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FLUORESCENT PROBES IN MODEL MEMBRANES

II. MONOLAYER STUDIES OF 2,2′-(VINYLENEDI-*p*-PHENYLENE)BIS-BENZOXAZOLE, *d*-3-AMINODESOXYEQUILENIN AND *N*-OCTADECYLNAPHTHYL-2-AMINO-6-SULFONIC ACID WITH HOST-LIPID TETRADECANOIC ACID

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Summary

Film studies at the air-water interface have been carried out for pure films of 2,2′-(vinylenedi-*p*-phenylene)bisbenzoxazole (VPBO), d-3-aminodesoxy-equilenin (EQ) and *N*-octadecylnapthyl-2-amino-6-sulfonic acid (ONS), and for mixed films with tetradecanoic acid for the first two fluorescent probes. Pure film isotherms indicate highly rigid non-monomolecular films for both VPBO and EQ, revealing the presence of strong intermolecular forces. In mixed films with tetradecanoic acid VPBO rapidly segregates with resultant film loss over a wide concentration range. EQ, however, can be stabilized by the host-lipid at low concentrations. This, coupled with an ability to only slightly affect the host-lipid liquid-condensed/liquid-expanded phase change, suggests that EQ can be regarded as "non-perturbing" and should be retained in condensed lipid phases.

ONS, because of its unusual polar headgroup, resembled hexadecanoic acid more than octadecanoic acid. While difficulties in spreading ONS precluded the study of mixed films, the indications are that it would be a satisfactory expanded lipid state probe if mixing can be brought about.

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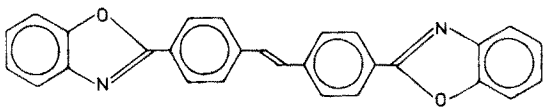
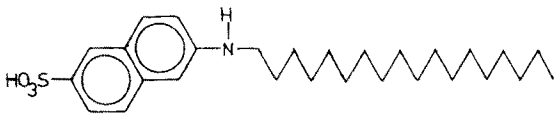
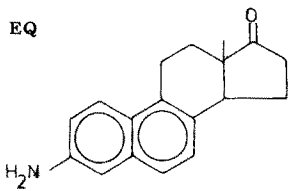
Abbreviations: VPBO, 2,2′-(vinylenedi-*p*-phenylene)bisbenzoxazole; EQ, *d*-3-aminodesoxyequilenin; ONS, *N*-octadecylnapthyl-2-amino-6-sulfonic acid; 2-AP, 2-(9-anthroyl)palmitic acid; 12-AS, 12-(9-anthroyl)-stearic acid; 16-AP, 16-(9-anthroyl)palmitic acid.

TABLE I

RELEVANT PROPERTIES OF THE FLUORESCENT PROBES STUDIED

Probe	Structural formula	Thin layer chromatography solvent system	m.p.obs (°C)	m.p.lit (°C)	Spreading solvent
VPBO		—	362	362—363 [a]	Chloroform [b]
ONS		Chloroform/ methanol/sulfuric acid (conc.) (5 : 5 : 0.5, v/v)	—	—	Chloroform/ dichloroacetic acid (9.6 : 0.4, v/v) [c]
EQ		Ethyl acetate/cyclo-hexane/ethanol (9 : 9 : 2, v/v)	217.5—218	218—219 [d]	Chloroform

[a] From Chemical Abstracts 70: 38906h.
[b] In very dilute solution due to limited solubility of VPBO.
[c] Due to the insolubility of ONS in common spreading solvents, this rather unconventional spreading solvent was necessitated.
[d] From ref. 4.

Introduction

In a recent article [1], a detailed study was made of the behavior of three anthroyl fatty acid fluorescent cell membrane probes (2-(9-anthroyl)palmitic acid (2-AP); 12-(9-anthroyl)stearic acid (12-AS) and 16-(9-anthroyl)palmitic acid (16-AP)), both in monolayer and bilayer systems. The monolayer work measured the "perturbation" of a host-lipid, dipalmitoylphosphatidylcholine as indicated by an incrase in the pressure of the liquid condensed/liquid expanded phase change. It also measured the ability of the dipalmitoyl phosphatidylcholine to "accommodate" these probes, that is to show negative deviations from ideal behavior in mean molecular area plots. Both perturbation and accommodation decreased in the order 2-AP > 12-AS > 16-AP. This means that in the expanded state the ease of incorporation decreases 2-AP > 12-AS > 16-AP, but that formation of a condensed state is increasingly hindered by the same probe order. The bilayer data confirmed the order of perturbation. Here we present monolayer data for three additional probe molecules which have been used in fluorescence studies of cell-membrane-related systems [2,3].

