## GENERAL DISCUSSION

**Prof. P. Fromherz** (*University of Ulm, West Germany*) said: Stretching and breaking of a macroscopic elastic sheet is described by a force/elongation curve. The integral of this relation is some measure of the energy required to create a fracture. By analogy is it possible to estimate from Prof. Evans' data the energy required to form the open edge of a lipid bilayer?

**Prof. E. Evans** (*University of British Columbia, Vancouver, Canada*) replied: At first glance, it does appear that our empirical fracture energy model could be viewed as a crack propagation model for failure of a solid surface which is characterized by an 'edge' energy as you have indicated. However, careful examination of the origin of the crack model (see Landau and Lifshitz, *Theory of Elasticity*) shows that this model is a poor model for bilayer rupture. Specifically, the crack propagation problem is analysed for shear failure in a solid, whereas the lipid bilayer above the acyl chain transition has no shear rigidity (in other words, membrane rupture is more like cavitation than crack propagation). Secondly, the crack failure problem is based on the assumption that there exists a stationary crack distribution (*i.e.* an empirical length $L$) from which the failure emanates. Again, this is a solid-like property not representative of the lipid bilayer in the liquid state.

**Dr D. S. Dimitrov** (*Bulgarian Academy of Sciences, Sofia, Bulgaria*) said: The very interesting experimental fact for me is that the critical membrane tension of rupture is proportional to the square root of the elastic area compressibility modulus. This is in agreement with the theoretical formula[1] for the critical membrane tension $T_{cr}$:

$$T_{cr}^2 = 24\sigma G h$$

where $\sigma$ is the membrane surface tension, $G = E/3$ for incompressible bodies, $E$ being Young's modulus. In the case of an incompressible membrane subjected to expansion in one direction it is equivalent to the elastic compressibility modulus $K$ multiplied by the membrane thickness. Therefore, the slope of the dependence $T_{cr}K^{1/2}$ is equal to $(8\sigma)^{1/2}$. The experimental value for the slope is 0.4 (dyn cm$^{-1}$)$^{1/2}$. This leads to a surface membrane tension equal to 0.03 dyn cm$^{-1}$, which is a high but reasonable value. I would like to point out, however, that this does not prove that the theoretical formula and especially the underlying mechanism of action of the surface tension is entirely correct. It is interesting to note that the critical surface energy corresponds to the membrane surface tension.

1 D. S. Dimitrov, *J. Membr. Biol.*, 1984, **78**, 53; D. S. Dimitrov and R. K. Jain, *Biochim. Biophys. Acta*, 1984, **779**, 437.

**Prof. E. Evans** (*University of British Columbia, Vancouver, Canada*) said: Your comment brings to light an interesting possibility for the use of your theory of failure for thin liquid films to model bilayer rupture behaviour. However, a key point to keep in mind is that your model is not an appropriate representation of the stable (condensed) bilayer structure, since no constant tension exists as for a film, only an area dependent tension. What is appealing is that the empirical 'fracture energy density' derived from fig. 3 could represent a transition from our condensed state to a liquid-like film modelled by your theory, *i.e.* similar to a transition for an ideal plastic from a solid to a liquid.

**Prof. J. F. Nagle** (*Carnegie-Mellon University, Pittsburgh, PA*) said: These are very important measurements to which theories should be compared. Here let me just make contact with one point. In your paper you mention that the enthalpy of expansion is often *ca.* 100 erg $cm^{-2}$, and you then note that this is just twice the surface free energy of hydrocarbon–water interfaces. However, on the next page you note that the enthalpy of expansion is elevated for ten degrees above $T_m$, and from table 1 one would calculate *ca.* 210 erg $cm^{-2}$. My statistical-mechanical model (specifically case 6 in my table 1) gives an enthalpy of expansion that starts out *ca.* 500 erg $cm^{-2}$, just above $T_m$. As temperature is increased by 35 K, the enthalpy of expansion gradually decreases by a factor of two. The fact that the values are too large overall is a reflection of the fact that the values of area thermal expansion in these models are generally too large. This should not be allowed to obscure the important temperature dependence, which is a manifestation of what I call post-critical phenomena (which we have also seen experimentally by a decreasing volume expansivity $\alpha_T$ in DMPC). The calculation also shows that all the interactions in the system, not just the interfacial term, contribute to the enthalpy of expansion. In the model over 70% of the enthalpy of expansion at constant temperature comes from the van der Waals interaction between chains, *ca* 20% comes from the *trans–gauche* energy, and only *ca.* 5% comes directly from the interfacial term, although it might be noted that this latter percentage increases by a factor of about two, from 4 to 7%, as the temperature increases by 35 K from $T_m$. Also, if I use the larger $\gamma$ that you prefer in my interfacial term in my eqn (4), these latter percentages will be roughly tripled. Therefore, while I would not wish to suggest that the model is capable of predicting precise numbers for these quantities, nevertheless it strongly suggests that the interfacial water term is not the exclusive source of the enthalpy of expansion. This agrees with my intuition that a lateral expansion of the membrane at constant temperature is also going to increase the volume, thereby doing work against the van der Waals cohesive energy, and that more *gauche* conformations will have to be introduced in order for the chains to spread out to fill the larger area, even at constant temperature. Since these interactions dominate the enthalpy of transition, at which area increases by *ca.* 25%, it is not surprising that they would continue to contribute significantly to the enthalpy of expansion in the single-phase region.

