Chiral Aggregation Phenomena. 4. A Search for Stereospecific Interactions between Highly Purified Enantiomeric and Racemic Dipalmitoyl Phosphatidylcholines and Other Chiral Surfactants in Monolayers, Vesicles, and Gels

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Cell membranes are composed primarily of phospholipids, cholesterol, and globular proteins. Since all of these components are chiral and only one natural enantiomer is commonly found, one might suppose that stereochemical factors could be important in the way chiral components pack together in the membrane bilayer. Furthermore, since many of the metabolites and wastes that traverse the membrane are optically active, there is the interesting possibility that the membrane of the cell wall could act as a stereospecific screen toward biomolecules of the "wrong" chirality. However, the potential significance of chiral discrimination factors in phospholipid aggregates has been largely ignored in most published studies, which frequently have used commercial racemic materials interchangeably with the natural L isomer. If chiral discrimination factors were significant, serious questions would arise regarding the value of many reports of phospholipid research.

To test the question, van Deenen and co-workers[1] reported a small, but definite difference between the force-area isotherms of D- or L-1-stearoyl-2-lauroylphosphatidylcholine compared to racemic material. Cadenhead and co-workers[2] noted a difference between their force-area isotherm of L-dimyristoylphosphatidylcholine (DMPC) and van Deenen's[1] of racemic DMPC. Phillips and Chapman[3] were unable to detect any difference between the L- and racemic forms of dimyristoylphosphatidylethanolamine when spread on 0.1 M NaCl at 22 °C. Cadenhead,[4] in his authoritative review of phospholipid monolayers as biomembrane models, considered the question unresolved. Recently, Miñones et al.[5] were unable to detect any difference between force-area isotherms of L- and racemic DPPC on water at 20 °C. A very recent electron diffraction study on single microcrystals of dipalmitoylphosphatidylcholine shows clear differences in the two-dimensional lattices of the optically pure L-isomer compared to the racemic modification.[6] It would be reasonable to suppose that energetic differences might accompany structural differences in packing.

The present communication reports first our attempts to detect differences between pure D or L isomers[7] of dipalmitoylphosphatidylcholine (DPPC) and their 1:1 racemic mixture by ultrahigh field NMR spectroscopy, differential scanning calorimetry, and several monolayer techniques. We then report our results for diastereomeric interactions of the optical isomers of DPPC with several other chiral lipids.

All general chemical and instrumental methods used in this work have been described previously[8] except as noted below. Racemic and L-DPPC and phospholipase A_2 were obtained from Sigma Chemical Co., St. Louis, MO. The purity of the phospholipids was checked by TLC (silica gel G, 115:45:4:4 $CHCl_3/CH_3OH/NH_4OH/H_2O$). The samples were chromatographed under an N_2 atmosphere on a column of activated (prewashed with 4:1 $CHCl_3/CH_3OH$ and dried at 120 °C, 6 h in vacuo) silica gel (Woelm, 32-63 μm). Pure DPPC was eluted from the column with chloroform-methanol mixtures ranging from 3:1 to 2:1, v/v. All phospholipids were stored under nitrogen at -85 °C.

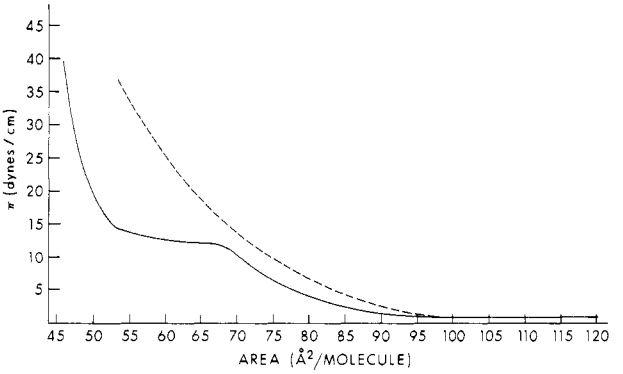

Figure 1. Force-area isotherms for L-DPPC at the air-water (pH 5.5) interface at 25 ± 0.2 (—) and 44.5 ± 0.5° (- - -). The isotherm shown is for a compression rate of 7.2 Å²/molecule/min. Compression rates of 18.0 and 27.7 Å²/molecule/min were also used, giving the same results as shown here.

