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COMMENTARY

CHARACTERIZATION OF DRUG–MEMBRANE INTERACTIONS USING THE LIPOSOME SYSTEM*

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Most drugs encounter biological membranes at some point in their interaction with organisms, prior to reaching the ultimate metabolic target. This encounter may be nonproductive if any membrane, particularly that enclosing the target receptor, repels the drug and hence does not allow its entry into the space bounded by the membrane. Alternatively, the drug may pass through the membrane if solubility properties are favorable or if an appropriate transport mechanism exists. Thus, pharmacologists have been concerned with the importance of cell membranes in the action of medicinal agents at this level for many years. The enormous increase in our understanding of the composition and function of cellular membranes has, however, stimulated a variety of investigations which have suggested that a number of drugs may exert at least a portion of their pharmacological effects through alteration of membrane function(s) by either interaction with the membrane or interference with its biogenesis. Consequently, an understanding of the physicochemical basis by which drug–membrane interactions occur is of great importance to a definition of the mechanism of action of certain drugs.

A large number of studies exist which are concerned with the effect of drugs on the properties of membranes or membrane-supported activities. By and large these studies are of a descriptive nature with relatively little information on the molecular mechanisms of interaction. This situation is largely the result of the enormous complexity of cellular membranes. Thus, it is exceedingly difficult to define in detailed molecular terms the interaction of a pharmacological agent with a three-dimensional array of phospholipids and proteins probably organized into specific neighborhoods and underpinned by a complex cytoskeleton of microtubules and microfilaments.

Fortunately, a good model system for the phospholipid bilayer of membranes exists, namely phospholipid vesicles or liposomes. Liposomes are easily prepared by simply suspending phospholipids in aqueous solutions; the resulting liposomes consist of large multilamellar cell-like structures which, upon being subjected to high intensity ultrasound, form single walled (unilamellar) vesicles (see Ref. 1 for a review of techniques). Liposomes prepared so as to contain drugs are being widely investigated as tools for the delivery of these agents to cellular sites [2]. Relatively less effort has been applied to the employment of the liposome as a model membrane for which to study drug interactions with phospholipid bilayers. This Commentary directs itself to a discussion of some of the methodology which either has been or could be utilized to provide structural and/or dynamic information on drug–membrane interactions using liposome vesicles. Many of the techniques and studies described involve equipment which is generally available in biological laboratories and could be utilized by pharmacologists to investigate the particular drug class in which they are interested.

The liposome system has several advantages over natural cell membranes for defining the physicochemistry of drug action. Liposomes are exceedingly simple; sonicated dispersions of phospholipids produce small vesicles of fairly uniform size (on the order of 300 Å) consisting of a single bilayer surrounding an aqueous space [3]. The membrane composition both in terms of the polar head group and the fatty acid side chains is accurately known, since pure phospholipids can be employed as starting materials. In this way, both the surface charge density and interior fluidity can be controlled. Furthermore, membranes of practically any lipid composition can be prepared and purified proteins or other molecules can be incorporated into either the aqueous or hydrocarbon phases. Such vesicles can be prepared on the large scale necessary for certain types of physical measurements without the complex manipulations required to purify cellular membranes. Most importantly, liposomes would appear to be relevant models of biological membranes, as they have the basic phospholipid bilayer structural element of the membrane.

As indicated previously, most drugs must interact with membranes in some manner to exert their biological action. This interaction can be either a transient phenomenon while the drug passes through the membrane, or the pharmacologically important act itself. That is, the drug may alter some property of the membrane to produce its characteristic pharmacological response.

Several distinct membrane properties are potential targets for a drug molecule. One of the most widely discussed of these properties is fluidity. Fluidity in this sense is a measure of the resistance to lateral diffusion of molecules in the membrane plane. A membrane in the solid (or gel) phase possesses hydrocarbon chains with a high energy barrier to bond rotation. These hydrocarbons, therefore, form an all *trans* geometry about the carbon–carbon bonds and pack into an ordered hexagonal array. In contrast, in the fluid (or liquid crystalline) phase the barrier to bond rotation is much lower and the hydrocarbon

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chains possess liquid-like disorder, thus disrupting the ordered array. The transition from one phase to the other occurs, at least for pure phospholipids, at a characteristic temperature called the T_m (sometimes referred to as the T_c). This critical temperature is a function of membrane composition and solution conditions and can be studied by a variety of physical techniques. In principle, drugs could affect the fluidity characteristics of a membrane in either direction, i.e. by making it more fluid or more solid and thus either enhancing or inhibiting membrane functions which require translation of molecules within the bilayer. Examples of both cases are known and will be discussed.

