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CHOLESTEROL MODULATES THE EFFECTS OF MEMBRANE PERTURBERS IN PHOSPHOLIPID VESICLES AND BIOMEMBRANES

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Summary

The order parameter of spin-labeled phosphatidylcholine vesicles has been shown to increase upon incorporation of cholesterol, cannabinol, chlorpromazine and pentobarbital. Cannabinol was as effective on a mole basis as cholesterol in increasing the order parameter of 5-doxyl stearic acid in phosphatidylcholine: 4% phosphatidic acid bilayers at low concentrations. The average increase in order parameter with chlorpromazine and pentobarbital was two to three times less than that of cholesterol. Relative to cholesterol these compounds were less effective at ordering 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine.

The ordering effect with any given membrane perturber became smaller when increasing amounts of cholesterol were incorporated in the phospholipid bilayers until a disordering effect was finally observed. The cholesterol composition at which this cross-over from ordering to disordering occurred varied with the perturber, being 26 mole % for chlorpromazine, 23 mole % for cannabinol and 14 mole % for pentobarbital. The ability of cholesterol itself to increase the order parameter of the bilayer was decreased in the presence of these perturbers.

These compounds may exert their ordering effect on the interfacial region of phospholipid bilayers in an analogous manner to cholesterol. However, at higher cholesterol contents their additional ordering effect is more than counterbalanced by a weakening of the cholesterol-acyl interaction and a net disordering effect results.

In biological membranes a similar role for cholesterol in modulating the effect of perturbers was observed. Cannabinol decreased the order of erythrocyte membranes but increased that of mitochondrial membranes, while octanol disordered both of these biological membranes.

Abbreviations: PC (7,6), 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine; PC (10,3), 1-acyl-2[5(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine.

Introduction

Cholesterol is a major component in mammalian membranes and yet its content varies from one membrane to another; it ranges, for example, from 40 mole % in myelin to 6 mole % in mitochondria [1]. The exact function of cholesterol in the membrane is still unknown, but biophysical studies on model membranes show that one role of cholesterol in membranes is to regulate the degree of order and mobility of the acyl chains of lipids, i.e., above the transition temperature of the phospholipid, cholesterol reduces the mobility of the acyl chain while below the transition temperature, it has the opposite effect. Cholesterol thus acts as a modulator of the packing of the acyl chains of phospholipids.

On the other hand many studies [2—4] have shown that small lipophilic molecules fluidize membranes. Many of these compounds are pharmacological agents such as general and local anesthetics and their ability to fluidize membranes has been related to their pharmacological potency. However, more recently a few studies have shown that lipophilic molecules may also exert a condensing effect on lipid bilayers. Thus, cannabinol increases the order parameter of dipalmitoyl phosphatidylcholine-cholesterol bilayers [5], and local anesthetics have a similar effect on decholesterolized ox-brain lipids [6]. In a preliminary communication [7] we have shown that pentobarbital and some other anesthetics may exert either a condensing or a disordering effect on phosphatidylcholine bilayers depending on their cholesterol content. Thus, under some circumstances these compounds mimic the effect of cholesterol in reducing the mobility of the acyl chains in phospholipid bilayers. In this study we compare the ability of a number of compounds to order phospholipid bilayers and we examine in detail the role of the cholesterol content of bilayers in modulating their ability to be either ordered or disordered. We also show that our conclusions are applicable to biological membranes.

Materials and Methods

The effect of the lipid-soluble compounds on membrane structure was generally monitored from the electron spin resonance (ESR) spectra of 5-doxyl stearic acid (Synva, Calif.) and 1-acyl-2[8(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine (PC (7,6)) label which was synthesized in this laboratory by Dr. M. Pringle according to the method of Hubbell and McConnell [8]. Egg yolk phosphatidylcholine and phosphatidic acid were from Lipid Products, Surrey, U.K. and used without further purification. Cholesterol (Sigma) was recrystallized in methanol. Chlorpromazine hydrochloride and pentobarbital were from Sigma. Octanol was purchased from Applied Science, State College, Pa. One sample of cannabinol was a gift from the National Institute on Drug Abuse, and was 98% pure. Another was purchased from Poly Science, Warrington, Pa at 95% purity and further purified by thin layer chromatography [9].

