MOLECULAR PHARMACOLOGY, 12, 263-278

Nuclear Magnetic Resonance Studies of Barbiturate-Phospholipid Interactions

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(Received April 22, 1975)

SUMMARY

NOVAK, R. F. & SWIFT, T. J. (1976) Nuclear magnetic resonance studies of barbiturate-phospholipid interactions. *Mol. Pharmacol.*, 12, 263-278.

Interactions between phenobarbital or pentobarbital with the phospholipids phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and cardiolipin were indicated by the effects of added phospholipid on the ^{1}H magnetic resonance spectra of these barbiturates. Similar studies with phosphatidylserine gave no indication of interaction between phospholipid and the barbiturates at a comparable phospholipid concentration. Of the two barbiturates studies, phenobarbital produced the greater spectral change at a given phospholipid to barbiturate ratio. The interactions of the barbiturates with the phospholipids were further characterized using ^{31}P and ^{13}C magnetic resonance. Addition of barbiturate to phospholipid produced marked upfield shifts in the respective ^{31}P peaks of the phospholipids. ^{13}C studies verified that protonation of the basic phosphate group of the phospholipids by the weakly acidic N$-H$ proton of the barbiturate did not account for the observed spectral changes. The direction, magnitude, and temperature dependence of the observed changes in chemical shifts in the proton and phosphorus magnetic resonance spectra are consistent with hydrogen bonding as the mode of association of the barbiturates with the phospholipids. These studies specifically identified the molecular sites of interaction as an N$-H$ moiety of the barbiturate and the phosphate group of the phospholipid, and allowed determination of the association constants for the interactions in chloroform: K_{assoc} for the various barbiturate-phospholipid pairs ranged from 175 to 25, the latter value corresponding to the phosphatidylethanolamine-phenobarbital interaction. The results are discussed in terms of a model for the interaction of barbiturates with membranes.

INTRODUCTION

A large number of drugs, especially those affecting the central nervous system, e.g., the barbiturates, require a considerable degree of membrane permeability or membrane solubility to achieve the desired clinical effect. Barbituric acid itself produces no anesthetic effect, and it is only with the addition of various substituents in the C-5 position (Fig. 1) that it derives its general depressant nature. The pharmacological properties of the barbiturates have been related to their membrane solubility (i.e., the more highly membrane-soluble compounds are the shorter-acting agents with a rapid onset of action) (1).

Since the barbiturates exist in significant concentrations in both ionized and un-ionized forms at pH 7 in aqueous media, it becomes necessary to consider

This work was supported by National Institutes of Health Grant NS-07758-06.

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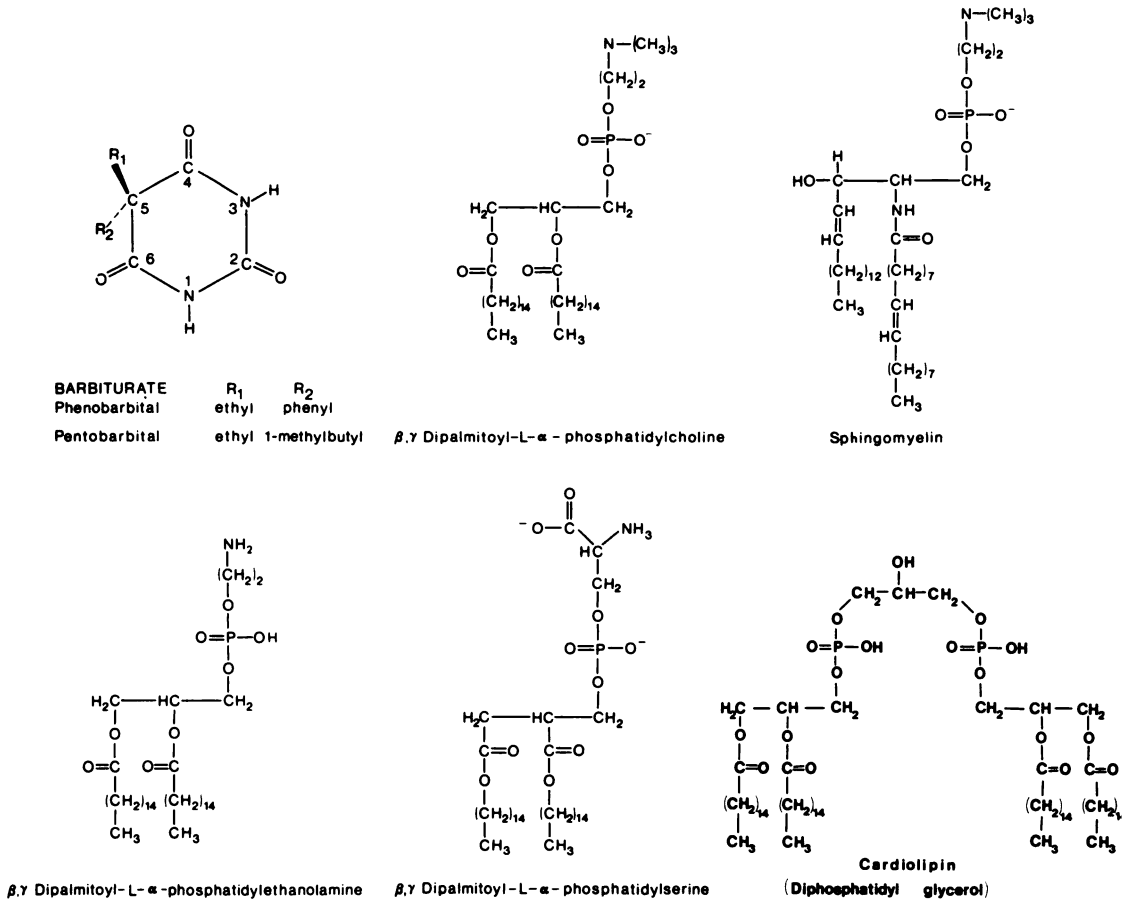


FIG. 1. *Barbiturate and phospholipid structures*

which form is responsible for anesthetic action. Clowes *et al.* (2), evaluating the effect of pH change in both extracellular and intracellular phases on the potency of barbiturates in inhibiting cell division of fertilized eggs and movement of the larvae of *Arbacia,* concluded that the uncharged form of the barbiturate was responsible for anesthetic action. Blaustein (3), however, using giant axons of the lobster, found that pentobarbital, when applied extracellularly, was more effective in blocking the action potential at external pH 8.5 than at pH 6.7 and concluded that the anionic form of pentobarbital was the active form. In contrast, Krupp *et al.* (4) observed that both pentobarbital and phenobarbital were more effective at external pH 6.8 than at pH 8.8 in blocking the action potential of demyelinated frog nerve. Further studies by Narahashi *et al.* (5), using internally and externally perfused squid giant axon, support the conclusion that pentobarbital blocks the action potential in the uncharged molecular form.

