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# The formation and Langmuir-Blodgett deposition of monolayers of novel photochromic azobenzene-containing phospholipid molecules

S.S. Sandhu [a], Y.P. Yianni [a], C.G. Morgan [a,*], D.M. Taylor [b] and B. Zaba [b]

[a] *Department of Biological Sciences, University of Salford, Salford, M5 4WT and* [b] *School of Electrical Engineering Science, University College of North Wales, Dean Street, Bangor, Gwynedd, LL57 1UT (U.K.)*

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**A group of novel phospholipid molecules has been synthesised, in which one or both acyl chains has been replaced with an azobenzene-containing acid. These lipids are photochromic, isomerising from the *trans* form in which the azobenzene unit is almost linear to a *cis* form with a bent chain. The influence of this isomerisation on the properties of monolayers of these lipids on an aqueous subphase has been investigated using the Langmuir trough. The lipids are shown to form compact monolayers when in the *trans* form, but to have greater molecular areas after isomerisation. All lipids could be transferred to a glass substrate by Langmuir-Blodgett deposition. Evidence suggestive of phase equilibria is seen for surface monolayers of one of the lipids, which also shows evidence of a temperature-dependent phase transition by light scattering when dispersed as vesicles in water. Marked hysteresis is seen between cycles of compression and expansion for surface monolayers of azo-lipids before and after photoisomerisation.**

## Introduction

Amphipathic molecules, including important membrane components such as phospholipids, readily form organised arrays by self assembly. On the surface of water under appropriate conditions, such molecules form monomolecular films [1], and the study of such monolayers using the Langmuir trough gives information on molecular area and packing [2]. Surface monolayers can be transferred to a solid substrate, and multilayer structures built up by 'Langmuir-Blodgett' (L-B) deposition [3]. Although L-B deposition is a well established technique with a long history, there has recently been an upsurge of interest in this area. The current interest has its origin in part in the (as yet) speculative area of 'molecular electronics' [4] and similar 'engineering' applications including biologically-responsive sensors. Langmuir-Blodgett multilayers are extremely suitable for studies on oriented molecules, and have given rise to a host of ingenious physical studies of fundamental importance, including fluorescence energy transfer measurements [5], magnetic effects in two dimensions [6], and electron tunnelling [7].

In this paper we discuss the synthesis of novel phosphatidylcholine derivatives (Fig. 1) where one or both acyl chains has been replaced by an azobenzene-containing moiety, and investigate the surface properties of monolayers of these molecules. In addition, using Langmuir-Blodgett deposition, we show that oriented multilayer films can be formed from these lipids. Azobenzene-containing compounds such as these are photochromic [8], isomerising to a photostationary state mixture of '*cis*' and '*trans*' isomers with a com-

* To whom correspondence should be addressed.

position determined by the illumination wavelength and temperature. Illumination with ultraviolet light produces a mixture where *cis* isomer predominates, while exposure to heat or visible light reforms the *trans* isomer. Isomerisation changes both molecular shape and polarity. The *trans* azobenzene unit is approximately planar with no molecular dipole moment, while the *cis* isomer has benzene rings oriented at an angle and has a significant molecular dipole moment [9]. The 'bent' chain of a *cis* azobenzene-containing molecule resembles an unsaturated fatty acid chain, and isomerisation has effects on molecular packing and associated properties such as refractive index. Oriented films of photochromic materials are of interest as possible high density information storage media [10] and though azobenzene-containing chromophores are not ideally suited to practical application because of thermal reversal of isomerisation, they are very useful as model systems to investigate effects of molecular close-packing on photoisomerisation. Such changes in molecular packing and polarity might be exploited to control the activity of membrane-bound molecules of biological interest (enzymes, channel-forming peptides etc.) to form sensors, and the work described here is a necessary preliminary to such experiments.

## Materials and Methods

Except where stated, all chemicals used were purchased from Aldrich Ltd.

4-(4-*n*-Butylphenylazo)phenylbutyric acid (I) was prepared by coupling equimolar amounts of 4-aminophenylbutyric acid (prepared by catalytic hydrogenation of 4-nitrophenylbutyric acid) with 4-nitroso-*n*-butylbenzene (from 4-*n*-butylaniline by peroxybenzoic acid oxidation) in glacial acetic acid. Coupling was complete after standing overnight at room temperature. The azo-acid was purified by chromatography on silica gel (Kieselgel 60 from Merck). Details of the synthesis have been given elsewhere [11].

1-Palmitoyl-2-(4-(4-*n*-butylphenylazo)phenylbutyroyl)phosphatidylcholine (II) (palmitoyl-Azo-PC) and 1-oleoyl-2-(4-(4-*n*-butylphenylazo)phenylbutyroyl)phosphatidylcholine (oleoyl-Azo-PC) (III) were prepared by acylation of the appropriate lysophosphatidylcholines (from Sigma Ltd.) using a mixed anhydride prepared from the acid (I) (as triethylamine salt) and pivaloyl chloride in dichloromethane containing 4-(*N*,*N*-dimethylamino)pyridine. The procedures and purification were as previously described for the synthesis of a fluorescent phospholipid derivative of diphenyl-1,3,5-hexatriene [12]. All operations were carried out under dry nitrogen in the case of compound (III). The lipids were stored in the dark as solids under nitrogen at −4°C. An azo-containing phospholipid was also prepared from egg-yolk lysophosphatidylcholine, and this is referred to as 'Azo-PC' in the text.

