipid phosphorous content and for which the linear regression line passes through the origin were used in all studies. Phosphatidylcholine concentrations in vesicle solutions are expressed in terms of lipid phosphorous (P_i) as determined spectrophotometrically (Huang, 1969). Employing the modified procedure, homogeneous vesicle fractions collected directly from the column effluent were concentrated enough for our purpose so that the final ultrafiltration step reported earlier could be eliminated.

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received October 25, 1971.* This research was supported by U. S. Public Health Service Grants GM-17452 and GM-14628.

* To whom correspondence should be addressed.

[1] Abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl.

Gel Filtration Studies. The binding of TNS by phosphatidylcholine vesicle at various temperatures was studied by the gel filtration method of Hummel and Dreyer (1962). The gel filtration column, equipped with a constant-temperature water jacket, was connected to a Zeiss MZ2D microflow cell. The outflow tube of the microflow cell was connected to a peristaltic pump (Holter pump, Model RL175) which could be adjusted to give the desired flow rate (0.3–0.5 ml/min). A Zeiss PMQII spectrophotometer equipped with the automatic sample changer and a multipoint recorder was used to monitor successively four microflow cells. In most cases, two pairs of columns equilibrated with two desired concentrations of free ligand were measured simultaneously. The column (1.0 × 30 cm) was first packed with Sephadex G-50 (medium) and then saturated with 0.1 M KCl–0.01 M Tris solution (pH 8.0) containing the desired concentration of TNS until a stable background absorbance corresponding to that of the free ligand (TNS) was obtained. Samples of 75–300 μl containing 1.3–4.5 μmoles of P_i in 0.1 M KCl–0.01 M Tris (pH 8.0) were applied to the top of the column with a micropipet (H. E. Petersen, Denmark). When the sample had entered the column bed, it was rinsed and followed by buffered ligand solution containing the same TNS concentration used in the column equilibration. The column effuent was continuously monitored at 317 nm. The resulting elution profile contains a peak and trough separated by a plateau region of the constant-background absorbance. The bound TNS migrates with the excluded phosphatidylcholine vesicle–TNS complex to produce a peak in excess of its background concentration. For mass to be conserved this excess must be matched by a corresponding deficiency, the trough, at the elution volume of the TNS. The trough, however, has to be corrected by the dilution factor resulting from the application of sample volume. The number of moles bound can thus be calculated from eq 1, where A is the area of the trough (in.^{2}) measured

$$\text{moles of bound ligand} = (\text{trough ligand deficiency}) - (\text{deficiency loaded}) = \left(\frac{AF}{ab \times \epsilon \times 10^3}\right) - (cv) \quad (1)$$

with a planimeter, F is the constant flow rate (ml/min) of the column, a is the pen excurison in inches per unit change in absorbance for a 1-cm optical path, b is the chart rate (in./min) of the recorder, ϵ is the molar extinction coefficient of buffered TNS solution at 317 nm, c is the concentration (moles/l.) of the free ligand, *i.e.*, the concentration of TNS with which the column was saturated, and v is the volume (l.) of the vesicle sample.

The molar extinction coefficient for TNS in the pH 8.0, buffered 0.1 M KCl solution was found to be 1.88×10^4, in agreement with the literature value (Wong and Edelman, 1971). Beers Law was checked for the TNS solution over the concentration range studied and no significant deviations were observed.

For determination of the binding parameters of phosphatidylcholine–TNS associations, the basic Scatchard equation, $\bar{\nu}/c = k(n - \bar{\nu})$, derived from the law of mass action was used (Scatchard, 1949), where $\bar{\nu}$ is the mole of ligand (TNS) bound per mole of lipid phosphorous, c is the concentration of the free ligand, n is the apparent maximum number of binding sites, and k is the apparent association constant.

