C. B. (1972b), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3790.
Scatchard, G. (1949), *Ann. N.Y. Acad. Sci. 51,* 660.
Yalow, R. S., and Berson, S. A. (1970), in Statistics in Endocrinology, McArthur, J. W., and Colton, T., Ed., Cambridge, Mass., MIT Press, pp 327-344.
Young, N. S., Curd, J. G., Eastlake, A., Furie, B., and Schechter, A. N. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4759.

Structure in the Polar Head Region of Phospholipid Bilayers: A ^{31}P $\{^1H\}$ Nuclear Overhauser Effect Study†

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ABSTRACT: The structure of the head-group region of some phospholipid bilayers in vesicle form has been studied and an intermolecular association of the *N*-methyl protons of phosphatidylcholine (PC) with the phosphate of phosphatidylethanolamine (PE) in mixed vesicles has been identified. Observation of a ^{31}P $\{^1H\}$ nuclear Overhauser effect (NOE) in the phosphorus nuclear magnetic resonances of both PC and PE in mixed vesicles demonstrates an intimate dipolar interaction between some protons and the phosphorus nuclei. Substitution of deuterium for the *N*-methyl protons of PC eliminated the majority of the effect and necessitated the construction of a model of the bilayer surface in which the *N*-methyl protons of PC could interact closely with the phosphates of neighboring PE molecules. The predominant orientation of the head group must then be parallel to the bilayer surface. The amino protons of PE do not contribute significantly to the observed NOE. A corollary of these results is that there is little if any tendency for either PC or PE in the mixed vesicles to segregate into separate domains. A decrease in NOE in sphingomyelin vesicles on going from H_2O to D_2O suggests that an exchangeable proton contributes to the NOE. In addition the low value of the NOE observed in D_2O suggests that the head-group conformation of sphingomyelin differs from that of PC.

Polar head groups of lipids in model and natural membranes constitute both surfaces of a bilayer and exert considerable influence over membrane properties. Differing chemical structures of the various phospholipid head groups control shapes of hydrophobic aggregates (Tanford, 1973), induce asymmetric lipid distributions in small vesicles (Litman, 1973; Michaelson et al., 1973; Berden et al., 1975), and either cause or result from marked asymmetries in natural membranes (Bretscher, 1973; Verkleij et al., 1973; Gordesky et al., 1975). In the process of penetrating a membrane bilayer, molecules must contend with two charged polar regions. Cation binding to phospholipid head groups in a bilayer can order or disorder the hydrocarbon chains (Trauble and Eibl, 1974), and effect lateral phase separations (Ohnishi and Ito, 1974). Polar head groups undoubtedly play a role in membrane fusion and have been implicated in certain pathological processes (Weissman and Rita, 1972). Further, orientation of the head-group charges is important in defining membrane surface potential which affects the local water structure and may influence protein orientation.

Unfortunately, though the polar head region is of importance, little conformational information is available, due to the lack of suitable structural probes. In the particular case of phosphatidylcholine (PC[1]) and phosphatidylethanolamine (PE), a gauche conformation around the C_α to C_β bond in the head groups has been assigned from x-ray crystal structures and NMR solution studies (Birdsall et al., 1972; Richard et al., 1974; Andrieux et al., 1972; Abrahamsson and Pascher, 1966; DeTitta and Craven, 1973). With this restriction the zwitterion dipole can be aligned parallel or perpendicular to the bilayer surface or somewhere in between.

A previous report (Yeagle et al., 1975a) described a new probe of surface structure in PC bilayers, the ^{31}P $\{^1H\}$ nuclear Overhauser effect (NOE), which demonstrated a strong interaction between the *N*-methyl groups of the choline moiety and the phosphate group. Though the observation of the interaction could not of itself distinguish between an inter- or intramolecular mode, model building suggested an intermolecular interaction as the most reasonable explanation. Exploring this question further we have examined the nature of the ^{31}P $\{^1H\}$ NOE in other lipid systems. A detailed study of the effect in PC/PE mixed vesicles demonstrates, by the observation of a NOE in PE due to the *N*-methyl protons of PC, that the interaction is intermolecular. In addition, other lipid systems that exhibit ^{31}P $\{^1H\}$ NOE are described.