**Prof. E. Evans** (*University of British Columbia, Vancouver, Canada*) said: It is important to recognize that there are two components of the heat of expansion of a bilayer: the surface pressure plus the heat of expansion of the hydrocarbon–water interface. Thus, if the hydrocarbon–water interaction is constant, changes in the heat of expansion (given by the product of temperature × elastic area compressibility modulus × thermal expansivity) would represent variations in surface pressure which would be expected to decrease with temperature in the $L_\alpha$ phase (as shown for DMPC in table 2). However, interfacial interactions could be temperature-dependent, which makes the comparison vulnerable.

**Prof. J. F. Holzwarth** (*Fritz-Haber Institüt, Berlin, West Germany*) said: In your paper you examine the influence of cholesterol and peptides on the compressibility of lipid bilayer systems. We have examined similar preparations by looking into the dynamics of these systems between 1 $\mu$s and 100 ms. One important result of these experiments was that the relaxation phenomenon around 10 $\mu$s is broadened, the relaxation around 1 ms is weakened, the relaxation around 25 ms becomes broader and its amplitude decreases by a factor of two (see figures in our contributed paper). As can be seen from the equilibrium fluorescence anisotropy measurements in fig. 1, we explain this behaviour by the preference of cholesterol for lipids in an intermediate state of order. This intermediate state is clearly preserved even above the phase-transition temperature. When we incorporated a similar peptide (24) mentioned in your paper

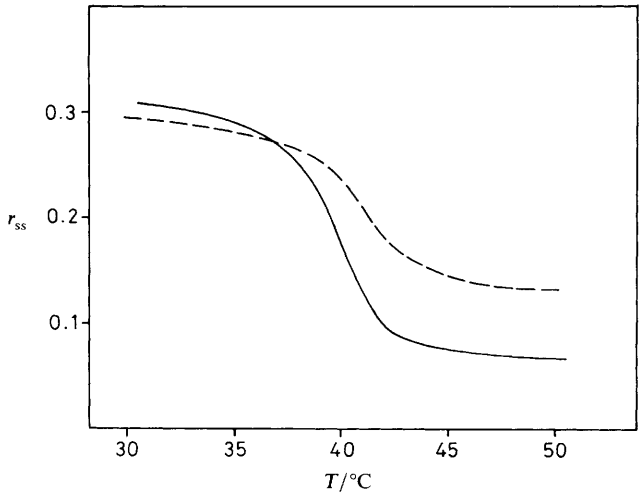


**Fig. 1.** Temperature dependence of the equilibrium fluorescence anisotropy $r_{SS} = (I - I_{\perp})/(I + 2I_{\perp})$ for unilamellar vesicles of DPPC containing diphenylhexatrian (DPH) as a probe without further additives (——) and with 15% CHOL incorporated (- - -). $\lambda_{exc} = 360$ nm, $\lambda_{obs} = 430$ nm, $c_{DPPC} = 2.7$ mmol dm$^{-3}$.

into the bilayer we found a much smaller effect, but this might be due to the low concentration of the peptide which can be dissolved in the bilayer structure without causing precipitation. We could handle preparations of SUVs containing up to 20% CHOL but only 2% peptide could be incorporated without precipitation.[1] From our results we conclude that the peptide is 10 times more effective in causing cohesion in SUVs than CHOL is. If one incorporates a channel-forming substance like Gramicidin into DPPC vesicles one finds a very dramatic effect on the slowest relaxation process around 20 ms which almost disappears at a ratio of 15/1. We are still puzzled about the dramatic differences between CHOL, peptide 24 and Gramicidin A. Could you give an explanation from your results?