Competitive Binding Studies. The TNS binding equilibrium at pH 8.0, 20°, in the presence of constant concentration (6×10^{-5} M) of spin-label Tempo was also studied by the zonal method of Hummel and Dreyer (1962). The Sephadex G-50 medium column was first saturated with 0.1 M KCl–0.01 M Tris–6×10^{-5} M Tempo containing desired concentration of TNS, samples of phosphatidylcholine vesicles were then applied to the column. Column elution was monitored continuously at 317 nm. Since the absorbance at 317 nm of Tempo at the concentration under study was negligible, the amount of TNS bound by the phosphatidylcholine vesicle could be determined from eq 1 by measuring the area of the depletion trough as described in the previous section on gel filtration studies.

Sedimentation Velocity Measurements. In order to determine s^0 for the phosphatidylcholine vesicle–TNS complex, an extrapolation to zero complex concentration is necessary. The extrapolated value is meaningful only if the composition of the complex is constant. From the Scatchard equation it can be easily shown that $\bar{\nu}$ is constant provided c, the concentration of free ligand, is constant. It is also apparent that the complex is at equilibrium with the free ligand in the Hummel and Dreyer experiments provided that the peak of the complex in the elution diagram is clearly separated from the trough by a plateau region. The eluted fractions from the phosphatidylcholine vesicle–TNS complex peak of a Hummel and Dreyer experiment have equal $\bar{\nu}$ but different lipid concentrations. Thus, these fractions can be used to determine the sedimentation coefficient of the complex at infinite dilution with a constant value of $\bar{\nu}$. In sedimentation velocity experiments, the concentrations of the complex usually ranged from 2 to 12 μmoles of P_i per ml. The highest concentration was obtained from the central fraction of the peak from a modified Hummel and Dreyer experiment as follows. Concentrated vesicle sample (0.3–0.5 ml; P_i, ~40 μmoles/ml) was loaded on a water-jacketed column immediately following the addition of 2 ml of ligand solution with a molar concentration corresponding to the amount of TNS bound by the concentrated sample. This concentration of TNS can be calculated from the known binding isotherm. The column (10 mm × 30 cm) was packed with Sephadex G-50 which had been exhaustively washed and saturated with the desired concentration of free-ligand solution. When the concentrated vesicle sample had entered the column bed, it was rinsed and connected to a reservoir containing the same concentration of free ligand as that with which the column was initially saturated. The absorption of column effluent at 317 nm was continuously recorded. The effluent solution, with a constant flow rate as controlled by a peristaltic pump, was collected in eight small fractions (approximately 0.5 ml each) covering the whole elution peak.

Sedimentation velocity studies were carried out at 20 ± 0.05° in a Beckman-Spinco Model E analytical ultracentrifuge, equipped with a schlieren optical system fitted with a phase plate and a RTIC temperature-control unit. The An-D rotor, operated at 42,040 rpm, was employed; a double-sector, capillary-type synthetic boundary cell with a 12-mm optical path was used in all the experiments. The boundary was formed by layering TNS solution of equilibrium concentration over the phosphatidylcholine vesicle–TNS complex collected from individual fractions across the elution peak obtained in the modified Hummel and Dreyer experiments. The sedimentation coefficient was calculated from the least-squares slope of a curve 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.

TABLE I: Results of TNS Binding Studies by the Gel Filtration Method of Hummel and Dreyer.