Stock solutions (36 mg/ml) of phosphatidylcholine: 4% phosphatidic acid containing various mole percentages of cholesterol were made up in chloroform/methanol (9 : 1, v/v) to avoid variation between experiments. 0.5 ml of

stock solution was mixed with the spin label in methanol and with drugs in chloroform/methanol or ethanol and dried down in a rotatory evaporator. Residual solvent was removed by pumping on a vacuum line for at least 2 h. Tris buffer (0.01 M, pH 7) in 0.15 M KCl was added and liposomes were formed by vigorous vortexing for 1 min. The final concentration of lipid was 15—25 mg/ml, with spin label constituting about 1 mole % of the lipid. Drugs were equilibrated with liposomes for up to 24 h at 25°C before being sealed in 1 mm glass capillaries. All the drugs in the study are highly lipophilic, for example, the partition coefficient of octanol is 152 in erythrocytes [10] and 670 in dipalmitoyl phosphatidylcholine [11] and, thus, are expected to be mainly concentrated in the lipid phase. Spectra were recorded on either a Varian E-9 or E-109 electron spin resonance spectrometer operating at 9.5 GHz with the variable temperature control unit set at 23°C or 25°C. Order parameters, S, were calculated from the spectra according to the method of Hubbell and McConnell and the $T_\perp$ value was corrected by the method of Gaffney [8,12].

Mitochondria from rat liver were prepared using the method of Parsons and Williams [13]. Erythrocyte ghosts were prepared from out-dated human blood according to the procedures of Dodge et al. [14]. The protein concentration of membranes was determined by the method of Lowry [15]. To label the membranes, 1 ml of a membrane solution was added to a test tube containing an appropriate amount of 5-doxyl stearic acid label previously deposited in a thin film, and was incubated overnight at 4°C. The spin-labeled membrane solutions were spun down (9750 × g for 10 min for mitochondria; 20 000 × g for 30 min for erythrocyte ghosts) and resuspended in Tris buffer. Aliquots of labeled mitochondrial membranes were incubated for 1 h at 23°C with the desired amount of cannabinol or octanol, which were previously deposited on test tubes. For erythrocyte ghosts, the incubation with drugs was usually carried out at 4°C overnight. ESR spectra were obtained at 23°C.

Results

The order parameter, S, in phosphatidylcholine: 4% phosphatidic acid bilayers increases when either cholesterol, cannabinol, chlorpromazine or pentobarbital are added (Fig. 1, A and B). Similar results have been reported for cholesterol in phosphatidylcholine bilayers [16—21] and for pentobarbital and chlorpromazine in decholesterolized ox-brain lipids [6]. None of the other compounds produced such a large absolute ordering as cholesterol. Cannabinol was the strongest orderer of the other three. It ordered 5-doxyl stearic acid as strongly as cholesterol on a mole basis, but the effect appeared to saturate above 17 mole %. No such saturation was observed up to 34 mole % by PC (7,6), possibly reflecting different interactions at the interface and the interior of the bilayer. There is also a suggestion, particularly with 5-doxyl stearic acid, of curvature in some of the other data, but the effect is not large compared to our errors and because of the difficulties of solubilizing these compounds we did not pursue it in more detail. Nor did we find any consistent and reproducible non-linearities in other experiments even at high concentrations (e.g. Figs. 2 and 5). With these reservations we have assumed linearity in our analysis.

