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# Immobilization of Opiates by Membrane Lipids

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SUMMARY

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The polarization of the intrinsic fluorescence of levorphanol and related morphinans was used to investigate the physical and chemical nature of opiate-lipid complexes in both organic solvents and aqueous buffers. Phosphatidylserine, but neither phosphatidylethanolamine nor stearic acid, produced a large fluorescence polarization of levorphanol in a cyclohexane solvent system, suggesting that the drug became immobilized by phosphatidylserine micelles. Aqueous suspensions of phosphatidylserine and phosphatidylinositol produced a maximal fluorescence polarization of levorphanol, whereas the same concentrations of cerebroside sulfate and phosphatidic acid were much less effective and phosphatidylethanolamine, not at all. Thus phosphatidylserine and phosphatidylinositol greatly restricted the rotational mobility of the bound opiate while the other lipids did not. A modified Scatchard treatment of the data indicated single binding sites with dissociation constants of $8.8 \times 10^{-5}$ and $5.7 \times 10^{-5}$ M for the serine and inositol phosphatides, respectively. The accessibilities of chloroform and $I^-$ to the drug bound to phosphatidylserine liposomes were determined from fluorescence quenching experiments. Chloroform was about 10 times more accessible than $I^-$, suggesting that levorphanol was intercalated in the hydrophobic interior of the lipid bilayer and remote from the aqueous/lipid interface. Stereospecificity was absent, since the (+) enantiomer of levorphanol, dextrorphan, had the same binding affinities as levorphanol for both phosphatidylserine and phosphatidylinositol. A hydrogen bond between phosphatidylserine and the phenolic hydroxyl of opiate derivatives was demonstrated by infrared techniques in an organic solvent. The finding that the fluorescence of dextromethorphan could be polarized by the addition of phosphatidylserine liposomes demonstrated that hydrogen bonding between the phenolic hydroxyl and the lipid was not required for immobilization of the bound drugs. It is suggested that phosphatidylserine or phosphatidylinositol could be a site for the nonspecific binding of opiates and related compounds to nerve membranes.

## INTRODUCTION

The affinity of opiates for a number of membrane lipids, including cerebroside sulfate (1) and the serine and inositol phosphatides (2), has been demonstrated by a variety of chemical and physical techniques. Morphine *in vivo* increases the turnover of phospholipids in brain (3), and levorphanol alters phospholipid metabolism of the squid giant axon at a concentration which blocks electrical conduction (4). The present study was undertaken in

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order to define more clearly the chemical and physical nature as well as the specificity of opiate-lipid interactions.

Many opiates and related compounds are fluorescent because they contain a phenolic moiety having fluorescence lifetimes on the order of $10^{-9}$ sec. For dilute solutions of low viscosity, fluorescence polarization is a measure of rotational diffusion during the lifetime of the excited singlet state (5). During such a time domain, the orientation of molecules the size of opiates in solution at room temperature is virtually randomized by Brownian motion. On the other hand, immobilization by a particle of sufficient radius, estimated to be about 25 A from the Stokes relation, to make rotation negligible during the excited state lifetime would cause maximal polarization. Since even the smallest lipid microvesicle is expected to be an order of magnitude larger, fluorescence polarization of opiates could be used as an index of the degree of their immobilization and calculation of binding constants with lipid micelles and liposomes.

## MATERIALS AND METHODS

*Materials.* Phosphatidylserine and phosphatidylethanolamine were prepared from bovine brain by fractionation on DEAE-cellulose columns and identified by thin-layer chromatography as described previously (2). Phosphatidylinositol was prepared from baker's yeast by the method of Trevelyan (6), involving column chromatography on silicic acid, or purchased from Koch-Light. Other lipids were obtained from commercial sources as follows: sulfatide, Analabs; arachidonic acid, Sigma; stearic acid, Eastman; phosphatidic acid, Koch-Light. Lipids used in these studies were pure by chromatographic criteria. Levorphanol and dextrorphan tartrate were gifts from Hoffmann-La Roche.

Solvents used for fluorescence and infrared spectroscopy were obtained from standard sources and had been purified especially for that purpose. Other chemicals were of analytical reagent quality, including chloroform (Mallinckrodt), which was distilled prior to use as a chromatographic solvent.