Models presented for ion flow in excitable membranes postulate that the polar groups of phospholipids may undergo configurational transitions producing changes in membrane permeability and nerve function (6–8). Certain agents have been demonstrated to influence cation permeability of lipid bilayer model systems (9), and the barbiturates have been demonstrated to affect the electrical properties of nerve *in vitro* (3–5). While Blaustein and Goldman (10) have suggested a possible drug-phospholipid complex, no investigation of these interactions on a molecular level has been made. If barbiturates are

active in the uncharged form, as experiments of Krupp *et al.* (4) and Narahashi *et al.* (5) have indicated, then one mechanism of drug-membrane interaction might be complex formation with phospholipids through a combination of hydrogen bonding and hydrophobic interactions.

^{1}H, ^{31}P, and ^{13}C magnetic resonance spectroscopy has been employed to investigate directly the nature and strength of barbiturate-phospholipid interactions as a model for possible drug-membrane interactions. While the membrane itself is amphipathic and exposed to water at its hydrophilic surfaces, the interior is relatively nonpolar and hydrophobic in nature. These studies were conducted using a weakly polar medium, chloroform, in order to simulate the internal polarity of the membrane, as well as to provide optimal conditions for observing an interaction by nuclear magnetic resonance spectroscopy. To investigate the possibility of an interaction occurring between barbiturates and phospholipids in a more polar environment such as water, a "wet" phospholipid (one in which the water concentration was 5 times the concentration of the phospholipid) was made, and barbiturate-phospholipid association was demonstrated. While experimental requirements preclude investigation of this phenomenon in a totally aqueous environment, the wet phospholipid approach was taken to demonstrate that association can in fact occur in an environment containing a considerable concentration of water.

MATERIALS AND METHODS

Phenobarbital was purchased from Merck and used without further purification. Pentobarbital was obtained from K & K Laboratories as the sodium salt and converted to the acid form. The sodium pentobarbital was dissolved in distilled water and acidified with nitric acid before extraction with chloroform. The pentobarbital was purified by recrystallization from hot water. Tri-*n*-butyl phosphate was obtained from Fisher and used without purification. An alcoholic solution of sodium hydroxide was prepared by the addition of sodium hydroxide to anhydrous ethanol. The concentration of the resulting solution was determined by titration. Phosphatidylethanolamine (bacteria) was purchased from Calbiochem. Phosphatidylcholine (egg) and sphingomyelin (bovine brain) were purchased from Sigma Chemical Company. Cardiolipin (bovine brain) and phosphatidylserine (bovine brain) were ordered specially prepared in the acid form from Applied Science Laboratories. The purity of these phospholipids was checked by thin-layer chromatography on silica gel plates, using solvent systems composed of $CHCl_3$–CH_3OH–H_2O (70:30:4) and $CHCl_3$–CH_3OH–CH_3CO_2H–H_2O (75:25:5:2) (11). The compounds were located by iodine vapor or by ashing with an H_2SO_4 solution saturated with $Na_2Cr_2O_7$. Each method produced a single, well-defined, darkened area. Thin-layer chromatography performed on samples after completion of NMR experiments showed virtually no decomposition.

The experimental solutions were prepared with deuterochloroform (Diaprep) which had been dried over molecular sieves. A ^{1}H Fourier transform spectrum of the $CDCl_3$ was taken to check the purity of the solvent. After 300 acquisitions no signals other than $CHCl_3$ were present. All solutions were stored over molecular sieves (type 4A, Fisher) for at least 2 days prior to use.

The "wet" phospholipid was made by addition of aliquots of deionized water to a solution of phospholipid in chloroform until the desired concentration ratio of H_2O to phospholipid was achieved. No change in the physical characteristics of the solution were observed.

The ^{31}P spectra of the phospholipids in deuterochloroform were recorded at 24.3 MHz on a Varian HA 60 spectrometer equipped with a probe which accepts 15-mm sample tubes and is modified for Fourier transform technique. A 20% solution of tetraethylammonium phosphate (pH 10–12) contained in a concentric 4.5-mm NMR tube served as an external lock standard. The observed changes in the ^{31}P chemical shift are reported in parts per million and are considered accurate to ± 0.1 ppm.

The ^{31}P variable-temperature studies were performed on the Varian HA 60 instrument using 12-mm sample tubes; temperatures were measured to ±1°. The changes in chemical shift as a function of increasing temperature are reported for phosphatidylcholine in Hertz, using a 20% solution of phosphoric acid as the external lock standard.[2, 3] The addition of barbiturate to phosphatidylcholine sufficiently shifted the ^{31}P peak upfield to allow the use of a 20% phosphoric acid lock. Phosphoric acid does not shift appreciably with temperature (≦5 Hz over the temperature range 30–60°) as determined by measuring the change in chemical shift of the 20% phosphoric acid lock with a solution of 0.4 M tri-*n*-butyl phosphate in carbon tetrachloride; therefore the downfield shift of the complexed phosphatidylcholine peak with increasing temperature could be obtained. Teflon inserts were used in the 15-mm and 12-mm NMR tubes to assure that the external lock remained centrally located while spinning. The direction, values, and thermal effects of all ^{31}P studies were verified using the Varian XL 100 spectrometer operating in the ^{31}P Fourier transform mode at 40.5 MHz. Spectra obtained were proton-decoupled, and the instrument was locked internally on the signal from deuterochloroform. Temperatures were measured directly to ±1° in the probe with a thermometer.

The ^{1}H magnetic resonance results were recorded at 60 MHz and 100 MHz using Varian A 60A and HA 100 spectrometers, respectively. Shifts are reported downfield from tetramethylsilane, the internal reference, in parts per million, with an accuracy of ±0.03 ppm. Variable-temperature studies were performed using the A 60A spectrometer, and temperatures were measured to ±1°.

^{13}C studies were performed with a Varian XL 100 spectrometer operating at 23.5 MHz in the Fourier transform mode. The instrument was locked internally on the deuterium signal from deuterochloroform, with the ^{13}C deuterochloroform signal serving as the internal reference. Changes in chemical shifts are reported in parts per million, with the negative sign indicating a downfield shift from $CDCl_3$.

Treatment of data. The nonlinearity of the ^{31}P plots of the change in chemical shift vs. increasing concentration of barbiturates allows stability constants to be calculated. The method by which these stability constants may be calculated employs the following equation (12, 13):

$$\delta_{obs} = x_a\delta_a + (1 - x_a)\delta_b$$

where δ_a and δ_b represent the chemical shift in parts per million of the unassociated species and the complexed species, respectively, and x represents the mole fraction of nuclei that are not complexed. If the changes in chemical shift which occur as a result of complexation are measured from the peak of the free species, then δ_a can be taken as the reference point and set equal to zero. The equation now reduces to

$$\Delta\delta_{obs} = (1 - x_a)\delta_b = x_b\delta_b$$

where x_b is the mole fraction of complexed nuclei.

The association constant K_{assoc} (assuming a 1:1 complex) is given by

$$K_{assoc} = \frac{[C]}{[\text{barbiturate} - C]\,[\text{phospholipid} - C]}$$

[2] Although the accepted convention for reporting ^{31}P chemical shifts is to use 85% phosphoric acid as a standard, the experimental conditions imposed by the Fourier transform technique and instrumentation as well as the observed ^{31}P chemical shift of the samples precluded use of 85% phosphoric acid as the external lock standard.