1 A. Genz, J. F. Holzwarth and T. Y. Tsong, *Chem. Phys. Lipids*, 1986, in press.

**Prof. E. Evans** (*University of British Columbia, Vancouver, Canada*) said: In our experiments, the addition of cholesterol (0–50 mol%) to PC lipids caused a decrease in bilayer compressibility over a wide temperature range. Also, tensions for vesicle lysis (rupture) increased with cholesterol concentration. Together, these results indicate an increase in bilayer cohesion *via* the formation of a 1:1 lipid cholesterol complex. At all concentrations above 12.5 mol%, the bilayers behaved in a liquid-like manner (no resistance to shear deformation). In this respect, the lipids appear to be in an 'intermediate state of order' as you put it, *i.e.* low compressibility due to head-group and outer-chain condensation, but still fluid as a result of peripheral chain expansion at the bilayer centre. Our lipid–polypeptide system showed a slightly higher compressibility and lower lysis tension than for pure SOPC bilayers. This result indicated a decrease in cohesion, consistent with a lipid wetting model where the shorter peptide causes chain compression in the adjacent lipid. In your experiments, the mismatch between peptide–lipid hydrophobic length is opposite to our case; so, the larger peptide would be expected to decrease compressibility by extension of the shorter acyl chains. Your observation that the peptide is 'ten times more effective' in increasing cohesion than cholesterol may reflect the different stoichiometry and nature of the two interactions.

For example, cholesterol appears to form a 1:1 complex with lipid whereas each polypeptide molecule interacts with several nearby lipids (*ca.* 7–10) in each monolayer.

**Dr G. Cevc** (*University of Essen, West Germany*) asked Prof. Sackmann three questions.

(1) Some of the bilayer surface invaginations that your elegant experiments have demonstrated look very much like closed vesicles. Surely, if they were such their formation would be irreversible; could you give us an estimate of the degree to which the opposing bilayers come towards a complete closure?

(2) The vesicle shape-transformations which you report are characterized by a steep temperature dependence, similar to that of lipid chain-melting. Is the relative rate at which these macroscopic transformations occur similar to that of the lipid chain fluidization; have you considered performing $T$-jump experiments to investigate this or to obtain further insight into the molecular origin of the vesicle shape transformations?

(3) How do the rate of vesicle shape-transformation and the final vesicle form depend on the degree of 'surface polymerization'?

**Prof. E. Sackmann** (*University of Munich, West Germany*) replied. (1) The neck connecting the two vesicles and the distance of approach of the opposing bilayers are beyond the limit of resolution of the microscope. (2) The rate of the shape changes is an interesting point since it is directly related to the question whether the shape changes are first order transitions. The transitions are indeed instantaneous within the response time of our camera (*ca.* 0.01 s). (3) Since it is very difficult to determine the progress of polymerization of the individual vesicles we cannot answer this question yet.

**Dr W. S. Bont** (*Amsterdam, The Netherlands*) said: In the introduction it is stated that endocytosis takes place *via* invagination of the plasma membrane and exocytosis by protrusion. In general this is not correct, as demonstrated by the secretion of hormones. Hormones and many other products are stored in vesicles inside the cells. The 'export' of these substances takes place by fusion of the membrane of the vesicles with the plasma membrane, resulting in an omega-shaped membrane; the material is now outside the cell. This demonstrates that both endocytosis and exocytosis occur *via* invagination of the plasma membrane. The protrusion shown in fig. 1(*b*) leads to shedding of vesicles, a phenomenon observed with tumour cells.

**Prof. E. Sackmann** (*University of Munich, West Germany*) replied: I agree with your remark that the initial step of exocytosis is preceded by the fusion process. Thus fig. 1(*b*) refers to shedding of vesicles or to the process of budding.

**Prof. B. Žekš** (*Institute of Biophysics, Ljubljana, Yugoslavia*) addressed Prof. Sackmann.

(1) You have clearly demonstrated that the variation of temperature can induce shape transformations of vesicles made of charged lipids. As one can see in plate 2 the vesicle attains a two-vesicle shape with large average curvature at increased temperature (43 °C), goes through a discoid shape at intermediate temperature (42 °C) and develops an internal vesicle with small average curvature at lower temperature (41 °C), which agrees with theoretical predictions. I would only like to point out that the shapes with external and internal vesicles, respectively, do not belong to the same class of shapes, and therefore the transition between these two shapes cannot be expected to be continuous. Somewhere between the shapes (*a*) and (*b*) in plate 2 one would expect to observe a discontinuous shape transformation analogous to a first-order phase transition. On the other hand it would be interesting to know what happens with a vesicle if the temperature is increased above 43 °C or decreased below 41 °C.