Materials and Methods

Egg phosphatidylcholine and phosphatidylethanolamine were purified by silicic acid chromatography (Huang, 1969; Litman, 1973). Sphingomyelin (bovine brain) was kindly provided by Dr. Y. Barenholz. Phosphatidylglycerol was a gift of Dr. W. Kundig. Deuterated PC (PC-*Me*-d_9) was synthesized as described previously (Yeagle et al., 1975a).

Vesicles were prepared by sonication under nitrogen with a Heat Systems W-350 sonifier at 2 °C until clear in a deoxygenated, pH 4, 10 mM acetate buffer, 100 mM NaCl, 2 mM EDTA, for the mixed PC/PE systems, and in 100 mM NaCl

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[1] Abbreviations: DPL, dipalmitoyllecithin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOEE, nuclear Overhauser effect enhancement; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; EDTA, ethylenediaminetetraacetic acid; CW, continuous wave; PC-*Me*-d_9, deuterated PC.

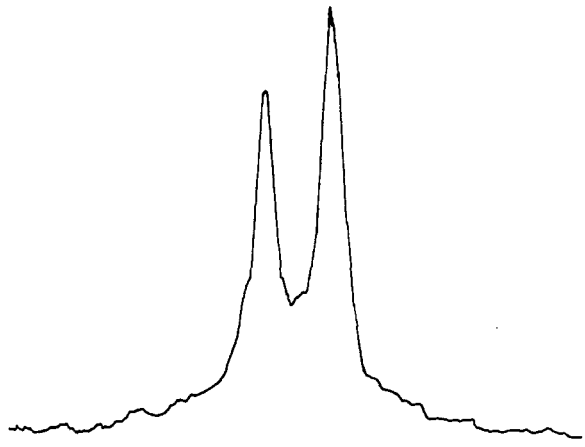

FIGURE 1: ^{31}P NMR spectrum of 1.2:1 PC/PE mixed vesicles in pH 4, 10 mM acetate buffer, 100 mM NaCl, 2 mM EDTA. Total lipid concentration is 32 mM. Peak separation is 24 Hz.

for the other lipids. For mixed lipid systems, the two lipids were co-lyophilized from benzene before sonication to ensure homogeneous dispersions. The vesicle solutions were used for NMR experiments within 48 h and were stable during that period of time. Phosphate concentration was determined by the method of Gomori (1942).

^1H and ^{31}P NMR spectra were measured using a JEOL-PS100/EC-100 Fourier transform spectrometer at 23.5 kG and 23 °C with a JEOL 5-kHz RF crystal filter for signal-to-noise enhancement. ^1H spectra of the vesicles in H_2O were obtained using the "WEFT" technique, waiting at least $3T_1$ for the choline protons to selectively eliminate the water resonance (Patt and Sykes, 1972; Benz et al., 1972). ^{31}P spectra were obtained using a 5-kHz spectra range and pulse repetition rates of 3 to $5T_1$ with a 90° pulse width. T_1 measurements were made using the 180°–τ–90° pulse sequence with proton decoupling and T_1 values obtained from least-squares analysis of the data.

NOE enhancements were determined by comparison of intensities of fully decoupled spectra and spectra with the decoupling frequency gated (JEOL OA-1M/SD-HC gated decoupler) in order to eliminate the NOE (Freeman et al., 1972). Intensities were evaluated by cutting and weighing copies of the spectra and by triangulation. NOEE values are reported to the nearest 10%.

Theory

The nuclear Overhauser effect arises from dipole-dipole interactions between two or more spins and manifests itself in a change in the intensity of one spin upon the saturation of the other spin or spins with which the observed spin is interacting (Noggle and Schirmer, 1971). If the motion modulating the interaction between the observed spin and saturated spin can be described by a single isotropic correlation time, τ_c, then the enhancement is given by (Doddrell et al., 1972):

$$f_I(S) = \frac{\gamma_S}{\gamma_I} \frac{\left[\frac{6\tau_c}{1+(\omega_I+\omega_S)^2\tau_c^2} - \frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau_c^2}\right]}{\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{\tau_c}{1+(\omega_I-\omega_S)^2\tau_c^2} + \frac{6\tau_c}{1+(\omega_I+\omega_S)^2\tau_c^2}\right]} \quad (1)$$