[3] Tetraethylammonium phosphate was also tested in the temperature study; however, the ^{31}P peak of this standard shifted dramatically with temperature as determined against a 0.4 M solution of tri-*n*-butyl phosphate in carbon tetrachloride. However, when the known values for the shift of tetraethylammonium phosphate as a function of temperature were established, a standard curve could be constructed. When this chemical shift was taken into consideration in measuring the difference in the downfield shift of the phosphatidylcholine peak as a function of temperature, the values were essentially the same as those determined with respect to the 20% phosphoric acid reference (differences in $\Delta\delta$ ≦2 Hz).

with the mole fraction

$$x_b = \frac{[C]}{[\text{phospholipid}]}$$

where C is defined as the concentration of the phospholipid-barbiturate complex. It is possible via a reiterative procedure to evaluate K_{assoc} and δ_b. By using various stability constant values and values of δ_b, a reasonable fit of the data points may be obtained. The results of these calculations for the systems phosphatidylcholine-phenobarbital, phosphatidylcholine-pentobarbital, sphingomyelin-phenobarbital, and sphingomyelin-pentobarbital are given in Table 1, and typical examples of the curve fit obtained are shown in Fig. 5A, B, and C. These results are summarized in Table 1.

RESULTS

^1H NMR studies. Proton magnetic resonance spectra of certain barbiturates in $CDCl_3$ have been reported previously (14, 15). Phenobarbital (0.04 M) gives a PMR spectrum consisting of a singlet at δ 8.2, indicative of protons bound to the ring nitrogen atoms. The phenyl group gives rise to a singlet at δ 7.4, with the ethyl substituent producing a quartet at δ 2.6 and a triplet at δ 1.0. The PMR spectrum of pentobarbital (0.04 M) has a singlet at δ 8.2 produced by the protons of ring-bound nitrogen, as well as signals of various multiplicities in the range 1–2 ppm resulting from the ethyl and 1-methylbutyl substituents.

The chemical shift of the signal corresponding to the protons attached to the nitrogen atoms of the ring varies with temperature and solvent; as the polarity of the solvent increases, the position of the N$-H$ peak is shifted downfield (15, 16). The self-association constants of phenobarbital and pentobarbital in $CDCl_3$ are 8.1 M^{-1} and 7.0 M^{-1}, respectively (16).[4]

Addition of aliquots of phosphatidylcholine (0.11 M) and sphingomyelin (0.045 M) in $CDCl_3$ to a solution of 0.04 M pentobarbital in $CDCl_3$ produced significant line broadening[5] and a considerable downfield shift in the N$-H$ peak of the barbiturate, with no change in the remainder of the spectrum. A plot of the chemical shift of the N$-H$ peak of phenobarbital against the concentration of phosphatidylcholine and sphingomyelin is given in Fig. 2A and B. A phosphatidylcholine to phenobarbital concentration ratio of 1:1 corresponds to a 1.9-ppm downfield shift of the N$-H$ signal, while a 1:1 ratio of phosphatidylcholine to pentobarbital gives a shift of 0.9 ppm (Fig. 2A and B). Similarly, for sphingomyelin-phenobarbital and sphingomyelin-pentobarbital at unity concentration ratios, downfield shifts of 1.9 ppm and 0.9 ppm, respectively, were measured (Fig. 2A and B).

Addition of aliquots of 0.03 M cardiolipin (acid form) to samples of 0.04 M phenobarbital and 0.04 M pentobarbital produced large downfield shifts with signal broadening. At a concentration ratio of cardiolipin to phenobarbital of 0.43, a downfield shift of 1.4 ppm was observed, while the corresponding ratio of cardiolipin to pentobarbital gave a 0.9-ppm shift (Fig. 2A and B). Addition of aliquots of 0.07 M phosphatidylserine (acid form) to 0.04 M phenobarbital produced virtually no changes in linewidth or chemical shift of the barbiturate

[4] Calculation of the dimer concentration in 0.04 M phenobarbital and 0.04 M pentobarbital gives a value of about approximately 8 mM; 20% of 0.04 M barbiturate exists as dimers. While these concentrations of dimers may be present, it is not necessary to add an additional term to the first equation. The contribution of such a term would be small, since K_{assoc} for the barbiturate-phospholipid complex is larger than that for dimer formation.

[5] Quantitative data on linewidth changes of the N$-H$ peak with addition of the phospholipids phosphatidylcholine, sphingomyelin, and cardiolipin were not determined. These linewidth changes may occur as the result of chemical exchange of the barbiturate between the free and complexed species, or may be due to additional ^{14}N quadrupolar relaxation effects arising from complexation of the barbiturate. These changes are more difficult to monitor accurately and are not as dramatic as the changes occurring in chemical shift. In the case of phosphatidylethanolamine, however, quantitative data were determined, since the linewidth changes, measured at 100 MHz, caused by addition of this phospholipid were marked, changing by a factor of 5 over a small concentration range; these changes were the principal manifestation of the interaction in this case.

TABLE 1

System	K_{assoc}[a]	δ_b[b]
	M^{-1}	*ppm*
Phosphatidylcholine-phenobarbital	115	2.05
Phosphatidylcholine-pentobarbital	50	1.08
Sphingomyelin-phenobarbital	175	1.7
Sphingomyelin-pentobarbital	100	1.2
Phosphatidylethanolamine-phenobarbital	25	0.5

[a] Estimated error in these values is approximately ±10%.

[b] δ_b is the limiting chemical shift value for the fully complexed species.

N–*H* peak. Addition of aliquots of 0.03 M phosphatidylethanolamine to a solution of 0.04 M phenobarbital produced considerable line broadening and a small downfield shift of the N–*H* peak. A plot of the change in chemical shift of the phenobarbital N–*H* peak against increasing phosphatidylethanolamine concentration is given in Fig. 2A (inset). The full linewidth at half-maximum of the barbiturate N–*H* peak with addition of phosphatidylethanolamine changed by a factor of approximately 5, going from about 5.6 Hz to 25.6 Hz (at 100 MHz) at a relatively small phosphatidylethanolamine to barbiturate ratio of 0.1. Aliquots of 3.65 M tri-*n*-butyl phosphate added to solutions of 0.04 M phenobarbital and 0.04 M pentobarbital also resulted in large downfield shifts of 2.1 ppm and 1.9 ppm, respectively, at a tri-*n*-butyl phosphate to barbiturate ratio of 34.5. However, it is important to note that the concentration of tri-*n*-butyl phosphate was considerably larger (i.e., 100-fold) than that reached in the cases of phospholipid additions. At a barbiturate to phospholipid concentration ratio of 0.1, the change in the N–*H* linewidth upon the addition of phosphatidylcholine, sphingomyelin, cardiolipin, or tri-*n*-butyl phosphate was negligible compared to that observed for the phosphatidylethanolamine-phenobarbital system.[5]

Hydrogen bonding in PMR is characterized by a downfield shift and displays a marked temperature dependence, the peak shifting back toward the non-hydrogen-bonded position with increasing temperature, i.e., upfield (17). As noted above, the N–*H* peak of the barbiturates was shifted downfield with the addition of phospholipid or tri-*n*-butyl phosphate. The effect of temperature was monitored over the range 34–65°, and the results were similar for all the reactant pairs. For example, the temperature dependence of the barbiturate-phosphatidylcholine interaction is given in Fig. 3, where the log of the change in chemical shift of the N–*H* peak is plotted against reciprocal temperature. The upfield shift produced by the 30° increase in temperature was 30 Hz (0.5 ppm) for phosphatidylcholine-phenobarbital at a concentration ratio of 0.4, and 8 Hz (0.08 ppm) for phosphatidylethanolamine-phenobarbital at a concentration ratio of 0.1. The slopes were virtually identical for all the barbiturate-phospholipid pairs at various concentration ratios, thus indicating that the ΔH values of formation of the complexes were all approximately equal. This result might be expected, since hydrogen bond formation typically yields ΔH values of 2–10 kcal/mole (18).