(2) I would expect similar but smaller effects for uncharged lipids. Because of the anisotropic thermal expansion of the two monolayers the relative area difference $\Delta A/\Delta A_s$ should change with temperature and therefore induce shape transformations.

(3) Have you observed some changes of shapes with time? A non-spherical shape with given $\Delta A$ has a larger bending energy than the corresponding sphere, and this energy difference represents a driving force for water flow into a cell coupled to lipid flip-flop processes. Such shape transformations are expected to be slow because of large flip-flop relaxation times and because of small membrane permeability for water. Nevertheless, this is in my opinion a process which makes small vesicles spherical and could also be important for large vesicle shape changes which do not require large volume changes.

**Prof. E. Sackmann** (*University of Munich, West Germany*) replied: (1) The transitions between shapes *d* and *e* or *b* and *c*, respectively, occur spontaneously within the response time of our TV camera, and the same holds for the discocyte-to-stomatocyte transition. These are thus of first-order. The transition from shape *b* to the discocyte is difficult to observe since the two states are hard to distinguish in the microscope. (2) I agree with you. However, we did not succeed in observing the transitions for giant uncharged vesicles. It may be that the range of temperature over which the shape changes occur is too large. (3) The giant vesicles of non-spherical shapes transform into spherical shapes after some hours which may be due to a flip-flop process as suggested by you.

**Prof. T. W. Healy** (*University of Melbourne, Australia*) said to Dr Jones: The conclusion that the authors arrive at, *viz.* that the phospholipids are adsorbed with their head groups uppermost, is perhaps too simplistic in the light of earlier studies on adsorption of alkylethoxylate non-ionic surfactants by Ottewill's group at Bristol and in my own group. Thus for adsorption of such non-ionics on hydrophobic silica (contact angle 85 °) and hydrophilic silica (contact angle 0 °) the detailed isotherm is a high-affinity type on the hydrophobic surface and an S-shaped (autocatalytic) type on the hydrophilic silica. The plateau adsorption is identical on both substrates at a notional coverage of *ca.* 'one monolayer'. The adsorption on hydrophobic silica is 90% of the plateau value at $C/\text{c.m.c.} = 1$ and *ca.* 15% for the hydrophilic surface at $C/\text{c.m.c.} = 1$. At $C/\text{c.m.c.} > 1$ the adsorption process generates the same contact angle of *ca.* 28 ° for both surfaces.

Given that the forces controlling micelle (or vesicle) formation and transfer into an adsorbed layer at the solid-water interface are essentially the same, all one can conclude is that the surfactant forms a layer at the solid-liquid interface which best balances the hydrophobic-hydrophilic energies of the surfactant in water. With the hydrophilic surface it appears more difficult to nucleate adsorbed areas or (hemimicelles) into which the hydrophobic chains can transfer. Further analysis of the low-coverage part of the isotherms of Jackson *et al.* may indeed reveal S-shaped behaviour. I refer to the points above the line at low concentration in fig. 3. In contrast, the adsorption of CTAB on hydrophilic silica, mica *etc.* is able to produce an ordered monolayer anchored electrostatically followed by bilayer formation with the head groups out.

For non-ionic alkylethoxylates and zwitterionic phospholipids adsorbed on hydrophilic silica, the adsorbed layer is perhaps better described as a partly ordered, part hydrophobic, part hydrophilic layer of an area per molecule corresponding to apparent monolayer coverage.

**Dr M. N. Jones** (*University of Manchester*) replied: These are interesting comments and we agree that the apparent monolayer coverage we find for DPPC and the other phosphatidylcholines could have an alternative interpretation as you suggest. We have deposited phospholipid films from monolayers by the Langmuir-Blodgett technique and from vesicle dispersions onto glass slides and measured the advancing contact angle

of water. Both techniques gave very similar contact angles. The contact angle for DPPC deposited on hydrophilic ($\theta \approx 0°$) soda-lime glass is 45 °, suggesting either a monolayer with heterogeneous orientation or the formation of islands or clusters of DPPC interspersed by 'clean' surface. However, deposition of lipid onto glass from mixed DPPC-PI monolayers results in a decreasing contact angle with increase in PI to DPPC ratio, and for pure PI deposition the contact angle is *ca.* 9 °. This suggested to us that the orientation of the lipid was changing with PI content in the system and in the extreme case (pure PI) the head groups were largely uppermost. In this case island formation perhaps seems less likely in view of the negative charge on the phosphate group and the hydrophilic nature of the pentahydroxyinositol group. Both these factors would inhibit the nucleation of PI clusters with head groups down on a negatively charged glass surface.