1,2-Bis(4-(4-*n*-butylphenylazo)phenylbutyroyl) phosphatidylcholine (IV) (Bis Azo-PC) was prepared by acylation of L-α-glycerophosphorylcholine (as the cadmium chloride complex from Sigma Ltd.) with the mixed anhydride of (I) as described elsewhere for other phospholipids [13]. Cadmium chloride and 4-dimethylaminopyridine were removed from the product by passage of a solution in methanol/chloroform/water (5 : 4 : 1, v/v) through mixed bed ion exchange resin (mixture of Amberlite CG400 and CG20 precleaned with eluting solvent mixture), and the free phospholipid was purified by chromatography on Kieselgel 60 eluting with chloroform/methanol/acetic acid/water (75 : 25 : 7.5 : 2.5, v/v). Thin-layer chromatography in a variety of solvent systems showed that this, and each of the other lipids was free of lysophosphatidylcholine contamination, and that no free acid (I) was present.

Monolayer studies were carried out on a Langmuir trough constructed in the School of Electrical Engineering Science, University College of North Wales, Bangor. The apparatus, which has been described in detail elsewere [14], is basically of constant perimeter design equipped with a high sensitivity film balance and servo/control electronics allowing highly precise measurement of surface pressure/area isotherms. In addition, a dipping mechanism allows monolayers to be transferred to substrates while surface pressure is held constant under servo control, and the change in surface monolayer area is continuously recorded. In this way, transfer efficiencies from monolayer to support can be precisely measured, since the surface area of the support is known.

The trough was filled with pure water at 23°C

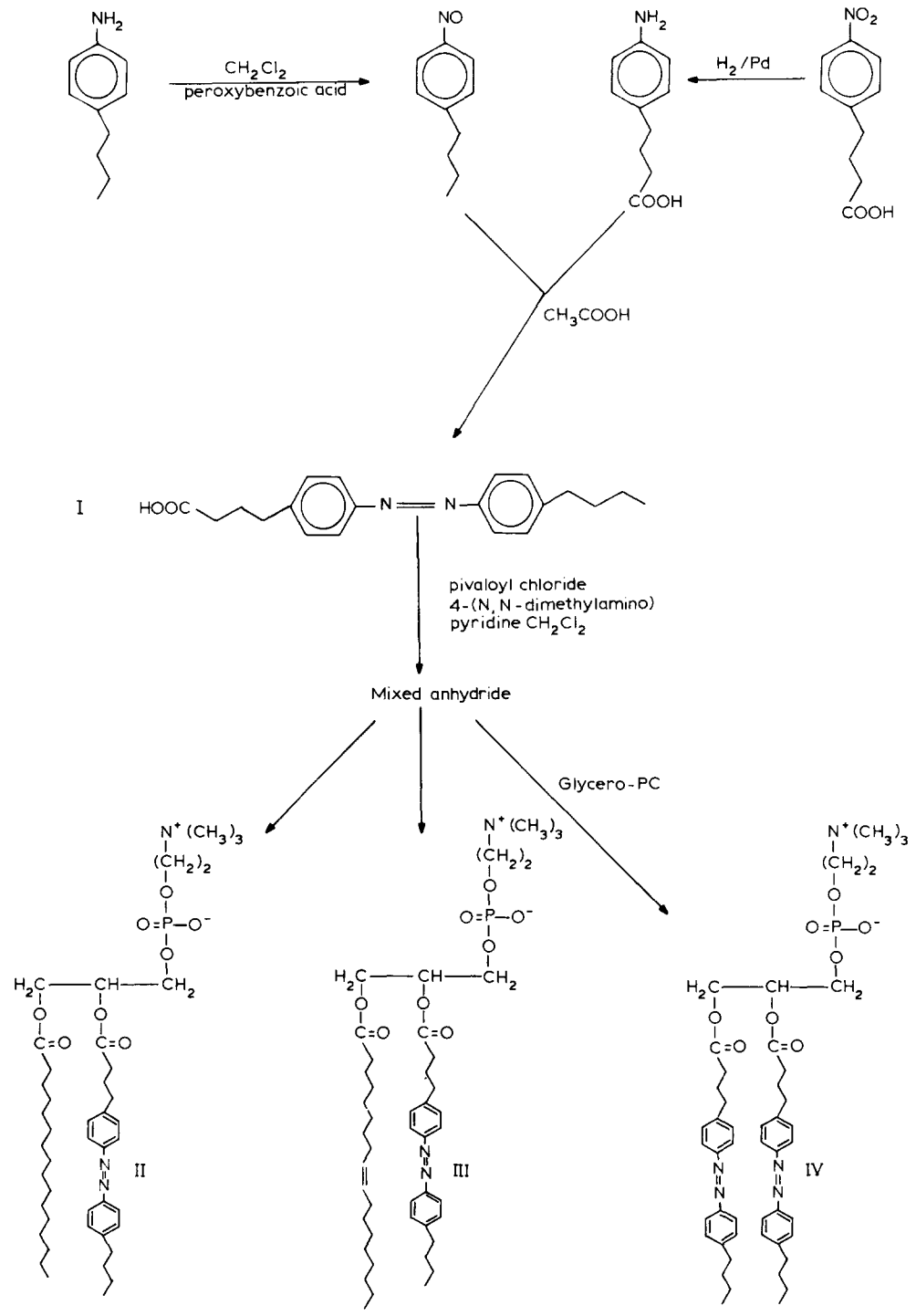


Fig. 1. Scheme showing the synthesis of the Azo-acid (I), palmitoyl-Azo-PC (II), oleoyl-Azo-PC (III) and Bis Azo-PC (IV). The synthesis of Azo-PC prepared from egg-yolk lysophosphatidylcholine has been described previously [11].