^{31}P NMR studies. In order to investigate the involvement of the phosphate moiety of the phospholipids in the interaction with the barbiturates, ^{31}P magnetic resonance studies were conducted. The ^{31}P chemical shift of phosphatidylcholine is approximately 3 ppm (75 Hz) upfield from the signal of a 20% solution of tetraethylammonium phosphate (external reference), while those of sphingomyelin and phosphatidylethanolamine are approximately 2 ppm (45 Hz) and 2.6 ppm (63 Hz), respectively. Some approximate values for the ^{31}P signal positions of the phospholip-

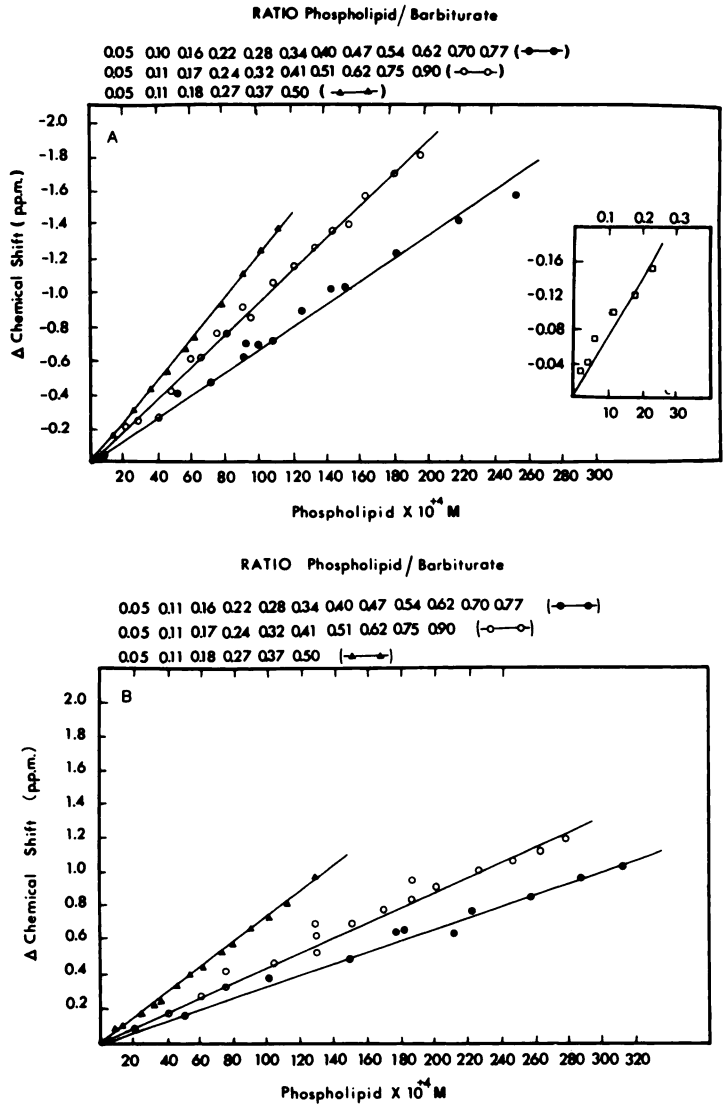


FIG. 2. *Change in chemical shift of the barbiturates phenobarbital (A) and pentobarbital (B) as a function of increasing concentration of cardiolipin (▲), sphingomyelin (○), phosphatidylcholine (●), and phosphatidylethanolamine (□, inset in A)*

The concentration of barbiturates used was 0.04 M, the maximum solubility of phenobarbital in chloroform.

ids as related to a solution of 20% phosphoric acid are: phosphatidylcholine, about 0 ppm; sphingomyelin, about −1 ppm; phosphatidylethanolamine, about −0.6 ppm. A typical spectrum of 0.045 M phosphatidylcholine is given in Fig. 4.

Addition of weighed amounts of solid phenobarbital or aliquots of a 0.04 M solution of pentobarbital to a solution of 0.11 M and 0.045 M phosphatidylcholine or 0.045 M sphingomyelin produced significant upfield shifts in the phosphorus peaks of the phospholipids. There was virtually no change in the spin coupling constant J_{PH} of phosphatidylcholine. A plot of the chemical shift of the ^{31}P peak of phosphatidylcholine against increasing concentrations of phenobarbital and pentobarbital is

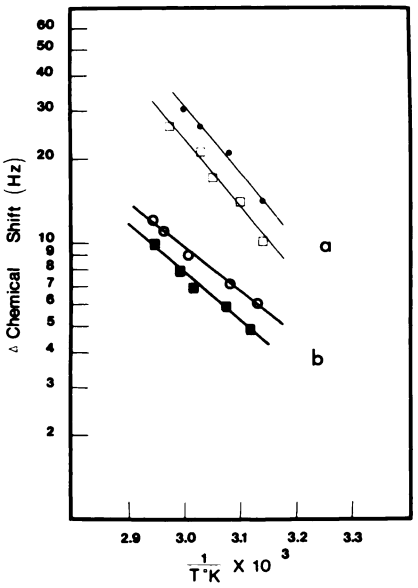


FIG. 3. *Chemical shift temperature dependence*

a. Typical change in the N—*H* chemical shift (upfield) for the systems phosphatidylcholine-phenobarbital (●) and phosphatidylcholine-pentobarbital (□) with increasing temperature. The concentrations of phosphatidylcholine in the phosphatidylcholine-phenobarbital and phosphatidylcholine-pentobarbital systems were 14 and 31 mM, respectively.

b. Downfield change in the ^{31}P chemical shift of 0.11 M phosphatidylcholine, 68 mM in phenobarbital (○) and 150 mM in pentobarbital (■), as a result of increasing temperature.

