THE BEHAVIOR OF OXONOL DYES IN PHOSPHOLIPID DISPERSIONS

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ABSTRACT The interaction of a class of oxonol dyes with sonicated phospholipid vesicles was followed optically. The spectra of vesicle-associated dyes resemble those found for the dyes in organic solvents, indicating that the oxonols occupy a hydrophobic region of the membrane. At equilibrium the affinity of the oxonols for the vesicles depends on the structure of the dye, the physical and chemical composition of the vesicles, and the ionic strength of the medium. The oxonols occupy soybean lipid vesicles to a level of 147.9 ± 17.1 nmol/mg lipid with a dye membrane dissociation constant of 3.33 ± 0.54 μM. The interaction of the oxonols with soybean lipid vesicles is biphasic. The fast phase has a second order rate constant of 9.04 ± 0.36 × $10^6 M^{-1} s^{-1}$ and the number of "fast" binding sites, 68 ± 8 nmol/mg lipid, was determined from the ratio of the second order rate constants obtained with lipid and with dye in excess. The dissociation of oxonols from soybean lipid vesicles is also biphasic, and the fast process has a rate constant of 17 ± 2 s^{-1}, yielding a dissociation constant for the fast sites (k_{-1}/k_2) of 1.88 ± 0.15 μM. The slow phases of oxonol association with, and release from, soybean lipid vesicles are not second order and have half times of between 0.2 and 5 min, depending on the physical and chemical composition of the membrane lipids. The amplitudes of the slow phases are sensitive to the composition of the aqueous media on each side of the vesicle membranes, which suggests that the slow processes represent the permeation of the membrane by the oxonols. The importance of the properties of the oxonol dyes in the interpretation of their behavior in natural membranes is discussed.

INTRODUCTION

Biological membranes possess many different biochemical activities which may interact in a very complex manner. Analysis of the functions of the membrane can be simplified by the observation of specific components which respond uniquely to particular biochemical events. These useful components are "probes" of membrane function and may either occur naturally, intrinsic probes, or have to be added to the membrane, extrinsic probes. The important criterion is that they have readily accessible physical characteristics sensitive to changes in their local environment.

Extrinsic probes offer greater flexibility than intrinsic probes of membrane function for two reasons. They are not limited by their natural location and can be applied to a variety of membrane systems with common biological properties. Secondly, extrinsic probes can be designed and synthesized to respond to particular features of their membrane environment (Radda, 1971). The disadvantage of extrinsic probes is that they necessarily perturb the situation that they are designed to observe.

Chance and co-workers (1974, 1975; Smith et al., 1976) have recently described a class of oxonol dyes that can be useful indicators of membrane activity in a wide variety of energy-transducing membranes. Oxonol dyes appear to be probes of transmembrane potential (Cohen et al., 1974; Waggoner, 1976; Ross et al., 1977; Waggoner et al., 1977) and have recently been used to monitor membrane potential during oxidative phosphorylation catalyzed by mitochondrial membranes (Bashford and Thayer, 1977). At another level, oxonols also appear to follow the electrical activity of tissues, for example the rat brain cortex during anoxia and spreading depression (Chance et al., 1976).

Oxonol and other optical probes of membrane function are valuable because either or both of their absorption and fluorescence reflect the function of the membrane to which they are bound. The relative ease with which optical properties can be monitored affords an instantaneous and continuous readout of membrane function during the course of the experiment. A complete interpretation of the behavior of optical probes in natural membranes will depend in part on an understanding of their properties within the membrane phase. In the present investigation the behavior of the oxonols has been studied in a simple model membrane system. The use of a model system allows the experimental variation of some of the factors which influence the oxonol-membrane interaction.

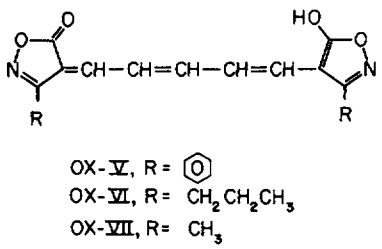
The model system employed was that of sonicated dispersions of phospholipid. In aqueous media containing dispersed phospholipids sonication generates small vesicles 250–500 Å in diameter, bounded by a single phospholipid bilayer (Huang, 1969; Berden et al., 1975). The advantage of such dispersions is that they are optically clear, which facilitates the analysis of the spectral properties of the oxonols when they occupy the lipid phase.

MATERIALS AND METHODS

The oxonol dyes OX-V, OX-VI, and OX-VII were synthesized as described by Smith et al. (1976). Their structures are illustrated in Fig. 1 and were confirmed by absorbance and nuclear magnetic resonance spectroscopy. The elemental analysis for the new probes OX-VI and OX-VII are given in the legend for Fig. 1 and are consistent with the proposed structures.

Soybean phospholipids (azolectin) partially purified by the method of Kagawa and Racker (1971) were the kind gift of Dr. P. Chein (Eastern Pennsylvania Psychiatric Institute, Philadelphia, Pa.). DL-α-dipalmitoyl lecithin, egg lecithin, and cholesterol were from Sigma Chemical Co. (St. Louis, Mo.), and all other chemicals were of the highest purity commercially available.

Soybean lipids containing the desired fraction by weight of added cholesterol were stored as a stock solution in chloroform under nitrogen at −20°C. Vesicles were prepared by evaporating an aliquot of the stock under a stream of nitrogen. The residue was dispersed in a small volume of diethyl ether and formed into a dry film by evaporation under nitrogen. Aqueous medium was added and liposomes were formed by agitation on a vortex mixer. The liposome suspension was sonicated either in a Branson sonifier cell disruptor model W185 (Branson Sonic Power Co., Danbury, Conn.) under medium power or in a Laboratory Supply Co. sonicator model G112 SP1 (Laboratory Supply Co., Indianapolis, Ind.). Sonication was considered complete when the turbidity of the vesicle suspension, monitored at 450 nm, had reached a minimum, which usually took 5–10 min in the Branson sonifier or up to 45 min in the Laboratory Supply Co. instrument. A typical time-course is shown in Fig. 2; the half time for the loss of turbidity was 25 s. Longer sonication times were required when cholesterol was present. Fractionation of the sonicated lipid suspensions at 23°C on a Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) column (Huang, 1969) indicated that <15% of the applied phospholipid eluted with the void volume, so that the majority of the lipid was in the form of unilamellar vesicles. The "large vesicle" fraction was not diminished



OX-V, R = ⬡
OX-VI, R = $CH_2CH_2CH_3$
OX-VII, R = CH_3

FIGURE 1

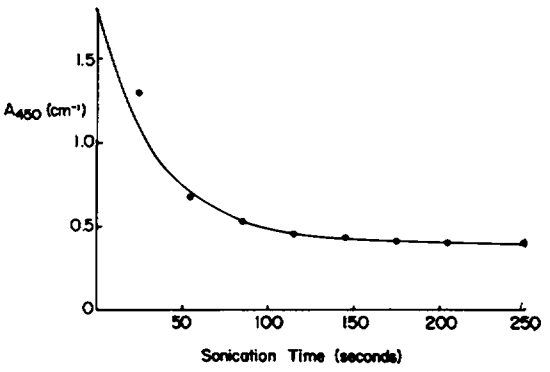


FIGURE 2

FIGURE 1 The structures of the oxonol dyes OX-V, OX-VI, and OX-VII. The dyes were synthesized and their structures determined as described by Smith et al. (1976). OX-V, R = phenyl; OX-VI, R = propyl; OX-VII, R = methyl. The elemental analysis for the dyes OX-VI and OX-VII were:

		C	H	N	O
		%	%	%	%
OX-VI ($C_{17}H_{20}N_2O_4$)	Found:	64.42	6.49	8.90	—
	Expected:	64.56	6.33	8.86	20.25
OX-VII ($C_{13}H_{12}N_2O_4$)	Found:	59.77	4.70	10.61	—
	Expected:	60.00	4.62	10.77	24.62

The expected compositions were calculated from the empirical formulas derived from the structures proposed in the figure. The elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, Tenn.

FIGURE 2 Time-course for the sonication of soybean lipids. Soybean lipids (10 mg/ml) were suspended in a medium containing 100 mM Na_2SO_4 and 10 mM Na^+/Hepes pH 7.5 and sonicated in a Branson sonifier cell disruptor model W185 (Branson Sonic Power Co.) under medium power. The temperature of the suspension during sonication was 50 ± 10°C. The curve drawn is that expected for an exponential process with $k_1 = 0.02\ s^{-1}$.

by further sonication. In all the experiments described here, essentially similar results were obtained from either the fractionated or unfractionated vesicles, and the latter were used routinely.

The dyes were stored as concentrated ethanolic solutions at −20°C in the dark. Aliquots of the stock dye solutions were added directly in vesicle suspensions to yield the desired final concentration of the dye. The ethanol concentration was not allowed to rise above 1% vol/vol final concentration. Absorbance measurements were made in a Perkin-Elmer 356 two-wavelength double beam spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.), in a Cary 15 spectrophotometer (Cary Instruments, Fairfield, N.J.) and in a Johnson Foundation scanning double beam spectrophotometer (Johnson Research Foundation, Philadelphia, Pa.) equipped with a digital memory (Chance et al., 1975). Fluorescence measurements were made in a Hitachi Perkin-Elmer MPF-2A spectrofluorometer (Perkin-Elmer Corp.) modified to include a zero offset capability and a binary scale expansion.