T (°C)	c Concn (M × 10^5)	Bound TNS (Mole × 10^7)	Lipid P_i (Mole × 10^6)	$\bar{\nu}$ (× 10^2)	$\bar{\nu}/c$ (× 10^{-3})	T (°C)	c Concn (M × 10^5)	Bound TNS (Mole × 10^7)	Lipid P_i (Mole × 10^6)	$\bar{\nu}$ (× 10^2)	$\bar{\nu}/c$ (× 10^{-3})
5	0.500	0.643	1.85	3.47	6.94	25	0.500	0.319	1.88	1.70	3.39
5	0.500	0.650	1.85	3.50	7.01	25	0.500	0.335	1.88	1.78	3.56
5	1.50	2.05	2.96	6.91	4.60	25	1.50	0.656	1.88	3.49	2.32
5	1.50	1.26	1.81	6.95	4.64	25	1.50	0.682	1.88	3.63	2.42
5	1.50	1.27	1.81	7.01	4.67	25	3.00	1.06	1.88	5.63	1.88
5	3.00	2.81	2.82	9.96	3.32	25	3.00	1.03	1.88	5.46	1.82
5	6.00	1.92	1.55	12.36	2.06	25	6.00	1.91	2.43	7.86	1.31
5	6.00	1.87	1.55	12.02	2.00	25	6.00	1.92	2.43	7.91	1.32
10	0.500	0.560	1.85	3.03	6.06	30	0.500	0.498	3.71	1.34	2.69
10	0.500	0.566	1.85	3.06	6.12	30	0.500	0.480	3.71	1.30	2.59
10	1.50	0.904	1.48	6.10	4.07	30	1.50	0.635	1.96	3.23	2.15
10	1.50	0.908	1.48	6.13	4.09	30	3.00	1.39	2.82	4.92	1.64
10	3.00	2.53	2.82	8.97	2.99	30	3.00	1.37	2.82	4.87	1.62
10	3.00	2.53	2.82	8.97	2.99	30	6.00	1.03	1.55	6.64	1.11
10	6.00	1.75	1.55	11.24	1.87	30	6.00	1.01	1.55	6.54	1.09
10	6.00	1.71	1.55	11.01	1.83	35	0.500	0.479	4.94	0.968	1.94
15	0.500	0.494	1.85	2.67	5.33	35	0.500	0.474	4.94	0.958	1.92
15	0.500	0.488	1.85	2.63	5.26	35	0.500	0.303	3.01	1.01	2.01
15	1.50	1.18	2.26	5.23	3.48	35	1.50	0.708	3.01	2.35	1.57
15	1.50	1.18	2.26	5.24	3.49	35	1.50	0.740	3.01	2.46	1.64
15	3.00	2.15	2.82	7.63	2.54	35	1.50	0.710	3.01	2.36	1.57
15	3.00	2.11	2.82	7.50	2.50	35	3.00	1.08	2.82	3.83	1.28
15	6.00	1.56	1.55	10.02	1.67	35	3.00	1.12	2.82	3.99	1.33
15	6.00	1.60	1.55	10.29	1.72	35	6.00	1.72	2.91	5.92	0.987
20	0.500	3.86[a]	17.82	2.16	4.33	35	6.00	1.69	2.91	5.81	0.968
20	0.500	3.78[a]	17.82	2.12	4.24	40	0.500	0.421	4.94	0.851	1.70
20	1.00	7.00[a]	18.77	3.73	3.73	40	0.500	0.412	4.94	0.834	1.67
20	1.50	5.60[a]	12.40	4.52	3.01	40	1.50	0.556	2.96	1.88	1.25
20	1.50	5.82[a]	13.02	4.47	2.98	40	1.50	0.615	2.96	2.08	1.39
20	2.00	6.79[a]	12.33	5.51	2.75	40	1.50	0.872	4.52	1.93	1.29
20	3.00	8.38[a]	12.33	6.80	2.26	40	1.50	0.929	4.52	2.06	1.37
20	3.00	1.43	2.26	6.32	2.11	40	3.00	1.03	2.82	3.67	1.22
20	3.00	1.46	2.26	6.47	2.16	40	3.00	0.999	2.82	3.55	1.18
20	6.00	1.38	1.55	8.88	1.48	40	6.00	1.58	2.91	5.43	0.905
20	6.00	1.36	1.55	8.77	1.46	40	6.00	1.55	2.91	5.32	0.886

[a] From 3.2×10^{-7} to 5.7×10^{-7} mole excess TNS preloaded on columns.