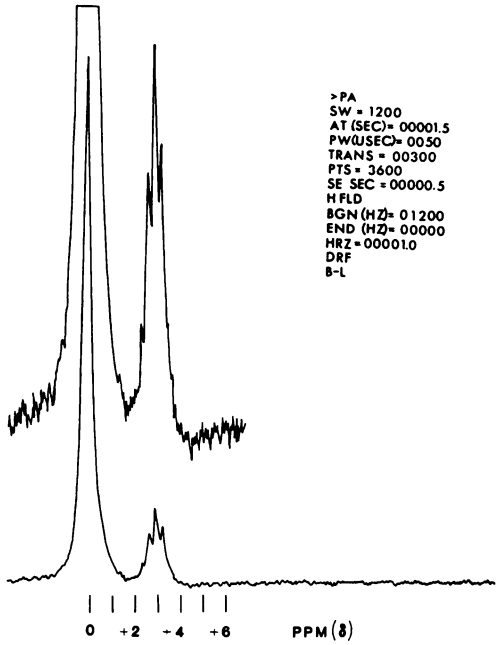


FIG. 4. *^{31}P Fourier transform spectrum of 0.045 M phosphatidylcholine consisting of a peak split into five components with spin coupling constant J_{PH} of 7 Hz*

The large peak occurring at 0 ppm is the ^{31}P lock/reference signal of tetraethylammonium phosphate.

given in Fig. 5A and B; the corresponding plot for the addition of phenobarbital and pentobarbital to sphingomyelin is given in Fig. 5C. Addition of weighed amounts of phenobarbital to 7 mM cardiolipin produced a small upfield shift of the cardiolipin ^{31}P peak (Fig. 5D). This shift is much less than that observed for phosphatidylcholine or sphingomyelin because of the concentration difference as well as the state of the phosphate group in the phospholipid. The phospholipid structures are presented in Fig. 1.

Addition of weighed amounts of phenobarbital to a solution of 0.03 M phosphatidylethanolamine produced a small shift of the phosphorus peak (Fig. 5D), while the addition of phenobarbital to tri-*n*-butyl phosphate solution produced virtually no change in position of the ^{31}P peak.[6]

The direction of the ^{31}P chemical shifts might be the result typically expected for protonation of a phosphate ester group and could be verified by comparison with the ^{31}P titration curve of the phosphate groups in ATP in aqueous media (19, 20). Since our studies were performed in nonaqueous media, it was necessary to document that the same qualitative change occurred for protonation of phosphates in a weakly polar solvent such as chloroform. This was accomplished by the addition of aliquots of glacial acetic acid to solutions of 0.045 M phosphatidylcholine, 0.045 M sphingomyelin, 0.03 M phosphatidylethanolamine, and 0.365 M tri-*n*-butyl phosphate.

If phosphatidylcholine and sphingomyelin in $CDCl_3$ solution do contain ionized phosphate groups, as suggested (21, 22), the chemical shift should occur in an up-

[6] Since these spectra were recorded without proton decoupling, the concentration of tri-*n*-butyl phosphate was necessarily much greater than the concentration of phenobarbital, 0.365 M vs. 0.04 M, and therefore no significant shift was observed.

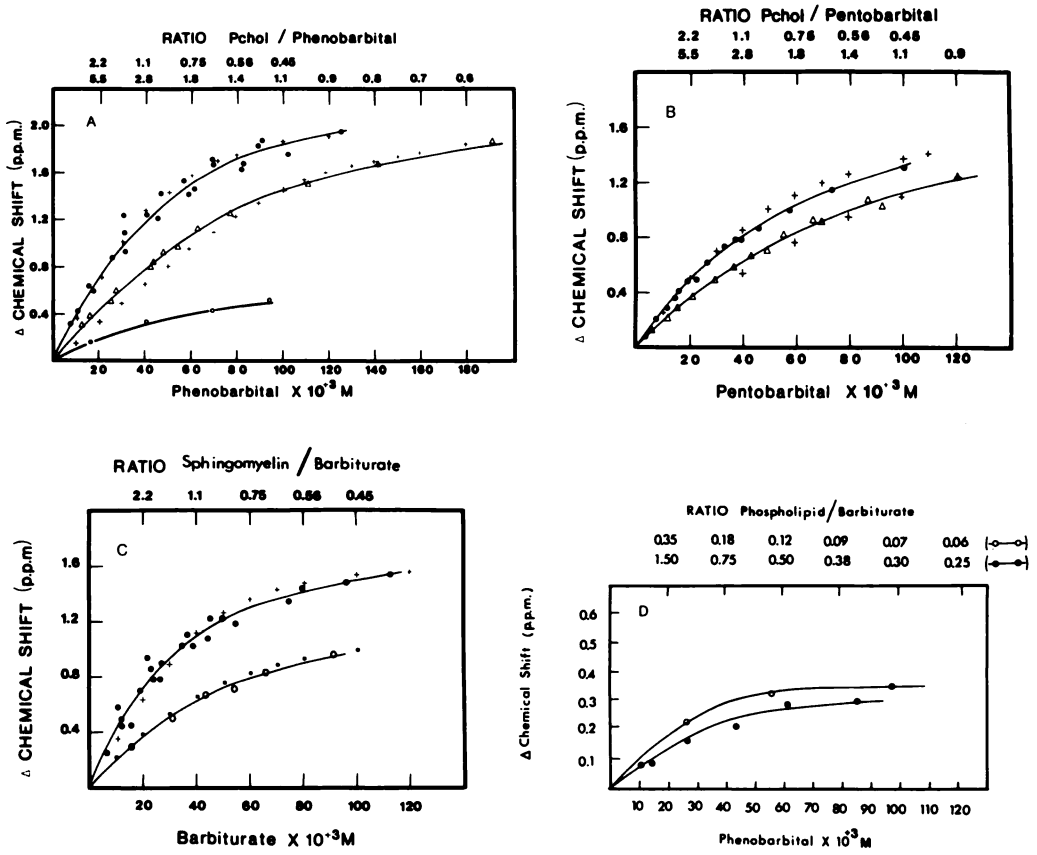


FIG. 5. *Upfield change in ^{31}P chemical shift of phospholipids as a function of increasing barbiturate concentration*

A and B. Change in the ^{31}P chemical shift of 0.11 M (△) and 0.045 M (●) phosphatidylcholine (PChol) with increasing concentrations of phenobarbital (A) and pentobarbital (B). +, Δδ values calculated from the association constants (Table 1) and data fit obtained for the phosphatidylcholine-phenobarbital and phosphatidylcholine-pentobarbital complexes. (○) change in ^{31}P chemical shift of hydrated phosphatidylcholine (0.045 M phosphatidylcholine containing 0.225 M H_2O) upon addition of phenobarbital.

C. Upfield change in the ^{31}P chemical shift of 0.045 M sphingomyelin with the addition of phenobarbital (●) and pentobarbital (○). + and ■, Δδ values obtained from the association constants of sphingomyelin-phenobarbital and sphingomyelin-pentobarbital, respectively (Table 1).

D. Upfield change in the ^{31}P chemical shift of 7 mM cardiolipin (○) and 0.03 M phosphatidylethanolamine (●) with increasing phenobarbital concentration.

field direction as a result of addition of acetic acid and the observed Δδ value of these phospholipids should be much greater than that of tri-*n*-butyl phosphate, which contains a neutral phosphate group. This was in fact observed. The results are illustrated in Fig. 6A and B, with phosphatidylcholine and sphingomyelin producing the greatest Δδ, upfield, in agreement with protonation studies conducted in aqueous media (19, 20).

In addition to determination of specific sites and mechanisms of association, it was also possible to evaluate the association constant (K_{assoc}) for the phospholipid-barbiturate pairs. The ^{31}P concentration plots were nonlinear, and the shift appeared to approach a limiting value, which allowed stability constants to be calculated for these systems (13). These values were determined, and ranged from 175 for sphingomylin-phenobarbital to 25 for phosphatidylethanolamine - phenobarbital (Table 1).

