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The Molecular Composition of Some Lipid Bilayer Membranes in Aqueous Solution

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Summary. An analytical technique is described for direct determination of the molecular composition of lipid bilayer membranes in aqueous solution. Membranes formed from chemically pure, radioactively labeled components, were sampled by pipetting a mercury droplet through the bilayer-water interface. During this procedure, the membrane remains intact but decreases in area with a concomitant increase in the area of the surrounding bulk phase. It is shown that each mercury droplet is covered with a fragment of the bilayer membrane in the form of a closed vesicle. The chemical composition of the bilayer is determined from an analysis of the readioactivity on the mercury droplet.

Bilayers generated from glyceryl monooleate in *n*-decane or *n*-hexadecane contain $(4.7 \pm 0.4) \times 10^{14}$ molecules of monoglyceride per cm^2 and a minimum of $(2.8 \pm 0.7) \times 10^{14}$ molecules of solvent (*n*-hexadecane) per cm^2. It is estimated from these numbers that 37 vol % of the hydrocarbon core of the bilayer is occupied by solvent.

The composition relationships between the bilayer and bulk membrane-forming solution were determined for mixtures of glyceryl monooleate (GMO) with cholesterol (Chol) or glyceryl monostearate (GMS). It was found that $[GMS/GMO]_{bilayer} \simeq [GMS/GMO]_{bulk}$, and $[Chol/GMO]_{bilayer} = 0.5\,[Chol/GMO]_{bulk}$. While the molecular areas of glyceryl monooleate and glyceryl monostearate are unchanged in the mixed system, the average area for mixtures of cholesterol and glyceryl monooleate is decreased, suggesting a condensing effect of the sterol in the bilayer analogous to that observed in lipid monolayers.

Extensive studies on phospholipid model systems in aqueous solution [16, 19] and the recent use of physical techniques to probe the organization of natural membranes [11, 18] strongly support the existence of a bimolecular lipid lamella as a mosaic element in many biological membranes [8]. Indeed, a number of important membrane-associated functions including facilitated diffusion [15], cation discrimination [21], and electrical excitability [12] have been observed in synthetic lipid bilayer membranes separating two aqueous phases either in unmodified systems or in membranes

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altered by interaction with a suitable protein or polypeptide component. However, it is clear that a complete description of these phenomena at the molecular level in the model system requires knowledge of the membrane composition, the number of molecules per unit area of each component present in the membrane. Such information is not obtainable from the composition of the bulk solution from which these membranes are generated because the membrane components are organized into a two-phase system, the lipid bilayer membrane and a surrounding bulk phase. While the composition of the latter is known, that of the bilayer is difficult to determine because of its microscopic mass and extreme fragility. In principle, however, its composition may be deduced indirectly from the properties of the membrane-forming species at an appropriate bulk oil-water interface, or obtained directly from studies of bilayers prepared from radioactively labeled components. Using the former approach, Haydon and co-workers [2, 4] have calculated the composition of several two- and three-component bilayer systems. It should be noted that calculations of the content of hydrocarbon solvent in this method ignore any local fluctuations in membrane thickness, and the results of this procedure, in general, have yet to be verified experimentally. Moreover, these authors point out that their approach is not applicable to systems comprising complex mixtures, and that in such cases an alternative technique for studying membrane composition must be developed [2]. Henn and Thompson [9] have prepared bilayers from labeled phospholipids and analyzed the radioactivity of membrane fragments obtained after fixation by $La(NO_3)_3$ and $KMnO_4$. As discussed by these authors, this method is limited to some extent because of the possible alteration of the membrane composition during the fixation procedure and the uncertainty in the surface area of the membrane fragments which must be used in calculating the molecular areas of the component membrane species.

This paper describes an analytical technique, using radiotracer, for direct determination of the bilayer composition based on the encapsulation of a spherical object by the membrane. The technique is free of any artifacts due to chemical perturbation of the membrane structure, and has been used to study the solvent content of single-component bilayers, and the compositional relationships between the membrane-forming solution and the bilayer in several multicomponent systems.

Materials and Methods

The oleic acid, stearic acid, and cholesterol used in this study were obtained from Applied Science Laboratories, State College, Pa. Glyceryl monooleate was purchased from

Sigma Chemical Co., St. Louis, Mo. Glyceryl monostearate was synthesized and recrystallized as described below for the labeled derivative. All compounds were judged pure by thin-layer chromatography. *n*-Decane, a British Drug House reagent grade product, was distilled under reduced pressure before use. *n*-Hexadecane, a Koch-Light puriss grade, was used without further purification. Purified liquid mercury was distilled three times under vacuum, immediately prior to use. Water was first deionized by passage over an ion-exchange column, and then distilled in a Pyrex-glass still after addition of $KMnO_4$ to oxidize any organic material present. All other materials were of analytical reagent grade.

Tritiated stearic acid, oleic acid, cholesterol, and *n*-hexadecane, of high specific activity, and ^{14}C-labeled oleic acid were purchased from the Radiochemical Center, Amersham, England. The fatty acids and cholesterol were found to be chemically pure by thin-layer chromatography. Their radiochemical purity was checked following chromatography, by analyzing the radioactivity of the chromatogram; no radiochemical impurities could be detected. No analysis of the *n*-hexadecane was carried out in this laboratory, but the supplier states the radiochemical purity to be 98%, as determined by radio gas chromatography. Cholesterol and *n*-hexadecane were isotopically diluted to give final specific activities in the range of 20 to 60 mC/mmole, as required.