Results

Gel Filtration Studies. Figure 1 is a representative elution pattern for the Hummel and Dreyer type experiment which shows the elution peak of the phosphatidylcholine vesicle–TNS complex, the TNS depletion trough, and the free-ligand plateau region separating the trough from the peak. The results of the gel filtration studies on the phosphatidylcholine vesicle–TNS system are given in Table I, where $\bar{\nu}$ is the number of moles of TNS bound (calculated from eq 1) per mole of lipid phosphorus (P_i), and c is the concentration of free ligand. At each of the temperatures used determinations were made of $\bar{\nu}$ at a minimum of four concentrations (in the range of 5×10^{-6} to 6×10^{-5} M) and experiments were duplicated for each concentration of c.

The results of the gel filtration studies on the phosphatidylcholine vesicle–TNS system are also shown in the Scatchard plots, $\bar{\nu}/c$ *vs.* $\bar{\nu}$, of Figure 2. The linearities of the $\bar{\nu}/c$ *vs.* $\bar{\nu}$ plots were satisfactory, for the linear correlation coefficients of the least-squares lines for the Scatchard plots were around 0.994 ± 0.005. The maximum number of equivalent TNS binding sites per P_i of vesicles, n, and the apparent intrinsic association constant, k, can be estimated from the plots and the results are presented in Table II.

Since the anhydrous vesicle weight of the phosphatidylcholine vesicle is 2.13×10^{-6} dalton (Huang and Charlton, 1971), and the average molecular weight of the egg phosphatidylcholine is 770, the number of phosphatidylcholine molecules per vesicle is 2766. The maximum number, n, of equivalent TNS binding sites per mole of P_i can thus be converted to the maximum number of equivalent TNS binding sites per *vesicle* by multiplying n by 2766. For example, the maximum number of equivalent TNS binding sites per vesicle at 20° is approximately 324.

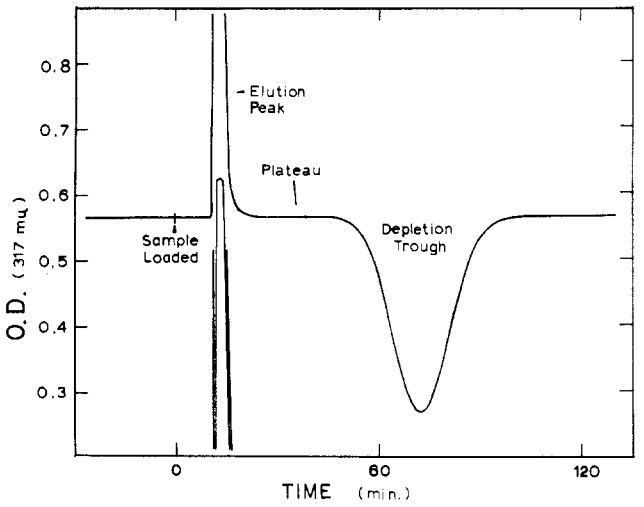

FIGURE 1: A representative elution profile of a Hummel and Dreyer experiment in which 2.26 × 10^{-6} mole of phosphatidylcholine vesicle (in terms of P_i) were placed on a Sephadex G-50 medium column (1.0 × 30 cm) equilibrated with 0.1 M KCl–0.01 M Tris–3.00 × 10^{-5} M TNS and the effluent monitored at 317 nm. The details of the experiment are given in the text.

Temperature Dependence of TNS Binding. The temperature dependence of the binding by phosphatidylcholine vesicles was studied at eight different temperatures ranging from 5 to 40°. A van't Hoff plot of ln k *vs.* T^{-1}, presented in Figure 3, is characterized by a gradual change in slope around 15°. If a linear function is assumed, a least-squares treatment of the experimental data obtained between 20 and 40° gives a $\Delta H'$ (the apparent standard enthalpy change), according to Gibbs–Helmholtz's equation, of −9.5 kcal/mole for binding of the TNS to phosphatidylcholine vesicle. At 25°, the $\Delta G'$ (the apparent standard free energy) is −6.2 kcal/mole, yielding an apparent entropy contribution of −11.1 eu/mole.