where $f_I(S)$ is the increase in intensity of the observed spin I due to saturating spin S, γ is the gyromagnetic ratio, and ω is the Larmor frequency for the particular nucleus in rad s^{-1}. When the extreme narrowing condition is satisfied, $\omega^2\tau_c^2 \ll 1$, and eq 1 reduces to:

$$f_I(S) = \gamma_S/2\gamma_I \quad (2)$$

In the present case, I is phosphorus, and S is hydrogen, and saturation of the proton resonances may produce a maximum increase in intensity from 1.00 to 2.24 (Noggle and Schirmer, 1971). The maximum nuclear Overhauser effect enhancement (NOEE) is then 124%.

If the extreme narrowing condition is not satisfied, then eq 1 indicates how the NOE can be attenuated by slow motion, assuming isotropic reorientation. For the $^{31}P\{^1H\}$ case, when $\omega^2\tau_c^2 \gg 1$, the NOEE is reduced nearly to zero. When the molecular motion is not isotropic, eq 1 becomes more complex, but is of a similar form. In the fully protonated PC vesicle, the maximum observed NOE is limited by motion and not by competing relaxation mechanisms (Yeagle et al., 1975a).

The NOE can also be attenuated by relaxation mechanisms contributing to the relaxation of the observed phosphorus not arising from the dipole-dipole interaction with the saturated proton. The NOE is therefore a measure of the extent to which the saturated proton or protons determine the relaxation mechanism of the observed phosphorus resonance (Breitmaier et al., 1975). It is important to eliminate paramagnetic metal ions from the solution which offer a powerful competing relaxation mechanism, and for this purpose EDTA was added in the present experiments. Further, since the dipolar interaction depends on the inverse sixth power of the internuclear distance, in general the saturated spin must be close in space to the observed spin to induce an NOE (Yeagle et al., 1975b).

Results

In order to show that in these sonicated systems the NMR measurements were sensitive to all the phospholipid in the sample, an experiment with an internal standard was performed. A known amount of the ion, HPO_3^-, whose chemical shift is well downfield of PC, was added to a vesicle solution whose phosphatidylcholine concentration had been determined by phosphate analysis. The relative areas of the two peaks were then measured by integration, and comparison with the phosphate analysis demonstrated that greater than 95%, or virtually all, of the lipid in the sample was contributing to the observed ^{31}P resonance. This experiment was performed with gated decoupling to remove the NOE, a procedure necessary for quantitative measurements.

The ^{31}P spectrum of PC and PE in mixed vesicles (1.2:1 PC/PE) at pH 4 can be seen in Figure 1; PC appears upfield of PE. The chemical shift difference is similar to that reported in organic solvents (Henderson et al., 1974). These spectra show good resolution between the PC and the PE peak at pH 4 to pH 6. The different behavior reported by Michaelson et al. (1974) is presumably due to the bacterial source of PE and the high pH. An attempt to produce vesicles with bacterial PE and egg PC at pH 6 yielded only precipitated aggregates. Furthermore, the use of proton decoupling enhances considerably the peak resolution in these systems. The NOEE for the two lipids at pH 4 is listed in Table I, measured with broad-band proton decoupling. In order to identify the source of the NOE in both peaks, the NOEE was measured as a function of continuous wave (CW) proton decoupling frequency at pH 4 and these results are graphed in Figure 2. The source of the NOE is the same in both PE and PC resonances. Identical results were obtained with a 2:1 PC/PE mixture at pH 6. Since the N-methyl protons of PC had been previously identified as

TABLE I: Phosphorus Nuclear Overhauser Effect Enhancements (NOEE) for Lipids in Vesicles.

	NOEE[a] (%)	T_1[b] (s)
PC	40	1.4
PC in PC/PE[c]	60	1.8
PE in PC/PE[c]	50	2.2
d-9 PC in d-9 PC/PE[d]	20	
PE in d-9 PC/PE[d]	20	
Sphingomyelin in H_2O	40	1.1
Sphingomyelin in D_2O	20	
Sphingomyelin in sphingomyelin/PC[e]	20	0.9
PC in sphingomyelin/PC[e]	40	1.3
PG	20	1.2

[a] Measured with broad-band decoupling and reported to the nearest 10%. [b] ±10%. [c] 1.2:1, PC/PE. [d] 1:1, d-9 PC/PE. [e] 1:1, sphingomyelin/PC.