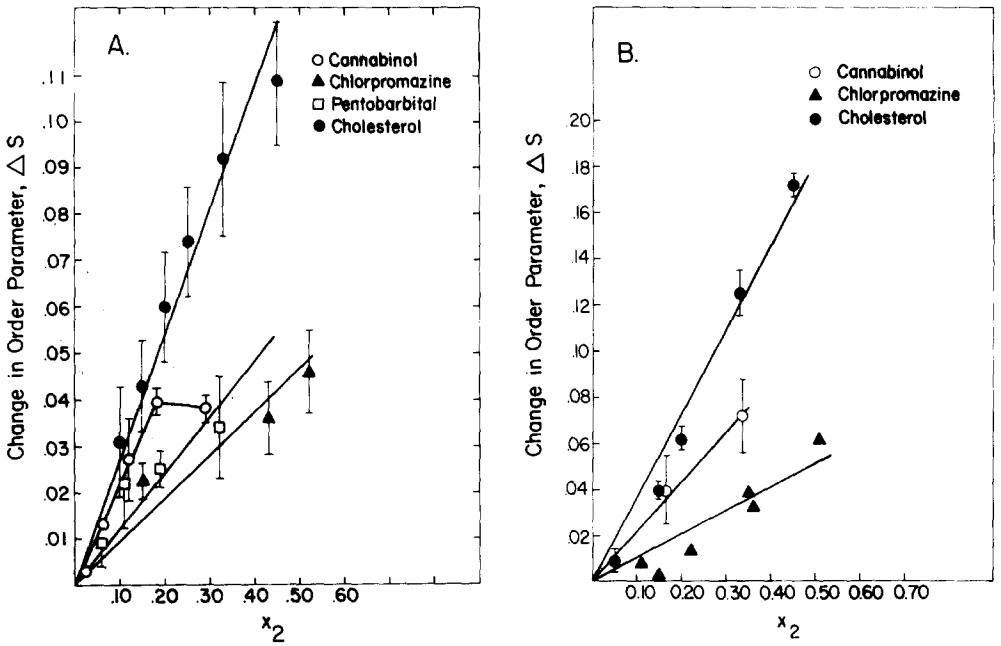

Fig. 1. A. Change in order parameter (ΔS) of spin labeled 5-doxyl stearic acid in phosphatidylcholine: 4% phosphatidic acid vesicles containing various mole fractions of cholesterol, cannabinol, chlorpromazine and pentobarbital. The slopes ($\Delta S/X_2$) were as follows: cholesterol, 0.27 ± 0.01 (S.D.), cannabinol, 0.23 ± 0.03; chlorpromazine, 0.09 ± 0.01; pentobarbital, 0.12 ± 0.01. Error bars are standard deviations, other points represent single experiments, and the regressions were weighted by the number of points. This procedure is followed in all subsequent figures. B. The corresponding results with PC (7,6) were: cholesterol, 0.37 ± 0.02; cannabinol, 0.22 ± 0.01; chlorpromazine, 0.10 ± 0.01. The mean order parameter for control vesicles with 5-doxyl stearic acid was 0.599 ± 0.009; with PC (7,6) was 0.499 ± 0.005.

Cholesterol increases the order of 5-doxyl stearic acid less than that of PC (7,6) (Fig. 1, A and B), as has been found by previous workers [17,22]. However, recent deuterium magnetic resonance studies show that cholesterol causes a uniform ordering of the acyl chains in this region [21,23,24]. A similar discrepancy is found between the two methods when the fluidity gradient is considered [21]. An additional uncertainty in our experiments is the location of 5-doxyl stearic acid compared to PC (7,6). We attempted to use 1-acyl-2[5(4,4-dimethyloxazolidine-*N*-oxyl)] palmitoyl phosphatidylcholine (PC (10,3)) instead but it gave such a high order parameter in the phospholipid bilayers that additional ordering was difficult to detect. Thus, comparisons between the absolute effects of perturbers on our two labels should be made with caution. On the other hand, these considerations are unlikely to invalidate our results when relative ordering effects are considered on a given label.