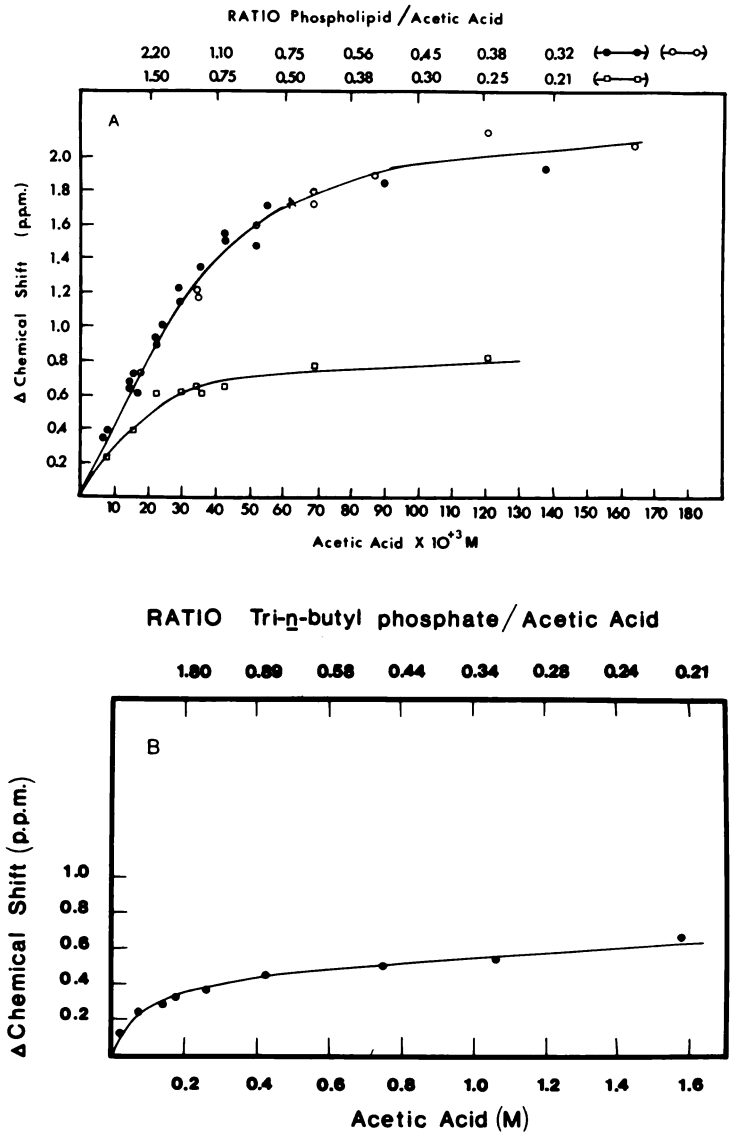


FIG. 6. *Effects of acetic acid upon ^{31}P spectra of phospholipids and tri-n-butyl phosphate*

A. Upfield change in ^{31}P chemical shift of the phospholipids phosphatidylcholine (0.045 M) (○), sphingomyelin (0.045 M) (●), and phosphatidylethanolamine (0.03 M (□) with increasing concentration of acetic acid.

B. Upfield change in ^{31}P chemical shift of 0.365 M tri-*n*-butyl phosphate with acetic acid.

To support the conclusion that hydrogen bonding is responsible for the upfield shifts, a temperature study was conducted. The phosphorus peak was shifted upfield by the addition of phenobarbital or pentobarbital, and the temperature was increased over the range 30–65°. The result of increasing the temperature was a downfield shift of the phosphorus peak in a manner reflective of hydrogen bond disruption. The downfield shifts of the phosphatidylcholine-phenobarbital and phosphatidylcholine-pentobarbital solutions with increasing temperature are shown in Fig. 3. The temperature study conducted on sphingomyelin-barbiturate gave a

downfield shift of approximately 5 Hz. No ^{31}P temperature studies were performed on phosphatidylethanolamine-barbiturate or cardiolipin-barbiturate solutions.

Since chloroform was used as the weakly polar medium to study barbiturate-phospholipid interactions, it was also desirable to investigate the association of barbiturate with phospholipids in the presence of some water, such as might exist at the surface of the membrane. A system consisting of phosphatidylcholine and water in chloroform was used.

Figure 5A shows that despite the presence of water (at 5 times the concentration of phosphatidylcholine), additions of phenobarbital to this system produced upfield shifts in the ^{31}P peak of the phospholipid. These shifts were on the order of 0.5 ppm (12 Hz) at a phosphatidylcholine to phenobarbital concentration ratio of unity, demonstrating that barbiturate-phospholipid association occurred even in the presence of an excess of water.

^{13}C NMR studies. The direction of the ^{31}P chemical shift produced by the addition of barbiturate was upfield, similar to that of protonation of phosphate groups in the ATP molecule (19, 20). Since the ionized phosphate group, reported to exist in phosphatidylcholine and sphingomyelin (21, 22), is a very basic site and may be capable of abstracting a proton from the barbiturate, it was necessary to verify that the observed ^{1}H or ^{31}P chemical shift changes were not the result of proton transfer. This was checked using ^{13}C magnetic resonance. A 0.1 M pentobarbital solution was titrated by the addition of aliquots of a 1.0 M solution of sodium hydroxide in ethanol. The results (Fig. 7) show that the ^{13}C downfield shifts obtained for the carbonyl position at a sodium hydroxide to pentobarbital concentration ratio of approximately 1 as a result of deprotonation of the barbiturate were very large (13–14 ppm). This demonstrates that if a proton is removed from an N–*H* group in the barbiturate, the corresponding increase in charge results in a large downfield shift of the signal corresponding to the carbonyl carbon. Hence, if the phosphate group is

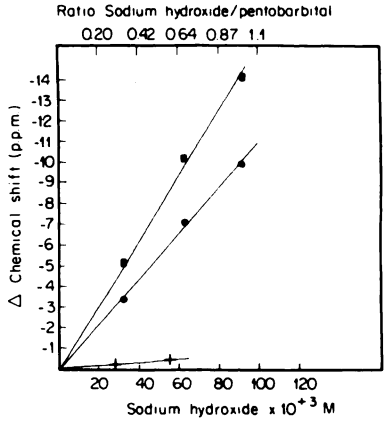


FIG. 7. *Change in ^{13}C chemical shift in carbonyl groups of pentobarbital (carbonyl group 2, ■; carbonyl groups 4 and 6, ●) (Fig. 1) with addition of alcoholic sodium hydroxide*

+, change in the ^{13}C carbonyl chemical shift with the addition of the phospholipid phosphatidylcholine at concentrations equal to those of the alcoholic sodium hydroxide.

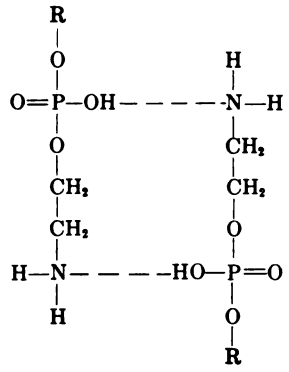
basic enough to remove the proton, a large ^{13}C downfield shift of the carbonyl signal should occur with the addition of phosphatidylcholine to barbiturate.