Experimental

Materials. 2,2′-(vinylenedi-*p*-phenylene)bisbenzoxazole, *d*-3-aminodesoxyequilenin, and *N*-octadecylnaphthyl-2-amino-6-sulfonic acid were kindly supplied by R.A. Badley (then at the National Research Council, Ottawa, Canada). The VPBO and ONS samples were used as supplied after having been determined to be pure by thin layer chromatography and/or a melting point determination (see Table I). The EQ samples was purified by vacuum sublimation and subsequent recrystallization from benzene [4]. Tetradecanoic acid was purchased from Applied Science Laboratories, State College, Pennsylvania and used as supplied.

Method. An automated Wilhelmy film balance which continuously records surface pressure and surface potential as a function of area per film molecule, as well as film handling techniques have been described in detail elsewhere [5,6]. Reproducibility of isotherms was ±0.5 Å^2/molecule; ±0.2 dynes/cm, and ±10 mV.

Results and Discussion

VPBO

The isotherm for VPBO is illustrated in Fig. 1. From CPK molecular models, the close-packed area for a monolayer of VPBO would be on the order of 25 Å^2/molecule (assuming the molecule aligns itself with the long axis perpendicular to the water surface). the fact that the actual experimental area is (reproducibly) on the order of 5 Å^2/molecule indicates that the film is not truly monomolecular. Evidently, some sort of stacked array of molecules is formed at the interface. From the apparent area/molecule observed, the stack would have to consist of approximately four layers of VPBO molecules. Alternatively, if it is assumed that, at the interface, the molecule has its long axis horizontally

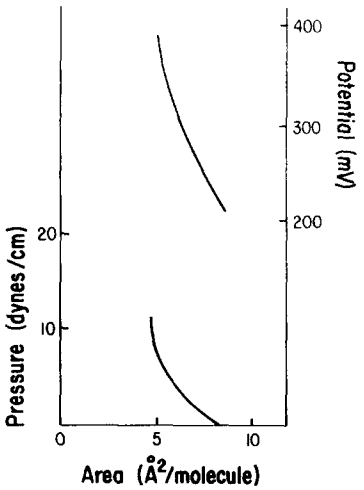


Fig. 1. Pressure vs. area and potential vs. area isotherms for VPBO on H_2O at 21°C.

oriented with respect to the interface (Area = 125 Å^2/molecule from molecular models), approximately 25 layers would be needed.

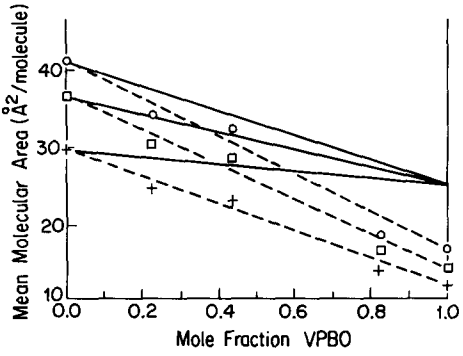
It should also be pointed out that the film was extremely rigid, i.e., upon compression, practically as soon as lift-off ocurred, the film becomes so viscous that the Wilhelmy plate was pushed out of its vertical orientation by the pressure of the film *. This fact indicates that extremely strong cohesive forces are present in the film, an indication that the VPBO molecules are oriented with their long axes perpendicular to the interface since this arrangement would seem likely to provide maximum lateral interactions among the film molecules.