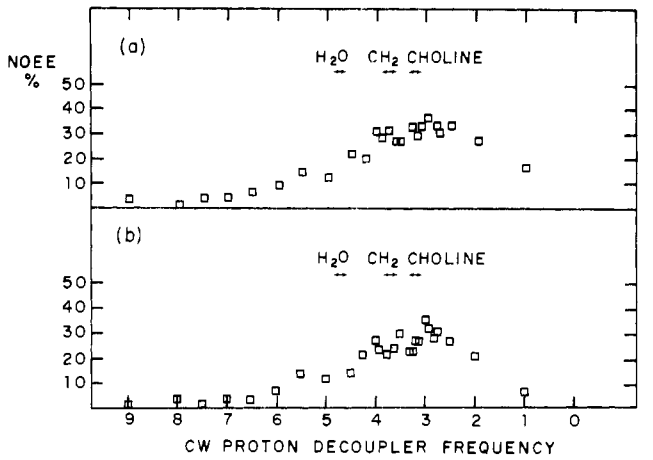

FIGURE 2: Frequency dependence of NOEE in a 1.2:1 PC/PE mixture. Continuous wave proton frequencies are ppm downfield from Me_4Si. (a) NOEE for PE; (b) NOEE for PC.

the source of the NOE in PC (Yeagle et al., 1975a), they must also be the source of the NOE in PE in the PC/PE mixture.

In order to verify the conclusion that the *N*-methyl protons are the source of the NOE, specifically deuterated PC (PC-*Me*-d_9) in which all *N*-methyl protons had been replaced by deuterium, was co-lyophilized with PE (1:1 PC/PE) and sonicated. The ^1H spectrum of this sample revealed the unique and conspicuous absence of the *N*-methylcholine resonance, while the rest of the spectrum was the same as for a PC/PE mixture in which the PC was fully protonated. However, the $^{31}P\{^1H\}$ NOEE in this sample for both peaks with broad-band proton decoupling is considerably less than in the fully protonated sample (see Table I). Thus the major source of the NOE in both PC and PE in the mixed vesicle is clearly identified as the *N*-methyl protons of PC.

The amino protons of PE do not appear to make a significant contribution to the NOE of either the PC or PE peak. At pH 4, a separate proton resonance for PE amino protons is discernible in the ^1H spectrum about 3 ppm downfield of H_2O, as previously reported (Lange et al., 1975). In the frequency dependence of the NOE, no significant NOEE is observable in that region. Chemical exchange of the amino protons with the solvent may prevent any effect from being seen, or motional parameters for that interaction may be somewhat different than for the *N*-methyl–phosphate interaction.

NOEE and T_1 results for several other lipid systems are listed in Table I. The difference between the NOEE of sphingomyelin in H_2O and D_2O is reproducible. This experiment was performed by measuring the NOE in H_2O and then dialyzing the same vesicles against D_2O so that differences could not be ascribed to sample preparation.

Discussion

In a previous paper (Yeagle et al., 1975a), we first described the $^{31}P\{^1H\}$ NOE in PC vesicles and established that the NOE derived from the *N*-methyl protons of the choline head group. Though in pure PC bilayers inter- and intramolecular effects could not be experimentally distinguished, it was suggested upon careful consideration of molecular models that the most reasonable interpretation of the NOE invoked an intermolecular interaction between the *N*-methyl protons on one PC molecule and the phosphate of a neighboring PC molecule. This model found immediate application in explaining the effects of cholesterol on this interaction. Since the NOE was substantially reduced in the presence of a high molar ratio of cholesterol, it was suggested that inclusion of large amounts of cholesterol in the bilayer prevented the intermolecular interaction between lipids from occurring and thereby eliminated the NOE.