*Organic solvent system.* Salts of the opiates were converted to the free base by the addition of 0.1 M $NaHCO_3$ and were extracted with three portions of ether. The combined ethereal extracts were dried ($MgSO_4$) and evaporated. Stock solutions of the drugs were made up using 10% methylene chloride in cyclohexane. Stock solutions of lipids were prepared in cyclohexane; the final solutions contained 1% methylene chloride by volume.

*Lipid suspensions.* Purified lipids were stored dissolved in organic solvents under nitrogen at −20°. Solutions were prepared approximately by weight, and the concentration of lipid was determined by phosphate assay (7). Stock suspensions of lipids were prepared essentially by the method of Bangham *et al.* (8). Thus 7–10 mg of lipid were evaporated from an organic solvent to form a thin film on the inside of a 50-ml Pyrex round-bottomed flask, using a rotary evaporator. Subsequently 3–4 ml of buffer plus several glass beads were added, and the mixture was shaken in a water bath at room temperature with PS[1] and PI, at 37° with PE, or at 60° with sulfatide. The suspension was then diluted with more buffer to yield a final stock suspension containing 1.0 μmole of lipid per milliliter and 0.1 mM EDTA, and was allowed to equilibrate at room temperature for at least 1 hr. PS microvesicles were prepared by sonication of a concentrated liposome suspension contained in a sealed tube under $N_2$, using a Bransonic-12 bath Sonifier. The resulting mixture was centrifuged at 100,000 × *g* for 30 min, and the clear supernatant was used as a stock suspension of microvesicles. Thin-layer chromatography of the microvesicles on silica gel H developed with heptane–ether–acetic acid (90:10:1) did not reveal any decomposition of the phospholipid to form free fatty acids.

*Fluorescence measurements.* Fluorescence intensities were obtained with a Perkin-Elmer model MPF-3 fluorescence spectrophotometer at the emission maximum, using high instrumental sensitivity and narrow slit widths (approximately 5-nm

[1] The abbreviations used are: PS, phosphatidylserine; PI, phosphatidylinositol, PE, phosphatidylethanolamine.

spectral bandpass) in order to minimize the overlap of the scattered excitation light with the fluorescence emission. In some experiments a filter which eliminated light of wavelength less than 290 nm was placed between the sample and the detector. A pair of film-type polarizing lenses of wide aperature (Perkin-Elmer) was employed for the measurement of fluorescence polarization, $P$, which was calculated according to the expression

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (1)$$

where $I_{\parallel}$ and $I_{\perp}$ refer to intensities obtained with the polarizers parallel and crossed, respectively. The observed intensities, $I_{obs}$, were corrected (9) for any residual scattering by subtracting the value obtained from a control sample containing no drug, $I_s$, as follows:

$$I = I_{obs} - I_s \quad (2)$$

Light scattering was expected to present additional difficulties for polarization measurements; the problem has been treated theoretically and solved experimentally using a system of filters and horizontal slits (10). Another approach has been the use of smaller cells to minimize scattering effects (11). In the present case identical results were obtained using cells with path lengths of 4 or 10 mm, and the larger cells were generally used.

*Binding constants.* Binding constants were calculated from the polarization data, using a modified Scatchard analysis (12). The number of moles of fluorescent ligand bound per mole of ligand, $\bar{S}$, is given by the expression

$$\bar{S} = \frac{P - P_{min}}{P_{max} - P_{min}} \quad (3)$$

where $P$ refers to polarization. The association constant, $K_a$ was determined from a plot of $\bar{S}/[L]$ vs $\bar{S}$ according to the equation

$$\frac{\bar{S}}{[L]} = K_a(1 - \bar{S}) \quad (4)$$

where $[L]$ is the concentration of unbound lipid. The reported values are averages of two or three independent determinations.