Rapid kinetic measurements were monitored in a Johnson Foundation Model B rapid mixing device (Chance, 1974). Oxonol absorbance was followed at 591-605 nm or at 620-650 nm with a double beam spectrophotometer. The rapid mixing device can monitor optical changes at four points along the flow path using fiber optic coupling to four photomultipliers with independently programmable power supplies. In practice, however, only one or two points along the flow path were monitored

because the binding reaction was so fast that no advantage was gained from monitoring optical changes at additional points nearer the point of mixing.

RESULTS

A. Dye-Vesicle Interaction at Equilibrium

The interaction of oxonols with phospholipid vesicles is characterized by changes both in the absorption and the fluorescence spectra of the dyes. Fig. 3 illustrates the changes in absorption of a constant concentration of the dye OX-VI when it is exposed to increasing concentrations of soybean lipid vesicles; above 20 mg lipid/ml the optical changes are complete, indicating that all the dye occupies the membrane phase. The fluorescence spectra of OX-V in solution and bound to lipid vesicles are shown in Fig. 4. The excitation and emission spectra shift approximately 25 nm to higher wavelengths, and the fluorescence yield increases as the dye binds to the membranes. The complete binding of OX-V to soybean lipid vesicles (Fig. 4) is found at much lower lipid concentrations (0.5 mg/ml) than that of OX-VI (20 mg/ml. Fig. 3). Furthermore, under similar experimental conditions no interaction can be detected for OX-VII with soybean lipid vesicles. However, all the dyes interact with egg lecithin vesicles and exhibit the characteristic optical changes outlined above. The spectral changes when the oxonols bind to lipid vesicles resemble those found when the dyes are transferred from aqueous to organic solvents. The spectral properties of the dyes in a number of organic solvents are summarized in Table I.

The oxonol-lipid interaction was followed quantitatively using dual wavelength absorption, 640-612 nm for OX-V, 630-603 nm for OX-VI, and 625-598 nm for OX-VII. The results of typical lipid titrations at constant dye concentration are shown in Fig. 5 in the

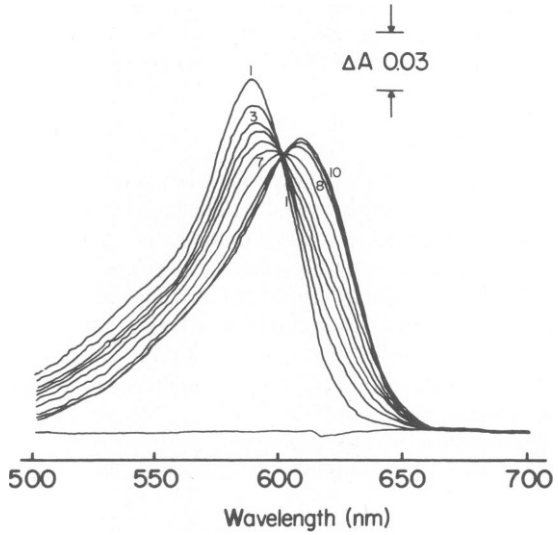


FIGURE 3 The effect of soybean lipid: cholesterol vesicles (4/1, wt/wt) on the absorption spectrum of OX-VI. Aliquots of a vesicle suspension (100 mg lipid/ml) in 50 mM Na_2SO_4, 10 mM Na^+/Hepes pH 7.4 were added to a similar medium containing 2 μM OX-VI at room temperature (21°C). Trace 1 is the free dye spectrum, traces 2–10 contain 0.1–17 mg lipid/ml final concentration.

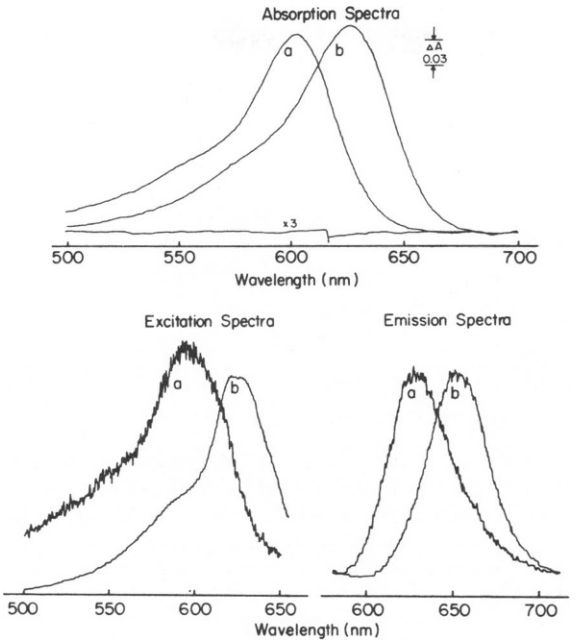


FIGURE 4 The effect of soybean lipid:cholesterol vesicles (4/1, wt/wt) on the optical spectra of OX-V. Traces labeled *a* show the spectra of 1 μM OX-V in 100 mM K_2SO_4, 10 mM Tris/Hepes pH 7.6. In traces labeled *b* 0.47 mg lipid/ml was also present. The fluorescence spectra were recorded using 10-nm excitation and emission slits. The excitation spectra were recorded from the emission at 670 nm with the instrument gain set at 16 in trace *a* and at 1 in trace *b*. The emission spectra were recorded using 550 nm excitation, and the gain settings were eight in trace *a* and four in trace *b*.

TABLE I

THE PROPERTIES OF OXONOL DYES IN ORGANIC SOLVENTS AND LIPID VESICLES

Dye	Absorption maximum					Lipid affinity	
	H_2O	Ethanol	Hexane	Chloroform	Vesicles	Soybean lipid: cholesterol (4/1)	Egg lecithin
			(*nm*)			(*mg/ml*)	
OX-V	600	614	615	625	625	0.042	0.0072
OX-VI	592	604	615	615	617	2.1	0.11
OX-VII	588	602	611	613	613	—	2.7

Dye concentration was in the range 1–10 μM. The aqueous media contained 100 mM Na_2SO_4 and 10 mM Tris/Hepes, pH 7.6. The dyes have a very low solubility in hexane, and the suspension contained particulate material. The lipid affinities were determined by titration of a fixed concentration of dye with sonicated lipid vesicles. The slope of the semireciprocal plots of absorbance change vs. absorbance change/lipid concentration that represents the reciprocal of the classic first association constant K_d/n (Scatchard, 1949; and see Appendix) and has the units of a dissociation constant was determined by a linear regression analysis.

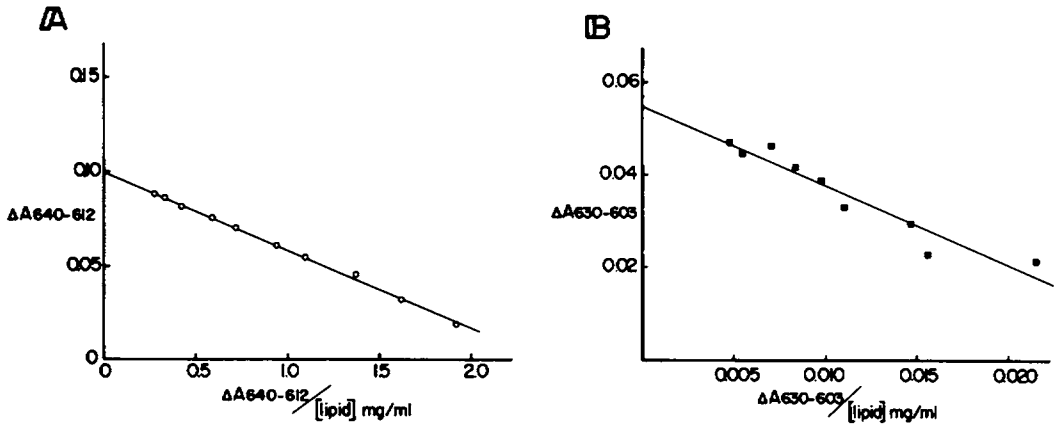


FIGURE 5 Titrations of OX-V and OX-VI with soybean lipid:cholesterol vesicles (4/1, wt/wt). 1 μM OX-V, ΔA 640-612 (A) or 1.5 μM OX-VI, ΔA 630-603 (B) were titrated with lipid vesicles (100 mg lipid/ml) in a medium containing 100 mM K_2SO_4, 10 mM Tris/Hepes pH 7.6 at room temperature. The absorption difference due to the free dye was adjusted to zero optically before the start of the titration. Linear regression analysis yields values for the slopes of the plots of -0.042 mg lipid/ml (A, OX-V) and -2.1 mg lipid/ml (B, OX-VI).

form of a semireciprocal plot. The plot represents an adsorption isotherm analogous to the Eadie-Hofstee plot used in enzyme kinetics (see Appendix). The maximal absorption change at infinite lipid concentration is given by the ordinate intercept and the slope of the plot represents the dye-lipid dissociation constant divided by the number of binding "sites" per unit of lipid. The slope of the semireciprocal plot provides a reliable estimate of the reciprocal of the classic first association constant for the dye-vesicle interaction (Scatchard, 1949) and thus of the relative affinities of the oxonols for lipid vesicles. The data presented in Fig. 5 and summarized in Table I indicate the OX-V binds to vesicles more strongly than OX-VI and much more strongly than OX-VII. Furthermore, all the dyes have a higher affinity for egg lecithin vesicles than for soybean lipid vesicles. The dye binding parameters determined fluorometrically were identical to those obtained monitoring dye absorbance.