In mixed films with tetradecanoic acid, two trends are noticeable: (1) In a mean molecular area vs. composition plot (Fig. 2), the experimental points initially lie along the "ideal" line [7], in this case, almost certainly an indication of immiscibility of the two components in the mixed film. With increasing VPBO concentration, film loss is clearly occurring. In Fig. 2 the area/VPBO molecule is shown as the minimum (25 Å^2) possible as evaluated from CPK models. If VPBO occupies a greater area/molecule than this, then segregation and film loss will occur at even lower concentrations. (2) The liquid-condensed/liquid-expanded phase transition of the host tetradecanoic acid is shifted to higher pressures upon addition of VPBO, indicating a perturbation of the host lipid.

EQ

As far as pure film behavior is concerned. EQ behaves quite similarly to VPBO, i.e., a rigid film of quite low apparent areas/molecule is formed (Fig. 3). It was thought initially that the low areas/molecule might have been caused

* It should be noted that this necessarily means that the pressure being recorded was not an equilibrium value. The fact that the Wilhelmy plate was moved indicates that a macroscopic pressure gradient existed in the film which could not be instantaneously relieved due to the extreme rigidity of the film. The result is that the recorded pressure should only be regarded as an approximation to the true film pressure.



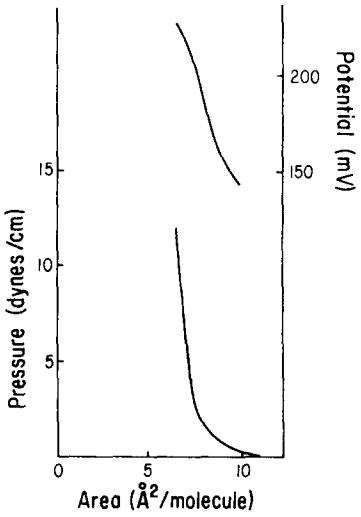


Fig. 2. Mean molecular area vs. composition for mixed monomolecular films of VPBO and tetradecanoic acid on H_2O at 22°C using a minimum (25 Å^2) area/molecule for the probe. The solid lines indicate ideal behavior. The broken lines indicate actual behavior. ○, 5 dynes/cm; □, 10 dynes/cm; +, 15 dynes/cm.

Fig. 3. Pressure vs. area and potential vs. area isotherms for *d*-3-aminodesoxyequilenin on H_2O at 21°C.

by a relatively high solubility of EQ, because of the two polar groups in the molecule. A check of film stability as a function of both compression speed and of the time between spreading and eventual compression indicated that these factors were not major problems, since essentially identical results were obtained under all conditions. The explanation for the low areas must again, therefore, lie in a non-monomolecular structuring of the EQ molecules at the air/water interface. Either this molecule does not spread as a monomolecular film or it collapses (reproducibly) to a multilayered aggregate as soon as pressure is applied to the film. On the basis of the relative hydrophilic strengths of the two polar groups, it can be assumed that the keto group would be the primary polar group [8]. It is also known that amino groups as primary polar groups in aromatic molecules cause the surface potential to drop dramatically [9]. This would not appear to be the case here, indicating that the amino group is probably not exclusively acting as the primary polar group, in spite of packing considerations which favor the placement of the primary polar groups at the 3-position rather than the 17-position [10]. It is also possible that the molecule as a whole has no uniquely preferred orientation at the air/water interface, this in turn resulting in steric problems in packing which led to collapse at very low pressures. It might also be noted that rigid films, though without the extremely low areas observed here, have been obtained with other polynuclear, sterol-like molecules [11].

As with VPBO, the meaningfulness of any mean molecular area vs. composition plot is open to question in view of the low areas/molecule for the pure probe film. It is interesting to note, however, that the experimental points for the system tetradecanoic acid-EQ all lie on a continuous curve indicating initial

positive deviations from ideality (Fig. 4). In this figure, instead of the unrealistic "monolayer" areas for the pure EQ film, more reasonable areas calculated from a CPK model are assumed (molecular dimensions = 12 Å × 5.6 Å as an upper limit with the keto group as the primary group). It should be noted that the positive deviations from ideality which occur at low pressures and low probe concentrations decrease at higher concentrations, presumably indicating film loss. The region of positive deviations also decreases with increasing pressure, but it must be remembered that the areas assumed for pure EQ are an upper limit. An assumption of lower pure EQ areas/molecule would inevitably increase the region of positive deviations. Two conclusions can be drawn from Fig. 4: (1) that the presence of the host lipid tends to stabilize the EQ molecules in a monomolecular array, and (2) that EQ and tetradecanoic acid are miscible, at least at low EQ concentrations.