for the experiments described. The purity of the subphase is crucial to the measurement of reproducible surface pressure/area isotherms, and water used was deionised and purified with an ultrafiltration system. Samples were applied to the trough by carefully adding a drop (50 $\mu$l, 1 mg/ml) of the solution in 'Spectrosol' grade chloroform to the subphase, ensuring that the chloroform remained on the water surface. After evaporation of the solvent, the film so formed was partially compressed and allowed to expand several times to ensure that the system was equilibrated before measurements. After measurement of properties of a given lipid, the subphase surface was carefully cleaned by vacuum aspiration of the surface layer, until no change in surface pressure was recorded on moving the barrier within the trough. Calibration of the trough was regularly checked by measurement of the surface pressure/area isotherm of pure stearic acid as a standard.

Where monolayers were deposited on glass slides, these were first cleaned by washing in pure water and, in chloroform, and further by immersion in a beaker of 'Spectrosol' chloroform in an ultrasonic cleaning bath for approximately ten minutes. Clean slides were used immediately for deposition. Monolayers were transferred by dipping the slide at constant speed through the surface of the subphase, while maintaining the surface pressure constant between 40–60 mN/m.

The effect of photolysis on molecular area was measured by irradiating lipid samples with the focused output of a high pressure mercury arc lamp. A heat filter was used in conjunction with an ultraviolet filter with a passband of approximately 60 nm centred at 365 nm, and the lipid was photolysed as a dilute (1 mg/ml) chloroform solution. Control experiments demonstrated that the photostationary state was achieved within minutes by such exposure. After photolysis, the samples were kept in the dark and immediately spread on the subphase in the trough, which was operated under dim red light to avoid possible photoisomerisation of the lipid back to the *trans* form.

## Results and Discussion

The lipids investigated were Azo-PC (mixed chains in the '1' position, from egg-yolk lysolecithin), palmitoyl Azo-PC, oleoyl Azo-PC and Bis Azo-PC. Each phospholipid readily formed a monolayer on the surface of pure water. In Fig. 2A are shown surface pressure/area isotherms for each phospholipid at 23°C, while Table I shows the molecular area for each. Fig. 2B shows the corresponding isotherms measured for each lipid after isomerisation to the photostationary state as described in Methods, and Table I also lists the molecular areas calculated in each case. Each of the lipid films was apparently stable under an applied pressure of 40 mN/m. Isotherms of all lipids showed some degree of hysteresis between compression and expansion cycles; surprisingly this was not generally more marked after photolysis. Comparison of the isotherms before and after photolysis shows that in all cases the monolayer rigidity (as judged by the slope of the curve) decreased after photolysis and the breakpoints in the low pressure region of the isotherms are abolished by photolysis. In particular, the (unphotolysed) Bis Azo-PC shows evidence of a 'knee' in the region of the breakpoint in the compressive cycle, but significant hysteresis in this pressure region on re-expansion. Fig. 3 shows an isotherm for the parent acid (I) from which the phospholipids were synthesised, with an isotherm of pure stearic acid for comparison. The 'knee' in the

TABLE I

SURFACE MONOLAYER MEASUREMENTS FOR AZO-LIPIDS

Molecular areas for both isomers of each azo-phospholipid and for the azo-acid (I) (data shown are the average in each case of four measurements and were calculated from the corresponding compression isotherm by extrapolation to zero pressure from a point close to collapse). In some cases, notably after photoisomerisation, films were elastic and 'molecular areas' so measured are upper limits.

| Azo-phospholipid | area/molecule (Å$^2$) |
|---|---|
| *trans* Bis Azo-PC | 58.9 |
| *cis* Bis Azo-PC | 84.6 |
| *trans* palmitoyl-Azo-PC | 61.2 |
| *cis* palmitoyl-Azo-PC | 98.1 |
| *trans* oleoyl-Azo-PC | 93.0 |
| *cis* oleoyl-Azo-PC | 122.6 |
| *trans* Azo-PC | 56.6 |
| *cis* Azo-PC | 78.6 |