Lipid titrations of the type illustrated above yield values of the ratio of the apparent dissociation constant and the number of binding sites. Estimates of both these parameters can be obtained by using the "extinction coefficient" determined from the ordinate intercept of a semireciprocal plot (Fig. 5) to calculate the concentrations of dye "bound" and hence of dye "free" (see Appendix). Experimentally, the bound dye extinction coefficient must be determined for a number of dye concentrations by a lipid titration (cf. Fig. 5), and a matrix of data is obtained from which the appropriate extinction coefficients can be obtained for the calculation of the concentrations of both bound and free dye at every point within the matrix. Data for the binding of OX-V to soybean lipid vesicles are presented in Fig. 6. The line labeled *a* represents the best fit Langmuir adsorption isotherm, assuming that the maximum number of binding sites, n, is 70 nmol/mg lipid determined by a kinetic analysis (Fig. 8) which yields a dye-vesicle dissociation constant, K_d, of 1.86 μM. The line labeled *b* represents the overall best fit Langmuir adsorption isotherm where n is 145 nmol/mg lipid and K_d is 4.41 μM.

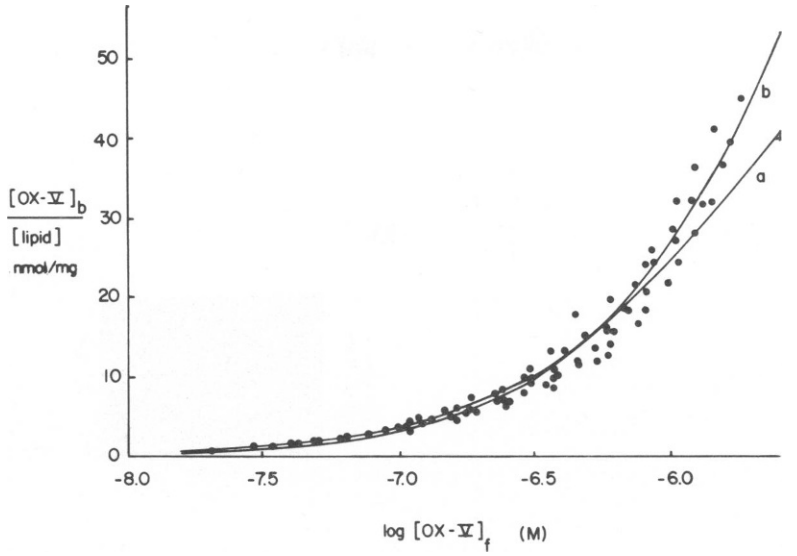


FIGURE 6 Equilibrium binding of OX-V to soybean lipid:cholesterol vesicles (4/1, wt/wt). The binding of OX-V was calculated from the absorption change at 640-612 nm. ΔA_{max} for completely bound dye was determined from lipid titrations at each dye concentration and was used to calculate [OX-V] bound and [OX-V] free for each point in the titration. The data from each titration are plotted as [OX-V] bound/[lipid] against log [OX-V] free. The line labeled *a* is the best-fit Langmuir adsorption isotherm, assuming that the maximum number of binding sites (*n*) is 70 nmol/mg lipid, which yields a K_d of 1.86 μM. The line labeled *b* is the best-fit Langmuir isotherm where *n* is 145 nmol/mg lipid and K_d is 4.41 μM.

B. Kinetic Analysis of the Oxonol-Lipid Interaction

The time-course of the interaction of OX-V with soybean lipid vesicles is shown in Fig. 7; similar time-courses were observed for both dye absorption and dye fluorescence. On a slow time scale when the components were mixed by hand, Fig. 7 A, there is a fast phase of binding, which is not resolved, and a slow phase that is nearly exponential and has a half time of approximately 1 min. The rapid phase of binding was monitored using a rapid-mixing, stopped-flow apparatus with a flow/delivery ratio of 160. A typical time-course of the fast phase of binding is shown in Fig. 7 B. The pseudo-first-order rate constant k_{app} is given by:

$$k_{app} = \frac{2.3}{t_m} \cdot \log (D_2/D_1),$$

where displacements D_1 and D_2 are defined in Fig. 7 B. t_m is related to t_f, obtained from the flow velocity trace: $t_m = (v/v_d) \cdot t_f$, where v is the volume from the point of mixing to that of observation and v_d is the volume discharged in time t_f.

The pseudo-first-order rate constant for the binding reaction is related to the true second-order rate constant for the reaction by the concentration of the reagent in excess: $k_{app} = k_{2nd} \cdot$ [reagent in excess]. k_{2nd} can be obtained directly from the slope of a plot of k_{app} vs. [reagent in excess]. Fig. 8 illustrates the results obtained for OX-V binding to soybean lipid vesicles when either dye or lipid is in excess. Significantly, the second-order rate con-

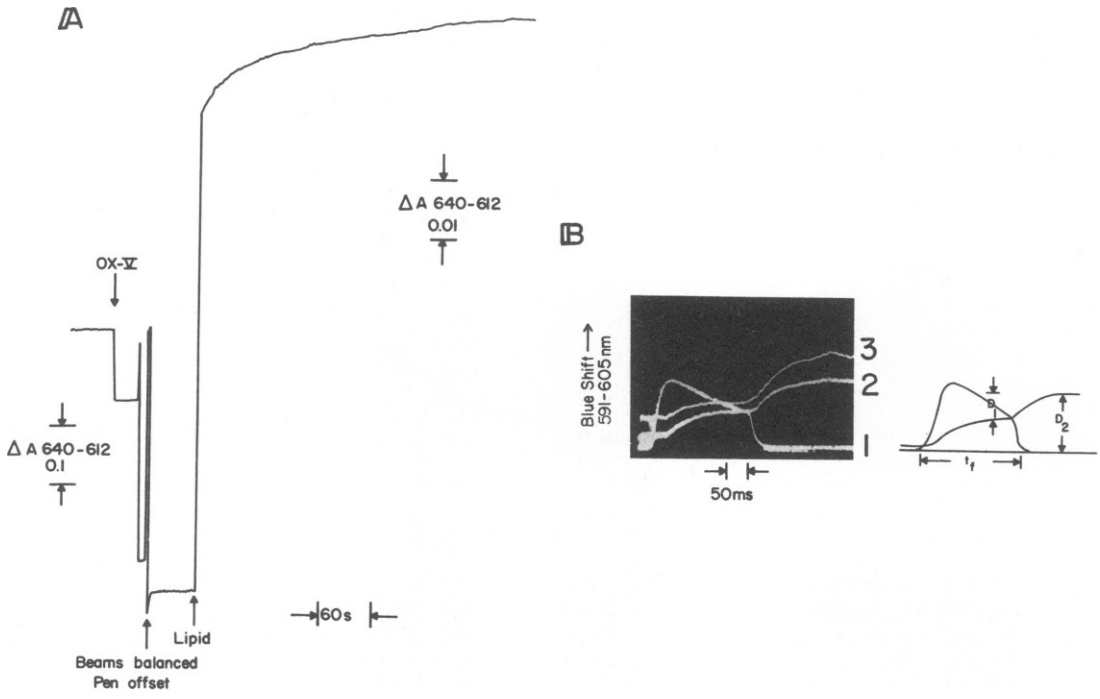


FIGURE 7 The time-course of OX-V binding to soybean lipid vesicles. (A) Soybean lipid:cholesterol vesicles (4/1, wt/wt) in 100 mM Na_2SO_4, 10 mM Na^+/Hepes pH 7.5 were added to a medium containing 100 mM K_2SO_4, 10 mM Tris/Hepes pH 7.6 and 1 μM OX-V at room temperature to give a final lipid concentration of 0.1 mg/ml. (B) A typical oscilloscope record of the rapid binding of OX-V to soybean lipid vesicles. Transmission changes were recorded at 591-605 nm. 1. the flow velocity trace. 2,3. the time-course of the reaction at two points along the observation chamber. 12 μM OX-V neutralized with Tris base, pH 7.5, was mixed with a solution containing 200 mg lipid/ml, 10 mM Na^+/Hepes pH 7.5. The dilution factor was 160 yielding a final mixture that contained 12 μM OX-V and 1.25 mg lipid/ml. The temperature of this experiment was 48°C.

stants obtained under the two conditions are not identical. This follows from the fact that the number of binding sites per unit of lipid is not known so that the concentration of sites is not available, only the bulk lipid concentration. However, the number of binding sites can be calculated from the kinetic measurements alone. The number of sites, n, is simply the ratio of the second-order rate constants: $n = k_{2nd}[\text{lipid}]^{-1}\,s^{-1}/k_{2nd}[\text{dye}]^{-1}\,s^{-1}$, which has units of nanomoles dye bound per milligram lipid when the lipid concentration is expressed in milligrams per milliliter. In experiments similar to those presented in Fig. 8 the number of "fast binding" sites was 68 ± 8 nmol/mg soybean lipid, approximately half the value obtained by equilibrium binding methods (Fig. 6).