Competitive Binding Studies. The electron paramagnetic resonance studies of spin labels with phospholipid vesicles have indicated that Tempo molecules do partition into the hydrophobic region of the vesicle (Hubbell and McConnell, 1968). Since TNS molecules also interact with the vesicle as shown by the gel filtration experiment, it seemed of interest, therefore, to examine the binding of phosphatidylcholine–TNS system in the presence of Tempo.

If there exists a unique binding region in the phosphatidylcholine vesicle for the Tempo molecule, and if this binding region or equivalent binding site is the same as the equivalent binding site for the TNS molecule, then the Scatchard equation for the competitive binding, a modification of the equation derived by Klotz *et al.* (1948), is $\bar{\nu}_1/c_1 = [k_1/(1 + k_2c_2)] \times (n - \bar{\nu}_1) = k^*(n - \bar{\nu}_1)$. Thus, if $\bar{\nu}/c$ *vs.* ν, plotted for simple binding, is compared to a similar plot for binding in the presence of a competitor, the line should have the same intercept on the $\bar{\nu}$ axis but the competitive plot should have a smaller slope. The results of the competitive binding studies are summarized in Figure 4. Within the experimental accuracy, some Tempo molecules appear to compete with TNS for binding to the same equivalent site in the vesicle. This is indicated by a very small decrease in the slope of the Scatchard plot (Figure 4) with no change in the intercept on the $\bar{\nu}$ axis.

Sedimentation Velocity Measurements. The extrapolated value of the sedimentation coefficient of the phosphatidylcholine–TNS complex at infinite dilution, s^0_c, at constant $\bar{\nu}$

TABLE II: Binding Parameters for the Interaction of TNS with Phosphatidylcholine Vesicle Obtained from Scatchard Plots.

T (°C)	n	$k \times 10^{-4}$ (M^{-1})
5	0.1561 ± 0.0034 (SD)[a]	5.56 ± 0.24 (SE)[b]
10	0.1456 ± 0.0034	5.11 ± 0.24
15	0.1301 ± 0.0054	4.73 ± 0.38
20	0.1170 ± 0.0040	4.29 ± 0.25
25	0.1090 ± 0.0059	3.38 ± 0.37
30	0.1039 ± 0.0014	2.92 ± 0.09
35	0.1043 ± 0.0027	1.99 ± 0.11
40	0.1021 ± 0.0053	1.53 ± 0.19

[a] Standard deviation. [b] Standard error.

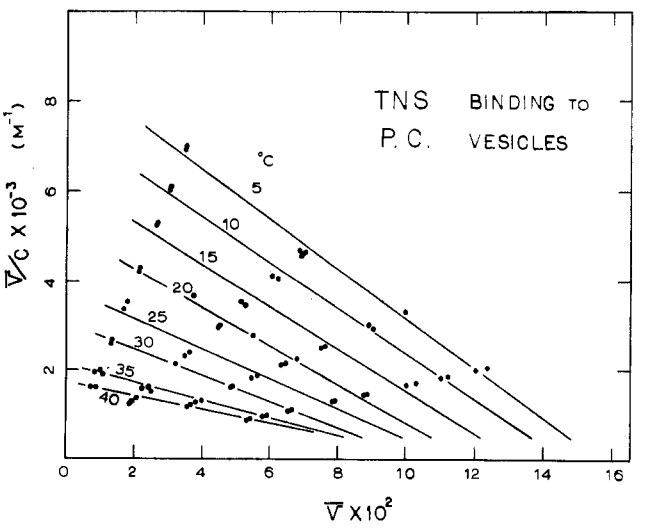

FIGURE 2: Scatchard plots of binding of TNS to the phosphatidylcholine vesicle. Data points, calculated from the trough areas of elution profiles according to eq 1, were taken from Table I.