D-DPPC was prepared by enzymatic hydrolysis of racemic DPPC with phospholipase A_2 from *Crotalus adamanteus*. This enzyme reacts only with the L isomer, leaving the D isomer untouched.[9] Racemic DPPC (3 g) was dispersed in 350 mL of distilled, peroxide-free ether to which 20 mL of 5 mM aqueous $CaCl_2$ solution had been added. To 1 mL of 5 mM aqueous $CaCl_2$ solution, 1000 units (~1 mg) of phospholipase A_2 was added and the reaction allowed to proceed for 24 h.

The resulting gel was ultracentrifuged (Beckman J-21) at 14000 rpm for 30 min. The etheral layer, containing palmitic acid, was discarded. The aqueous layer was dissolved in methanol and taken to dryness by rotary evaporation. The dried sample was column chromatographed, as described above, to yield 1.29 g (86%) of pure D-DPPC. Cholesterol (Aldrich) was recrystallized from 95% ethanol. Cholestanol and epicholestanol (Research Plus, Inc., Bayonne, NJ) were used as received.

Methyl esters of each lot of DPPC were prepared by the method of O'Connor and co-workers[10] and analyzed by GLPC, as previously described.[8] All of the samples analyzed were ≥99% C_{16} fatty acid.

Optical rotations were measured as previously described:[8] L-DPPC, $[\alpha]^{25}_D$ +6.5 ± 0.1°; D-DPPC, $[\alpha]^{25}_D$ -6.4 ± 0.1° (*c* 5.6, $CHCl_3$). Racemic DPPC exhibited no optical rotation. Previously reported values for L-DPPC range from $[\alpha]^{25}_D$ +7.0° (*c* 5.6, $CHCl_3$)[11] to $[\alpha]^{25}_D$ +6.65° (*c* 8, 1:1 $CHCl_3/CH_3OH$).[12]

Differential scanning calorimetry was performed on a Perkin-Elmer DSC-1B interfaced to a Gould 105 strip chart recorder equipped with an event marker. The calorimeter was used with a liquid nitrogen-filled Dewar cap for low-temperature work. Aqueous dispersions were prepared by weighing dry phospholipid

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(7) The enantiomeric forms of dipalmitoylphosphatidylcholine shall be referred to as D-DPPC and L-DPPC, which correspond, respectively, to the 1-*sn* and 3-*sn* isomers using the conventions of IUPAC-IUB Stereospecific Numbering or *S* and *R* using Cahn-Ingold-Prelog absolute configurational designations.
(8) See Arnett et al. (Arnett, E. M.; Chao, J.; Kinzig, B.; Stewart, M. V.; Thompson, O.; Verbiar, R. J. *J. Am. Chem. Soc.*, in press) for experimental details of equipment and purification of materials. That paper is third in this series on chiral aggregation.
(9) de Haas, G. H.; Mulder, I.; van Deenen, L. L. M. *Biochem. Biophys. Res. Commun.* **1960**, *3*, 287-291.
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Table I. Static Surface Tension Lowerings of L-, D-, Racemic, and a 1:1 Mixture of L- and D-DPPC's on Pure Water (pH 5.5) (Fisher Autotensiomat, DuNouy Ring Method)[a]

temp, °C	area, Å²/mol	L-DPPC	D-DPPC	rac-DPPC	1:1 D-DPPC + L-DPPC
15	40	12.7 ± 1.7 (7)	13.1 ± 2.1 (8)	12.8 ± 1.4 (7)	
	50	0.7 ± 0.7 (6)	0.0 ± 0.1 (5)	0.5 ± 1.1 (5)	
35	40	25.8 ± 1.1 (10)	26.2 ± 1.8 (5)	23.7 ± 1.6 (12)	28.4 ± 0.8 (6)
	50	16.9 ± 0.6 (10)	16.0 ± 1.0 (5)	17.9 ± 0.6 (5)	18.3 ± 0.5 (6)
	65	10.9 ± 3.7 (68)	11.6 ± 4.1 (64)	12.1 ± 2.8 (40)	13.4 ± 2.9 (40)
45	65	14.7 ± 0.6 (7)	14.3 ± 0.4 (7)	14.3 ± 0.9 (6)	15.9 ± 0.9 (6)

[a] The number of measurements per value is in parentheses.

and water into tared volatile sample pans, which were sealed and heated in the calorimeter to several degrees above the transition temperature of the sample. The unit was then cooled to −20 °C, and programmed heating of the sample was begun.