Relative to cholesterol none of these compounds ordered PC (7,6) as much as they did 5-doxyl stearic acid. Therefore, their relative ordering effects are probably strongest near the bilayer surface. Unfortunately 12-doxyl stearic acid yields too fluid a spectrum for order parameters to be measured. However, in one experiment 24 mole % of pentobarbital gave a spectrum identical to the control, tending to confirm the impression that the ordering effect gets weaker

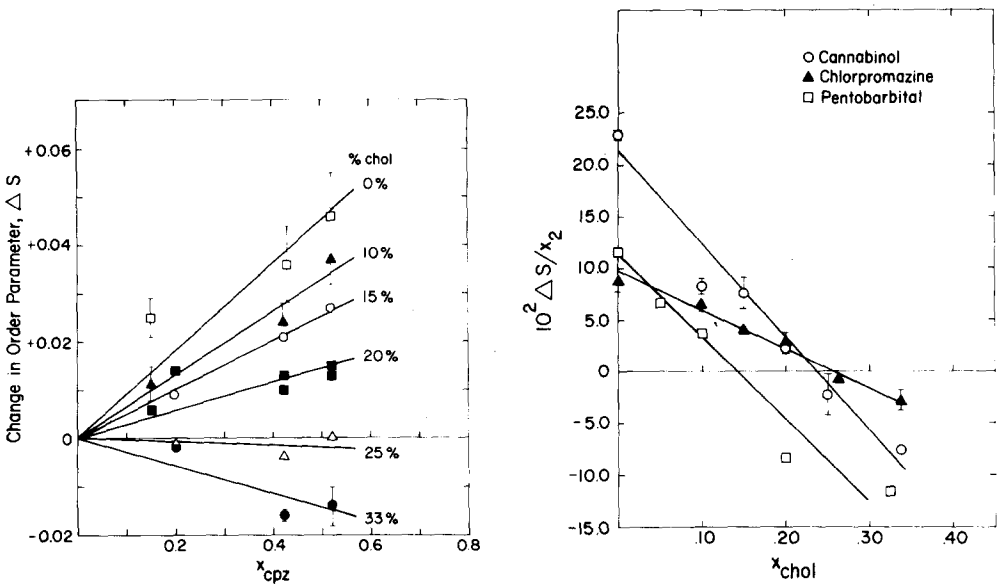

Fig. 2. The effect of chlorpromazine on the change in order parameter (ΔS) in phosphatidylcholine: 4% phosphatidic acid containing various mole % of cholesterol. X_{cpz} is chlorpromazine/chlorpromazine + phospholipid. Percent cholesterol is 100 (cholesterol/cholesterol + phospholipid).

Fig. 3. The effect of cholesterol on the change in order parameter per mole of membrane perturbers in phosphatidylcholine: 4% phosphatidic acid vesicles. The mole percentage of cholesterol at the cross-over point, ($\Delta S = 0$) were as follows: cannabinol, 23 ± 1.0; chlorpromazine, 26 ± 1.2; pentobarbital, 14 ± 1.8. X_{chol} is cholesterol/cholesterol + phospholipid.

further from the interface. The remainder of our work was carried out with 5-doxyl stearic acid because it gives the largest effects.

When one of the membrane perturbers was added to bilayers containing cholesterol the magnitude and sign of the change in order parameter was dependent on the cholesterol content. Thus, Fig. 2 shows that chlorpromazine orders bilayers with cholesterol content up to 20 mole %, but disorders those with greater than 25 mole % cholesterol. Similar results (not shown) were obtained with cannabinol and pentobarbital. If the change in order parameter per unit concentration of perturber in the bilayer (i.e., the slope in Fig. 2) is plotted versus the mole % of cholesterol in the lipid, the cholesterol content at which ΔS is zero may be defined. In Fig. 3 this cross-over composition of cholesterol is seen to be 26 mole % for chlorpromazine, 23 mole % for cannabinol and 14 mole % for pentobarbital.

In biological membranes a similar role for cholesterol in modulating the effects of perturbers was observed (Fig. 4). Cannabinol decreased the order of erythrocyte ghost membranes which have about 40 mole % cholesterol [1], but increased that of mitochondrial membranes which have an average lipid composition of 6 mole % cholesterol [1]. Previously we have shown [7] that octanol disorders phosphatidylcholine: 4% phosphatidic acid bilayers regardless of cholesterol content. Fig. 4, A and B, show that it also disorders both the biological membranes examined.