Drugs can also alter the permeability of a membrane. Indeed, one of the chief functions of a membrane is to act as a permeability barrier to restrain movement of desirable or undesirable molecules. Any alteration in membrane permeability characteristics produced by a drug could have profound effects on cellular activity. Polyene antibiotics are examples of agents which make certain kinds of membranes leaky, whose molecular action at this level has been probed using liposomes [4].

Spatial organization is another important feature of membrane design that is a possible target for drug action. Several kinds of experimental evidence exist which suggest that most biological membranes are not simply a homogeneous sea of molecules randomly drifting about (for a relevant review see Ref. 5). Rather, certain proteins, which include receptors and antigens as well as more complex assemblies, are ordered into specific environments. Lipids too probably exist in defined local environments and, in fact, show an asymmetrical distribution on the two sides of the bilayer [6]. Relatively little is known of the structural details of such organization, but pharmacologic disruption is certainly a possible mode of drug action. Detergents are perhaps the most drastic example of agents which alter membrane spatial organization [7].

The fusion of one membrane with another is an additional physiological function of membranes which could be altered by chemical agents. Since fusion is an important component of biological events ranging from secretion to virus infection, alteration of this mechanism could profoundly influence living cells. The liposome system would appear to have considerable usefulness in studies of membrane fusion. Gent and Prestegard [8], for example, have shown that free fatty acids promote vesicle fusion, with the maximum rate occurring at the transition temperature. Interestingly, prostaglandins, which are complex derivatives of fatty acids, do not stimulate vesicle fusion (T. R. Tritton and C. A. Briggs, unpublished results); however, other kinds of molecules promote liposome fusion (e.g. antidepressants [9] and Ca^{2+} [10]), but apparently no compound has been described which inhibits the effects of such fusogens.

Charge distribution and density are increasingly being recognized as yet another important characteristic of membrane surfaces [11]. This property is interrelated with fluidity, permeability, fusion and structural organization and most certainly is important in drug interactions with membranes, particularly as a determinant of binding specificity. All of these properties of biological membranes can be studied in the relatively simple liposome system. To demonstrate the utility of these vesicles, selected examples of appropriate physicochemical approaches using liposomes, which have assisted in the definition of the mechanisms of chemical interactions with membranes, will be described.

ANAESTHETICS

Anaesthetics, as a class, have been extensively studied in liposome systems. Unlike many other drugs, anaesthetics appear to be quite nonspecific, acting on a variety of cell types and cell functions. In addition, different anaesthetic drugs possess structures with no obvious chemical similarities. Instead, the unifying feature of these agents is that anaesthetic action appears to occur at the membrane, although the actual molecular details of this interaction are controversial.

High resolution nuclear magnetic resonance has been usefully employed in the study of anaesthetic–membrane interactions by several workers. Modern Fourier transform multinuclear instruments are certainly capable of giving a large amount of detailed information, but are expensive and complex and are not readily available to many research workers. On the other hand, the low field continuous wave proton machines available in most laboratories can yield useful information, as exemplified by the studies of Hauser *et al.* [12] and Cerbón [13] on liposome–anaesthetic interactions. The basic principle in this kind of experiment is that a macromolecular assembly has much slower molecular motions in solution than a small molecule. When a small molecule binds to a large one, however, it takes on the motional correlation time of the macromolecule. The result is a shortening of the magnetic relaxation times (i.e. the so-called T1 and T2) of the small molecule and a resultant line broadening. Thus, an increase in an NMR line width is an indication of a molecular interaction. Fischer and Jardetzky [14] were the first to apply this approach, and Jardetzky and others have subsequently developed the technique, expanding its range of application over the last 10 yrs. A recent book gives appropriate details and pertinent references [15].

As an example of this technique, Hauser *et al.* [12] showed that interaction of procaine and tetracaine with liposomes occurred only with those composed of acidic phospholipids, but not with zwitterionic neutral ones, indicating that the interaction was dominated by electrostatic and Van der Waals forces. Consistent with this idea, they also observed a differential effect on the line broadening of various regions of the anaesthetic molecule. Cerbón [13] further showed that the presence of a hydrophobic tail on the anaesthetic, as in butacaine or pantocaine, resulted in interaction with membranes, even in the absence of a net negative charge, presumably by insertion of the hydrophobic portion of the drug into the hydrocarbon region of the membrane.