Addition of aliquots of 0.1 M phosphatidylcholine to 0.1 M pentobarbital up to a phosphatidylcholine to pentobarbital ratio of 1:2 produced a small ^{13}C chemical shift of the carbonyl groups (0.5 ppm) (Fig. 7). These observed changes in chemical shift were too small to have resulted from the deprotonation of the barbiturate and may have been due to hydrogen bonding of the N–*H* moiety to the phospholipid.

DISCUSSION

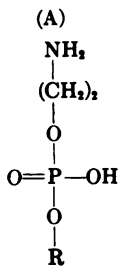
The proton magnetic resonance studies show that a hydrogen bonding interaction may have occurred between the barbiturates and phosphatidylcholine, sphingomyelin, cardiolipin, phosphatidylethanolamine, and tri-*n*-butyl phosphate; in addition, virtually no interaction was indicated between the barbiturates and phosphatidylserine at comparable concentrations. One rationale for the failure to observe any interaction between phosphatidylserine and barbiturates is that the K_{assoc} for this system is small and therefore

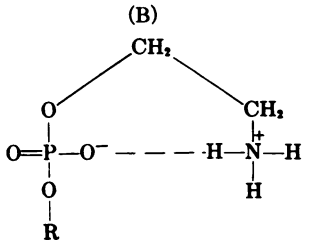
higher concentrations of phosphatidylserine would be required. In all cases the observed downfield shift of the N–*H* peak of pentobarbital was markedly less than that of phenobarbital. This can be predicted from pK_a values of the two barbiturates studied. The pK_a values of phenobarbital and pentobarbital are 7.3 and 8.0, respectively (23), and hence the observation that pentobarbital produces smaller shifts than phenobarbital is consistent with the ability of these barbiturates to serve as proton donors. Clearly the ionized phosphate group provides a basic site that might be expected to participate in hydrogen bonding. However, it becomes necessary to consider these shifts and linewidth changes as possibly resulting from interactions not directly related to formation of a barbiturate-phospholipid complex. Infrared studies have shown that phosphatidylcholine and sphingomyelin exist in an ionic form, characteristic of a dipolar ion or internal salt, in a nonaqueous medium such as CCl_4, and have 1 strongly associated water molecule per molecule of phospholipid (21, 22). Cardiolipin was ordered specially prepared in the acid form; the state of phosphatidylethanolamine was less well defined.[7] Since the position of the associated water peak or P–O*H* peak was not observed in PMR, possibly because of



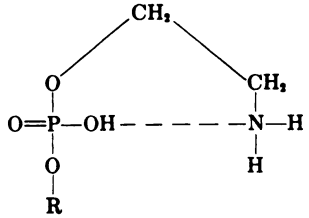
A more recent publication by Akutsu and Kyogoku (27), using infrared and Raman spectroscopy, indicated that phosphatidylethanolamine exists in a dipolar ionic form in tetrachloroethylene and carbon disulfide solutions. Our ^{31}P NMR results show that the phosphate group in phosphatidylethanolamine is not the same as the phosphate groups in phosphatidylcholine or sphingomyelin. For example, addition of acetic acid to phosphatidylcholine or sphingomyelin in $CDCl_3$ gives a total shift of approximately 2.0 ppm at a concentration ratio of 0.32, while a concentration ratio of 0.21 produces a shift of only about 0.8 ppm in phosphatidylethanolamine. The addition of acetic acid to tri-*n*-butyl phosphate (a neutral phosphate) in $CDCl_3$ produces a change in chemical shift of approximately 0.6 ppm at a concentration ratio of 0.21.

Since ^{31}P NMR directly monitors the phosphate group, one would conclude that the phosphate group of phosphatidylethanolamine appears to be different from that of phosphatidylcholine or sphingomyelin. This difference may be due to several factors, such as the ionic state of the phosphate group or the micellar structure of the phospholipid. Our interpretation of the data would be to suggest that phosphatidylethanolamine exists in solution either in a nonionic form (A) or in a strongly intra- or intermolecularly hydrogen-bonded complex (B or C).





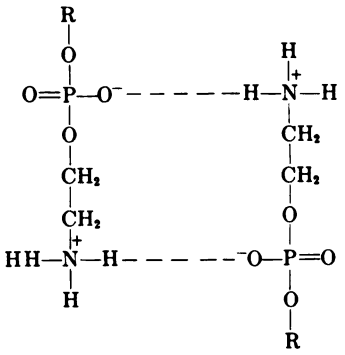
[7] The exact nature of the ionic structure of phosphatidylethanolamine in a weakly polar solvent would seem at present to be a somewhat controversial issue. Chapman *et al.* (24, 25) have interpreted their PMR data and the infrared data of Abramson *et al.* (22) as being suggestive of the existence of a $-NH_3^+$ group in phosphatidylethanolamine. Other authors (21, 26), however, have reported infrared data indicating that the phosphate group in phosphatidylethanolamine exists in the un-ionized form. A structure for phosphatidylethanolamine was suggested by Abramson *et al.* (22) with this phospholipid existing in a hydrogen-bonded configuration (intramolecular), i.e.,



although another possibility might be (intermolecular)

severe broadening or masking by the proton spectra of the phospholipid, the observed downfield shifts and linewidth changes could merely be reflective of, or contain a sizable contribution from, an exchange process (28). Such exchange processes may be present in the observed changes of proton chemical shifts upon addition of cardiolipin or phosphatidylethanolamine to barbiturate (Fig. 2A and B). The PMR results might also be indicative of hydrogen bonding between the barbiturate and water strongly associated with the phospholipid. While the ^1H temperature studies demonstrated that a hydrogen bonding interaction may well be the source of the observed downfield shifts, it was necessary to perform ^{31}P magnetic resonance studies on the phospholipids to investigate other potential mechanisms (i.e., exchange, barbiturate interactions with water) as well as to identify the site of interaction of the phospholipid.

(C)



This intra- or intermolecular complexation within a micelle may also account for the observed dipole moment of 2.86 Debye rather than the expected value of 20 Debye for the dipolar form (27). Furthermore, this intra- or intermolecular complexation may permit retention of the dipolar ionic characteristics observed in the infrared studies. However, the ionic state of the phospholipid phosphatidylethanolamine is not the major issue of this paper and does not affect our conclusions in a qualitative (mechanistic) or quantitative (K_{assoc}) fashion. The use of small molecules to obtain information on the molecular structure of larger molecules is the forte of the NMR method. Perhaps the use of ^{31}P NMR spectroscopy in conjunction with other forms of spectroscopy may resolve the controversy of the nature of the ionic state of phosphatidylethanolamine.

If the observed ^1H shifts were in fact a result of chemical exchange, one mechanism might conceivably be exchange of the barbiturate N–*H* with the proton of the P–O*H* groups, should the phospholipid exist in a protonated state (i.e., phosphatidylethanolamine, cardiolipin). Since it has been demonstrated by infrared studies (21, 22) that phosphatidylcholine and sphingomyelin exist in the ionized state in a CCl_4 medium, P–O*H* exchange for these cases does not seem reasonable. Furthermore, for phospholipids in the acid form, the observed direction of the ^{31}P chemical shifts also serves to rule out this possibility. Other mechanisms, such as hydrogen bonding or exchange between the barbiturate N–*H* and water molecule(s) possibly associated with the phospholipid, remain.