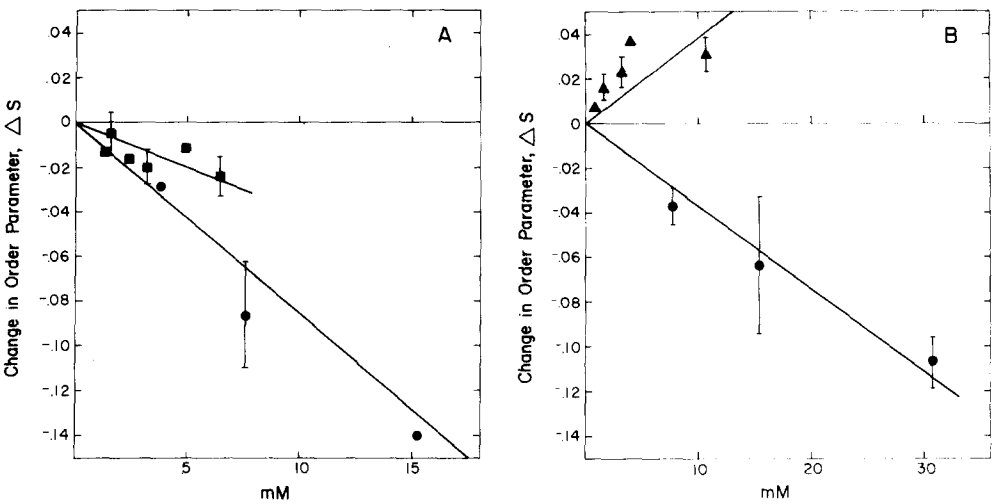

Fig. 4. Effect of increasing total concentrations of cannabinol (■) and octanol (●) on the change in order parameter (ΔS) of 5-doxyl stearic acid in A: erythrocyte membranes, protein concentrations 1—4 mg/ml. The mean order parameter of the control was 0.714 ± 0.033. B: mitochondrial membranes, protein concentrations 23 mg/ml. The mean order parameter of the control was 0.598 ± 0.012.

Discussion

The change in order produced by cholesterol probably reflects the orientation of the rigid steroid nucleus parallel to the phospholipid acyl chains, with the 3 β-hydroxyl group located in the interfacial region [21,24—26]. All the compounds which order phospholipid bilayers [4—7] also contain chemical ring structures, so they may act in a manner analogous to cholesterol. However, one might expect that they would produce a less extensive ordering than cholesterol because of their less favorable geometry. Thus, both chlorpromazine and cannabinol have three fused rings, but those of the former are folded about the N-S axis at 139.4° [27], and the presence of a charge may also limit its penetration into the bilayer. On the other hand, the rings of cannabinol should not deviate seriously from coplanarity, but the proximity of the hydroxyl and pentyl substituent groups should mitigate against orientation of the long axis of the fused ring structure parallel to the acyl chains. These considerations are consistent with our finding that these perturbers are less effective orderers at the eighth than at the fifth carbon. Similarly the deuterium magnetic resonance data show that the ordering effect of cholesterol declines beyong the 12 carbon [21], and it is possible with the other perturbers examined here that their ability to order might fall off even more rapidly. Preliminary studies of fluorescence depolarization with 1,6-diphenyl hexatriene in this laboratory show a decrease in microviscosity in pentobarbital-phosphatidylcholine liposomes. This result lends support to the suggestion that it is possible that our perturbers simultaneously order the ester terminal of the acyl chains whilst disordering the methyl terminal. Deuterium magnetic resonance studies could confirm this prediction.