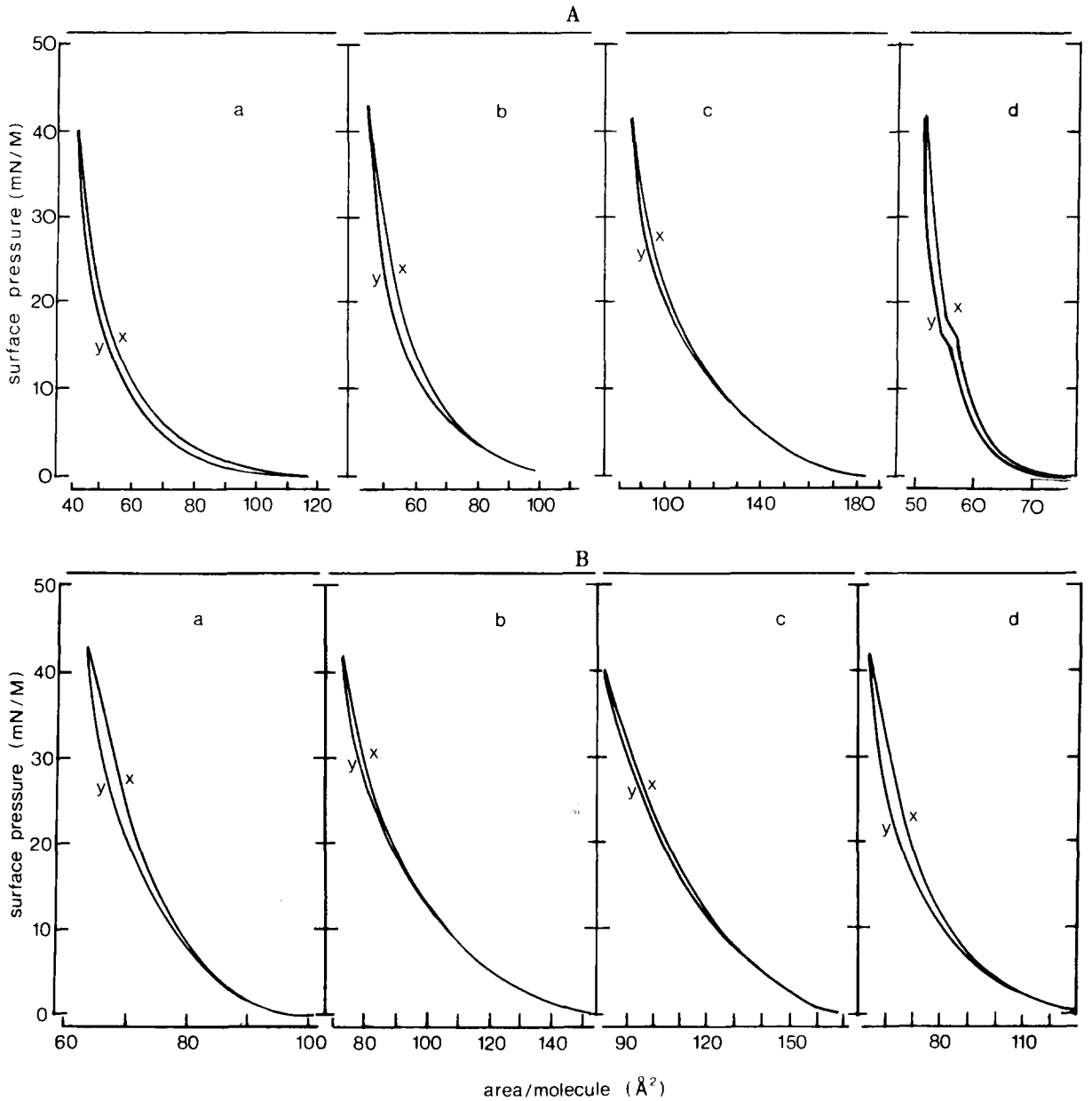


Fig. 2. (A) Surface pressure/area isotherms (on compression ($x$) and expansion ($y$)) for Azo-phospholipids at 23°C; isotherms for (3) Azo-PC, (b) palmitoyl-Azo-PC, (c) oleoyl-Azo-PC, (d) Bis Azo-PC. (B) Surface pressure/area isotherms (on compression ($x$) and expansion ($y$)) for Azo-phospholipids at 23°C after photoisomerisation to the photostationary state; isotherms for (a) Azo-PC, (b) palmitoyl-Azo-PC, (c) oleoyl-Azo-PC, (d) Bis Azo-PC.

azo-acid transition is especially clear, and there is marked hysteresis. A stable film did not form from photolysed samples.

A molecular model of the *trans* form of the azo-acid (I) is shown in Fig. 4a with a model of palmitic acid for comparison. A similar model of the *cis* form of the azo-acid is shown in Fig. 4b. It is clear that the *trans* acid is a compact molecule which might be expected to be compatible with a close-packed array such as a lipid monolayer or bilayer. The similarity to palmitic acid in overall size suggests also that the molecule might form mixed layers with the latter. The *cis* acid has some similarities to oleic acid in overall shape and size,

and might be expected to behave similarly to this in ordered systems. Fig. 5 shows molecular models of oleoyl Azo-PC for both isomers of the azo-containing chain.