The model of intermolecular interactions between head groups acquires considerable strength from the present detailed study of the mixed PC/PE vesicle system. This system has been shown to consist of vesicles of structure similar to that of pure PC vesicles, providing the PC/PE ratio is greater than 1:2, though some asymmetry in the distribution of PE between the inside and outside surfaces has been noted (Litman, 1973). Substantial NOEE was observed in the resonances due to each of the lipids. As shown in the Results section, the major source of the NOE in both PC and PE in the mixed vesicle is the *N*-methyl protons of PC. This result requires that the *N*-methyl protons be close in space to the phosphorus nuclei. Since the *N*-methyl protons make the major contribution to the NOE, they are more effective at relaxing the phosphorus nuclei than are the nearby methylene protons.

These results are difficult to interpret any way other than as an intermolecular interaction. The PC *N*-methyl groups interact with phosphate groups of neighboring phospholipids, which in this case include both PC and PE molecules, and dominate the dipolar relaxation mechanism of the phosphorus to produce the observed NOE. Almost by definition of the term, the interaction must be intermolecular in order for protons on one molecule to dominate the relaxation mechanism of the phosphorus in another, different, molecule. The surface of the bilayer in the PC/PE system, in order to produce an NOE, is envisioned to consist of interacting zwitterionic head groups, the positively charged *N*-methyl moiety of each PC molecule associating with the negatively charged phosphates of neighboring phospholipid molecules. This head-group conformation, which is electrostatically favorable, identifies the predominant orientation of the electric dipole as parallel to the bilayer surface. It must be emphasized that this is not a static model, but very much a dynamic one in which one head group can interact with many different neighboring phosphates in a short time. This model still allows for considerable freedom of movement about the various bonds in the head group (Gally et al., 1975), including major excursions of limited probability from the favored configuration, and also can incorporate the gauche conformation about the choline C_α to C_β bond. This result strengthens the original suggestion that, in pure PC bi-

layers, the NOE is also due to intermolecular interactions.

In the x-ray crystal structure of PE (Hitchcock et al., 1974), intermolecular interactions occur between the amino groups and neighboring phosphates. Two other relevant crystal structures are those of glycerophosphorylcholine (Abrahamsson and Pascher, 1966) and glycerophosphorylethanolamine (DeTitta and Craven, 1973). In neither case are there any intramolecular interactions; all hydrogen bonds, including those between amine and phosphate in the latter structure, are intermolecular. Thus the model of intermolecular interactions in vesicles is supported by crystal structure determinations of component molecules.

As a corollary of the NOE results with PC/PE mixtures, it is possible to assess the surface distribution of lipids in the PC/PE mixture. In some systems lateral phase separations can be induced which sequestor one type of lipid, or one phase of two lipids, in pools segregated from the rest of the lipid (Ohnishi and Ito, 1974; Wu and McConnell, 1975). If the two lipids in the PC/PE vesicles were strongly segregated into separate domains, the NOEE in PE would be only the 20% due to methylene hydrogens observed in the experiment with PC-*Me*-d_9. However, the observed NOEE in the fully protonated mixed vesicle is 50% for PE which is comparable to the 60% for PC. In order to obtain a NOE from *N*-methyl groups in the PE resonance, PE molecules require PC molecules as neighbors. Thus there is little if any tendency for either component to form separate domains.

If the result that water is not responsible for the NOE in PC vesicles (Yeagle et al., 1975a) may be applied to sphingomyelin vesicles, then the greater NOEE in H_2O over D_2O (Table I) may be ascribed to an exchangeable proton (though a tightly bound water molecule cannot be absolutely ruled out). Sphingomyelin contains two exchangeable protons not present in lecithins: an amide proton and a hydrogen proton on the glycerol moiety. In order to account for the greater NOE in H_2O, one or both of these protons spend a considerable fraction of their time hydrogen bonded to the phosphate group. Since an exchangeable proton contributes to the NOE in sphingomyelin, and since in the absence of that proton the NOEE is only 20%, the head-group conformation evidently differs from PC, even though both contain the phosphorylcholine group.

Though substantial information has been gleaned from NOEE differences in this paper, there are similarities in T_1 and NOEE in Table I among the several lipid systems. Apparently, despite differing chemical structures motional parameters for these lipids in bilayers are similar, a concept compatible with the Singer–Nicholson membrane model of a sea of lipid.