Labeled α-monoglycerides were synthesized according to the method of Hartman [6], using a mixture of the labeled and unlabeled fatty acids in the esterification step. In the synthesis of glyceryl monooleate, esterification and subsequent purification were carried out in a nitrogen atmosphere. The final products were dissolved in 40 to 60 volumes of petroleum ether and crystallized at 0 and −25 °C for glyceryl monostearate and glyceryl monooleate, respectively [10]. The compounds were stored as a benzene solution at 0 °C. The synthesized α-monoglycerides were indistinguishable by thin-layer chromatography from monoglyceride standards purchased from commercial sources and appeared to be chemically pure by this criterion. However, when their chromatograms were analyzed for radioactivity, a small contaminant was found which did not correspond to either the free fatty acid, di- or tri-glycerides. This contaminant represented less than 2% of the total radioactivity in the case of glyceryl monooleate, and about 4% for glyceryl monostearate. No attempt was made at a further purification of the product or identification of the trace contaminating species. The specific activity of each product was determined to within 2% error from the sample weight and the radioactivity of aliquots taken from the stock solutions. The values obtained for both products were greater than 20 mC/mmole.

All membrane-forming solutions contained ~10 mg lipid per ml of hydrocarbon solvent. Mixtures of glyceryl monooleate with glyceryl monostearate or cholesterol were prepared in the desired molar ratios from stock solutions of these compounds. Some of the mixtures containing high proportions of glyceryl monostearate or cholesterol were not completely soluble in the hydrocarbon solvent at room temperature. In those cases, gentle heating to about 40 °C was required to produce a clear solution, and care had to be taken to form the membranes quickly before the temperature of the solution reached that of the aqueous phase.

The apparatus for the composition studies reported in this paper is shown in Fig. 1. It consists of an all-glass, water-jacketed cell into which is placed a teflon disc. The disc is fitted with two sections of hollow glass tubing, one for accomodating a single disposable glass cup (~12 × 3 mm ID) the second for addition of solution to the cell. A horizontally oriented teflon ring (3 mm ID) which is attached to a supporting glass rod by a fine platinum wire is used for membrane formation. A micrometer (Metrohm, Ltd., Switzerland) and an attached glass capillary (~0.3 mm tip diameter) are filled with mercury and positioned over the cell by a micromanipulator. The entire apparatus is placed on the base of a low-power dissecting microscope and the membrane observed with reflected light.

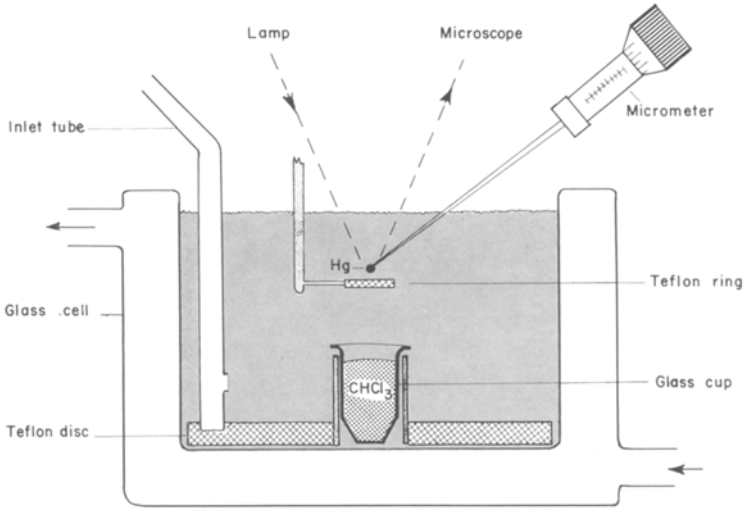
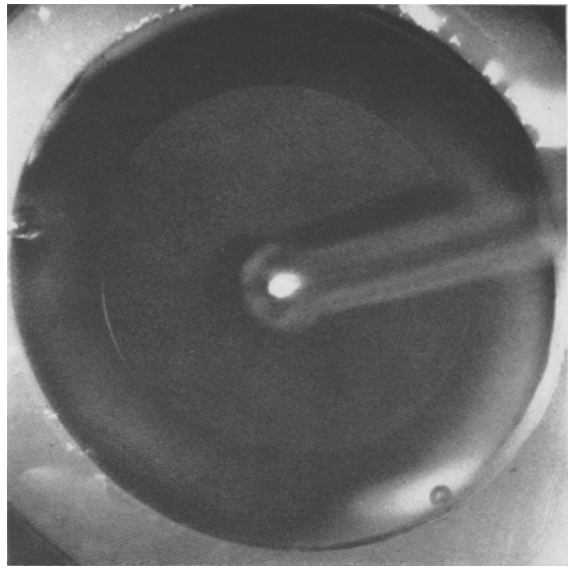


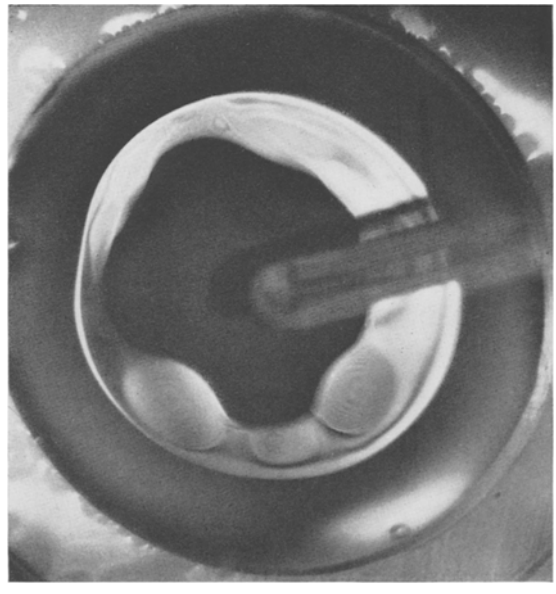
Fig. 1. Apparatus for bilayer composition measurements. *See* text for description

The temperature in all experiments was controlled to 25 ± 0.05 °C by circulating water from a constant temperature bath through the glass cell.