^{1}H NMR spectra were obtained at 90, 250, 300, and 600 MHz[13] at ambient probe temperature, except for study of the D_2O vesicles, which were analyzed at 52 ± 1 °C[14] on a JEOL FX-90Q. Vesicles were prepared by ultrasonication of D_2O/phospholipid mixtures at 52 °C with a Heat Systems Model W-375 sonicator equipped with a standard microtip probe. Mixed phospholipid/steroid vesicles were prepared by combining the lipids in chloroform. The solvent was evaporated, and the dry sample was sonicated as described above. Chemical shifts are taken relative to an external Me_4Si reference.

^{31}P NMR spectra were obtained at 101.2 MHz on the Mellon Institute (Pittsburgh, PA) superconducting spectrometer. Chemical shifts are relative to an external H_3PO_4 reference.

Monolayers were spread from hexane–ethanol, 9:1 (v/v). Hexane (Fisher spectrograde) was distilled from 4-Å molecular sieves and then passed through silica gel (Fisher, 60–200 mesh) and alumina (Woelm, basic). Absolute ethanol was distilled from magnesium methylate.[15] Blank runs of spreading solvent gave no measurable surface pressure after evaporation. Mixed monolayers were prepared by syringing together various amounts of lipid solutions with an Agla micrometer syringe (Wellcome Reagents, Ltd., England, Model MS-01) and then syringing the mixed solutions onto the clean water surface. Phospholipids were dried at 90 °C in vacuo with P_2O_5 for 8–12 h and weighed on a Cahn RG microbalance in a nitrogen-filled glove bag before dissolution in the spreading solvent.

Water used for all experiments was purified by reverse osmosis (Millipore RO-4), deionization (Millipore Milli-Q) and double distillation, first from alkaline permanganate and then from dilute sulfuric acid, in an all glass apparatus.[8]

Figure 1 shows our isotherms for carefully purified L-DPPC monolayers on water.[16] Although our automated film balance is sensitive to ±0.005 dyn/cm and has easily detected enantiomeric/racemic differences in other systems,[8] no detectable chiral recognition was found for DPPC at 25.0 ± 0.2 or 44.5 ± 0.5°.[17] Again, static surface tension lowering by DPPC films on water was measured at several molecular areas and several temperatures (see Table I), since force-area isotherms of phospholipids may involve dynamic factors as well as reversible thermodynamic properties. No significant difference due to stereochemistry could be established.

Many measurements were made with samples of D and L isomers and their mixtures, which were prepared repeatedly and purified from separate batches of DPPC. In our hands, the

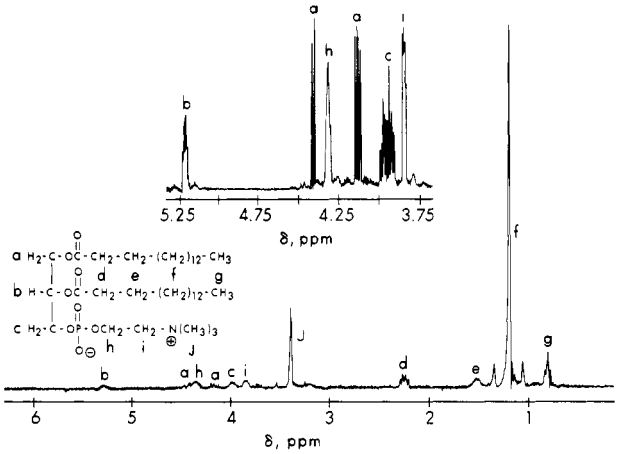

Figure 2. Proton NMR spectrum of L-DPPC in $CDCl_3$ at 250 MHz. The inset shows the region of 3.75–5.25 ppm amplified at 600 MHz. Letters a–j indicate peak assignments.[22]