Various kinds of approaches have been employed to examine the effects of anaesthetics on the fluidity of membranes. For example, Trudell *et al.* [16] have used electron spin resonance (ESR) to show that vola-

tile anaesthetics produce disorder in a lipid bilayer. This kind of information is readily obtainable from these (ESR) kinds of studies because the conformational mobility of nitroxide spin labeled fatty acids (commercially available from Syva Corp., Palo Alto, CA.) can be deduced from the splittings in the ESR spectrum. The practical details of such an analysis can be found in the review of Griffith and Jost [17]. Trudell *et al.* [16] were able to show the probable pharmacological relevance of their studies by demonstrating a dose–response relationship between anaesthetic concentration and liposome membrane disorder over concentrations similar to those required for clinical anaesthesia.

Likewise, Papahadjopoulos *et al.* [18] showed that local anaesthetics also increase the fluidity of membranes and that this fluidizing effect was even more pronounced in the presence of Ca^{2+}. These conclusions were derived from differential scanning calorimetric (DSC) and fluorescence polarization measurements. In the former type of experiment, the sample under study is heated at a programmed rate and the amount of heat absorbed or released in a thermally induced transition is measured. Fluorescence polarization measurements, on the other hand, require a probe molecule embedded in the membrane and, like ESR experiments, sense the mobility of the probe and hence indirectly the degree of fluidity or disorder in the membrane. Using both techniques, Papahadjopoulos *et al.* [18] found that the critical temperature (T_m) of the gel–liquid crystal transition of the liposomes decreased in the presence of dibucaine, indicating that this agent caused the membrane to become more fluid.

CHOLESTEROL

The widespread occurrence of cholesterol in biological membranes suggests that this relatively small molecule plays an important role in modulating membrane structure and function. Although not technically a drug, cholesterol is included in this discussion because it exemplifies several principles of small molecule–liposome interaction. A variety of techniques have been employed to determine the effects of cholesterol on phospholipid liposomes; these studies have yielded a fairly detailed picture of their interaction. The dominant concept is that cholesterol modulates the packing of the hydrocarbon chains such that in the fluid phase chain flexing is inhibited, while in the solid phase chain packing into a rigid array is prevented. Relatively little information is available on the interaction of other steroids, such as the steroid hormones, with liposomes and this would appear to be a fruitful area for future research.

To study the effects of cholesterol on the lipid phase transition, a variety of techniques including differential scanning calorimetry have been employed. Ladbrooke *et al.* [19] and Hinz and Sturtevant [20] showed that addition of cholesterol to lecithin liposomes lowered the transition temperature and decreased the specific heat absorbed in direct proportion to the mole per cent of cholesterol incorporated. When membranes contain between 33–50 per cent cholesterol, the solid–fluid transition is completely eliminated. NMR spectroscopic study of the protons on the lecithin molecules in liposomes shows a reduction in signal for the methylene protons, suggesting that cholesterol makes the membrane less fluid above the T_m [21, 22]. In this type of experiment, the only observable resonance signals are those from protons on the lecithin itself, i.e. the resonance signals from cholesterol are obscured by the high lipid background. To overcome this problem, Kroon *et al.* [23] have synthesized > 99 per cent deuterated lecithin; use of vesicles consisting of this material allows the observation of some of the resonances of the cholesterol present in the liposomes. From these studies, it was concluded that the cholesterol molecule has a differential mobility in the bilayer. The motion of the aliphatic tail is essentially unrestricted, while the steroid nucleus is more immobilized. These results, however, are in conflict with some previous models of cholesterol–membrane interactions, which postulate a specific stoichiometric association [23]. Nevertheless, the success of these studies makes it likely that the use of deuterated phospholipids will be an increasingly important tool for NMR spectroscopic studies of other small molecules which interact with membranes.