A typical experiment was carried out as follows. A glass cup was filled practically to the top with distilled $CHCl_3$. The remaining space, representing $<10\%$ of the capsule volume was filled with 0.1 M NaCl. The cup was then placed in the teflon disc, and the glass cell filled with 0.1 M NaCl. During this initial setting-up procedure, the teflon ring and supporting glass rod are not immersed in the cell. A droplet of the membrane-forming solution was applied to the teflon ring, which was then transferred to the aqueous phase by passing it at an oblique angle through the air-water interface. Spreading of some of the bulk phase membrane-forming solution could be seen to occur at the air-water interface during this transfer. The teflon loop was positioned at a fixed distance of about 7 mm from the top of the cup, and the resulting membrane, observed with reflected light, was seen to become completely black. The capillary was centered and lowered to <1 mm of the surface of the black membrane (*see* Fig. 2*a*), and a droplet of mercury was expelled from the capillary and trapped in the glass cup. Generally, the surface area of the expelled mercury drop was 20 to 30% of the total surface of the black membrane. In experiments in which multiple passes were made through a single membrane, the black membrane was allowed to thin to its original area and the procedure was repeated again. After collecting the desired number of drops, the teflon ring was removed from the cell, and the contaminated air-water interface cleaned by aspiration of the surface. This was accompanied by successive additions of distilled water through the inlet tube (Fig. 1) and finally by overflow of the water in the cell. In all, about ten times the cell volume, or 500 ml of distilled water was used in the cleaning procedure. The cell was then emptied of solution, and the entire glass cup with the mercury droplet and $CHCl_3$ was removed and deposited into a liquid scintillation vial containing 10 ml of scintillation liquid. One liter of scintillation fluid contained 4 g 2,5-diphenyloxazole, 200 mg 2,2-*p*-phenylene-bis(5-phenyloxazole), 60 g naphthalene, 175 ml methanol, 40 ml ethylene glycol, and 700 ml distilled dioxane.



a



b

Fig. 2. Photograph of a planar bilayer membrane (*a*) before, and (*b*) after penetration by a mercury droplet. Internal diameter of the teflon ring is 3 mm

In blank experiments in which either the membrane broke before attempting to sample it, or in which a membrane was made and removed from the cell without sampling it, no radioactivity was found in the cup *provided* membrane formation and cleaning were carried out as described above.

All samples were counted in a Packard Tricarb liquid-scintillation counter, model 3380, for times sufficient to give less than 3% standard deviation in the counting rates. After determination of the radioactivity, the mercury from each scintillation vial was removed, dried on a piece of filter paper, and weighed. The surface area of the droplets was calculated from the drop weight and density of mercury at 25 °C.

Results and Discussion

Mechanism for Penetration of a Mercury Droplet through a Lipid Bilayer Membrane

We have made the observation that it is possible to pass large macroscopic objects such as mercury droplets or glass beads, through lipid bilayer membranes without causing membrane rupture. During this procedure, the bilayer remains intact, but decreases in area. Concomitantly, surplus lipid material is pulled into the plane of the membrane from the lipid bulk phase in the form of a relatively thick colored film (Fig. 2*b*). This procedure can be repeated many times without any apparent effect on membrane stability. To utilize these observations in the study of membrane composition, it is of primary importance to establish the mechanism by which such objects pass through the bilayer. The quantitative studies reported in this paper were restricted to the use of liquid mercury because very clean surfaces may be obtained with this material and it can be manipulated conveniently.

If some reproducible fraction of material is removed from the bilayer on each successive pass through the membrane, then there should be a quantitative relationship between the change in area of the bilayer (black) membrane ΔA_{memb}, and the surface area of the mercury droplets passed through the membrane A_{Hg}. This can best be realized if one considers a hypothetical experiment in which a single mercury droplet whose surface area just equals that of the bilayer is passed through the thin lipid membrane. If a bimolecular lipid layer is removed on the droplet, then after a single passage, the entire black membrane would be removed, leaving only a colored film, i.e. $\Delta A_{\text{memb}} = A_{\text{Hg}}$. On the other hand, if only a monolayer were removed, then two mercury droplets would be required to produce the same change in area, or $\Delta A_{\text{memb}} = \frac{1}{2} A_{\text{Hg}}$. This analysis assumes that the rate of membrane thinning is negligibly slow compared to the extension of the bulk phase which is observed when the mercury droplet passes through the bilayer membrane.

To test these possibilities, two kinds of experiments were carried out to measure the relationship between ΔA_{memb} and A_{Hg} in bilayers formed from glyceryl monooleate and *n*-decane. In both cases, the surface area of the

Table 1. Correlation of surface area of mercury droplet A_{Hg} with increment in bilayer area ΔA_{memb}

Experiment	$(\Delta A_{memb}/A_{Hg})$	No. of Exps.
1. Single sample	0.94 ± 0.11	9
2. Multiple samples	0.93 ± 0.10	10

mercury A_{Hg} was calculated from the mass of the collected mercury as described in Materials and Methods. In one series of experiments, the change in black membrane area was determined from photographs similar to those shown in Fig. 2. In the second series of experiments, n-drops were passed, always through the black area of the membrane, until the entire membrane became colored. The results of these experiments, summarized in Table 1, clearly show that within about a 10% error, the relation $\Delta A_{memb} = A_{Hg}$ holds. The fact that the ratio $(\Delta A_{memb}/A_{Hg})$ was in all cases slightly less than one, suggests that a small systematic error may have been introduced in the determination of ΔA_{memb} or A_{Hg}. If the rate of bilayer formation is not negligibly slow, then in the finite time required for photographing the membrane, the area of the black film would increase, giving an apparently lower value for ΔA_{memb}. That such a process is occurring is evidenced from the blurring seen in Fig. 2*b*, which required a 30-sec exposure time. In the second series of experiments, errors may also have been introduced from the fact that a small number (3 to 5) of mercury drops were required to reach the end point of a completely colored membrane; consequently, the quantity A_{Hg} was overestimated. That is, the final drop always had a surface area greater than that required to remove the remaining black membrane.