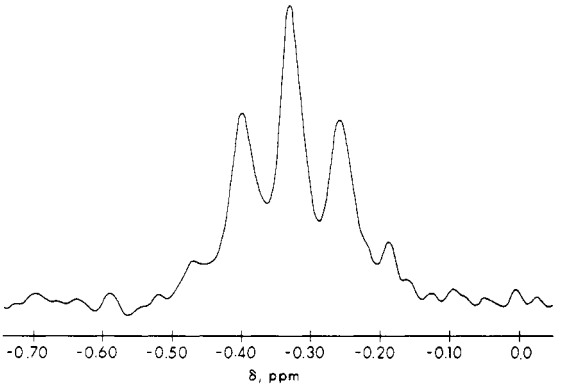

Figure 3. ^{31}P NMR spectrum of L-DPPC in $CDCl_3$ at 101.2 MHz (proton coupled, J_{PH} = 7.02 Hz). The convention of increasing chemical shift with decreasing field strength is used here.

day-to-day and batch-to-batch variations of results for L isomer vs. racemic material were so large that entirely conflicting conclusions would have been possible if only small samples of data had been compared. Even highly purified DPPC (and probably most other phospholipids) is quite unstable, especially with regard to its surface properties. The absolute stereochemical check of the properties of pure (unnatural) D against the L isomer and of their 1:1 mixture against purified racemic material has been applied only very rarely in phospholipid surface chemistry. In view of the stability problems which we have uncovered, we recommend strongly that the stereochemical test be applied whenever experiments examining subtle effects on phospholipid systems are attempted.

After many comparisons of D-, L-, and racemic DPPC, both by static surface tension lowerings and force-area isotherms, we are unable to report a statistically significant difference (at greater than 95% confidence limits) between enantiomeric and racemic samples of DPPC as monolayers on water.

Differential scanning calorimetry (DSC) has been used often to determine differences in the phase transitions of phospholipids

(13) These spectra were obtained at Duke University (90 MHz), the University of Pittsburgh (300 MHz), and the Mellon Institute (250 MHz and 600 MHz). The 600-MHz instrument presently has the highest resolution of any spectrometer in the world.

(14) Levine, Y. K.; Birdsall, N. J. M.; Lee, A. G.; Metcalfe, J. C. *Biochemistry*, **1972**, *11*, 1416–1421.

(15) Lund, H.; Bjerrum, J. *Chem. Ber.* **1931**, *64*, 210–213.

(16) Force-area isotherms and NMR spectra are shown for L-DPPC. D- and racemic DPPC's gave isotherms and spectra which were superimposable with those shown.

(17) These measurements were made above the gel point where temperature control was very difficult and the results less reliable than at lower temperatures.

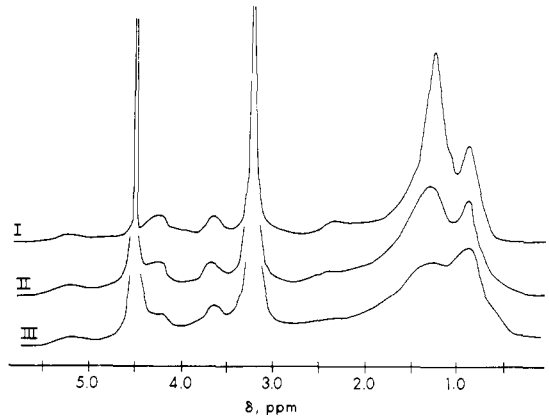

Figure 4. Proton magnetic resonance spectra of D_2O vesicles prepared from L-DPPC alone (I) and combined with cholestanol (20%, II; 50%, III). Spectra were obtained at 52 °C on a JEOL-FX90Q spectrometer.