ESR, too, has been used effectively to probe the interactions of cholesterol with liposomes. For example, using a spin labeled cholesterol derivative, Schreier-Muccillo *et al.* [24] demonstrated that (a) the steroid orients itself with its long axis perpendicular to the plane of the bilayer, and (b) the molecule rotates rapidly about this axis. When a nitroxide spin label was placed at different positions along the hydrocarbon chains, Hubbell and McConnell [25] were able to discern a "fluidity gradient" corresponding to increasing motion toward the center of the bilayer. Addition of cholesterol to the fluid bilayer (i.e. above the T_m) decreased the flexibility of the chains until at equimolar stoichiometry the hydrocarbons were almost completely rigid [24]. Conversely, introduction of cholesterol into solid membranes (i.e. below the T_m) actually increases chain flexibility. Other physical techniques, such as X-ray diffraction [26] and fluorescence polarization [27], support these conclusions.

Finally, cholesterol also affects the permeability of liposomal membranes. Papahadjopoulos *et al.* [27] showed that the presence of cholesterol produces a decrease in the rate of self-diffusion of Na^+ ions across liposome membranes at all temperatures. Moreover, at equimolar levels of cholesterol and lecithin the increase in permeability with increasing temperature showed no discontinuity and gave linear Arrhenius plots in direct contrast to the pure phospholipid alone. This result demonstrates that cholesterol inhibits the phase transition associated changes in Na^+ permeability.

ADRIAMYCIN

In our own laboratories, we have been interested in the interaction between the antineoplastic agent adriamycin and membranes. Although DNA has usually been considered to be the prime target for the action of this drug, recent evidence suggests that membrane effects are important as well [28]. These studies demonstrated that the rate of agglutination of Sarcoma 180 cells by concanavalin A was enhanced

by levels of adriamycin which inhibit cell growth and that this effect occurred in the absence of a change in the rate and extent of binding of labeled concanavalin A to these cells. In an attempt to understand the details of the action of adriamycin at the level of the membrane, we are employing a variety of physicochemical approaches using the liposome system.

Although of great utility in studying the fluidity characteristics of membranes, scanning calorimeters and electron spin resonance instrumentation are not routinely available to many investigators; therefore, we have successfully used a common spectrophotometer to measure phase transitions (fluidity) in liposomes by monitoring changes in turbidity as a function of temperature. Turbidity is a measure of light scattered in the forward direction. When a dispersion of phospholipid vesicles undergoes a solid–fluid phase transition, the scattered light intensity is altered [29] and measurement of this phenomenon provides a convenient monitor of this transition. Using this technique, we have obtained evidence that adriamycin fluidizes pure lecithin membranes; however, when small amounts of cardiolipin are incorporated into these membranes, the drug causes them to become less fluid (i.e. to have a higher T_m). This is apparently not a nonspecific effect of acidic phospholipids, since phosphatidyl serine containing liposomes are rendered more fluid by adriamycin [30].

We have also used 60 MHz continuous wave proton NMR to ascertain whether adriamycin alters the permeability and fusion characteristics of liposomes. The $N^+(CH_3)_4$ proton resonance of lecithin is a well-resolved single peak in the NMR spectrum. In the presence of the paramagnetic ion Pr^{3+}, the resonance position of the choline protons is shifted downfield [31]. Since the liposomes are normally impermeable to Pr^{3+} only those protons on the outside of the bilayer are accessible to the shift reagent, and hence the $N^+(CH_3)_4$ resonance is split into an inside-outside doublet. If a drug molecule makes the membrane leaky and thereby permeable to Pr^{3+}, then the doublet structure will collapse due to contact of the paramagnetic ion with choline protons on both the inside and outside of the vesicles. Addition of adriamycin to such a Pr^{3+}-liposome system causes no apparent NMR signal change, and thus we conclude that the drug does not make the membrane permeable to ions like Pr^{3+}.

Membrane fusion is also easily monitored in liposome suspensions using proton NMR [8]. The basic concept in this approach is that fusion of small vesicles to form larger ones slows down the molecular motions of the lipids. This, in turn, causes the proton resonances to broaden such that, as larger liposomes are formed, the motion becomes slower, and finally no resonance is observed. That is, the signal intensity will decrease with time as fusion proceeds. In pure lecithin liposomes, fusion occurs very slowly, if at all, and addition of adriamycin does not cause any change. In cardiolipin containing lecithin liposomes, however, adriamycin markedly enhances the rate of vesicle–vesicle fusion, as determined by the loss in signal intensity of the choline proton resonance. These results suggest that adriamycin interacts in a specific manner with cardiolipin containing vesicles to alter fluidity and fusion characteristics.