*Limiting polarization.* The limiting polarization of levorphanol, $P_0$ was estimated by extrapolating a plot of $1/P - 1/3$ vs. $1/\eta$ to zero according to the expression

$$\frac{1}{P} - \frac{1}{3} = \left[\frac{1}{P_0} - \frac{1}{3}\right]\left[1 + \frac{RT}{\eta V}(\tau)\right] \quad (5)$$

where $V$ is the molar volume of the fluorophore, $\tau$ is the lifetime of the excited singlet state, and $R$ and $T$ have their usual significance (5).

The viscosity was varied at room temperature by changing the relative proportions of methanol and propylene glycol used as the solvent (13). The values of $\eta$ were calculated as follows:

$$\log \frac{1}{\eta} = X_m \log \frac{1}{\eta_m} + X_g \log \frac{1}{\eta_g} \quad (6)$$

where $X$ is the mole fraction and the subscripts $m$ and $g$ denote methanol and propylene glycol, respectively (13). The fluorescence intensities, and presumably the lifetimes, were invariant over the range of solvent composition used. Moreover, the instrumental contribution to polarization was negligible under these conditions.

*Fluorescence quenching.* Quenching constants, $K_Q$, were determined from slopes of $I_0/I$ vs $[Q]$ according to the Stern-Volmer (14) relation:

$$\frac{I_0}{I} = 1 + K_Q[Q] \quad (7)$$

where $I_0$ is the fluorescence intensity in the absence of quencher and $[Q]$ is the concentration of quencher. Values obtained from two independent determinations were averaged. Conditions were chosen so that about 90% of the drug was initially bound to liposomes, and the ionic strength was maintained at a constant value by the addition of NaCl. Apparent accessibilities were estimated from the ratio $K_Q$ (liposome)/$K_Q$ (aqueous), where $K_Q$ (aqueous) is the value determined for the drug in the absence of lipid (15).

*Divalent ion competition.* For these experiments lipids were first washed with EDTA, which was omitted from the final

suspensions. To suspensions of liposomes or microvesicles containing bound drugs prepared as described above were added small volumes of concentrated divalent metal chloride solutions. In other experiments the salts and drugs were added simultaneously; about 1 hr was required for complete equilibration.

*Infrared measurements.* Infrared spectra were recorded from dilute solutions of the drugs (20 mM), using a 1.0-mm KBr liquid cell with a Perkin-Elmer model 257 spectrophotometer. Salts of the opiates were converted to the free base as described above, taken up in carbon tetrachloride, and finally dried with anhydrous sodium sulfate.

## RESULTS

*Cyclohexane solvent system.* Upon excitation at 280 nm the fluorescence emission of levorphanol free base in cyclohexane was observed at 305 nm, and the fluorescence polarization was minimal (0.02). The energy of maximum fluorescence remained unchanged after the addition of lipids that were soluble in cyclohexane. The addition of phosphatidylserine to a solution of 80 μM levorphanol increased the fluorescence polarization, $P$, which finally attained a maximum net increase in polarization, $P - P_{min}$, of 0.24 at 1 mM lipid concentration (Fig. 1); it remained at that value up to a PS concentration of 3 mM, which was the largest concentration used. Both phosphatidylethanolamine and stearic acid were much less effective over the same concentration range, attaining final $P - P_{min}$ values of 0.04 and 0.01, respectively. A Scatchard plot of the binding of levorphanol to PS revealed a single binding site with a dissociation constant of $1.1 \times 10^{-4}$ M (Fig. 1, inset).

The fluorescence intensity of levorphanol was approximately doubled after the addition of saturating amounts of PS. That this effect was caused by protonation of the free form of the drug was shown by titration with acetic acid in cyclohexane (data not shown).

*Aqueous suspensions.* The addition of an aqueous suspension of PS to a solution of 10 μM levorphanol tartrate in 20 mM Tris-Cl, pH 7.5, in the presence of 0.1 mM EDTA caused large increases in the fluorescence polarization of the drug, which became maximal ($P - P_{min} = 0.16$) when 0.50 μmole/ml was added (Fig. 2). The value of the dissociation constant was estimated to be $8.8 \times 10^{-5}$ M (Fig. 2, inset). Phosphatidylinositol was somewhat more effective than PS, reaching a maximum $P - P_{min}$ value of 0.20 at 0.25 μmole/ml of PI added; a value of $5.7 \times 10^{-5}$ M for $K_a$ was obtained (Fig. 2).