Kinetic methods can be used further in studies of oxonol-lipid binding. The rate at which dye leaves the vesicles can be measured directly if the equilibrium (dye + vesicles = complex) can be shifted sharply to the left. This can be done experimentally by the rapid dilution of an equilibrium mixture of dye and lipid (Chance, 1974). As with the forward reaction, two distinct phases of dye exit from soybean lipid vesicles can be observed. In Fig. 9 A, a mixture of OX-V and soybean lipid vesicles in a sodium-containing medium were diluted

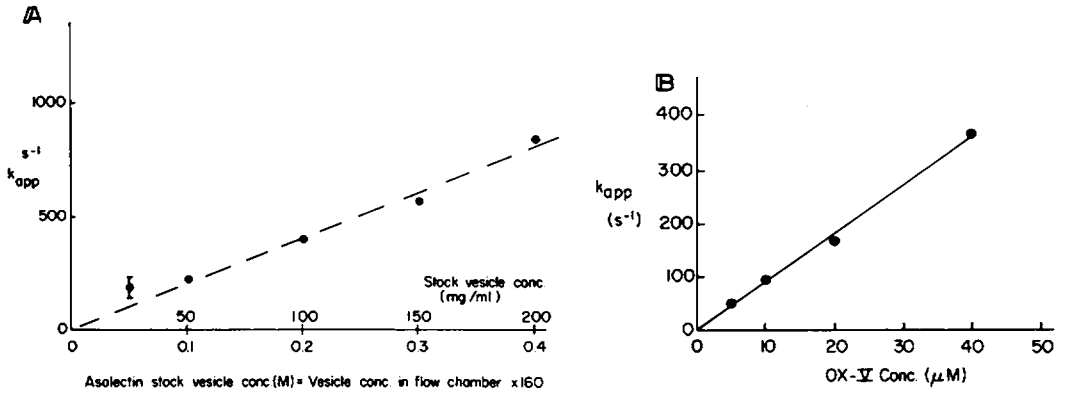


FIGURE 8 The rapid binding of OX-V to soybean lipid vesicles. The concentration dependence of the apparent first-order rate constant for the fast phase of OX-V binding to soybean lipid vesicles was determined under two sets of conditions: (A) Lipid vesicles in excess were mixed with a solution containing 10 μM OX-V neutralized with Tris base pH 7.5 at 22°C. Linear regression analysis yields a slope (the second-order rate constant) of $3.2 \pm 0.23 \times 10^5\ M^{-1}\ s^{-1}$, assuming a molecular weight of 500 for soybean lipids; in weight units, the slope has a value of 6.4×10^2 ml $mg^{-1}s^{-1}$. (B) OX-V in excess. Stock lipid (50 mg/ml) in 10 mM Na^+/Hepes pH 7.5 was mixed with OX-V solutions neutralized with Tris base pH 7.5 yielding a final lipid concentration of 0.31 mg/ml at 22°C. Linear regression analysis yields a slope of $9.04 \pm 0.36 \times 10^6\ M^{-1}\ s^{-1}$. In this pair of experiments the number of fast binding sites is given by the ratio of the second-order rate constants: $n = (6.4 \times 10^2)/(9.04 \times 10^6)$ mol/g = 70.8 nmol/mg. The mean values of k_{2nd} and the number of fast binding sites are summarized in Table II.

100 times with a similar medium. The half time for dye exit as determined by the blue shift in absorption was about 1.5 min. In an experiment of the type depicted in Fig. 8 A the low absorbance of the solvent before the addition of the dye-vesicle mixture makes the determination of an accurate base line difficult, preventing an unequivocal demonstration of a fast phase of dye exit from the vesicles. However, when a mixture of dye and lipid vesicles is diluted in the flow apparatus, a fast dissociation process with a half time of only 20–50 ms is observed (Fig. 9 B), the mean value being 41 ± 5 ms. If only the fast phases of binding and dye exit from the vesicles are considered then the dissociation constant for the dye-fast site complex is: $K_d = k_{off}(s^{-1})/k_{on}(M^{-1}s^{-1})$. The data from experiments similar to those presented in Figs. 8 and 9 indicate that K_d for the fast sites is 1.88 ± 0.15 μM, close to the value found in the equilibrium binding measurements (Fig. 6). A summary of the kinetic data for the binding of OX-V to phospholipid vesicles is included in Table II.

The extent of the slow phase of oxonol binding to lipid vesicles depends on the lipid/dye ratio, and on the relative composition of the external and internal aqueous phases. In Fig. 10 the fraction of the slow phase of OX-V binding to soybean lipid vesicles is expressed as its percentage of the total absorbance change accompanying the binding reaction. The proportion of the slow phase increased as either $[H^+]$ or $[K^+]$ inside the vesicles decreased with respect to the external phase, when the ionic strength was held constant by replacing K^+ by Na^+ in either medium. As the extent of the fast phase of binding is constant, the increase in slow phase binding represents an overall increase in dye binding. Lipid titrations performed under conditions that favor the slow phase binding yield semireciprocal plots of decreased slope, indicating the increased affinity of the dye for the membrane phase.

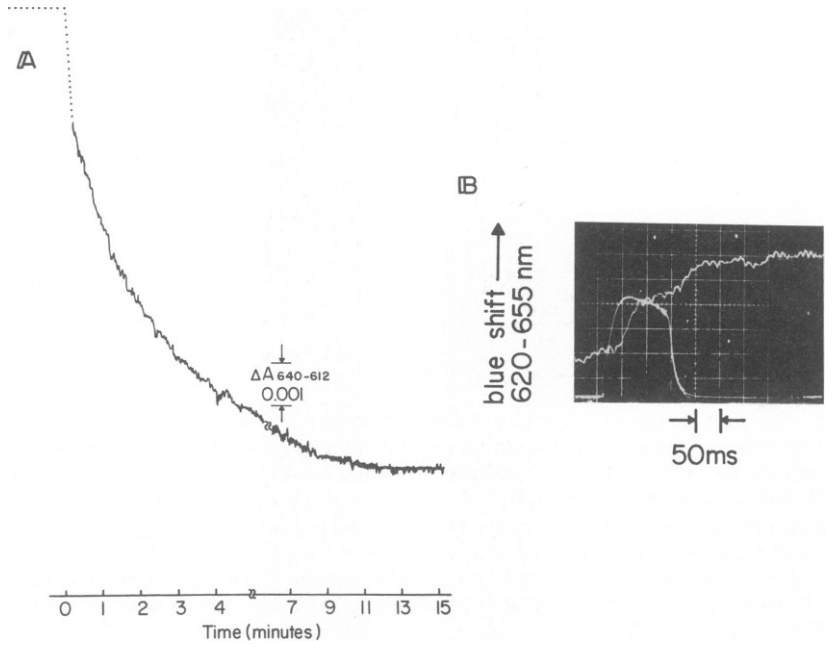


FIGURE 9 The dissociation of OX-V from soybean lipid vesicles. (A) A solution containing 10 mg soybean lipid:cholesterol vesicles (4/1, wt/wt)/ml, 100 mM Na_2SO_4, and 10 mM Tris/Hepes pH 7.5 and 0.1 mM OX-V was diluted 100 times in a similar medium at 23°C. (B) A typical oscilloscope record of the dissociation of OX-V from soybean lipid vesicles. 40 μM OX-V, 250 mg lipid vesicles/ml, and 2 mM Na^+/Hepes were diluted 160 times in water pH 6.8; the final mixture contained 0.25 μM OX-V and 1.56 mg lipid/ml at 27°C. The reaction was monitored at 650-620 nm, and the flow velocity was adjusted so that the dissociation occurred after the flow had stopped. The initial optical change is due to the injection of absorbing material (lipid + dye) into the diluting medium. k_1 was obtained from the half time of the absorption change, $k_1 = (\ln 2)/t_{1/2}$. In this experiment $t_{1/2}$ was 22 ms and k_1 31.5 s^{-1}. Mean values of k_1 are given in Table II.

TABLE II

THE RAPID PHASES OF THE INTERACTIONS OF OX-V WITH LIPID VESICLES

	Temperature	k_2		n	k_{-1}	K_d
		Excess OX-V	Excess lipid			
	(°C)	($M^{-1}s^{-1}$)	($ml \cdot mg^{-1}s^{-1}$)	(nmol/mg)	(s^{-1})	(M)
Soybean lipids	22	$9.04 \pm 0.36 \times 10^6$	$6.40 \pm 0.46 \times 10^2$	71	17 ± 2	1.88×10^{-6}
DL-α-dipalmitoyl lecithin	54	$3.38 \pm 0.08 \times 10^7$	$7.40 \pm 0.10 \times 10^3$	219	—	—

*The data presented in the table were obtained under the experimental conditions described in the legends of Figs. 8 and 9. The rate constants for the oxonol-lipid interaction are defined as follows: oxonol + lipid $\underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}}$ complex. The number of fast binding sites, n, is given by the relation: $n = k_2$ (excess lipid)$/k_2$ (excess dye), and the oxonol-lipid dissociation constant, K_d, by: $K_d = k_{-1}/k_2$ (excess dye).