Interactions in the system containing phospholipid-cholesterol-perturber are

more complex. Thus, the ordering effects of cholesterol and perturber are clearly not additive (Figs. 2 and 3). The favorable stereochemistry, which allows a strong van der Waals interaction between cholesterol and the acyl chains [21,24—26], will tend to be disrupted by the presence of the perturber in the same region of the bilayer. The net effect of the perturber on the packing of phospholipid-cholesterol bilayers will reflect the balance between its own ordering effect on the acyl chain and its disruptive effect on the acyl-cholesterol interaction. As the cholesterol content of the bilayer is increased, cholesterol-perturber interactions become more frequent. Eventually the resultant disruption of acyl-cholesterol interactions outweigh the ordering effect of the perturber and a cross-over to a net disordering effect is observed (Fig. 2). This apparently may occur at any cholesterol composition, depending only on the balance of these effects and not on any discrete phospholipid to cholesterol ratio. Pentobarbital, the weakest orderer, crosses over at the lowest cholesterol content (Fig. 3). In phospholipid bilayers, where only the perturber's ordering effect is important, larger changes in order parameter occur than in high cholesterol membranes where the changes result from a balance between two opposing effects (Fig. 2). Chlorpromazine and cannabinol cross-over from ordering to disordering at similar cholesterol contents even though chlorpromazine, like pentobarbital, is a less effective orderer of phospholipid bilayers. This results from the less steep slope exhibited by chlorpromazine in Fig. 3 and may reflect the additional role of chlorpromazine's positive charge which would limit its ability to penetrate fully into the region where cholesterol exerts its ordering effect.

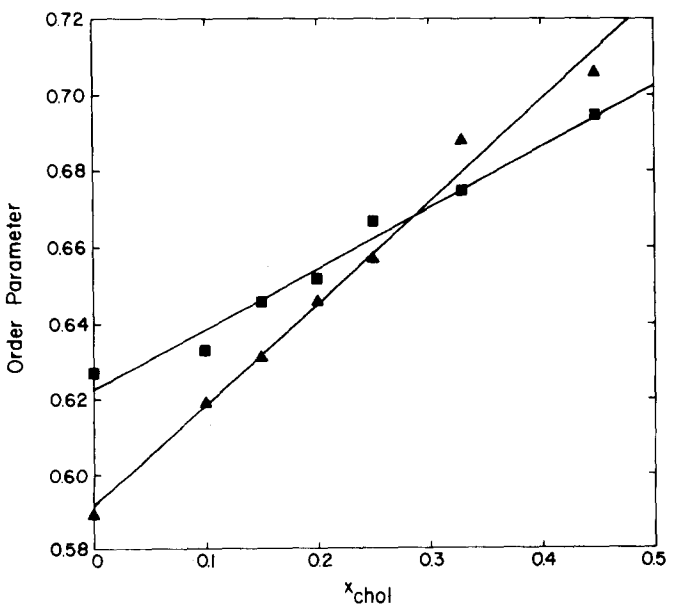

Fig. 5. Effect of cholesterol (chol) on the order parameter of phosphatidylcholine: 4% phosphatidic acid liposomes in the absence (▲) and in the presence, (■) of 17 mole % cannabinol. The slopes of the lines are 0.270 ± 0.001 and 0.160 ± 0.001, respectively.

This ability of perturbers to reduce the effectiveness of cholesterol to order bilayers is clearly illustrated in Fig. 5. Here cholesterol orders a phospholipid bilayer containing 17 mole % of cannabinol 1.7 times less effectively than in the unadulterated bilayer.

Thus, we may conclude that, in addition to its well known property of regulating membrane fluidity, cholesterol also modulates the effect of membrane perturbers in both lipid bilayers and biological membranes. If more extensive studies confirm this there are a number of interesting corollaries. Thus, many authors [28—31] have emphasized the possible relationship between the action of anesthetics and their ability to fluidize membranes. Our work here on biomembranes, together with previous work on general anesthetics in lipid bilayers [7], suggests that for this hypothesis to be correct the model must be restricted to biomembranes containing a high proportion of cholesterol. This would be consistent with the known composition of most neural membranes [32].

Changes in fluidity related to cholesterol content have been noted upon malignant transformation of normal lymphocytes [33], the fusion of muscle cells [34] and the complement lysis of antibody-sensitized cells with normal rabbit serum [35]. If there is a causal relationship between these phenomena and membrane fluidity, then the ability to selectively modify biomembrane fluidity by pharmacological agents may not be without application.