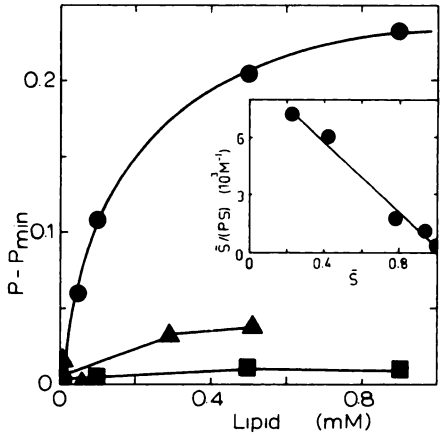


FIG. 1. *Effects of PS* (●– –●), *PE* (▲– –▲), *and stearic acid* (■– –■), *on fluorescence polarization of 80 μM levorphanol in cyclohexane containing 1% $CH_2Cl_2$*

Inset: Modified Scatchard plot of binding of levorphanol to PS micelles according to Eq. 4. The value of $K_D$ (−1/slope) was $1.1 \times 10^{-4}$ M.

Phosphatidic acid and cerebroside sulfate were much less effective than either PS or PI, reaching maximal polarizations equal to only 25% of that obtained with PI (Fig. 2). The same result was obtained when cerebroside sulfate was suspended in the presence of levorphanol. Phosphatidylethanolamine was the least effective lipid tried; a final net polarization of 0.02 was attained. A suspension prepared from a 1:1 mixture of PS and PE polarized the fluorescence of levorphanol to the same degree as PS alone when twice the total amount of lipid was added.

The fluorescence polarization of tyrosine, which has spectroscopic properties very similar to levorphanol but was not expected to bind to the lipids, served as an

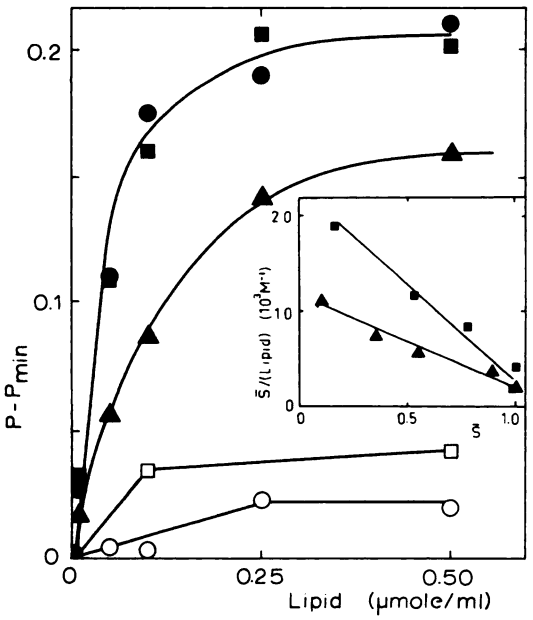


FIG. 2. *Effects of PS* (▲– –▲), *PI* (■– –■), *PE* (○– –○), *and cerebroside sulfate* (□– –□) *suspensions on fluorescence polarization of 10 μM levorphanol, and of PI on fluorescence polarization of 10 μM dextrorphan* (●– –●)

Final suspensions contained 20 mM Tris-Cl, pH 7.5, and $10^{-4}$ M EDTA. Inset: Modified Scatchard plot of the binding of levorphanol to PS (▲– –▲) and PI (■– –■) liposomes according to Eq. 4. The values of $K_D$ (−1/slope) were $8.8 \times 10^{-5}$ M and $5.7 \times 10^{-5}$ M, respectively.

additional control for light scattering effects. Serine and inositol phosphatides caused only small polarizations of the fluorescence of 10 μM tyrosine under conditions that resulted in large polarizations of levorphanol.

*Limiting polarization.* How did the observed polarizations compare with the maximum possible value? The viscosity of 10 μM solutions of levorphanol was varied at constant temperature by changing the composition of the solvent. Mixtures of methanol and propylene glycol, which have the same dielectric constant but differ by a factor of 60 in viscosity, were used (13). The limiting polarization of levorphanol fluorescence was estimated from a Perrin plot to be 0.17 (Fig. 3) and represents the mean of three independent experiments. The maximum polarizations obtained with PS and PI liposomes were near the limiting value.