OTHER DRUGS

Several other investigations of drug–liposome interactions exist in the literature. Much of this work is concerned with the effects of drugs on membrane fluidity. For example, using DSC and ESR, Cater *et al.* [32] reported that morphine-like compounds and tricyclic antidepressants altered the tilt of the hydrocarbon chains at low concentrations and then fluidized the membrane at higher drug concentrations. Likewise, Jain *et al.* [33] showed by DSC that a series of aliphatic alcohols, uncouplers of oxidative phosphorylation, and tranquilizers broadened the transition profile and decreased the T_m of dipalmitoyl phosphatidyl choline liposomes. Not all drugs fluidize membranes, however. For example, Lyman *et al.* [34] showed, again by DSC, that agents like dimethyl sulfoxide, dimethyl formamide, pyridine *N*-oxide and tetramethylurea, which induce differentiation in Friend erythroleukemia cells, cause an increase in the transition temperature of dimyristoyl phosphatidyl glycerol liposomes, indicating a tendency to promote less fluid or more ordered membranes.

It appears that practically any drug or other small molecule can produce fluidity changes in membrane phospholipids, although concentrations which are much higher than clinically relevant ones are often necessary to cause changes in physical properties of the membrane. Consequently, membrane fluidization by itself may not be the pharmacologically important event in the action of a drug on a cell, especially if high concentrations of the agent are required to produce the effect. Nonetheless, local membrane effects due to specificity of a drug for a particular membrane component or a differential effect on separate components of the membrane could be an important determinant of drug action. Thus, it appears reasonable to predict that future fruitful efforts in drug development may be attained by achieving such specificity in membrane active drugs. There are two general means by which such specificity might be obtained. The first is the conventional approach of compound design and synthesis followed by evaluation in a proper test system. A second possibility, however, is to use liposomes of defined composition to increase specific components of surface membranes of target cells through fusion and hence change the inherent binding specificity of that membrane toward selected small molecules. Thus, if a given drug has a high affinity for a certain membrane component or is particularly effective against cells whose membranes contain that component, then fusion of cells with appropriate liposomes could be used to introduce this material into cell membranes. The therapeutic potential of this approach has not been overlooked and much effort has been devoted to defining the mechanisms of liposome uptake by cells (reviewed in Refs. 1 and 35).

PROGNOSIS

Physicochemical studies have had a significant impact on our understanding of the structure and function of biological membranes. In this Commentary, we have outlined how such approaches can also yield information about membrane interactions with drugs that is not readily available by other kinds of tech-

niques. At least some of these approaches can be systematically exploited by nearly all pharmacologists. A major goal of these studies is a definition of the molecular details that govern the ways in which small molecules interact with membranes. Since interaction of chemical agents with membranes is so common and important in biological systems, it is likely that much future effort will be applied in this area. A second, more practical, goal of studies of drug–membrane interaction is to employ the findings obtained to suggest new ways in which optimal chemotherapeutic use can be attained. It seems reasonable to assume that a description of the mechanisms of drug–membrane interaction will assist in the rational design of new agents capable of achieving desired phenomena.

A limitation in most physical studies on liposomes, including all of the methodologies described in this report, is that only the bulk or average property of an entire system is observed in the experiment. Consequently, localized effects, which may be of critical importance in an organized membrane system, are not readily discernible. This is an inherent limitation of our methodology at present and represents a major challenge for developing new techniques in the future.

Some progress has been made in examining membrane properties of single cells, such as (a) the use of fluorescence polarization using microscope-optics [36], and (b) the application of intense highly focused laser beams to measure fluorescence photobleaching recovery as a means of detecting lateral motion of fluorescent cell surface components [37]. The value of the liposome system, however, lies in its simplicity and, therefore, it is highly useful for design and interpretation of physical studies on membranes. Such studies, however, should be viewed only as a starting point from which to proceed to biological membranes.