If water is added to the phospholipid so that the water is 5 times the concentration of the phospholipid, the ^{31}P peak shifts upfield by approximately 0.5 ppm. Since the addition of water to the phospholipid produces no separate peaks in the ^{31}P spectrum, we can assume that the exchange is rapid enough so that only an average signal is observed on the NMR time scale. Furthermore, if the effects giving rise to the shift in the ^{31}P were purely those of exchange, an increase in temperature should produce a change in the linewidth of the ^{31}P peak, with little or no change in the ^{31}P chemical shift (28). The addition of barbiturate to the hydrated phospholipid, if this were an exchange process, should now cause the ^{31}P peak to shift downfield or produce a dramatic change in the ^{31}P linewidth, indicative of a loss of water molecule(s) in the exchange process, i.e.,

$$\text{Phosphatidylcholine}\cdot x\ H_2O + \text{barbiturate} \rightleftharpoons \text{phosphatidylcholine} + \text{barbiturate}\cdot x\ H_2O$$

Since the addition of barbiturate to the hydrated phospholipid produces additional upfield shifting of the ^{31}P peak (0.5 ppm), this demonstrates that exchange involving water does not contribute to the observed ^{31}P shift and that the source of ^1H chemical shifts is not caused totally by water. The association of barbiturate with

the phospholipid phosphatidylcholine is sufficiently strong to occur in the presence of an excess of water.

The changes in ^{31}P chemical shift as a result of hydrogen bonding were in an upfield direction, which might not immediately be predicted. It should be noted that the NMR chemical shift of any particular nucleus is a complex function of the orbital motion of the surrounding electrons and is determined by a quantity σ:

$$H_{app} = (1 - \sigma)H_0$$

where H_{app} and H_0 are the applied and static magnetic fields, respectively, and σ is termed the screening constant. The parameter σ is further defined by

$$\sigma = \sigma_d + \sigma_p + \sigma'$$

where σ_d and σ_p are the diamagnetic and paramagnetic contributions, respectively, to the screening constant, and σ' is the contribution from neighboring nuclei (29). The screening constant is also a function of the energy of the lowest-lying excited state (Δ) (29) and, in certain cases, the geometry of the molecule (30, 31).

Because of the inherent complexity in evaluating the effects of hydrogen bonding upon the screening constant (i.e., Δ, σ'), it is not possible to predict which term will predominate and in which direction the NMR signal corresponding to a given nucleus will shift as a result of hydrogen bonding (29). Calculations of changes in proton chemical shifts occurring as a result of hydrogen bonding lead to the prediction that normally these changes will occur in a downfield direction (32). However, these calculations are extremely complex and the prediction may not apply to all nuclei (29, 32). In certain cases upfield chemical shifts as a result of hydrogen bonding have been reported (33). ^{14}N resonance studies performed on 5- and 6-membered nitrogen heterocycles have shown that proton donors make the ^{14}N resonance shift upfield (33). It is certainly plausible to interpret the observed ^{31}P upfield chemical shifts as resulting from hydrogen bonding.

The barbiturate-phospholipid studies reported here were conducted in a weakly polar medium, $CDCl_3$, in order to provide an environment which would maximize the possibility of observing the interaction as well as simulate the internal polarity of the membrane. Phospholipids form micelles in a weakly polar medium and have the inverted micelle structure; that is, the polar head groups are in the interior of the micelle with the fatty acid chains stretching out in the solvent (34). The use of chloroform as the solvent system in these investigations may have presented a major accessibility problem for the barbiturate. The barbiturate must penetrate the fatty acid chains in order to gain accessibility to the phosphate group. The size of the micelle has been reported as a function of the concentration and dielectric constant of the medium (35, 36). To remove the possibility of micellular interactions contributing to the observed phosphorus results, a system which has been suggested to be intermediate between C_6H_6 and water in terms of type of micelle and molecular aggregation of phosphatidylcholine was investigated (36). Phosphatidylcholine was reported by Elworthy and McIntosh (36) to exist as a trimer in methanol, while Kellaway and Saunders (37, 38) reported it to exist as a monomer. Whether these differences can be attributed to the experimental methods is not known; however, it is clear that very little, if any, aggregation of phosphatidylcholine occurs in methanol. The addition of methanol to a sample of phosphatidylcholine in $CDCl_3$ produced a chemical shift (approximately 0.35 ppm) in the phospholipid ^{31}P peak, showing that the observed ^{31}P shifts as a result of barbiturate addition neither were the result of, nor contained sizable contributions from, micellular interactions. The concentration of methanol required to produce this effect was 1.0 M, giving a phospholipid to methanol ratio of 0.11. This change in chemical shift (approximately 0.35 ppm upfield) observed upon the addition of methanol was probably indicative of hydrogen bonding between the methanol and the phospholipid. Although the methanol system has been suggested to represent a midpoint between aqueous systems and higher alcohols and

benzene (36), we felt that it was necessary to evaluate the association of barbiturates with phospholipids in the presence of water. The membrane itself is amphipathic, with the polar portion of the phospholipids exposed to water, protein, and a hydrophobic region containing the fatty acid chains. Therefore a model system was required which would incorporate water into the phospholipid and maintain the necessary requirement for the magnetic resonance experiment (i.e., well-defined ^{31}P signal). The model of the "wet" phospholipid was prepared with the water at 5 times the concentration of the phospholipid phosphatidylcholine. Phosphatidylcholine has been shown to contain at least three hydration layers, the first two, tightly bound layers constituting 4–6 molecules of water (39–41). Hence, at a ratio of 5 water molecules per molecule of phosphatidylcholine used in the phospholipid model, one would roughly expect both hydration layers to be present. The water is associated with the polar head group of the phospholipid and causes changes in micellular asymmetry, going from oblate ellipsoid to spherical at high water content (42).

The addition of phenobarbital to the system of the hydrated phospholipid produces an upfield shift in the ^{31}P peak (approximately 0.5 ppm), demonstrating that barbiturate association occurs in the presence of an excess of water. This association is sufficiently strong that the barbiturate must displace water in order to have access to the phosphate group.

The data presented here are consistent with the conclusion that the N–*H* of barbiturates can form a hydrogen bond with the phosphate moiety of phospholipids in nonaqueous media in the presence and absence of water. Although the PMR results indicate an electrostatic type of interaction, ^{31}P and ^{13}C magnetic resonance results were required to distinguish a hydrogen bonding interaction from other situations which might give rise to similar PMR results (exchange, ion pair formation) as well as establish the site of interaction of the phospholipids. Since barbiturates can bind to phospholipids, perhaps this is the mechanism by which they are capable of interacting with membranes. While it is beyond the scope of this paper to discuss in detail models dealing with mechanisms of ion flow in excitable membranes (6–8), the potential importance of observation of barbiturate-phospholipid interaction is evident.

ACKNOWLEDGMENTS

We thank Drs. John J. Mieyal and Cecil Cooper for their stimulating discussions and many useful suggestions.

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