The phospholipid derivatives of the *trans* azo-acid form vesicles both alone, and in admixture with DPPC. A typical electron micrograph of Bis Azo-PC vesicles is shown in Fig. 6a, while Fig. 6b shows molecular models of the *cis* and *trans* isomers of this lipid. These results are consistent with those of Shimomura et al. [15], who showed that alkyl(hydroxyethyl dimethyl)ammonium amphiphiles containing azobenzene chains formed closed bilayer vesicles. In the study cited, the alkyl chains were linked to the azobenzene chromophore as ethers, and the position of the azobenzene chromophore varied within a total chain length of 14–16 methylene units. Bilayers were formed from these lipids only when the alkyl 'tail'

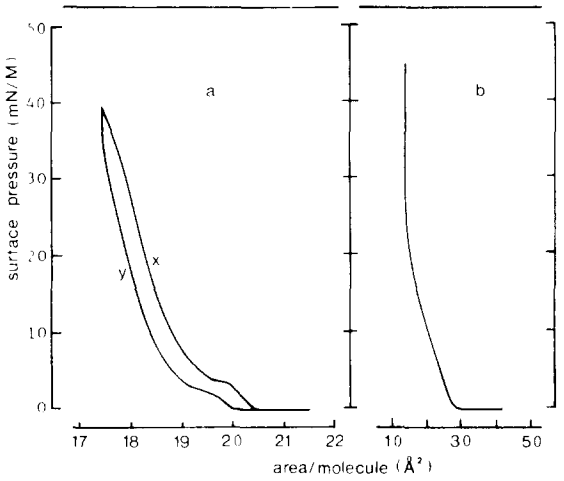


Fig. 3. Surface pressure/area isotherms at 23° C for (a) Azo-acid (I) on compression ($x$) and expansion ($y$), (b) pure stearic acid (compression only).

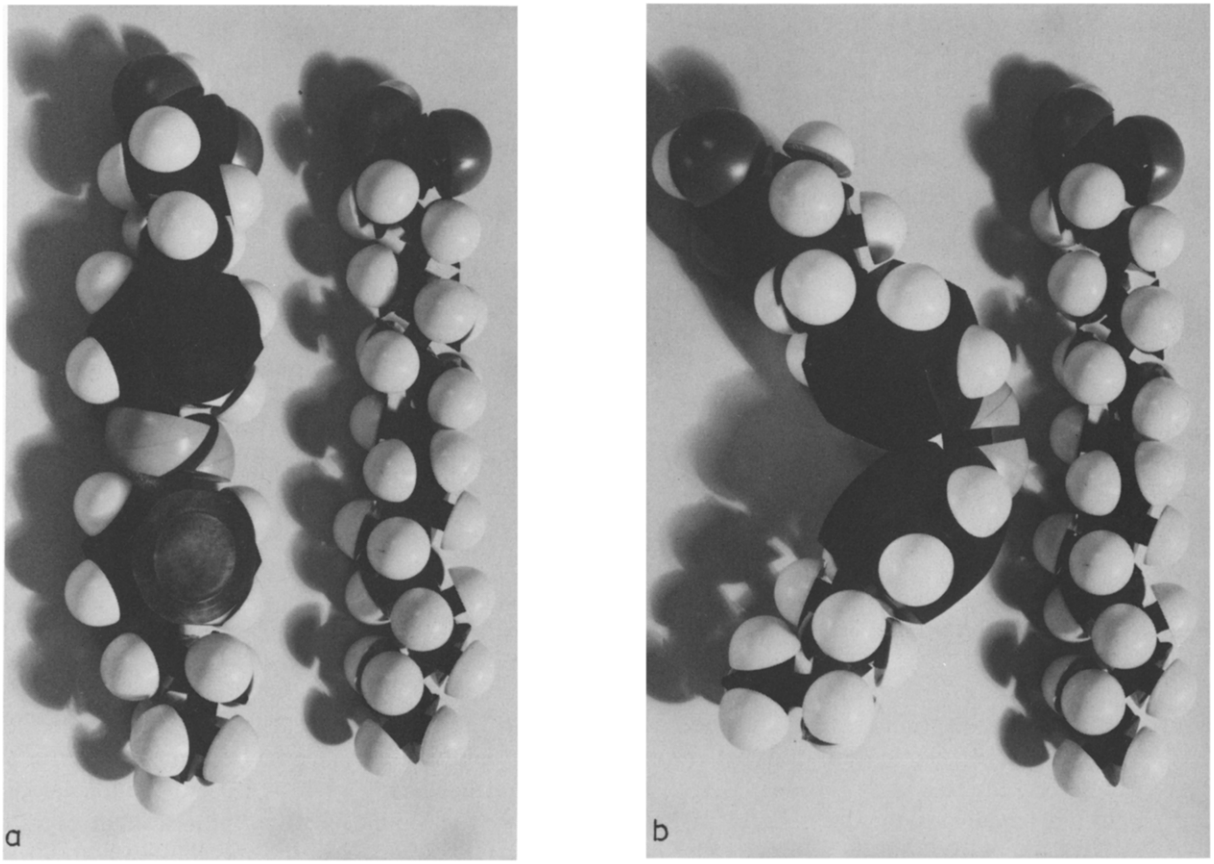


Fig. 4. (a) Molecular model of the *trans* form of the Azo-acid (I) (left), with a model of palmitic acid (right); (b) molecular model of the *cis* form of the Azo-acid (I) (left) with a model of palmitic acid (right).