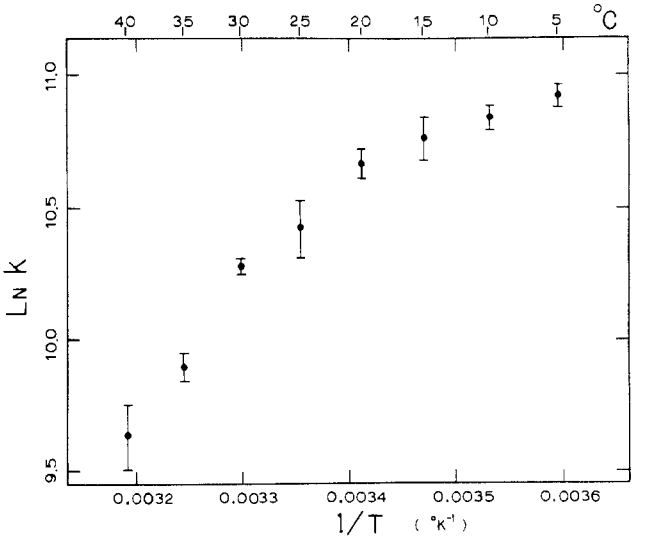

FIGURE 3: Temperature dependence of the binding of TNS by phosphatidylcholine vesicle from 5 to 40°. The vertical lines at each data point denote the standard error for that value of ln k.

TABLE III: Summary of Sedimentation Results of the Phosphatidylcholine Vesicle–TNS Complex Obtained at Varied Values of $\bar{\nu}$.[a]

Concn (M × 10^5)	$\bar{\nu}$	s^0	s_c^0	$\Delta s^{0b}/s^0$
0.0	0.0000	1.84 ± 0.09		0
0.5	0.0214		2.11 ± 0.03	0.15 ± 0.05
1.0	0.0373		2.49 ± 0.025	0.35 ± 0.05
1.5	0.0454		2.76 ± 0.04	0.50 ± 0.06
2.0	0.0551		2.84 ± 0.05	0.54 ± 0.06
3.0	0.0680		2.94 ± 0.04	0.60 ± 0.06

[a] The values of $\bar{\nu}$ were obtained by the gel filtration method of Hummel and Dreyer described in the text. [b] $\Delta s^0 = s_c^0 - s^0$ in Svedberg unit.

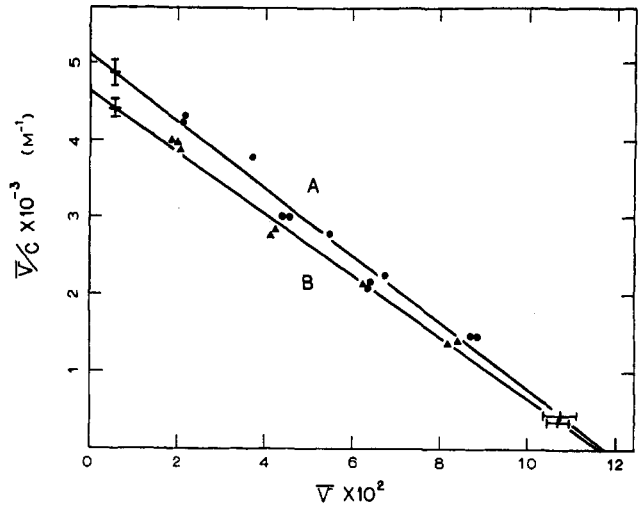

FIGURE 4: A comparison of the binding of TNS by phosphatidylcholine vesicle, at 20°, in the absence (curve A) and presence (curve B) of 6 × 10^{-6} M Tempo. The binding parameters of curve A are n = 0.117 ± 0.004 (standard deviation) and k = 4.29 ± 0.25 (standard error) × 10^4 M^{-1}, compared to n = 0.116 ± 0.003 and k^* = 3.98 ± 0.17 × 10^4 M^{-1} for curve B.