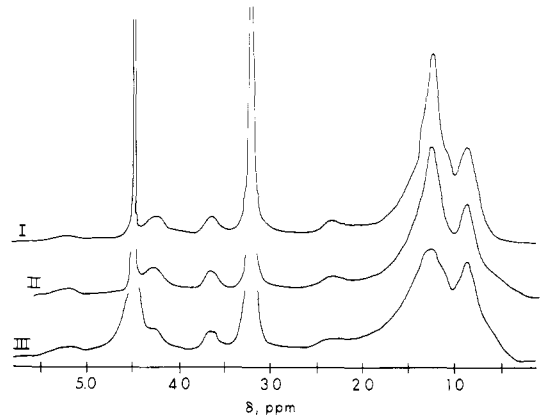

Figure 5. Proton magnetic resonance spectra of D_2O vesicles prepared from L-DPPC alone (I) and combined with epicholestanol (20%, II; 50%, III). Spectra obtained as described in Figure 4.

of different structures.[18,19] Thermograms for gels from our purified enantiomers of DPPC and their racemic mixture were superimposable and agreed closely with published figures.[20] Again many samples were analyzed and no significant differences were observed.[21]

NMR spectroscopy has been a powerful tool for detecting subtle structural differences between phospholipids. Proton spectra of the three stereochemical modifications of DPPC were run as vesicles in D_2O at 52 °C and as solutions in anhydrous chloroform, where DPPC presumably forms reverse micelles.[14] Again the spectra were superimposable—no enantiomeric recognition could be found at 90, 250, 300, or 600 MHz.[13] Finally, ^{31}P spectra were run at 101.2 MHz. Figures 2 and 3 are ^1H and ^{31}P spectra, which are in each case superimposable for D-, L-, and racemic DPPC.

Our final conslusion is that *by every important technique which has been applied to the study of phospholipid aggregates we are unable to demonstrate a significant difference between racemic DPPC and its enantiomers using the best tools available and material of high purity.*

Previous studies have shown clearly defined chiral discrimination between the enantiomers and the racemic modification for several chiral surfactants.[8,23] In contrast, our results with DPPC suggest that the chiral center of phospholipid molecules is sufficiently buried at the middle carbon of the glyceryl chain such that only a strong stereospecific perturbation is capable of distinguishing one enantiomer from the other.

Having demonstrated that the chiral discrimination factor between racemic DPPC and its two optically pure isomers was below the level of significant detection by several techniques commonly used for phospholipid study, we next tried to detect diastereomeric differences between mixtures of D- and L-DPPC with other chiral surfactants.

A good precedent is the attack of phospholipases on racemic phospholipids, which is highly stereospecific against the naturally occurring L isomer[9] and is, in fact, the basis for preparing the D isomer from synthetic racemic mixtures. With this in mind, there have been several studies of diastereomeric mixtures of optically active phospholipids with steroids.[24-27] In monolayer studies no difference was observed in the behavior of D- or L-DPPC with cholesterol.[24] No cross chiral checks could be made since the enantiomer of cholesterol is not accessible without enormous synthetic effort. However, epicholesterol is a diastereomer of cholesterol and might evoke a different response from the enantiomers of DPPC if stereospecific interactions of the phospholipid with the steroids were significant. Distinct differences were seen when L-1-oleoyl-2-stearoylphosphatidylcholine (L-OSPC) was mixed with cholesterol and epicholesterol at the air-water interface.[25] Differences in heats of transition were also observed when L-OSPC was combined with cholesterol and epicholesterol in H_2O dispersions and analyzed by differential scanning calorimetry.[26] In contrast, Chatterjie and Brockerhoff[27] found clear differences in 100-MHz ^1H NMR spectra of D-DPPC/cholesterol vesicles compared with L-DPPC/cholesterol vesicles. Once again no cross chiral check could be made.

Figures 4 and 5 present our ^1H NMR spectra of mixtures of L-DPPC with cholest*an*ol and epicholest*an*ol. No diastereomeric effect was seen for mixtures with either steroid. Our results closely parallel those of Demel et al.[25] in monolayers and DeKruyff et al.[26] in H_2O dispersions. The diastereomeric steroids have somewhat different effects on the packing of phospholipids in monolayers and vesicles. In view of the very small differences between the structures of our steroids and those studied by Chatterjie and Brockerhoff,[27] we doubt whether the difference reported by them is authentic. Figure 6 presents our force-area isotherms for mixtures of L-DPPC mixed with cholestanol and epicholestanol. As expected, these results are similar to those of Demel et al.[25] with mixed monolayers of L-OSPC and cholesterol or epicholesterol.