A plot of the shift in pressure for the tetradecanoic acid liquid-condensed/liquid-expanded phase transition ($\Delta\pi$) vs. composition (Fig. 5) indicates that the perturbation of the phase transition caused by the addition of EQ is extremely small. Up to a mol fraction of EQ 0.2, the phase transition is essentially unchanged, and it is still visible at concentrations as high as a mol fraction of EQ 0.6. The persistence of the phase change to quite high probe concentrations may in part be due to immiscibility and segregation in the mixed film, resulting in a lower effective probe concentration than is apparent, but the evidence from the mean molecular area vs. composition plot (Fig. 4) indicates that, at least at low EQ concentrations, the effect is real.

The small extent of the phase change perturbation is especially striking when

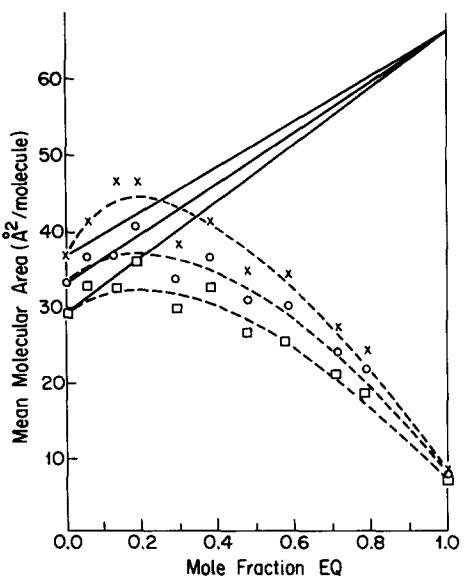


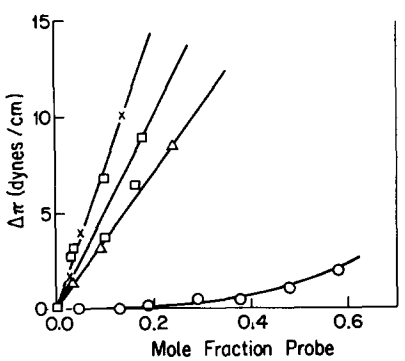
Fig. 4. Mean molecular area vs. composition for mixtures of *d*-3-aminodesoyequilenin and tetradecanoic acid on H_2O at 20°C using a maximum area/molecule (67 Å^2) for EQ calculated from CPK models. The solid lines indicate ideal behavior. The broken lines indicate actual behavior. ×, at 2 dynes/cm; ○, at 5 dynes/cm; □, at 10 dynes/cm.

compared with the $\Delta\pi$ vs. composition plots for mixtures of other cell-membrane probes with the same host lipid (see Fig. 5). It is also interesting that cholesterol, which has a similar sterol structure, also perturbs the phase transition to a much greater extent than EQ, at least at relatively low concentrations [13]. It would, therefore, seem that EQ constitutes less of an "impurity" than cholesterol.

ONS

A major difficulty with regard to monolayer experiments on this probe molecule is related to the problem of finding a suitable spreading solvent, because of the insolubility of this molecule in common solvents. The film formed upon spreading from the chloroform/dichloroacetic acid solvent, however, gave quite reasonable results, with a well-defined temperature for the appearance of the liquid-condensed/liquid-expanded phase transition of about 23°C (Fig. 6).