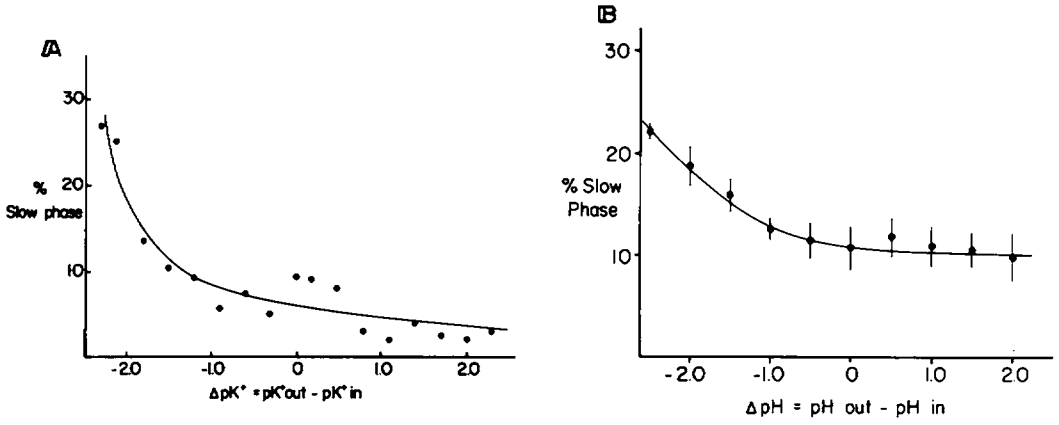


FIGURE 10 The solvent dependence of the slow phase of OX-V binding to soybean lipid:cholesterol vesicles (4/1, wt/wt). The extent of the slow phase of binding is expressed as its percentage of the total absorption change at 640-612 nm caused by the binding reaction. (A) 1 μM OX-V binding to 0.05 mg lipid vesicles/ml in a medium containing 100 mM X_2SO_4 (X = Na or K) and 10 mM Tris/Hepes pH 7.6 at room temperature. The vesicles contained either 100 mM Na_2SO_4 or 100 mM K_2SO_4. (B) 1.3 μM OX-V binding to 0.08 mg lipid vesicles/ml in a medium containing 50 mM KCl and 25 mM K^+ phosphate pH 5.5–8 at room temperature. The pH of the vesicles was either 6 or 8.

The rate of the slow phase of oxonol binding depended on the physical character of the membrane lipids. Fig. 11 shows the results of increasing the cholesterol content of soybean lipid vesicles on the half-time of the slow phase and on the overall affinity of OX-V binding. As the cholesterol content rose, the rate of the slow phase was much diminished, although the relative proportion of fast and slow phases was unaltered. The interaction of oxonol dyes with lipid vesicles alters dramatically when the phospholipid acyl chains adopt the all *trans*-configuration and form a highly ordered crystalline gel phase (Chapman, 1975). Dipalmitoyl

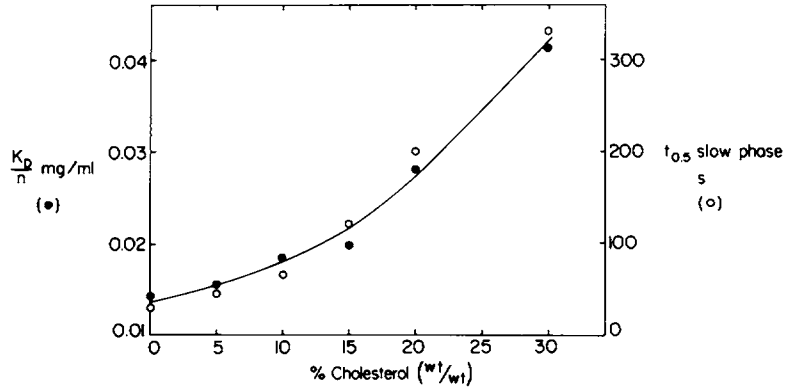


FIGURE 11 The effect of cholesterol on the interaction of OX-V with soybean lipid vesicles. The affinity (K_d/n, •) of OX-V (1 μM) for lipid vesicles containing different proportions of cholesterol was determined from the slope of semireciprocal plots of the type illustrated in Fig. 4. The half time for the slow phase ($t_{0.5}$, ○) was determined after the addition of 0.05 mg vesicles/ml (final concentration) to a solution containing 1 μM OX-V. The medium contained 100 mM Na_2SO_4, 10 mM Na^+/Hepes pH 7.5 at 23°C.

TABLE III

THE RETENTION OF OX-V BY DIPALMITOYL LECITHIN VESICLES AT VARIOUS TEMPERATURES

Incubation temperature	Column temperature	OX-V Retained with vesicle fraction
°C	°C	%
22	12	2.5
47	22	18.7
47	47	37.2

OX-V and sonicated dispersions of dipalmitoyl lecithin were incubated in a medium containing 40 μM OX-V, 2.4 mM dipalmitoyl lecithin, 20 mM Tris-SO_4 at pH 7.4. The column was of Sephadex G-25 equilibrated with 20 mM Tris-SO_4 and had a bed volume of 25 ml (25 cm × 1 cm).

lecithin undergoes the gel-liquid crystalline phase transition at 42°C (Hinz and Sturtevant, 1972), and the binding of OX-V to such vesicles is strongly temperature dependent. Below 42°C OX-V interacted weakly with dipalmitoyl lecithin vesicles (K_d/n > 1 mg/ml), and there was no slow phase of dye binding; above 42°C the binding of dye was extensive (K_d/n < 0.05 mg/ml) and had the biphasic kinetics observed for the binding to soybean lipid vesicles (Fig. 7). When a mixture of OX-V and dipalmitoyl lecithin was passed down a Sephadex G-25 column (Pharmacia) incubated at a temperature above 42°C a significant fraction of the dye eluted with the vesicle fraction (Table III); if the dye and lipid were incubated above 42°C but the column was maintained at room temperature, about half as much OX-V remained "trapped" in the vesicle fraction; if the dye and lipid were mixed at room temperature and the column was also at room temperature very little dye remained with the vesicle fraction. The proportion of the dye "trapped" after incubation above 42°C and fractionation at 23°C depended on the composition of the internal and external aqueous media. The

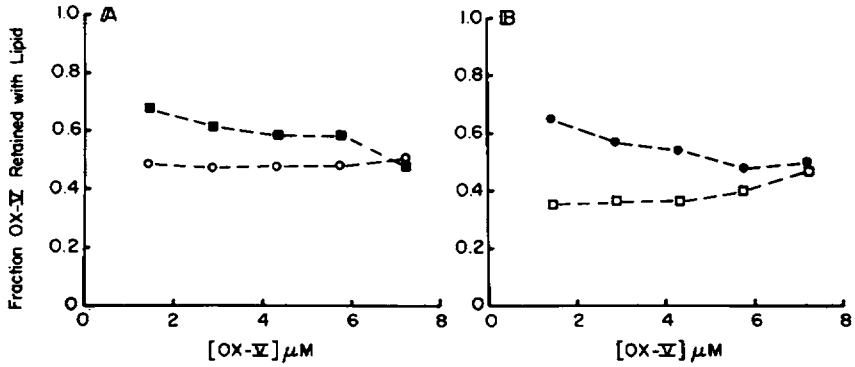


FIGURE 12 Retention of OX-V by dipalmitoyl lecithin vesicles. 0.1 mg lipid vesicles/ml were incubated with media containing OX-V (10 μM) at 52°C. The tubes were cooled on ice before passage down a Sephadex G-25 column (5 cm × 1 cm) equilibrated with medium at 23°C. (A) The medium contained 100 mM X_2SO_4 (X = Na or K) and 10 mM Hepes (Na^+ or K^+) pH 7.5. Incubation was for 25 min. (■) Na_2SO_4 inside and K_2SO_4 outside the vesicles; K_2SO_4 inside and Na_2SO_4 outside the vesicles. (B) The medium contained 75 mM Na_2SO_4 and 25 mM Na^+ phosphate pH 6 or 8; incubation was for 25 min. (●) pH 8 inside and pH 6 outside the vesicles; (□) pH 6 inside and pH 8 outside the vesicles.

results of the experiments illustrated in Fig. 12 indicate that significantly more OX-V was "trapped" in dipalmitoyl lecithin vesicles under conditions that promote the slow phase of oxonol binding. After the samples had been chilled, the fraction of dye remaining with the vesicles was not affected by changes in the external medium, indicating that adsorption of the dye to glass or to Sephadex did not significantly contribute to the observed results.

DISCUSSION

The use of dyes to probe membrane phenomena requires an understanding of the nature of the dye-membrane interaction. The oxonol dyes used in the present investigation interact noncovalently with phospholipid vesicles and natural membranes. The value of oxonols as probes of membrane function depends both on the location of the dye binding sites in the membrane phase and the level to which these sites are occupied. The experiments described above yield much basic information on the forces that govern the interaction of oxonol dyes with lipid membranes.

The optical spectra of the oxonol dyes in the presence of lipid vesicles indicate that the dyes occupy a hydrophobic region of the lipid bilayer. The spectra of the bound dyes closely resemble those found when the oxonols are dissolved in organic media such as hexane or chloroform (Figs. 3 and 4; Table I). In titrations of fixed dye concentrations with lipid vesicles (Fig. 3), the shift of the absorption spectrum 25 nm to higher wavelength with little change in extinction and an approximate isosbestic point midway between the bound and the free dye spectra suggests that the dye distributes between the hydrophobic and aqueous phases by a relatively simple adsorption mechanism. There is no indication that the dyes undergo a change of aggregation when they bind to lipid membranes; indeed, this particular class of oxonol dyes does not appear to form aggregates, as the optical spectra are independent of dye concentration over a fairly wide range (Smith et al., 1976). The difficulty of assigning a hydrophobic binding site for the oxonols is that at the pH values employed throughout these experiments the dyes are wholly anionic; the pK_a of OX-V has been determined as 4.2 (Smith et al., 1976) and both OX-VI and OX-VII have similar pK_a values. Preliminary evidence concerning both the location and the orientation of all the oxonol dyes in highly ordered lipid bilayer arrays[1] indicates that the long axis of the oxonol molecule makes a small angle with the plane of the bilayer membrane. The simplest model places the anionic charge of the dye at the membrane-water interface (Chance, 1975) and the long axis of the molecule penetrating the hydrophobic core of the membrane at a small angle.