Since the diastereomeric interactions of DPPC with steroids appear to be too subtle to be detected readily, we have examined mixtures of D- and L-DPPC with enantiomers of *N*-(α-methylbenzyl)stearamide. This surfactant alone exhibits strong chiral discrimination between its enantiomers and their racemic mixture.[8,23g,h] However, concentrated (>6 N) solutions of aqueous sulfuric acid are required as a subphase in order to expand this highly aggregated crystalline material into a monolayer. We were therefore intrigued to find that DPPC mixtures with the stearamide enantiomers produced excellent monolayers across a wide

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(19) Ladbrooke, B. D.; Chapman, D. *Chem. Phys. Lipids* **1969**, *3*, 304-367.
(20) Hinz, H. J.; Sturtevant, J. M. *J. Biol. Chem.* **1972**, *247*, 6071-6075.
(21) Clear differences in melting behavior and transitions have been seen in DSC thermograms of other chiral surfactants. See Stewart and Arnett (Stewart, M.; Arnett, E. M. *Top. Stereochem.*, in press) and ref 8 for examples.
(22) Birdsall, N. J. M.; Feeney, J.; Lee, A. G.; Levine, Y. K.; Metcalfe, J. C. *J. Chem. Soc., Perkin Trans. 2* **1972**, 1441-1445.
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(25) Demel, R. A.; Bruckdorfer, K. R.; van Deenen, L. L. M. *Biochim. Biophys. Acta* **1978**, *255*, 311-320.
(26) De Kruyff, B.; Demel, R. A.; van Deenen, L. L. M. *Biochim. Biophys. Acta* **1972**, *255*, 331-347.
(27) Chatterjie, N.; Brockerhoff, H. *Biochim. Biophys. Acta* **1978**, *511*, 116-119. Dr. Brockerhoff in a private communication has confirmed the difficulty in reproducing their original result.

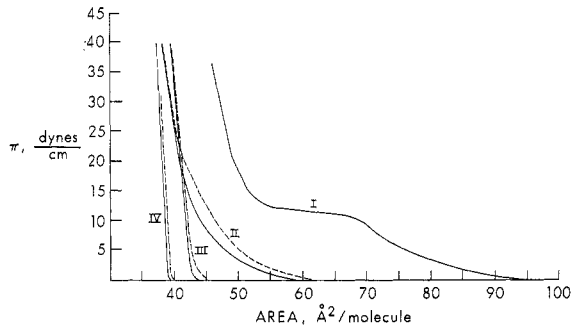

Figure 6. Force-area isotherms of DPPC alone (I) and combined with cholestanol (—) or epicholestanol (---). Ratios of DPPC/steroid are 2:1 (II), 1:1 (III), and 0:1 (IV). Isotherms were obtained at the air–water interface at 25 ± 0.2 °C.

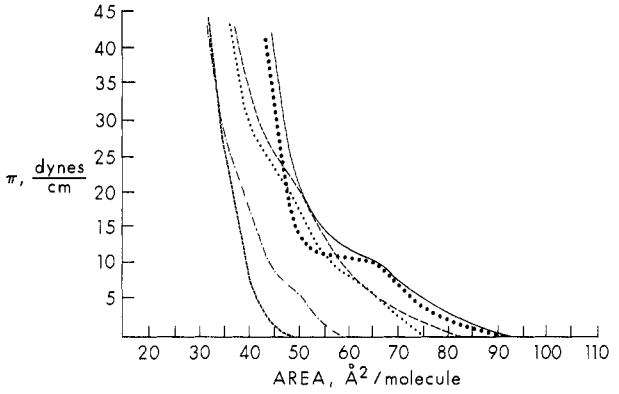

Figure 7. Force-area isotherms for mixtures of L-DPPC with (*S*)-*N*-(α-methylbenzyl)stearamide. Ratios of DPPC/stearamide are 1:0 (●●●), 10:1 (—), 2:1 (– – –), 1:1 (···), 1:2 (-·-·-) and 1:4 (- - -). (*R*)-*N*-(α-methylbenzyl)stearamide behaved the same as the (*S*)-stearamide when mixed with DPPC at the air–water interface.