Bilayer membranes were also formed from tritium-labeled glyceryl monooleate in *n*-decane, and the membrane sampled according to the technique described in Materials and Methods. The counting rate of the material trapped in the glass cups was plotted as a function of the total surface area of the mercury droplets used in the sampling. The results, seen in Fig. 3, show that the radioactivity is a linear function of the surface area of the collected drops. The plot is required to go through the origin since all blank experiments gave zero counting rate. Each point in Fig. 3 represents data from either one, two, three or four mercury droplets passed through a single bilayer membrane. From the slope of this line and the activity of the glyceryl monooleate used, it is calculated that the number of molecules of glyceryl monooleate removed from the bilayer was $(4.7 \pm 0.4) \times 10^{14}/cm^2$ Hg surface, corresponding to an apparent molecular area of 21 ± 2 Å^2 for the

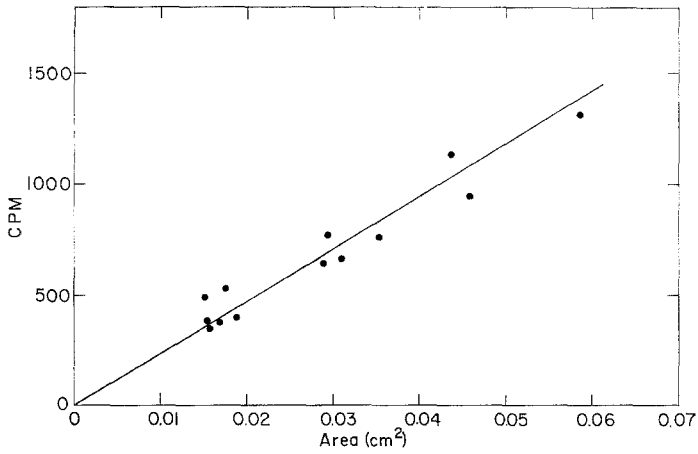


Fig. 3. Radiotracer data for bilayers formed from ^{3}H-glyceryl monooleate in *n*-decane. Radioactivity in cpm is plotted *vs.* the total surface area of the *n*-mercury droplets used in the sampling procedure

monoglyceride. Since this latter value is one-half the limiting area of 40 Å^2/molecule reported for glyceryl monooleate at the bulk *n*-decane/0.1 M NaCl interface [4], it is concluded that a bilayer fragment of area A_{Hg} is removed from the membrane by each droplet. Thus, the radiotracer experiments confirm the results of the optical experiments summarized in Table 1.

In initial attempts to carry out the radiotracer experiment, membrane formation was accomplished by the brush technique [12]. Large contamination was found in blank experiments in which membranes were formed but not sampled. On close examination it was found that the use of the brush dispersed small quantities of lipid into the aqueous phase, which adsorbed to the surface of the glass cup and gave rise to a high spurious background. For the purpose of the composition studies reported here, it is essential that this technique be avoided. However, the implications of this finding should also be considered in other bilayer studies. Dispersing the lipid material into microscopic droplets in the aqueous phase must create a large surface area of lipid. This dispersed material may considerably affect the results of any binding studies in which the minimal concentration of modifying components is assessed.

While both the optical measurements and the radiotracer experiments require that each mercury droplet remove enough material to cover its surface with a bimolecular lipid leaflet of material, these experiments say nothing about the organization of the lipid on the droplet. If the experiment was carried out in an identical fashion to that described in Materials and

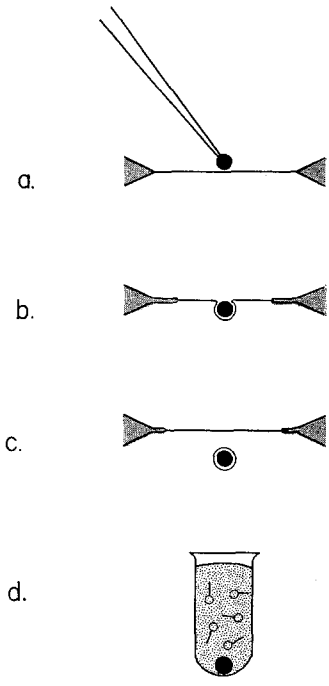


Fig. 4. Schematic representation of the mechanism for penetration of a bilayer membrane by a mercury droplet. (*a*) Cross-section through a bilayer and adjacent bulk phase showing the mercury droplet to be pipetted through the bilayer-water interface. (*b*) As the membrane is extended, solution from the bulk phase is pulled into the plane of the bilayer, producing a relatively thick, colored membrane. (*c*) The mercury droplet removes a fragment of the bilayer membrane in the form of a closed vesicle and the colored membrane recedes into the bulk phase. (*d*) The mercury droplet and the membrane sample are trapped in a glass cup containing an aliquot of $CHCl_3$

Methods except that the glass cup (Fig. 1) contained no $CHCl_3$ and only 0.1 M NaCl, then the following observations were made. (1) The results of the optical experiment were unchanged. (2) The radioactivity found in the glass cup was very low and essentially independent of the number or area of the mercury droplets passed through the membrane. Taking even the highest counting rates, only enough glyceryl monooleate to form a small fraction of a single monolayer on the mercury droplet was found. (3) Between 0.5 and 2 min after sampling of the membrane, adsorption to the bilayer of some small quantity of lipid material was often observed. This adsorption manifested itself as the appearance of a small colored patch, generally less than 0.1 mm in diameter, which suddenly appeared in the middle of the black membrane. No such behavior was observed if $CHCl_3$ was present in the glass cup.