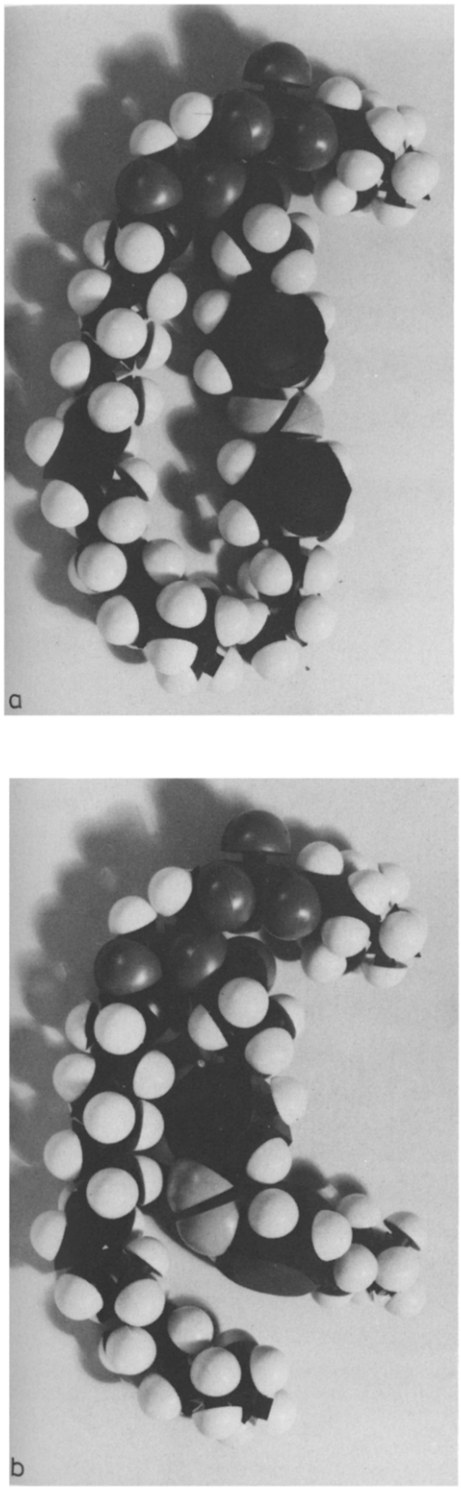


Fig. 5. (a) Molecular model of the *trans* form of oleoyl-Azo-PC; (b) molecular model of the *cis* form of oleoyl-Azo-PC.

was at least eight carbons long. A group of similar double chain dimethylammonium amphiphiles was also shown to form stable bilayers when one chain was octadecyl- and the other the azo-containing unit.

In a previous paper, we have shown that vesicles of Azo-PC are stable, and that the lipid acts as a substrate for phosphatidylcholine-specific exchange protein [11], demonstrating that the molecule is not grossly perturbed relative to a natural phospholipid. At the time of the above study, the molecules palmitoyl-Azo-PC and Bis Azo-PC had not been synthesised. The 'natural' Azo-PC would not be expected to show very clear-cut phase behaviour, since it is heterogeneous in acyl chain composition in the 1- position. However, the analogues palmitoyl-Azo-PC and Bis Azo-PC might be expected to show some signs of cooperative interactions between the chains of the lipids if the similarity between the overall size of the azo-acid and palmitic acid is a reliable indicator of compact bilayer packing. The results in Fig. 7 show clearly that for Bis Azo-PC this is the case, since the light scattering data reveal a very clear phase transition centered at a similar temperature (41.5°C) to the phase transition of DPPC, and this has been confirmed by preliminary experiments using scanning calorimetry (not shown). Light scattering shows no clear transition for vesicles of palmitoyl-Azo-PC in the temperature range studied (20–55°C). This suggests that palmitoyl-Azo-PC has a lower cooperative unit than that of Bis Azo-PC, resulting in a much broadened temperature profile. Cooperativity is determined primarily by steric- and packing considerations. A lipid such as palmitoyl-Azo-PC with dissimilar acyl chains has a broader range of packing orientations than symmetrical lipids such as DPPC and Bis Azo-PC, and this might account for lower cooperativity. The Bis Azo-PC transition is particularly interesting, in that a very clear hysteresis between heating and cooling is evident. A thorough study of the phase behaviour of palmitoyl-Azo-PC and Bis Azo-PC has been conducted using spin label probes and will be reported elsewhere.

Absorption spectra of both palmitoyl-Azo-PC and Bis Azo-PC dispersed as vesicles show blue shifts which are known to relate to interchromo-