*Stereospecificity.* Since some stereospecificity in the ability of opiates to inhibit competitively the binding of [$^{14}$C]-morphine to PS spread at the air/water interface could be demonstrated (2), the ability of lipids to polarize the fluorescence of dextrorphan was investigated. The fluorescence of these enantiomers was polarized to the same degree in the presence of all lipids tested. For example, Fig. 2 illustrates the effect of aqueous suspensions of PI on the fluorescence polarization of both levorphanol and dextrorphan.

*Hydrogen bonding.* Infrared spectra of phosphatidylserine in the presence of levorphanol and 3-methoxymorphine revealed hydrogen bonding between the phenolic hydroxyl group and the lipid (Fig. 4). The free phenolic hydroxyl stretching band of levorphanol at 3610 $cm^{-1}$ was reduced in the presence of phosphatidylserine, while the intensity in the hydrogen bonding region (3100–3500 $cm^{-1}$) simultaneously increased. Addition of more PS further reduced the intensity of the free phenolic hydroxyl. No reduction was observed in the free alcoholic hydroxyl group of 3-methoxymorphine or

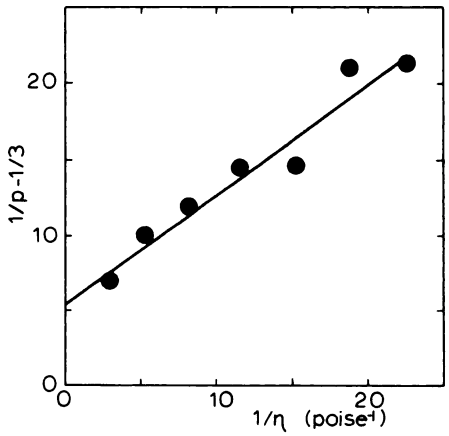


FIG. 3. *Perrin plot of fluorescence polarization of 10 μM levorphanol as a function of viscosity in mixtures of methanol and propylene glycol at room temperature*

The limiting polarization was 0.17 as determined from the intercept of the line (Eq. 5).

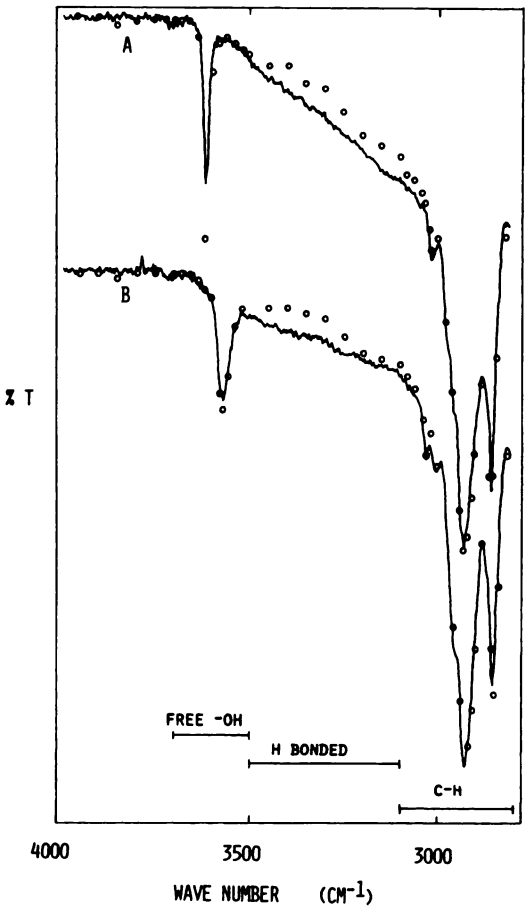


FIG. 4. *Infrared spectra of 0.02 M levorphanol (A) and 3-methoxymorhpine (B) in the presence of PS (3.5 mg/ml)*

The solid lines represent experimental records, and the points were calculated from the spectra of the individual components, assuming the additivity of absorbance and correcting for the small absorbance due to the cell plus solvent ($CCl_4$) alone.