was obtained from the intercept of the least-squares line derived from the s *vs.* [P_i] plot. The effect of addition of TNS on the sedimentation coefficient of the phosphatidylcholine vesicle is summatized in Table III. Here, the relative changes in sedimentation coefficients ($\Delta s^0/s^0$) increases linearly with $\bar{\nu}$, the mole ratio of bound TNS to phosphatidylcholine in vesicles. Δs^0 is the difference between the sedimentation coefficient of the complex, s_c^0, at constant $\bar{\nu}$ and that of the vesicle, s^0, determined in the absence of TNS. The linear regression line of the plot, $\Delta s^0/s^0$ *vs.* $\bar{\nu}$, as obtained by the method of least squares, passes through the origin with a linear correlation coefficient of 0.9824. Quantitative interpretation of these results is deferred to Discussion section, but it should be clear that qualitatively the sedimentation results can be taken as evidence to argue in favor of insertion of TNS, which is more dense than lipid or water, into the phosphatidylcholine vesicle.

Discussion

The value of the free energy of phosphatidylcholine vesicle–TNS complex formation, $\Delta G' = -6.2$ kcal/mole, is of special interest, since the same value has been calculated as the net energy change for a nonpolar interaction of *n*-heptane with benzene in water (Munck *et al.*, 1957). The contributions to the relatively strong binding energy of the phosphatidylcholine vesicle–TNS interactions have not yet been delineated, but the important fact that apparent standard free energy, enthalpy, and entropy of the complex formation are all negative strongly suggests that the association is of the "nonclassical" hydrophobic type interaction (Jencks, 1969). From the amphipathic character of TNS, one can speculate that TNS molecules are most likely to be located at the interfacial region perturbed by the hydrocarbon–water interface but portions of the TNS molecules may even be embedded deep into the hydrocarbon region of the phospholipid bilayer structure. Some water molecules originally present in the polar head region of the bilayer might thus be replaced by the TNS molecules. Likewise, some water molecules associated with TNS molecules in the aqueous environment must also be expelled because of the partial insertion of TNS molecules into the apolar hydrocarbon core of the bilayer structure. Thus, the main driving force for the phosphatidylcholine vesicles–TNS associations appears to be derived directly from the favorable enthalpy of recombination of water molecules which are displaced from the interacting portions of TNS and the vesicle wall. Similar small molecule–macromolecule associations with sizable negative changes in free energy, enthalpy, and entropy have been recorded for the interaction of the tryptophan derivatives with bovine serum albumin (Fairclough and Fruton, 1966).

Tempo was the first spin label used for the study of phospholipid vesicles and biomembranes (Hubbell and McConnell, 1968). It was shown that Tempo is associated with the long hydrocarbon chains of the phospholipid vesicle and undergoes a rapid and effectively isotropic motion in this hydrophobic region, with a correlation time of approximately 10^{-10} sec. Jendrasiak and Hayes (1970) showed that Tempo in the phosphatidylcholine vesicle can be displaced by addition of I_3^- and that benzene exhibits a barely noticeable effect on the electron spin resonance signal of the phosphatidylcholine–Tempo complex. Based on the spin-label experiment it was suggested that the spin label, Tempo, is located in the hydrocarbon region and also associated with the polar choline group of the bilayer (Jendrasiak and Hayes, 1970). Our competitive binding studies suggest that some Tempo molecules occupy the same equivalent TNS-binding site in the phosphatidylcholine vesicle. From the amphipathic nature of TNS, the sulfonate group of TNS may reasonably be considered to be located in the polar head region of the bilayer with its negative charge close to the quaternary nitrogen of choline, wheras the nonpolar toluidinyl group may be embedded into the outer part of hydrocarbon region of the bilayer. One can thus conclude that Tempo competes with TNS for the binding sites which are geometrically restricted to the neighborhood of the interface between the hydrocarbon core and the polar region of the bilayer.