All of the experimental observations on the penetration of bilayers by mercury droplets can best be explained in terms of the mechanism summarized in Fig. 4. Here the bilayer and surrounding bulk lipid phase are seen in vertical cross-section (Fig. 4*a*). Following release of the mercury droplet from the pipette, the bilayer membrane is extended, pulling lipid material

from the surrounding bulk phase into the plane of the membrane (Fig. 4*b*). Similar observations are made when planar bilayer membranes are stretched because of a hydrostatic pressure head on one side of the membrane [20]. As the droplet passes through the membrane, it is covered with a bilayer in the form of a closed vesicle, and the thickened edges of the membrane begin to recede as the black membrane thins to its original area (Fig. 4*c*). The vesicle covering the mercury droplet is fragile and can rupture when the mercury impacts with the glass cup. When $CHCl_3$ is present, all the lipid is solubilized in the organic phase and thereby retained in the cup (Fig. 4*d*), thus giving a correlation of the radioactivity with the surface area of the mercury drops. If $CHCl_3$ is omitted, the lipid material, being less dense than the aqueous support, floats upward toward the membrane where a fusion with the bilayer can take place, giving rise to a thickened, colored region of the membrane.

Solvent Composition

Bilayer membranes are generally formed from a solution of lipids dissolved in a hydrocarbon solvent in which the molar ratio of hydrocarbon to lipid is greater than 100:1. It is therefore of considerable importance to determine how much solvent, if any, is present in these ultrathin membranes after their generation from such a solution. For practical reasons, only *n*-hexadecane was used as a solvent in these studies. The specific activity of this product set a lower limit of detection at about 5×10^{12} molecules of solvent per cm^2 of bilayer.

The composition data obtained from sampling membranes of glyceryl monooleate generated from a solution of this material in labeled *n*-hexadecane is summarized in Table 2. From a series of 23 experiments, the data

Table 2. Solvent composition of bilayers formed from glyceryl monooleate and *n*-hexadecane in 0.1 M NaCl at 24 °C

Range of radioactivity (cpm/Hg drop)	No. of molecules of *n*-hexadecane/cm^2	No. of Exps.[a]
76,000–350,000	$(0.9–4.3) \times 10^{17}$	2[b]
1,500– 9,100	$(5.1 \pm 2.7) \times 10^{15}$	11
151– 298	$(2.8 \pm 0.7) \times 10^{14}$	10

[a] Each experiment represents a single sampling of a different bilayer membrane.
[b] Each droplet was passed through a region of the bilayer containing a "lens" (*see* text)

could be grouped into three distinct classes of values according to the range of radioactivity found with each mercury droplet. (1) Very large counting rates (7.6×10^4 and 3.5×10^5 cpm/Hg drop) were obtained after sampling two bilayers containing a small macroscopic lens of bulk liquid. The membranes were deliberately sampled so that the mercury droplet passed directly through the region of the black membrane containing this structure. These lenses of bulk membrane-forming solution which are in equilibrium with the bilayer [7], were observed to occur infrequently and are a result of improper drainage of the membrane during the thinning process, caused perhaps in part by the horizontal orientation of the membrane in our experiments. With the exception of the two measurements cited here, membranes containing these structures were not used in the studies reported in this paper. If it is assumed that the contribution to the measured radioactivity from the solvent in the bilayer region of the membrane is negligible compared to that from the lens, then it is possible to calculate the dimensions of the lens from the specific activity and molar volume of *n*-hexadecane and the measured counting rate. Such a calculation gives diameters of 120 and 200 μ for the two cases listed in the first entry of Table 2. While the exact diameters of these lenses were not measured, the calculated values are the right order of magnitude, and suggest that these lenslike structures were indeed included in the sample of the membrane. Furthermore, this observation confirms the mechanism proposed in Fig. 4 for the penetration of the bilayer by a mercury droplet. Alternative mechanisms involving either a physical adsorption process, or a process in which any solvent between the two monolayers comprising the bilayer structure is squeezed out during the sampling process are ruled out by this first set of observations. Such mechanisms would require the membrane sample to contain considerably less solvent than that present in the macroscopic lenses seen in these experiments. (2) The second class of values gave $(5.1 \pm 2.7) \times 10^{15}$ molecules of solvent per cm^2 of bilayer. This is more than 10 times the total number of molecules of glyceryl monooleate [$(4.7 \pm 0.4) \times 10^{14}/cm^2$] found in bilayers prepared from either *n*-decane or *n*-hexadecane solutions of this monoglyceride. The large value for the solvent content of these membranes suggests that microscopic lenses of solvent are part of the membrane structure and/or the membranes are not truly bimolecular in their transverse dimension, but are significantly thicker because of the inclusion of a layer of solvent between the faces of the bilayer. The most plausible explanation is the former one because no fluctuations in the bilayer thickness, similar to those seen for the solvent content of this group of membranes (Table 2, line 2), have ever been observed. The inclusion of such microscopic lenses

in the bilayer has also been observed in electron micrographs of these structures [9]. (3) The third class of values (Table 2, line 3) corresponds to membranes in which the solvent content [$(2.8 \pm 0.7) \times 10^{14}$ molecules/cm^2] was about the same as the total number of molecules of monoglyceride/cm^2 of membrane surface [$(4.7 \pm 0.4) \times 10^{14}$]. It is concluded that the values obtained for this group of membranes represent the minimum concentration of the *n*-hexadecane solvent in stable bilayers of glyceryl monooleate.