range of proportions from 10:1 to 1:4 DPPC/stearamide (Figure 7). No difference could be detected in any case between mixtures of D- or L-DPPC with (*R*)- or (*S*)-*N*-(α-methylbenzyl)stearamide. Nor was there any distinction between mixed monolayers of the stearamide with racemic DPPC as compared to those made with DPPC enantiomers. Thus again no diastereomeric chiral recognition could be found for this phospholipid with enantiomers of another chiral surfactant.

The most significant result from our study of the isotherms of the mixed monolayers of DPPC with the stearamide is the ability of DPPC to help the other surfactant to spread and to preserve chiral recognition between the stearamide enantiomers and their racemate. Even though the fatty acid chains of the stearamide are diluted 20:1 in the 10:1 mixture (Figure 8), the discrimination between enantiomeric and racemic mixtures is clear and is comparable to that for pure stearamide monolayers on aqueous acid.

If this phenomenon is general, it implies that within the mixed bilayers of the cell membrane, stereospecific packing interactions could occur between other nonphospholipid chiral components, such as peptides, even if those components did not engage directly

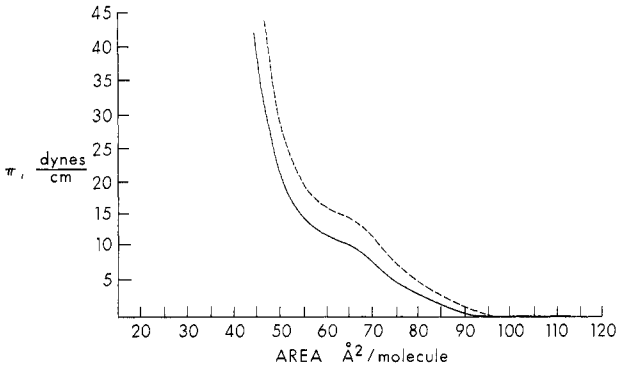

Figure 8. Force-area isotherms for a 10:1 mixture of L-DPPC with (*R*)-(—) or (±)- *N*-(α-methylbenzyl)stearamide (- - -).

in energetically significant diastereomeric packing with the phospholipids. There is the clear implication that in the monolayer state, unlike any other situation known to us, stereospecific interactions are well preserved or even transmitted in an essentially achiral medium at considerable surface dilution.[28]

The present study is the first that we know of to rigorously explore chiral recognition factors in mixed phospholipid monolayers, so generalizations are obviously premature. We hope to report other results from this new area of intermolecular stereochemistry in the near future.

Chiral discrimination factors which would produce significant differences between the properties of a pure racemic phospholipid and its enantiomers or between the enantiomeric phospholipids and other chiral molecules are of potential biochemical significance. We have searched for such enantiomeric and diastereomeric differences using highly purified D- and L-dipalmitoylphosphatidylcholine and can detect no significant chiral discrimination factors using the best tools and materials available. These results conflict with several previously published reports.

DPPC assists the strongly aggregated chiral surfactant *N*-(α-methylbenzyl)stearamide to spread as a film. We believe that it is quite significant that, although DPPC films behave in most ways as though they are achiral, mixed monolayers of DPPC with *N*-(α-methylbenzyl)stearamide maintain much of the chiral recognition factors which discriminate between the force-area isotherms of the pure chiral stearamides and their racemic modification. This offers the interesting possibility that chirality is being transmitted through several intervening molecules in the oriented milieu of the compressed monolayer.

Acknowledgment. This work was supported by the National Institutes of Health Grant R01-GM28757, for which we are most appreciative. We thank Eric Johnson and Robert J. Verbiar for help in preliminary studies and acknowledge the valuable assistance of the Mellon Institute NMR Facility for Biomedical Studies.

(28) While it is true that DPPC, like all other natural phospholipids, is a chiral surfactant, all of our results in this article indicate that it behaves in an essentially achiral manner as far as enantiomeric or diastereomeric interactions with other chiral surfactants are concerned.