The conclusion of the following analysis of the sedimentation velocity data for the phosphatidylcholine–TNS complex is that the mass per unit volume of the bilayer is increased by complex formation, which, in turn, can be taken as evidence to argue in favor of insertion of TNS into the bilayer of the phosphatidylcholine vesicle.

The sedimentation coefficient of the phosphatidylcholine–

TNS complex at infinite dilution in medium of constant $\bar{\nu}$ is given by

$$s_c^0 = M_c(1 - \phi_c'\rho)/Nf_c \quad (2)$$

where M_c is the molecular weight of the complex, ϕ_c' is the apparent specific volume of the complex, f_c is the frictional coefficient, ρ is the density of the solvent and N the Avogadro's number. With the assumption that both the molecular weight and apparent specific volume of TNS (M_{TNS} and ϕ'_{TNS}) and phosphatidylcholine vesicle (M and ϕ') are additive in the complex, we can then write

$$M_c = M + M_{TNS}\bar{\nu}K \quad (3)$$

$$\phi_c' = \frac{M\phi' + M_{TNS}\phi'_{TNS}\bar{\nu}K}{M + M_{TNS}\bar{\nu}K} \quad (4)$$

where $\bar{\nu}$ is the number of moles of bound TNS per mole of P_i, and K is a conversion factor that the product of $K\bar{\nu}$ is the number of moles of bound TNS per mole of phosphatidylcholine vesicle.

Substituting eq 3 and 4 into 2 and using $s^0 = M(1 - \phi'\rho)/Nf$ yields eq 5

$$s_c^0 = s^0\frac{f}{f_c}(1 + A\bar{\nu}) \quad (5)$$

$$A = K\frac{(1 - \phi'_{TNS}\rho)}{(1 - \phi'\rho)}\frac{M}{M_{TNS}}$$

Equation 5 can be transformed into eq 6

$$\frac{\Delta s^0}{s^0} = \frac{\Delta f}{f} + \frac{f}{f_c}A\bar{\nu} \quad (6)$$

where $\Delta s^0 = s_c^0 - s^0$ and $\Delta f = f - f_c$. The fact that the relative changes in extrapolated sedimentation coefficients, $\Delta s^0/s^0$, increase linearly with $\bar{\nu}$ (Table III) and that the linear regression line for the plot $\Delta s^0/s^0$ *vs.* $\bar{\nu}$ over the free-ligand concentration range studied (0.5×10^{-5} M– 6.0×10^{-5} M) passes through the origin indicates that Δf in eq 6 is either zero or a small varying function of $\bar{\nu}(\Delta f = af\bar{\nu})$. In either case, the smooth linear increase in sedimentation coefficient of phosphatidylcholine vesicles upon the addition of TNS over the concentration range studied cannot be attributed to a gross structural or conformational change of the vesicle. The increase in sedimentation coefficient of the system by the complex formation must, therefore, reflect essentially the increase in mass per unit volume of the bilayer. Since TNS is more dense than water or lipid, the most probable way the mass per unit volume of the bilayer can be increased when TNS is bound is that some of the bilayer region originally occupied by either water or lipid molecules be displaced by the more highly dense TNS molecules. Earlier work has demonstrated that there are sizable amounts of preferentially bound water associated with the vesicle, and has suggested that some of the preferentially bound water molecules may very well be present in the polar head region of the bilayer (Huang and Charlton, 1971). One region where TNS could cause an increase in mass per unit volume of the bilayer would thus be the polar head region of the vesicle, because the preferentially bound water molecules there can be displaced by the TNS molecules. It should be emphasized that this picture of the phosphatidylcholine–TNS complex is, indeed, consistent with our finding that the main driving force for the phosphatidylcholine–TNS complex formation is derived from the favorable enthalpy of recombination of water molecules which are displaced from the interacting surfaces.

Acknowledgments

We thank Dr. T. E. Thompson for helpful discussion throughout this work. Thanks are also due to Dr. Y. Toyoshima for his critical reading of the manuscript, and Dr. E. A. Dawidowicz for the preparation of Tempo.

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