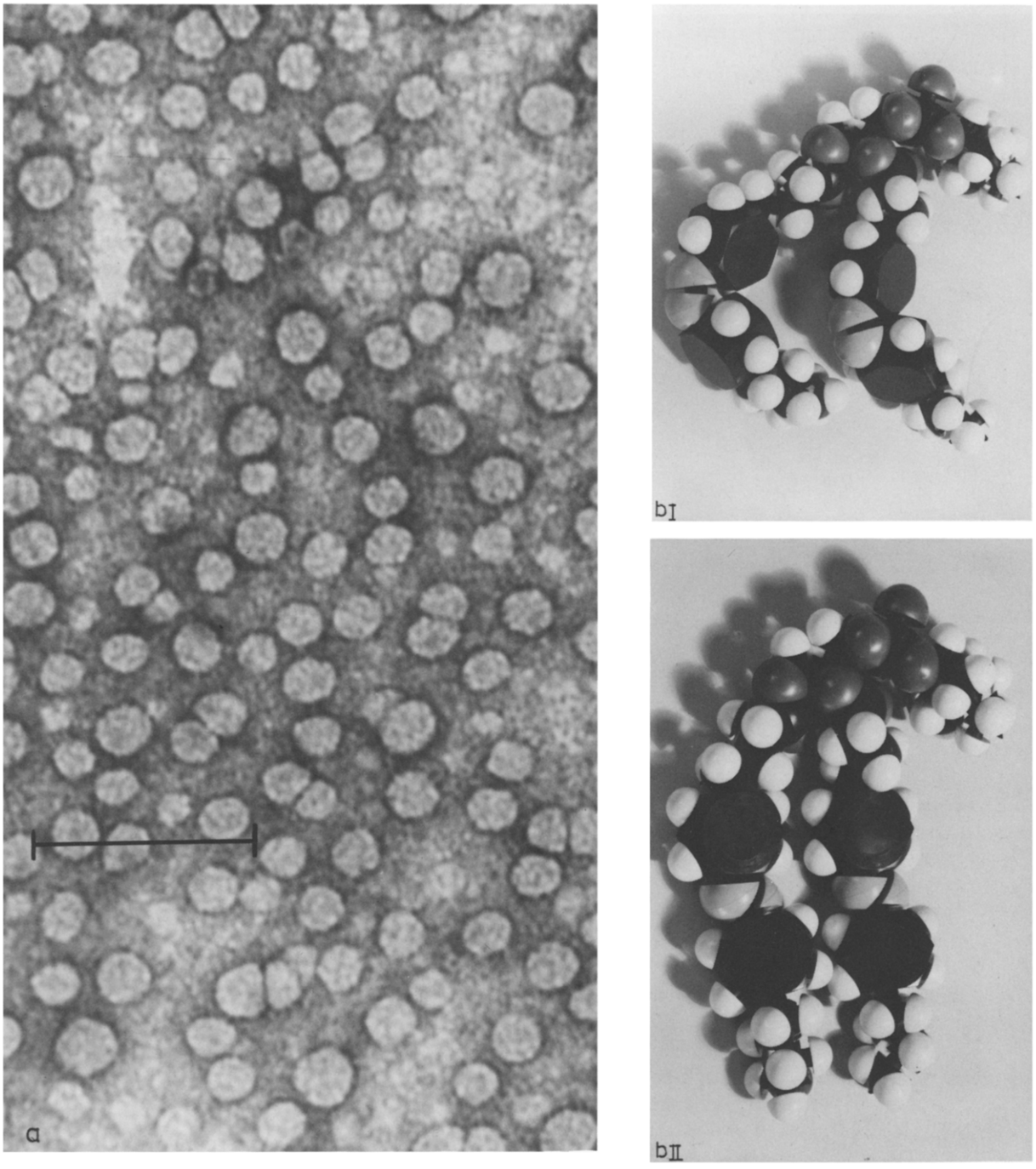


Fig. 6. (a) Electron micrograph of Bis Azo-PC vesicles. Magnification was ×120000, bar = 100 nm. (b) (I) molecular model of the *cis* form of the Bis Azo-PC; (II) molecular model of the *trans* form of the Bis Azo-PC.

phore interactions [15]. In chloroform solution the *trans* palmitoyl-Azo-PC has a maximum absorbance at 335 nm, while that of the *trans* Bis Azo-PC is at 334 nm. When dispersed as vesicles, the absorbance maxima are at 320 nm and 314 nm, respectively. In vesicles formed from DPPC

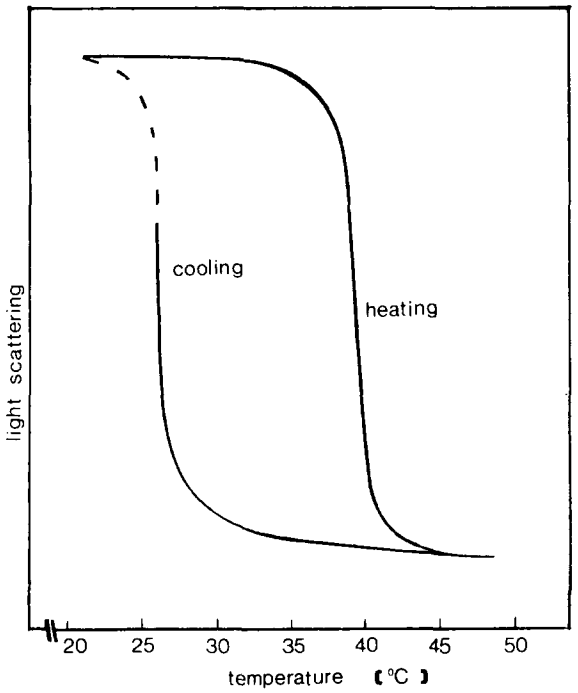


Fig. 7. Light scattering at 540 nm of small unilamellar Bis Azo-PC vesicles on heating and cooling. The slope of the dotted line (cooling curve) was a function both of time and of sample history. In some cases the scattering of the sample after cooling exceeded that before the experiment.

containing small amounts of the azo-lipids (2–5% wt./wt.), there is no evidence of inter-chromophore interaction, either above or below the phase transition temperature of the lipid, and absorption maxima are as in chloroform solution. This suggests that the presence of the azo-lipids does not grossly perturb the bilayer structure, since such perturbations commonly result in phase separations between lipid classes.