Hopefully, the model described in this report for the surface of PC and PC/PE bilayers will prove helpful in understanding those biological systems where PC and PE are major components. The structure of the bilayer surface should be important to molecules interacting with it; one example is the structured water near the membrane surface since that structure will be dependent upon the arrangement of charges on the surface. Both integral and peripheral proteins, which interact with the polar head-group region and may have effects on conformations of lipid head groups in the immediate vicinity of the protein, provide another example. Therefore the $^{31}P\{^1H\}$ NOE should be a valuable probe of the interactions of PC, in particular, with proteins and other lipids.

Acknowledgments

Sphingomyelin samples were kindly provided by Dr. Y. Barenholz and PG was the gift of Dr. W. Kundig.

References

Abrahamsson, S., and Pascher, I. (1966), *Acta Crystallogr. 21,* 79.

Andrieux, A., Dufourcq, J., and Lussan, C. (1972), *C. R. Acad. Sci. 274,* 2358.

Benz, F. W., Feeney, J., and Roberts, G. C. K. (1972), *J. Magn. Reson. 8,* 114.

Berden, J. A., Barker, R. W., and Radda, G. K. (1975), *Biochim. Biophys. Acta 375,* 186.

Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Metcalfe, J. C. (1972), *J. Chem. Soc., Perkin Trans. 2,* 1441.

Bretscher, M. S. (1973), *Science 181,* 622.

Breitmaier, E., Spohn, K.-H., and Berger, S. (1975), *Angew. Chem., Int. Ed. Engl. 14,* 144.

Bretscher, M. S. (1973), *Science 181,* 622.

DeTitta, G. T., and Craven, B. M. (1973), *Acta Crystallogr., Sect. B 29,* 1354.

Doddrell, D., Glushko, V., and Allerhand, A. (1972), *J. Chem. Phys. 56,* 3683.

Freeman, R., Hill, H., and Kaptein, R. (1972), *J. Magn. Reson. 7,* 327.

Gally, H.-U., Neiderberger, W., and Seelig, J. (1975), *Biochemistry 14,* 3647.

Gomori, G. (1942), *J. Lab. Clin. Med. 27,* 955.

Gordesky, S., Marinetti, G., and Love, R. (1975), *J. Membr. Biol. 20,* 111.

Henderson, T. O., Glonek, T., and Myers, T. C. (1974), *Biochemistry 13,* 623.

Hinz, H., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.

Hitchcock, P. B., Mason, R., Thomas, K. M., and Shipley, G. G. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3036.

Huang, C. (1969), *Biochemistry 8,* 344.

Lange, Y., Ralph, E. K., and Redfield, A. G. (1975), *Biochem. Biophys. Res. Commun. 62,* 891.

Litman, B. J. (1973), *Biochemistry 12,* 2545.

Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1973), *Biochemistry 12,* 2637.

Michaelson, D. M., Horwitz, A. F., and Klein, M. P. (1974), *Biochemistry 13,* 2605.

Noggle, J. H., and Schirmer, R. E. (1971), The Nuclear Overhauser Effect, New York, N.Y., Academic Press.

Ohnishi, S., and Ito, T. (1974), *Biochemistry 13,* 881.

Patt, S. L., and Sykes, B. D. (1972), *J. Chem. Phys. 56,* 3182.

Richard, H., Dufourcq, J., and Lussan, C. (1974), *FEBS Lett. 45,* 136.

Tanford, C. (1973), The Hydrophobic Effect, New York, N.Y., Wiley.

Trauble, H., and Eibl, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 214.

Verkleij, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D., and van Deenen, L. L. M. (1973), *Biochim. Biophys. Acta 323,* 178.

Weissman, G., and Rita, G. A. (1972), *Nature (London), New Biol. 240,* 167.

Wu, S., and McConnell, H. M. (1975), *Biochemistry 14,* 847.

Yeagle, P. L., Hutton, W. C., Huang, C., and Martin, R. B. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3477.

Yeagle, P. L., Hutton, W. C., and Martin, R. B. (1975b), *J. Am. Chem. Soc. 97,* 7175.