It is possible to calculate what volume fraction ϕ of the hydrocarbon core of the bilayer is occupied by solvent, from the relation

$$\phi = \frac{n_{HC}\,\overline{V}_{HC}}{n_{HC}\,\overline{V}_{HC} + n_{\mathrm{GMO}}\,\overline{V}_0} \tag{1}$$

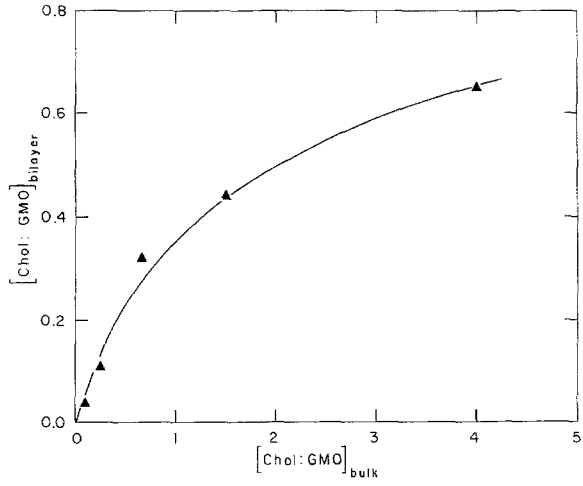
where $\overline{V}_{HC}$ and $\overline{V}_0$ are the partial molar volumes of the *n*-hexadecane and the oleyl side chains of the glyceryl monooleate in the membrane, and n_{HC} and n_{GMO} are the number of molecules of solvent (2.8×10^{14} cm^{-2}) and glyceryl monooleate (4.7×10^{14} cm^{-2}) found in the bilayer. It is assumed that $\overline{V}_{HC}$ and $\overline{V}_0$ can be approximated by the partial molar volume of *n*-hexadecane and 1-heptadecene in bulk [4]. The value of ϕ obtained in this calculation is 37%. From measurements of the electrical capacitance of glyceryl monooleate bilayers generated from solutions of this material in different solvents, Fettiplace, Andrews and Haydon [4] concluded that the solvent content of such membranes was a strong function of its hydrocarbon chain length, varying from 47% for *n*-decane to 17% for *n*-hexadecane. Taking even the lowest concentration of hexadecane measured in our experiments still leads to a volume fraction of 29% for the solvent. A partial explanation for the discrepancy in the calculated and measured values of ϕ may be that in the former method the thickness of the hydrocarbon region, calculated from the bilayer capacitance and an appropriate membrane dielectric constant ε, has been underestimated because of the choice of a bulk value of ε. While the results of a theoretical treatment of this problem [13] suggest that the anisotropy of the membrane components can introduce a correction in the bilayer thickness determined by this method, it is clear that these corrections are small and cannot completely account for the disparity in the values of ϕ. Thus, even the lowest values of the counting rates listed in Table 2 (line 3) most probably represent the presence of very minute lenses of solvent in the bilayer membrane which are an integral part of its structure. The fact that such a significant fraction of the membrane volume is hydrocarbon solvent is disturbing, and means that any discussion of the relationship between the properties of the artificial membrane to natural systems should be viewed with some caution.

Composition of Multicomponent Lipid Bilayers

Measurements of the composition of bilayers generated from mixtures of two lipid species dissolved in a hydrocarbon solvent were carried out to determine the relationship between the composition of the bilayer and that of the parent bulk phase from which the membrane is generated. In addition, these experiments also allow one to probe the bilayer system for phenomenon such as condensing and expanding effects [1, 17] and two-dimensional phase separation [14] which until now have been reported only in monolayer systems. If, for example, the molecular areas of the pure component species at the appropriate bulk oil-water interface are known, then from the composition data of the mixed lipid bilayer and the surface area of the membrane sample, it is possible to determine the molecular areas of the lipid components in the mixed bilayer. The bilayers used in the studies reported in this section were prepared from mixtures of glyceryl monooleate with either glyceryl monostearate or cholesterol.

The results obtained for mixed films of cholesterol and glyceryl monooleate are given in Fig. 5 in which the molar ratio, [cholesterol/glyceryl monooleate], found in the bilayer is plotted *vs.* the ratio in the membrane solution from which the bilayers were formed. In Fig. 5*a*, each molar ratio was obtained from a series of single-label experiments in which the number of molecules of cholesterol and glyceryl monooleate were determined separately. It is seen that the concentration ratio in the membrane is a continuous function of the ratio of the components in the parent bulk phase, but the amount of cholesterol found in the membrane tends to reach a constant value. Thus, for the largest ratio of cholesterol to glyceryl monooleate in bulk (4:1), the corresponding value in the bilayer was only 0.65:1. Bilayer compositions were also investigated in double-label experiments using ^{14}C-glyceryl monooleate and ^{3}H-cholesterol. The results, given in Fig. 5*b*, show that over this range of concentrations, the molar ratio, [cholesterol/glyceryl monooleate] in the bilayer is one-half the value in bulk. Several ratios from the single-label experiments are also given in Fig. 5*b* and show the excellent agreement between the two methods for determination of membrane composition.