3-ethoxymorphine under identical conditions. The data did, however, reveal some increase in hydrogen bonding from additional unknown sources, as shown in Fig. 4 by increased absorption in the 3200–3500 $cm^{-1}$ region.

In order to determine whether hydrogen bonding from the phenolic hydroxyl of the drugs was necessary to obtain the observed immobilization, the fluorescence polarization of 10 $\mu$M dextromethorphan in the presence of added PS liposomes was measured. As shown in Fig. 5, the affinity of dextromethorphan for PS was found to be about twice that of levorphanol.

*Divalent cations.* In order to determine whether divalent cations could compete with morphinans for lipid binding sites, their ability to reduce the polarization of levorphanol fluorescence in the presence of PS liposomes was measured over a concentration range of 1 $\mu$M–10 mM. In the presence of 1 mM $Ca^{2+}$ or 10 mM $Mg^{2+}$ the values of $P - P_{min}$ were reduced to 60% ± 13% and 52% ± 16% of their values in the absence of divalent cations, respectively (means ± standard deviations of four determinations). The source of the large variability is unknown. The ability of $Mn^{2+}$ to prevent the binding of levorphanol to PS liposomes was similar to that of $Ca^{2+}$, but no paramagnetic quenching of fluorescence was observed. Similar results were obtained if the divalent ions were added simultaneously with the drugs. When PS microvesicles were substituted for liposomes, 0.20 mM $Ca^{2+}$ and 0.30 mM $Mg^{2+}$ displaced 51% and 58% of the drug, respectively.

*Fluorescence quenchers.* The accessibilities of neutral and ionic fluorescence quenchers were measured in order to characterize the environment of the bound drug. Chloroform was found to be an efficient quencher of levorphanol fluorescence; as shown in Fig. 6, the value of

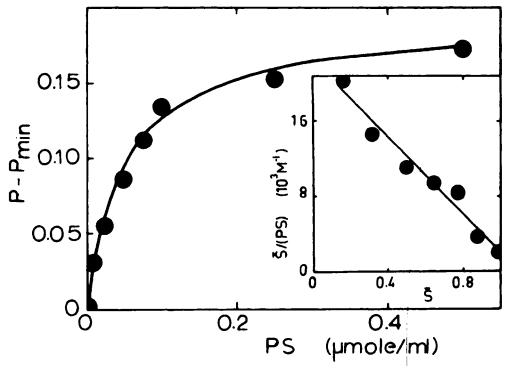


FIG. 5. *Effect of added PS liposomes on fluorescence polarization of 10 $\mu$M dextromethorphan*

See Fig. 2 for conditions. Inset: Modified Scatchard plot of the binding of dextromethorphan to PS liposomes according to Eq. 4. The value of $K_D$ (−1/slope) was $5.0 \times 10^{-5}$ M.

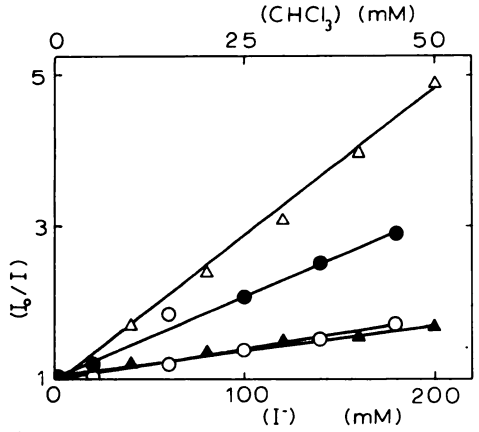


FIG. 6. *Quenching of fluorescence intensity of 10 μM levorphanol by chloroform in the presence (●– –●) and absence (○– –○) of 0.25 μmole/ml of PS liposomes, and by $I^-$ in the presence (▲– –▲) and absence (△– –△) of liposomes, according to Eq. 7*

The final solutions contained 20 mM Tris-Cl pH 7.5. The relative accessibilities of chloroform and $I^-$ to the bound drug, as estimated from the ratio of the slopes of the lines, were 2.6 and 0.17, respectively.

the quenching constant for the drug bound to PS liposomes was more than twice that observed with the drug alone. Although $I^-$ was also a good quencher, the value of the quenching constant for levorphanol bound to PS liposomes was only 17% of the value obtained in the absence of the lipid (Fig. 6). Virtually identical results were obtained by adding NaI to previously formed liposomes or by preparing the liposomes in the presence of the quencher.