Three major factors affect the equilibrium interaction of oxonol dyes with phospholipid vesicles: the structure of the dye, the physical chemistry of the vesicle, and the ionic strength of the aqueous medium. The determination of the reciprocal of the classic first association constant for the oxonol-lipid interaction by lipid titrations (Fig. 5 and Table I) indicates that the phenyl derivative (OX-V) has the highest and the methyl derivative (OX-VII), the lowest affinity for lipid vesicles, the propyl derivative (OX-VI) having an intermediate affinity. Furthermore, all the oxonols have a higher affinity for egg lecithin vesicles than they do for soybean lipid vesicles, so the chemical composition of the membrane contributes significantly to the interaction of the vesicles with oxonol dyes. The physical state of the mem-

[1] Smith, J. C., L. S. Powers, and B. Chance. Unpublished observations.

brane lipids also affects the dye-vesicle interaction. OX-V binds weakly to dipalmitoyl lecithin vesicles at room temperature where the acyl chains adopt a gel crystalline state, but interacts strongly with the vesicles when the ambient temperature exceeds that of the gel-liquid crystalline phase transition (~42°C). In soybean lipid vesicles, where the hydrocarbon region is very fluid, the addition of cholesterol, which may decrease the "fluidity" of such membranes (Chapman, 1975), also diminishes the binding of OX-V (Fig. 11). The binding of the dyes to both soybean lipid and egg lecithin vesicles is enhanced by the presence of electrolytes such as sodium or potassium sulfate. Indeed, in media of low ionic strength lipid titrations do not yield linear adsorption isotherms of the type illustrated in Fig. 5.

The description of the interaction of oxonol dyes with lipid membranes outlined above assumes that the dyes permeate membranes freely and that all of the membrane surface area is available to interact with the dyes. The oxonol dyes permeate black lipid membranes,[2] and the kinetic character of the vesicle-dye interaction strongly suggests that oxonol permeates lipid vesicles (see below). McLaughlin and Harary (1976) have reported that the adsorption of a nonpermeant anion to lipid vesicles can be adequately described by the Stern equation, which explicitly considers the surface potential induced by the adsorption of dye. In the case of permeant anions the relationship between the amount of dye adsorbed and the surface potential is more complex, as not all the dye is present at the outermost interface. However, at ionic strengths of 0.3, the linearity of simple adsorption isotherms (Fig. 5) suggests that the interaction can be approximated by the Langmuir adsorption isotherm, formally analogous to the Michaelis-Menten equation used in enzyme kinetics (McLaughlin and Harary, 1976). A number of lipid titrations are combined in Fig. 6, and a Langmuir adsorption isotherm can be found that fits the data well (see Appendix for the fitting procedures) yielding a value of 4.41 μM for the dissociation constant and 145 nmol/mg lipid for the number of binding sites. This result suggests that the oxonol interaction with lipid vesicles at moderate ionic strength is adequately described by a simple adsorption process. Indeed, it was not possible to find a satisfactory fit for the adsorption process under these conditions using the Stern equation (McLaughlin and Harary, 1976). At low ionic strength (<0.02) the interaction of oxonols with soybean lipid vesicles was complex and could not adequately be described either by the Langmuir adsorption isotherm or the Stern equation. Although the optical signals associated with oxonol adsorption to lipid membranes closely resemble those observed at ionic strengths in excess of 0.1, the physical description of the adsorption process at low tonic strength remains obscure.

In an attempt to overcome some of the uncertainties of equilibrium binding titrations (see Appendix), a detailed kinetic analysis of the oxonol-lipid interaction was undertaken. The binding reaction (oxonol + vesicle = complex) is a second-order process, and the second-order rate constant was determined under pseudo-first-order conditions for both dye and lipid (Fig. 9). The ratio of the second-order constants gives the number of fast binding sites directly. The value obtained for the number of fast binding sites for soybean lipid vesicles, 68 ± 8 nmol/mg, is about half that for the total number of sites found by equilibrium titrations (145 nmol/mg, Fig. 5). Furthermore, the value for the dye-lipid dissociation constant determined kinetically, 1.88 μM (Table II), is in reasonable agreement with the

[2]Krasne, S. Personal communication.

equilibrium dissociation constant, 4.41 μM (Fig. 6). The equivalent properties of the fast and the slow sites suggest that they have a similar physical character but differ in their accessibility to oxonol dyes. In the simplest model the fast sites represent those on the outer vesicle-water interface and the slow sites represent those in the internal aqueous phase (or phases) of the liposome system. Consistent with this model, the slow phases of dye binding and of dye release have similar time-courses (Figs. 7 and 9) that may represent permeation of the bilayer by the dyes. A similar discrimination of dye interaction with the opposite interfaces of vesicle membranes has been reported, and the permeation properties of oxonol dyes described here closely resemble those found for 1-anilino-8-naphthalenesulfonate (Haynes and Simkowitz, 1977). Oxonols do not permeate dipalmitoyl lecithin vesicles, as indicated by the binding kinetics, unless the hydrocarbon region is "fluid." Furthermore, in vesicles that possess very fluid bilayers, such as those of egg lecithin or soybean lipids (Chapman, 1975), the addition of cholesterol significantly reduces the rate of oxonol permeation (Fig. 11). The effects of temperature and chemical composition of the vesicle also suggest that the "slow" process is not a manifestation of the unstirred layer at the aqueous membrane interface. The properties of the unstirred layer are unlikely to be sufficiently affected by these changes to cause such a large alteration in the rate of the slow phase of oxonol binding to phospholipid vesicles. Oxonol anions permeate black lipid membranes rapidly (Waggoner, 1976), and a similar permeation of lipid vesicles would develop a diffusion potential, negative inside, that would inhibit further accumulation of the dye. The slow time-course of oxonol permeation (Figs. 7 and 9) suggests that, like 1-anilino-8-naphthalenesulfonate (Haynes and Simkowitz, 1977), cotransport of a cation forms part of the permeation mechanism. The diminution of the ion pair transport process by cholesterol (Fig. 11; and Haynes and Simkowitz, 1977) is in contrast to the stimulation of the electrically active, oxonol anion permeation of black lipid membranes by cholesterol (Waggoner et al., 1977).

The amplitude of the slow phase of the oxonol-vesicle interaction will depend on the size and number of layers of the vesicles. Chromatography on Sephadex 4B (Huang, 1969; Berden et al., 1975) provided vesicle fractions that exhibited slow phase amplitudes marginally less than those of unfractionated vesicles (cf. Berden et al., 1975), and the latter were used in all the experiments reported here. The solvent conditions that promote the slow phase of the dye-vesicle interaction are identical to those that favor the accumulation of the oxonols within lipid vesicles (Figs. 10 and 12). The physical basis for this phenomenon is uncertain. If the only permeant species were the free acid, then the oxonols would be expected to accumulate on the "basic" side of the membrane (Addanki et al., 1968). However, there is good evidence that the anion permeates (Waggoner, 1976), and the mechanism does not explain the accumulation of oxonols in a phase where sodium has replaced potassium. In terms of the ion pair transport model discussed above, the increased amplitude of the slow phase may indicate that the K^+/oxonol$^-$ ion pair is more permeable than the Na^+/oxonol$^-$ pair. In the presence of a suitable cation gradient, the oxonol will accumulate on the "sodium" side until the oxonol concentration gradient is sufficient to balance the differential permeability of the ion pairs. Haynes and Simkowitz (1977) report that the amplitude of dye transport is enhanced by external K^+ but found no discrimination between the alkali cations (except at LiCl concentrations in excess of 0.5 M).

A number of other mechanisms may explain the cation dependence of the slow interaction of oxonols with lipid vesicles. If the lipids themselves are selectively permeable to cations, then it is possible for diffusion potentials to develop across the membrane which would govern the distribution of the oxonol anions. In this case the rate of the slow phase probably represents the rate of formation of the membrane potential as the permeation of the oxonol anions is unlikely to be rate limiting. It is significant that the relative proportions of the fast and slow phases did not depend on the time of addition of the oxonol, so there appeared to be no slow development of membrane potential in the absence of the dye. In lipid vesicles the membrane potential can be modified by the use of ionophores (Bakker and van Dam, 1974). However, ionophores interact with some classes of oxonols (Waggoner, 1976), and there is some evidence that the oxonols employed here interact with valinomycin and K^+ in the membrane phase (Bashford and Smith, 1978). The use of ionophores to explore the response of oxonol dyes to membrane potential in phospholipid vesicles will be dealt with more fully in a forthcoming publication.[3] However, the results using ionophores clearly indicate that the formation of a membrane potential is associated with a relatively fast dye response, $t_{1/2}$ is less than 1 s, even when there is no interaction between the dye and the ionophore as is the case with protonophoric uncoupling agents. Furthermore, perturbation of the ion gradients with an electroneutral ionophore, such as nigericin, leads to changes in oxonol absorbance with half times on the time scale of minutes, very similar to the slow phase of the binding kinetics. These results suggest that a rapid movement of oxonol anions across membranes can occur in the presence of an electrical potential. Indeed, in photosynthetic bacteria the potential dependent migration of oxonols across the membrane dielectric can occur in milliseconds (Bashford et al., 1978). On the other hand, the response of the oxonols to an electroneutral change of the transmembrane ion gradients is rather slow, with a time scale of minutes, consistent with the cotransport model. Finally, the relative solubility of the oxonols in the aqueous phases separated by the vesicle membrane may explain the amplitude of the slow phase. OX-V is three times more soluble in 0.1 M Na_2SO_4 than in 0.1 M K_2SO_4 and its aqueous solubility declines dramatically at low pH values (Smith et al., 1976). It is possible that oxonols partition between the aqueous phases according to their solubility so that free dye concentration on the sodium side exceeds that on the "potassium" side. When the inner aqueous phase is either more basic or contains sodium rather than potassium the oxonols will tend to accumulate within the vesicles. Furthermore, as the internal volume of the vesicles is small, the effective membrane concentration of the internal phase is very high so that dye binding on the inner interface is strongly favored; indeed the spectrum of the vesicle-associated dye in the experiments reported in Fig. 12 indicates that most of the dye is membrane bound, presumably to the inner interface. This particular model makes no assumptions about the mechanism of permeation and has a general applicability. Additional experiments with other cations, such as ammonium and tris(hydroxymethyl)aminomethane, indicate that the amplitude of the slow phase binding always correlates with the relative solubility of the oxonol anions. However, the present experiments do not permit a defini-

[3] Bashford, C. L., B. Chance, and J. C. Smith. Anionic probes of membrane potential. An evaluation of their properties in phospholipid vesicles. Manuscript in preparation.

tive discrimination between the various models compatible with an asymmetric distribution of oxonol dyes across lipid bilayer membranes.