Similar experiments were carried out for mixtures of glyceryl monostearate and glyceryl monooleate. The results, given in Fig. 6, show that the molar ratios of glyceryl monostearate to glyceryl monooleate in bulk and in the bilayer are nearly equal, as is the case for points falling on the dashed line. However, at low bulk ratios, there seems to be an enhancement of the glyceryl monostearate concentration in the membrane, whereas at higher



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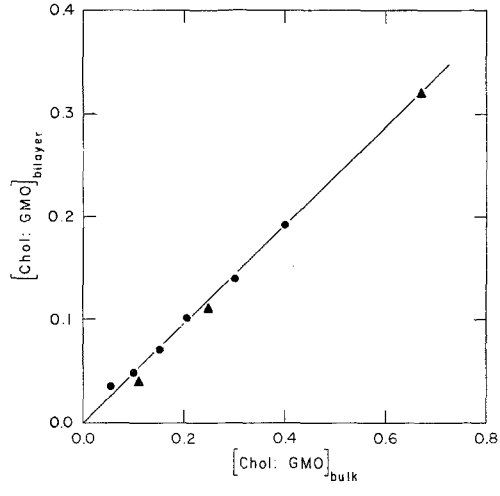

b

Fig. 5. Composition relationships for bilayer formed from mixtures of cholesterol (Chol) and glyceryl monooleate (GMO). The ratio [Chol/GMO] found in the bilayer is plotted *vs.* that in the bulk membrane-forming solution. (*a*) Data from single-label experiments (▲). Each molar ratio is the mean of 20 or more membrane samples. SD 15 to 20%. (*b*) Combined single- and double-label experiments for $[Chol/GMO]_{bulk} < 1.0$. Each molar ratio obtained from double labels (●) is the mean of 10 or more membrane samples. SD 8 to 10%

bulk ratios, the relative concentration of glyceryl monostearate to glyceryl monooleate in the bilayer decreases. Thus, the overall shape of the curve for these mixtures may be similar to that shown in Fig. 5*a* for cholesterol and

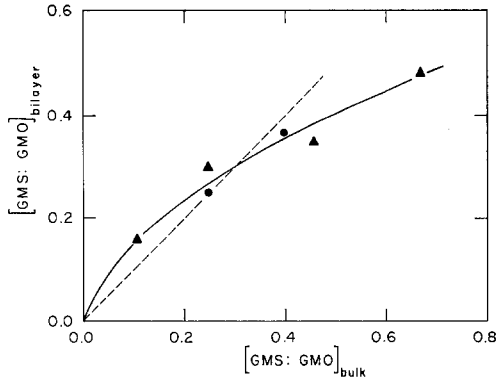

Fig. 6. Composition relationship for bilayers formed from mixtures of glyceryl monostearate (GMS) and glyceryl monooleate (GMO), using single (▲) and double (●) labels. The ratio [GMS/GMO] found in the bilayer is plotted *vs.* that in the bulk membrane-forming solution. SD as in Fig. 5

glyceryl monooleate, but because of the limited solubility of glyceryl monostearate in *n*-decane, no experiments at higher concentrations of this component could be carried out. Again, values for the mixed bilayer composition were determined in both single- and double-label experiments (*see* legend, Fig. 6) with good agreement between the two methods.

It is important to point out that for all the multicomponent bilayer systems studied, no deviation from the optical result, summarized in Table 1, could be observed. Thus it can be assumed that the same mechanism proposed in Fig. 4 for the single-component system also holds for the mixed lipid bilayers. This means the compositions determined for the mixed films must represent the true membrane composition, and that any artifacts due to the selective adsorption of one of the components on the mercury surface can be ruled out since the penetration of the bilayer by the mercury droplet involves a vesiculation of the membrane and not an equilibrium adsorption of the lipids onto the mercury surface. This latter problem has been studied using techniques similar to those described in this paper and will be presented elsewhere (R. E. Pagano & I. R. Miller, *in preparation*).

It is of interest to use the composition data for the mixed films to determine the molecular areas of the lipids in the bilayer. The surface area of the membrane sample A_{Hg} is related to the molecular area of glyceryl monooleate $\bar{A}_{GMO}$ and the second component $\bar{A}_2$ in the mixed lipid mem-

brane according to the equation

$$n_{GMO}\bar{A}_{GMO}+n_2\bar{A}_2=2A_{Hg}$$

$$\left(\frac{n_{GMO}}{A_{Hg}}\right)\bar{A}_{GMO}+\left(\frac{n_2}{A_{Hg}}\right)\bar{A}_2=2 \quad (2)$$

where (n_{GMO}/A_{Hg}) and (n_2/A_{Hg}) are the number of molecules/cm^2 of glyceryl monooleate and second component (cholesterol or glyceryl monostearate) found in the bilayer. The factor of 2 appears in Eq. (2) because the total surface area of the monolayers comprising the bilayer sample is twice the area of the mercury droplet. Eq. (2) can be solved only if the molecular area of one of the components in the mixed bilayer, $\bar{A}_{GMO}$ or $\bar{A}_2$, is known. If, for example, it is assumed that the molecular area of glyceryl monooleate is identical to that found in the single-component bilayers (40 Å^2), then the molecular area of the second component $\bar{A}_2$ can be calculated from the measured number of molecules per cm^2 of glyceryl monooleate and component 2, using Eq. (2). The results of such a calculation give a molecular area of 40 to 41 Å^2 for glyceryl monostearate in the mixed films, and 32 ± 2 Å^2 for cholesterol in mixtures with glyceryl monooleate. These calculations were made using data from both the single- and double-label experiments. No dependence of $\bar{A}_2$ could be seen on the amount of second component present in the membrane, and the values quoted above represent averages for all the mixed-film experiments carried out. If, on the other hand, $\bar{A}_2$ is fixed at 40 Å^2, for both glyceryl monostearate and cholesterol, then for mixtures of glyceryl monostearate and glyceryl monooleate, $\bar{A}_{GMO}$ is calculated to be 40 Å^2, while for mixtures of cholesterol and glyceryl monooleate, $\bar{A}_{GMO}$ is found to be $\leqq 40$ Å^2, depending on the quantity of cholesterol present in the membrane. For either assumption, it is seen that the average molecular area of the components in the bilayer is decreased for mixtures of cholesterol and glyceryl monooleate, and unchanged for the glyceryl monostearate-glyceryl monooleate system. Similar "condensing" effects in which the area of the mixture is significantly smaller than that calculated from the sum of the areas of each pure component at a given surface pressure have also been observed in mixed lipid monolayers containing cholesterol [1, 17].