## DISCUSSION

The polarization studies clearly demonstrated that opiates and related compounds become immobilized upon binding to PS micelles and to PS and PI liposomes as well as liposomes prepared from mixtures of PS and PE. Phosphatidylserine, which is the major acidic lipid in brain, is known to be closely associated with membrane proteins (16). In addition, it activates ($Na^+$ + $K^+$)-ATPase (17) and tyrosine hydroxylase (18) activities of brain. Although PI is present in much smaller amounts, its turnover is accelerated by both synaptic transmission (19) and the administration of opiates *in vivo* (3). Cerebroside sulfate, which has been shown to bind opiates stereospecifically with high affinity (1), could not effectively immobilize the drugs. Apparently binding is not sufficient to restrict the rotational mobility of the opiates.

Although some stereospecificity in the ability of opiates to compete with the binding of [$^{14}$C]morphine to PS surface films was demonstrated in a previous study (2), there was no selectivity between levorphanol and dextrorphan for PS or PI liposomes. It should be noted, however, that stereospecificity has only been demonstrated under conditions in which a large excess of drugs must compete for a small number of binding sites. In the present case the concentration of binding sites was in excess of the drug concentration, which was fixed at 10 μM.

Reduction in the intensity of the free phenolic hydroxyl vibrational band and a simultaneous increase in the hydrogen bonding region provided evidence for a hydrogen bond between levorphanol and PS in an organic solvent. Although it may also be a factor in the association between opiates and aqueous liposomes, hydrogen bonding was not required for immobilization. Dextromethorphan, in which the phenolic hydrogen is replaced by a methyl group, was also effectively immobilized by the addition of PS or PI liposomes.

Scatchard analysis of polarization data revealed binding constants that were several orders of magnitude weaker than those associated with the stereospecific binding sites, which have been well correlated with pharmacological potency (20). Immobilization of opiates by nerve membrane lipids could, however, occur with doses of opiates that produce analgesia. In attempting to use data obtained with liposomes for speculation about membranes, the availability of the lipids in the milieu of the membrane must be given consideration. This factor is presumably controlled by associations with other lipids, and especially with proteins.

Relatively large concentrations of divalent cations were required to displace the bound opiate from PS liposomes and microvesicles. This finding suggested that

opiates can effectively compete with $Ca^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ for acidic lipid binding sites. The ability of morphine to deplete $Ca^{2+}$ significantly from regional brain areas *in vivo* has been demonstrated (21), and both $Mn^{2+}$ and $Mg^{2+}$ have been shown to increase the binding of agonists to opiate receptors (22). These data do not, however, provide an accurate measure of relative affinities, since the lipid was in excess.

A question not directly addressed in this study concerns the extent to which differences in physical structure of the lipids under various experimental conditions contributed to the polarization measurements. For example, PS, PI, and phosphatidic acid readily form closed multilamellar liposomes by the procedures used here, but PE does not (23, 24). In addition, divalent cations can modify the physical properties of lipids (25). Nevertheless, it is apparent that the bilayer is a common structural feature of both natural and artificial membrane systems.

The finding that a neutral fluorescence quencher was an order of magnitude more accessible than an ionic one strongly suggested that the bound opiate was buried in the hydrocarbon region of the lipid bilayer. Immobilization of the drugs by PS and PI liposomes was not specific for analgesia-producing opiates. These lipids are, however, present in nerve membranes and could conceivably serve to incorporate a drug into a hydrophobic region near the stereospecific binding site, which has been shown to be sensitive to both protein- and lipid-degrading enzymes (26). The increased affinity of the most hydrophobic derivatives, such as etorphine (20), further supports the notion that the opiate receptor includes a hydrophobic component. The polarization data presented in this report suggest that complexation with PS or PI could provide a molecular basis for the nonspecific binding of opiates to nerve membranes.

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