Oxonol dyes respond to transmembrane potential by both fast (microseconds) and slow (seconds) mechanisms. Waggoner (1976) has proposed that the slow changes arise from changes in the partition of dye molecules between membrane vesicles and the external medium in a manner similar to that described for cyanine dyes (Sims et al., 1974). In photosynthetic bacteria (Bashford et al., 1978), it is apparent that this form of slow response can occur in the millisecond but not the microsecond time domain. Ultimately, the rate limit of the slow response is the second-order interaction of the dyes with the membrane vesicles which has a rate constant of around 10^7 M^{-1} s^{-1} (Table II) and in photosynthetic bacteria the second-order rate constant for the response of oxonols to membrane potential approaches this value (2×10^6 M^{-1} s^{-1}, Bashford et al., 1978). This result suggests that the permeation of the membrane by the oxonol anion can be reasonably rapid. The mechanism of the microsecond response of oxonols to membrane potential is less certain. Waggoner et al. (1977) propose an "on-off" mechanism at the membrane-aqueous interface. However, the "on" and "off" rate constants for oxonol interaction with membrane vesicles reported here, $t_{1/2}$ values in the millisecond time domain, do not seem consistent with an on-off mechanism in the microsecond time range unless the fast responses involve a fraction of the dye population with a very high local concentration. The problem is not one of dye structure, as we have found similar second-order rate constants for the interactions of the dyes used by Waggoner et al. (1977) with lipid vesicles as those reported here.

SUMMARY AND CONCLUSIONS

The oxonol dyes probe a hydrophobic region of the lipid bilayer that is probably close to the membrane-water interface. The dyes occupy the membrane with very different efficiencies depending both on their particular chemical structure and on the physical and chemical composition of the membrane. The oxonols bind rapidly to lipid membranes with a second order rate constant of about 10^7 M^{-1} s^{-1}. They permeate the membrane when the hydrocarbon core is fluid, probably in conjunction with a cation and with a half time of 0.2–5 min. The oxonols appear to distribute across the membrane according to the composition of the aqueous media at each membrane interface. The preference of the oxonols for sodium rather than potassium media may be significant for their use in physiological situations, as natural membranes often maintain high gradient of these cations. In vivo the selectivity of the oxonols will tend to keep them outside cells. It is in turn likely that the forces that control the distribution of oxonols across membranes may also contribute to the metabolite gradients found across natural membranes. For a complete understanding of the behavior of oxonols in natural membranes, both the extent to which the dye occupies the membrane and the properties of the membrane-associated dye must be considered.

We would like to thank Dr. Alan Waggoner for many helpful discussions and particularly for the gift of a number of oxonol dyes which have a different structure from those used here.

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APPENDIX

THE DETERMINATION OF OXONOL-MEMBRANE BINDING PARAMETERS BY SPECTROSCOPIC METHODS

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The adsorption of dyes to lipid membranes can be monitored by a number of different procedures. The most direct assay is the determination of the concentrations of adsorbed and free dye in equilibrium with the lipid membrane. A widely used experimental procedure is the physical separation of membrane and solvent by centrifugation or filtration. The concentration of adsorbed dye can be obtained from the fraction of the dye found in the membrane pellet (Nordenbrand and Ernster, 1971) or from the loss of dye from the incubation medium (Layton et al., 1974). A less direct, but often more convenient method of estimating dye binding is afforded by the optical changes that accompany binding. Two titrations are employed (Radda, 1971): membrane concentration is varied at constant dye concentration to obtain a calibration of the optical change at infinite membrane concentration when all the dye is bound; the calibration is used to calculate the fraction of dye bound at each point of a titration of dye at constant membrane concentration (Brocklehurst et al., 1970).

Consider the case of a small molecule binding to a membrane with a concomitant change in some optical parameter, P. The enhancement, ϵ, of the parameter accompanying binding is defined as:

$$\epsilon = P_{\text{observed}}/P_{\text{initial}} \tag{1}$$

where P_{initial} is the value of P in the absence of the membrane and P_{observed} the value in the presence of the membrane. If all the dye "binding sites" are identical, then we can define the parameter ϵ_b, the characteristic enhancement (Dwek, 1972) observed for dye binding, which will be a constant for defined dye and membrane systems. The enhancement parameters alone permit the calculation of the fraction of dye bound c:

$$P_{\text{observed}} = P_{\text{initial}}(1 - c) + P_{\text{initial}} \cdot \epsilon_b \cdot c \tag{2}$$

and

$$P_{\text{observed}}/P_{\text{initial}} = \epsilon = (1 - c) + \epsilon_b \cdot c; \tag{3}$$

rearranging,

$$c = (\epsilon - 1)/(\epsilon_b - 1) = (1 - \epsilon)/(1 - \epsilon_b). \tag{4}$$

The experimental determination of both ϵ and ϵ_b will then permit the calculation of the dye binding parameters. In experiments where the absorbance of the dye changes, the use of the enhancement parameter is not mandatory as the concentration of bound dye can be calculated directly from the extinction coefficient of the bound and free species (A_b and A_f, respectively):

$$A_{\text{observed}} = [\text{dye}]_b \cdot A_b + [\text{dye}]_f \cdot A_f. \tag{5}$$

Furthermore in dual wavelength experiments, A_f can be set equal to zero by balancing the beams in the absence of membrane and in this case:

$$\Delta A_{\text{observed}} = [\text{dye}]_b \cdot \Delta A_b. \tag{6}$$

The adsorption of a dye to a membrane can be represented by the equilibrium:

$$\text{dye} + \text{membrane} = \text{complex}. \tag{7}$$

If the initial concentration of the dye is d, the membrane concentration is m, the number of binding sites per unit of membrane is n and the concentration of occupied sites is b, and we can make the assumption that the total number of sites greatly exceeds the dye concentration (analogous to the assumption that [substrate] $\gg$ [enzyme] in enzyme kinetics and treating the dye as "enzyme"), then the dissociation constant, K_d, for the interaction described by Eq. 7 is given by:

$$K_d = (d - b)n \cdot m/b. \tag{8}$$

Rearranging:

$$b = d - K_d \cdot b/n \cdot m. \tag{9}$$

The fraction of dye that is bound, c, is b/d, hence:

$$c = 1 - K_d \cdot c/n \cdot m. \tag{10}$$

Substituting from Eq. 4 yields:

$$\epsilon - 1 = (\epsilon_b - 1) - \frac{K_d \cdot (\epsilon - 1)}{n \cdot m}. \tag{11}$$

Alternatively, substituting Eq. 6 in Eq. 9:

$$\Delta A_{obs} = d \cdot \Delta A_b - \frac{K_d \cdot \Delta A_{obs}}{n \cdot m}. \tag{12}$$

Eqs. 11 and 12 predict that a plot of $\epsilon - 1$ vs. $\epsilon - 1/m$ or ΔA_{obs} vs. $\Delta A_{obs}/m$ will be a straight line of slope $-K_d/n$ with an ordinate intercept of $\epsilon_b - 1$ or $d \cdot \Delta A_b$. Eqs. 11 and 12 are analogous to the Eadie-Hofstee equation in enzyme kinetics. In terms of a double reciprocal analysis (cf. the Lineweaver-Burke equation):

$$\frac{1}{\epsilon - 1} = \frac{1}{\epsilon_b - 1} + \frac{K_d}{(\epsilon_b - 1) \cdot n \cdot m} \tag{13}$$

and

$$\frac{1}{\Delta A_{observed}} = \frac{1}{d \cdot \Delta A_b} + \frac{K_d}{d \cdot \Delta A_b \cdot n \cdot m}. \tag{14}$$

Thus a titration of dye with lipid will allow the calculation of ϵ_b, ΔA_b, and K_d/n provided that the assumption $n \cdot m \gg d$ can be made. Zierler (1977) has made a detailed analysis of the intrinsic errors involved in double reciprocal and semireciprocal plots, and provides a rigorous demonstration that the value of ϵ_b or ΔA_b can only be obtained with large excess of membrane or binding protein. Under suitable experimental conditions the particular value of the semireciprocal plots is that they yield information on both the affinity of the dye for the membrane and on the properties of bound dye in a single titration.