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References

1 O'Brien, J.S. (1967) J. Theor. Biol. 15, 307—324
2 Hubbell, W.L., Metcalfe, J.C., Metcalfe, S.M. and McConnell, H.M. (1970) Biochim. Biophys. Acta 219, 415—427
3 Trudell, J.R., Hubbell, W.L. and Cohen, E.N. (1973) Biochim. Biophys. Acta 291, 321—327
4 Lawrence, D.K. and Gill, E.N. (1975) Mol. Pharmacol. 11, 280—286
5 Lawrence, D.K. and Gill, E.N. (1975) Mol. Pharmacol. 11, 595—602
6 Neal, M.J., Butler, K.W., Polnaszek, C.F. and Smith, I.C.P. (1976) Mol. Pharmacol. 12, 144—155
7 Miller, K.W. and Pang, K.-Y.Y. (1976) Nature 236, 253-255
8 Hubbell, W.L. and McConnell, H (1971) J. Am. Chem. Soc. 93, 314—326
9 Lombrozo, L., Kanter, S.L. and Hollister, L.E. (1976) Res. Commun. Chem. Path. Pharm. 15, 697—703
10 Seeman, P. (1972) Pharmacol. Rev. 24, 583—655
11 Hill, M.W. (1974) Biochim. Biophys. Acta 356, 117—124
12 Gaffney, B.J. (1976) in Spin labeling (Berliner, L.J., ed.), pp. 567—571, Academic Press, New York
13 Parsons, D.F. and Williams, G.R. (1976) in Methods in Enzymology (Estabrook, R.W. and Pulman, M.E., eds.), Vol. 10, pp. 443—448, Academic Press, New York
14 Dodge, J.T., Mitchell, C.E. and Hanahan, D.J. (1963) Arch. Biochim. Biophys. 100, 119—130
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol. Chem. 193, 265—275
16 Hsia, J.C., Long, R.A., Hruska, R.E. and Gesser, H.D. (1972) Biochim. Biophys. Acta 290, 22—31

17 Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I.C.P. (1973) Chem. Phys. Lipids 10, 11—27
18 Ladbrook, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333—340
19 Lecuyer, H. and Dervichian, D.G. (1969) J. Mol. Biol. 45, 39—57
20 de Kruijff, B., Demel, R.A., Slotboom, A.J., van Deenen, L.L.M. and Rosenthal, A.F. (1973) Biochim. Biophys. Acta 307, 1—19
21 Smith, I.C.P., Stockton, G.W., Tulloch, A.P., Polnaszek, C.F. and Johnson, K.G. (1977) J. Colloid Interface Sci. 58, 439—451
22 McConnell, H.M. and McFarland, B.G. (1972) Ann. N.Y. Acad. Sci. 195, 207—211
23 Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839—4845
24 Stockton, G.W. and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251—263
25 Demel, R.A. and the Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109—132
26 Rothman, J.E. and Engelman, D.M. (1972) Nat. New Biol. 237, 42—44
27 McDowell, J.J.H. (1969) Acta Crystalog. B25, 2175—2181
28 Metcalfe, J.C., Seeman, P. and Burgen, A.S.V. (1968) Mol. Pharmacol. 4, 87—95
29 Boggs, J.M., Yoong, T. and Hsia, J.C. (1976) Mol. Pharmacol. 12, 127—135
30 Johnson, S.M., Miller, K.W. and Bangham, A.D. (1973) Biochim. Biophys. Acta 291, 328—334
31 Trudell, J.R., Hubbell, W.L. and Cohen, E.N. (1973) Biochim. Biophys. Acta 291, 328—334
32 Whittaker, V.P. (1969) in Handbook of Neurochemistry 2 (Lajtha, A. ed.), pp. 327—364, Plenum Press, New York
33 Inbar, M., Shinitzky, M. and Sachs, L. (1974) FEBS Lett. 38, 268—270
34 Prives, J. and Shinitzky, M. (1977) Nature 268, 761—763
35 Mason, P.R., Giavedoni E.B. and Dalmasso A.P. (1977) Biochemistry 16, 1196—1201