Although ONS has an obvious hydrophilic group ($-SO_3H$) and hydrophobic group (the fatty acid chain), an unanswered question remains as to the effect of the secondary amino group on the surface properties of this molecule. The comparable fatty acid, octadecanoic acid, has a half-expansion temperature (1.4 dynes/cm) of 46°C [14]. ONS appears to compare more favorably with hexadecanoic acid (half-expansion temperature of 28°C [14]). Thus, the effect of the head group is analogous to a shortening of the chain of the fatty acid by two methylene groups. Given that the half-expansion temperature for *n*-hexa-



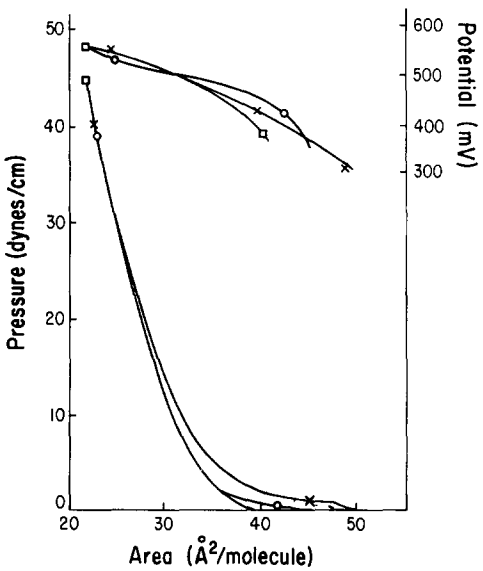


Fig. 5. Changes in pressure ($\Delta\pi$) for the appearance of the liquid-expanded/liquid-condensed phase transition in mixed films of tetradecanoic acid and various probe molecules at 21°C. ○, *d*-3-aminodesoxyequilenin; X, 2-(9-anthroyl)hexadecanoic acid [6]; □, 12-(9-anthroyl)octadecanoic acid [6]; △, 2-(10-carboxydecyl)2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl [12].

Fig. 6. Pressure vs. area and potential vs. area isotherms for ONS on H_2O at various temperatures: □, at 22.2°C; ○, at 23.5°C; X, at 25.0°C.

decylamine is 48°C (ref. 14, p. 227), i.e., 20° higher than for the corresponding C_{16} fatty acid, the expansion effect must be caused by the sulfonic acid group and/or the bulky naphthalene ring system. In this regard, it is worthy of note that 2-(9-anthroyl)hexadecanoic acid, which also has a bulky ring system incorporated into the region of the polar group of the molecule, does not undergo a liquid-condensed/liquid-expanded phase transition at all [1].

Unfortunately, the unusual spreading solvent selected effectively precluded a study of mixed monolayer films because of possible effects on the host-lipid component. There is every reason to expect, however, that, once formed, such films would be stable.

Conclusions

Two comments can be made with respect to a probe such as VPBO which exhibits great difficulty in forming a monomolecular array at the air/water interface, even when mixed with a host lipid. (1) When incorporated into a lipid bilayer, it presumably preferentially situates itself in the hydrophobic region of the bilayer. This is in agreement with a previous assumption [2]. (2) Given the extremely strong intermolecular cohesive forces evident in its film behavior, as well as in the bulk behavior (cf. the high melting point), it is quite likely that, when inserted as a probe into bilayer structures, VPBO will not be distributed randomly, but rather will tned to segregate out into probe-rich regions, with the result that fluorescence data obtained from this probe may well reflect the behavior of these probe-rich regions rather than the bilayer in general.

With EQ, in view of the probability discussed above that the primary polar groups is the keto group, the fluorescent chromophore is presumably located some 3.5 Å into the hydrophobic region of the bilayer. From the mixed film data, EQ would seem to be an excellent probe in that it is miscible with the host lipid, at least at relatively low concentrations, and, perhaps even more importantly, seems to perturb the liquid-condensed/liquid-expanded transition to a surprisingly small extent. This indicates that EQ should be capable of retention in a condensed state, either the liquid-condensed state in a monolayer, or the gel state in a bilayer [15].

The situation would seem to be different with regard to ONS. From its more normal monolayer film behavior, it can be predicted that, when incorporated in a bilayer system, this probe will be oriented with the chromophore at the interface and the fatty acid chain extending into the hydrocarbon region of the bilayer. Again this confirms previous speculation regarding this probe's probable location [3], as well as being in agreement with fluorescence [16] and X-ray data [17,18]. Based on our anthroyl probe studies [1], we would expect that ONS would be readily incorporated into an expanded lipid state, but would seriously affect any reading of a gel/liquid crystalline transition.

Acknowledgements

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