The slope of a semireciprocal plot of $\epsilon - 1$ vs. $(\epsilon - 1)$/[membrane] yields a dissociation constant, K_d/n, the reciprocal of the classic first association constant for the dye-membrane interaction (Scatchard, 1949), and has units of membrane concentration. To obtain estimates of K_d and n, further manipulation of the data is required. As the semireciprocal plot yields ϵ_b or ΔA_b directly, it is possible to use this value to calculate the concentrations of free and bound dye at each point of the membrane titration using Eq. 4. If a number of titrations are performed over a wide range of lipid-to-dye ratios, a matrix of data can be obtained providing the concentrations of free and

bound dye (Bashford et al., 1978) for differing membrane concentrations. If we define the number of dye molecules adsorbed per unit of membrane as σ then:

$$\sigma = b/m \tag{15}$$

and the binding data can be analyzed according to the Langmuir adsorption isotherm (McLaughlin and Harary, 1976):

$$\sigma/(n - \sigma) = f/K_d, \tag{16}$$

where f is the free dye concentration. The Langmuir isotherm contains two unknown parameters, K_d and n, and estimates of these parameters can be obtained by a number of different procedures.

A simple rearrangment of Eq. 16 yields the Scatchard relationship, derived to describe the binding of small molecules to proteins (Scatchard, 1949):

$$\sigma = n - K_d \cdot \sigma/f, \tag{17}$$

which predicts that a plot of σ vs. σ/f will be a straight line of slope $-K_d$ and having an ordinate intercept of n. Alternatively, Eq. 16 can be rearranged to yield:

$$1/\sigma = 1/n + K_d/n \cdot f \tag{18}$$

which indicates that K_d and n can be determined from a plot of $1/\sigma$ vs. $1/f$. For large numbers of data points a computer can search "parameter space" for the best-fit values of K_d and n (minimizing χ^2) in the Langmuir isotherm (Eq. 16; McLaughlin and Harary, 1976), using an iterative minimization procedure such as that described by Fletcher and Powell (1963). When there is significant scatter in the experimental points, the best fit values of K_d and n appear to depend rather significantly on the functional form of the Langmuir isotherm used in the minimization routine, presumably because the form used dictates the relative weight given to each determination. For example, in Fig. 6 of Bashford et al. (1978) and Fig. 2 here the best approximation of K_d and n by computer minimization was obtained by employing the logarithmic transformation of the Langmuir isotherm:

$$\log \frac{\sigma}{n - \sigma} = \log f - \log K_d. \tag{19}$$

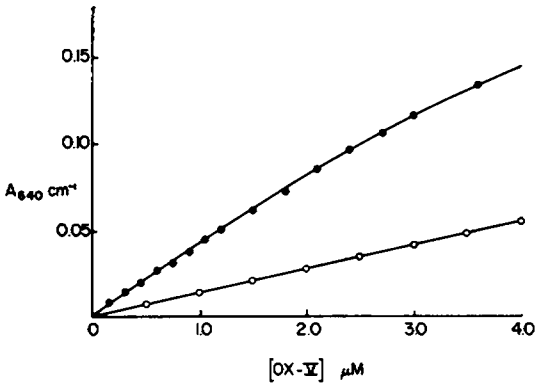

FIGURE 1 The interaction of OX-V with soybean lipid vesicles. The absorbance of OX-V at 640 nm in either the absence (o) or the presence of 0.02 mg/ml soybean lipid vesicles (•) was determined in a medium containing 100 mM Na_2SO_4 and 10 mM Na^+/Hepes pH 7.5 at 23°C. The line drawn through the closed circles was calculated assuming values of 147.9 nmol/mg lipid for n, the number of dye binding sites, 3.33 μM for K_d, the dye-lipid dissociation constant, and 5.87 for ϵ_b, the enhancement of oxonol absorption at 640 nm on binding to lipid vesicles.

A criticism of all these procedures is that the best-fit estimates of the parameters are determined by the method of least squares. Cornish-Bowden and Eisenthal (1974) have stressed that a least squares analysis requires a precise knowledge of the weighting factors for each determination, the assumption that the errors are normally distributed and that the independent variable is known precisely; these conditions certainly do not apply when σ and f are calculated from a single observation, as they are in the spectroscopic titrations described here.

A procedure that involves fewer assumptions about the nature of the experimental errors is the direct linear plot introduced by Cornish-Bowden and Eisenthal (1974) and developed by Porter and Trager (1977). In this case n and K_d can be estimated by the intersections of lines drawn through data points $(-\sigma_i,0)$ and $(0,f_i)$; for i data points there are $\frac{1}{2}(i)(i-1)$ intersections, and the best fit estimates are given by the median values of K_d and n, respectively. In the direct linear plot the principal assumption is that the errors of the estimates of n and K_d are as likely to be negative as they are to be positive. For large numbers of data points the intersections can be computed and the median values of the estimates can be determined by a sorting routine.

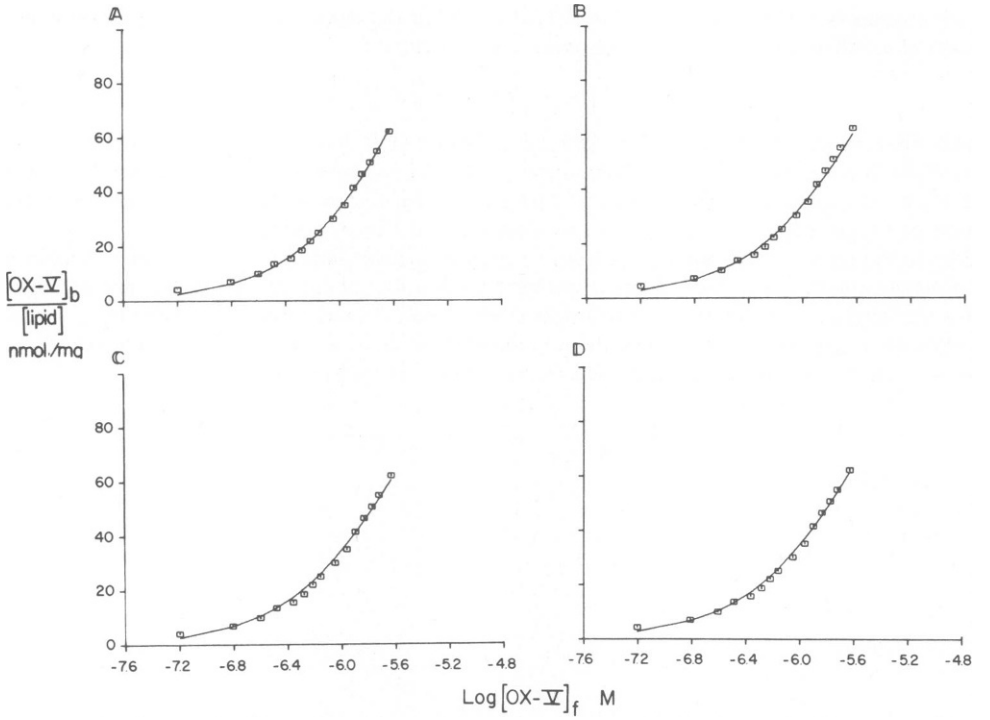

FIGURE 2 Estimates of the best fit binding parameters of OX-V with soybean lipid vesicles. The data presented in Fig. 1 were utilized to provide estimates of K_d and n according to the following procedures:

	K_d	n	K_d/n
	(μM)	(*nmol/mg*)	(*mg/ml*)
(A) Weighted least squares (Wilkinson, 1961)	4.11	172.6	0.024
(B) Scatchard plot (Scatchard, 1949)	3.09	138.5	0.022
(C) Langmuir isotherm (McLaughlin and Harary, 1976)	2.88	134.8	0.021
(D) Direct linear plot (Cornish-Bowden and Eisenthal, 1974)	3.23	145.6	0.022
Mean	3.33	147.9	0.022
SD	0.54	17.1	0.001

The results of a titration of OX-V against soybean lipid vesicles, 0.02 mg lipid/ml, are illustrated in Fig. 1. The values of σ and $[OX\text{-}V]_f$ were calculated using a value for $\epsilon_b - 1$ of the absorbance at 640 nm of 4.87, determined in a separate titration. The four plots in Fig. 2 represent the best fit of the experimental data to the Langmuir adsorption isotherm (Eq. 16) using the method of: (*a*) weighted least-squares (Wilkinson, 1961, Eq. 18); (*b*) Scatchard plot (Scatchard, 1949, Eq. 17), but omitting the outlying point at 0.15 μM OX-V added (Fig. 1); (*c*) a direct fit to the Langmuir isotherm by the minimization procedure of Davidon, Fletcher, and Powell (Fletcher and Powell, 1963) using Eq. 19; (*d*) the direct linear plot (Cornish-Bowden and Eisenthal, 1974; and Porter and Trager, 1977). The different fitting procedures provide a range of values of K_d (3.33 uM, SD 0.54) and n (147.9 nmol/mg lipid, SD 17.1) although the ratio K_d/n (0.022 mg lipid/ml, SD 0.001) shows very little variation. McLaughlin and Harary (1976) have noted that K_d and n in the Stern equation can be varied over a wide range and still provide a reasonable fit to the experimental data provided K_d/n was held constant. It thus appears that the different assumptions of the fitting procedures provide a range of estimates of K_d and n for the experiment illustrated in Fig. 1, although each procedure yields a similar value of the ratio K_d/n. This observation suggests that for comparative purposes K_d/n may provide a better indication of the affinity of the dye for the membrane than the more conventional parameter K_d, as the ratio seems less sensitive to the choice of analytical procedure. Indeed K_d/n can be determined readily by a lipid titration as long as the criterion of a large excess of binding sites over dye concentration applies. This condition also has the advantage that the perturbation of the membrane by the adsorbed dye is minimized.

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