Measurements of monolayer behaviour of the azo-lipids provides quantitative information on the molecular area and packing efficiency of these materials. The isotherm for the parent azo-acid (I) from which the phospholipids were synthesised shows an area/molecule of approximately 19 $Å^2$. This agrees well with the value of 20 $Å^2$ quoted by Möbius [16] for similar azobenzene-containing amphiphiles, and is comparable to values obtained for stearic acid monolayers. Azo-PC, palmitoyl-Azo-PC and Bis Azo-PC form compact monolayers when in the *trans* form, with oleoyl-Azo-PC giving a more compressible bilayer as might reasonably be expected in the presence of the *cis*-unsaturated oleoyl chain.

The monolayer formed from Bis Azo-PC is notably rigid, and shows interesting structure not seen with the other lipids. The 'knee' in the isotherm is strongly suggestive of a phase equilibrium in the monolayer, and is commonly seen for isotherms of synthetic lipids near phase transition temperatures. The shape of such isotherms has been discussed in detail by Albrecht et al. [17] in relation to phase equilibria detected by other methods. It is noteworthy that the effect is seen for Bis Azo-PC which shows a phase transition in vesicular form, but is not evident in monolayers of palmitoyl-Azo-PC which apparently lacks a similar transition. Using the approach described in Ref. 17, it is possible to calculate an approximate cooperative unit size for the transition. The relevant formula is:

$$N\Delta a_m = (4kT_m)/\Delta\pi$$

where $T_m$ is the temperature at which the isotherm is measured (K), $\Delta\pi$ is the difference in surface pressure between the turning points of the 'knee' of the isotherm (dyn/cm$^2$), $k$ is the Boltzmann constant ($1.38 \cdot 10^{-16}$ erg $\cdot$ K$^{-1}$) and $\Delta a_m$ is the difference in molecular area across the 'knee' region (cm$^2$).

Using this approach the cooperative unit is calculated as approximately 180 molecules, which is of the same order as that calculated by Albrecht et al. [17] for DPPC monolayers (120–190 molecules depending on temperature). This interpretation of the shape of the isotherm must, however, remain tentative in the absence of a full phase diagram since direct evidence of small domains is not yet available.

The isotherm of the Azo-acid (I) shown in Fig. 3 has a very pronounced 'knee' at much lower surface pressure than that seen for Bis Azo-PC. Möbius [16] has reported similar transitions for azobenzene amphiphiles, and suggests that at very low pressure molecules are oriented parallel to the surface plane. As the pressure is increased, molecules are forced to align perpendicular to this plane, after which further compression forms a close-packed film.

Photoisomerisation of surface monolayers of all of the azobenzene amphiphiles gave rise to large

changes in apparent molecular area (Table I). The photostationary state for such molecules is a *cis/trans* mixture with typically of the order of 70–80% of the mixture in the *cis* form. It is not expected that mixtures of such lipids will necessarily behave ideally in the thermodynamic sense, and some degree of phase separation is probable. In this context, considerable hysteresis seen in the isotherms of some lipids should be noted (Figs. 2A and 2B). Slight hysteresis is not uncommon, even for apparently pure lipids, and is expected if the system has not reached true equilibrium. The significant hysteresis seen argues for the existence of metastable states, which might be clusters of phase-separated lipid, for example. The significance of hysteresis in isotherms of lipids on surfaces has been discussed by other workers [18]. In an isotherm of a lipid undergoing a change of phase, hysteresis is also possible for pure material [17]. The isotherm for Bis Azo-PC shown in Fig. 2A shows clear evidence of hysteresis in the transition region. It is not clear at present whether this is intrinsic to the phase behaviour of the material, or whether this might be due to a small amount of *cis* isomer present at the photostationary state of azobenzene-containing molecules in normal room light. The marked extent of the hysteresis, especially in the 'transition' region of the isotherm, suggests that the former explanation is the more likely, and this is borne out by the similar hysteresis of photolysed and unphotolysed samples.

All of the lipids tested could be transferred to a glass substrate. Good surface coverage (> 95%) was achieved without special precautions, and multiple layers could be deposited. It was found that monolayers tended to be lost on removal from the subphase if more than a few layers were present. It is likely that this could be avoided with suitable control of surface pressure, dipping speed and subphase ionic composition.

The results presented demonstrate the value of surface monolayer measurements of photochromic amphiphiles as a means to characterise light-induced changes in molecular structure. It is hoped to extend preliminary results here obtained, by measurements of isobars for surface films at various temperatures. This will allow the nature of the transitions observed to be more fully investigated. Langmuir-Blodgett deposition of oriented multilayers on transparent substrates will also provide a means to characterise the relative orientation of azobenzene transition moments by measurement of dichroism in absorption. Such multilayers would also prove useful for future studies of lipid order using X-ray diffraction, and it is hoped to pursue such measurements using synchrotron radiation.

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