While, strictly speaking, the molecular areas in the bilayer membrane of either cholesterol, or glyceryl monooleate, or both, may be smaller than the values of the pure components at the hydrocarbon-water interface, it is physically more realistic that only the molecular area of cholesterol is reduced. This follows from a consideration of the ways in which it is possible

to pack the components into a bilayer structure, assuming that the most favorable arrangement energetically is that which minimizes the exposure of the hydrophobic parts of the cholesterol and glyceryl monooleate molecules to the water surface. Since the area of the polar group of cholesterol is considerably less than that of the cholesterol ring system (40 Å^2), the introduction of this molecule into a bilayer of glyceryl monooleate must expose nonhydrophillic regions of the molecule to the aqueous phase unless the cholesterol ring can occupy some of the volume normally available to the solvent molecules and oleyl side chains of the monoglyceride. In so doing, the area of cholesterol in the mixed bilayer would be reduced. Such a mechanism is in fact feasible because the cross-sectional areas of the hydrocarbon chain (~ 20 Å^2) and polar group (~ 40 Å^2) of glyceryl monooleate are sufficiently dissimilar to create a free volume normally filled by solvent, as suggested by the large volume fraction of neutral hydrocarbon found in the membrane.

It is interesting to see to what extent it is possible to predict the composition of the mixed lipid bilayer membranes (Figs. 5 and 6) from the bulk properties of the membrane-forming solutions, assuming that the membrane composition is given by that of the adsorbed monolayer at the bulk oil-water interface. Using the treatment developed for surface tensions of ideal solutions containing mixtures of molecules of similar size [3], it can be shown that the composition of an adsorbed film at the oil-water interface is given by

$$\frac{n_1^s}{n_2^s} = \frac{n_1^b}{n_2^b} \exp[(\sigma_2 - \sigma_1) A/kT] \tag{3}$$

where n_1^s, n_2^s, n_1^b, n_2^b are the number of moles of components 1 and 2 in the surface and in bulk, respectively, σ_1 and σ_2 are the interfacial tensions of the pure components at the oil-water interface, and A is the surface area occupied by each molecule at the interface. In nonideal cases, two additional contributions must be taken into account. First, the areas per molecule in the mixed monolayer may differ from those in the pure monolayer because of steric considerations, and second, there may be a difference in lateral interactions between like and unlike adsorbed molecules. This second contribution also comprises the interactions between the solvent molecules and the lipids. Taking into account these contributions [5], Eq. (3) will assume the form

$$\frac{n_1^s}{n_2^s} = \frac{n_1^b}{n_2^b} \exp\left[\frac{(\sigma_2 - \sigma_1) A^* + \Delta\chi}{kT}\right] \tag{4}$$

where A^* is the final area occupied by a molecule of component 1 after exchanging with a molecule of component 2 in the surface, and $\Delta\chi$ is the change in the lateral interaction energy brought about by this exchange.

It should be noted that for all the mixtures examined, the major component (glyceryl monooleate) was always present at a concentration greater than that required for micelle formation in the oil phase [4]. Thus, for low bulk concentrations of the second component (glyceryl monostearate or cholesterol) there is probably a distribution of this species between the micellar phase of glyceryl monooleate and the solvent. However, with increasing concentrations, the activities of the two components in bulk, and their ratio, must reach a constant value as saturation of the oil phase is approached. Since the activities in the bulk and surface phases are related by an expression of the form of Eq. (4), the ratio of the components in the surface must also reach a constant value at high bulk concentrations. Such a trend is seen in the data for both the cholesterol-glyceryl monooleate (Fig. 5*a*) and glyceryl monostearate-glyceryl monooleate (Fig. 6) systems.

Below saturation of the oil phase with both components, it may be assumed that there is a linear distribution of the solute components between the oil and micellar phase, and hence the activity ratio can be approximated by (n_1^b/n_2^b). In the case of glyceryl monooleate and glyceryl monostearate, it is reasonable to assume that the surface tension lowering of both the monoglycerides at the *n*-decane/0.1 M NaCl interface is about the same. Thus, the exponent in Eq. (3) becomes zero and any deviations from a glyceryl monostearate/glyceryl monooleate ratio of unity in the surface must be due to the $\Delta\chi$ term. For cholesterol and glyceryl monooleate, the ratio $[\text{Chol/GMO}]_{\text{bilayer}}/[\text{Chol/GMO}]_{\text{bulk}}$ is calculated to be 0.3 using Eq. (3), the literature values for σ_1 and σ_2, and a value of 40 Å^2 for the area of the components. Since, however, it was found that the molecular area of cholesterol is reduced in the mixed bilayer, it is reasonable to use a value of A^* of 32 Å^2 in Eq. (4). This gives a value of 0.4 for the ratio $[\text{Chol/GMO}]_{\text{bilayer}}/[\text{Chol/GMO}]_{\text{bulk}}$ which is in better agreement with the observed value of 0.5 (Fig. 5*b*). Again, the deviation is probably caused by contributions from $\Delta\chi$. While the agreement between the calculated and measured quantities can be considered to be fair, it would appear that apart from very simple systems, the radiotracer technique presented in this paper is the simplest and most direct method for elucidating the molecular composition of the bilayer. Hopefully, such information will lead to a better understanding of the relationship between the structure of these membranes and their physical properties.

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