REFERENCES

1. D. A. Tyrrell, T. D. Heath, C. M. Colley and B. E. Ryman, *Biochim. biophys. Acta* **457**, 259 (1976).
2. G. Gregoriadis, *New Engl. J. Med.* **295**, 704 (1976).
3. C.-H. Huang, *Biochemistry* **8**, 344 (1969).
4. D. B. Archer, *Biochem. biophys. Res. Commun.* **66**, 195 (1975).
5. S. J. Singer, *A. Rev. Biochem.* **43**, 805 (1974).
6. R. Latorre and J. E. Hall, *Nature, Lond.* **264**, 361 (1976).
7. A. Helenius and K. Simons, *Biochim. biophys. Acta* **415**, 29 (1975).
8. M. P. N. Gent and J. H. Prestegard, *Biochemistry* **13**, 4027 (1974).
9. J. Bermejo, T. Eirin and A. Barbadillo, *Fedn Eur. Biochem. Soc. Lett.* **58**, 289 (1975).
10. D. Papahadjopoulos and G. Poste, *Biophys. J.* **15**, 945 (1975).
11. H. Walter, in *Methods in Cell Biology* (Ed. D. M. Prescott), Vol. 9, p. 25. Academic Press, New York (1975).
12. H. Hauser, S. A. Penkett and D. Chapman, *Biochim. biophys. Acta* **183**, 466 (1969).
13. J. Cerbón, *Biochim. biophys. Acta* **290**, 51 (1972).
14. J. J. Fischer and O. Jardetzky, *J. Am. chem. Soc.* **87**, 3237 (1965).
15. T. L. James, *Nuclear Magnetic Resonance in Biochemistry*. Academic Press, New York (1975).
16. J. R. Trudell, W. L. Hubbell and E. N. Cohen, *Biochim. biophys. Acta* **291**, 321 (1973).
17. O. H. Griffith and P. C. Jost, in *Spin Labeling: Theory and Applications* (Ed. L. J. Berliner), p. 453. Academic Press, New York (1976).
18. D. Papahadjopoulos, K. Jacobson, G. Poste and G. Shepherd, *Biochim. biophys. Acta* **394**, 504 (1975).
19. B. D. Ladbrooke, R. M. Williams and D. Chapman, *Biochim. biophys. Acta* **150**, 333 (1968).
20. H-J. Hinz and J. M. Sturtevant, *J. biol. Chem.* **247**, 3697 (1972).
21. A. Darke, E. G. Finer, A. G. Flook and M. C. Phillips, *J. molec. Biol.* **63**, 265 (1972).
22. M. C. Phillips and E. G. Finer, *Biochim. biophys. Acta* **356**, 199 (1974).
23. P. A. Kroon, M. Kainosho and S. I. Chan, *Nature, Lond.* **256**, 582 (1975).
24. S. Schreier-Muccillo, D. Marsh, H. Dugas, H. Schneider and I. C. P. Smith, *Chem. Phys. Lipids* **10**, 11 (1973).
25. W. L. Hubbell and H. M. McConnell, *J. Am. chem. Soc.* **93**, 314 (1971).
26. Y. K. Levine and M. H. F. Wilkins, *Nature New Biol.* **230**, 69 (1971).
27. D. Papahadjopoulos, K. Jacobson, S. Nir and T. Isac, *Biochim. biophys. Acta* **311**, 330 (1973).
28. S. A. Murphree, L. S. Cunningham, K. M. Hwang and A. C. Sartorelli, *Biochem. Pharmac.* **25**, 1227 (1976).
29. P. N. Yi and R. C. MacDonald, *Chem. Phys. Lipids* **11**, 114 (1973).
30. S. A. Murphree, T. R. Tritton and A. C. Sartorelli, *Fedn Proc.* **36**, 303 (1977).
31. S. B. Andrews, J. W. Faller, J. M. Gilliam and R. J. Barrnett, *Proc. natn. Acad. Sci. U.S.A.* **70**, 1814 (1973).
32. B. R. Cater, D. Chapman, S. M. Hawes and J. Saville, *Biochim. biophys. Acta* **363**, 54 (1974).
33. M. K. Jain, Y-M. N. Wu and L. V. Wray, *Nature, Lond.* **255**, 494 (1975).
34. G. H. Lyman, H. D. Preisler and D. Papahadjopoulos, *Nature, Lond.* **262**, 360 (1976).
35. G. Poste, D. Papahadjopoulos and W. J. Vail, in *Methods in Cell Biology* (Ed. D. M. Prescott), Vol. 14, p. 33. Academic Press, New York (1976).
36. M. Inbar, *Fedn Eur. Biochem. Soc. Lett.* **67**, 180 (1976).
37. J. Schlessinger, D. E. Koppel, D. Axelrod, K. Jacobson, W. W. Webb and E. L. Elson, *Proc. natn. Acad. Sci. U.S.A.* **73**, 2409 (1976).