It should, however, be noted that the phospholipids probably initially adsorb with their head groups onto the glass and then subsequently change their orientation in aqueous media as was postulated many years ago by Langmuir.[1]

With regard to the possibility that the adsorption isotherms are S-shaped at low surface coverage, our most recent more detailed analysis of the shape of the isotherms has given no indication that they are S-shaped at low concentrations.

1 I. Langmuir, *Science*, 1935, **87**, 493.

**Prof. J. K. Thomas** (*University of Notre Dame, IN*) commented: In some recent work we have constructed 'bilayers' of various quaternary ammonium detergents on the surface of laponite clays. The detergent should be adsorbed in close proximity on the surface, yet no evidence of 'true' bilayers is found' *i.e.* no phase transaction in d.s.c., and the pyrene excimer formation is indicative of micelles, and not that explicit for bilayers as shown in our paper. An organic hemimicelle or similar structure is formed. Could similar structures form in your system? Such explicit behaviour in physical studies (pyrene excimer) lends much evidence to our earlier paper.

**Dr M. N. Jones** (*University of Manchester*) replied: This is a similar point to that raised by Prof. Healy, and we refer to the previous reply. However, with regard to the specific application of pyrene monomer–excimer equilibrium to the characterisation of the structure of adsorbed lipid, to observe a high excimer to monomer ratio requires restriction of the pyrene to localised regions of the surface (*e.g.* as in hemimicelles) or alternatively to pyrene-rich regions in a rigid bilayer structure which could arise as a result of lateral phase separation below the phase transition temperature. It is possible that a high excimer to monomer ratio could also occur by lateral phase separation in a tightly packed monolayer where excimer dissociation and pyrene diffusion is restricted. Adsorption at a solid surface has been shown to incease lipid phase transition temperatures indicative of restricted mobility [see ref. (1)]. While hemimicelle formation cannot be excluded as a possible explanation of our data, more detailed information on pyrene in other types of ordered lipid system (monolayers) is perhaps required before the technique can give firm evidence of hemimicelle formation.

**Dr L. R. Fisher** (*CSIRO, Sydney, Australia*) commented: It seems to me that your finding of monolayer adsorption on barium titanate is perfectly explicable if barium titanate glass has a hydrophobic surface. Have you determined the contact angle of water on this surface to check whether this is the case? Horn's results were obtained, of course, on mica, which is a hydrophilic surface with $\theta \approx 6°$ as measured by our methods.[1]

1 L. R. Fisher, *J. Colloid Interface Sci.*, 1981, **72**, 200.

**Dr M. N. Jones** (*University of Manchester*) answered: We have no direct measurements of the contact angle of water on the barium titanate beads. However, Mingins

and Scheludko[1] have studied the behaviour of these beads by attachment to the surface of a pendant drop. They report that the clean beads will not form a three-phase contact at the drop–air interface in the absence of adsorbed surfactant (in their case $C_8$TAB), which does suggest that the surface of the clean beads is completely wetted and hydrophilic. (The cleaning procedures used by us and by Mingins and Scheludko are essentially the same.) Perhaps what is more significant is that the contact angle for a glass surface with a deposited monolayer of phospholipid varies with the phospholipid composition (DPPC:PI ratio) which implies a change in lipid orientation on the same surface. Thus the nature of the lipid is critical in determining the orientation at the glass–aqueous solution interface, which suggests to us that even with a hydrophilic surface it is possible to orientate some lipids with their headgroups uppermost.

1 J. Mingins and A. Scheludko, *J. Chem. Soc., Faraday Trans. 1*, 1979, **75**, 1.

**Dr L. R. Fisher** (*CSIRO, Sydney, Australia*) continued: The extent of disruption of your vesicles (*e.g.*, fig. 5) seems much higher than is needed to provide the material for monolayer coverage of the bead surfaces. Can you suggest any mechanism to account for the additional disruption?

**Dr M. N. Jones** (*University of Manchester*) replied: This is indeed what we find, and it does appear that the beads catalyse vesicle disruption. The results could be interpreted by assuming that exchange between adsorbed lipid and vesicular lipid continues after surface saturation, resulting in disruption. Alternatively there is no reason to assume that adsorption of all the lipid from a single vesicle occurs, but rather that the build-up of surface coverage arises by transfer of a proportion of lipid from a larger number of vesicles with concomitant disruption.

**Dr D. S. Dimitrov** (*Bulgarian Academy of Sciences, Sofia, Bulgaria*) commented in introducing his own paper: I would like to point out several additional results we have obtained:

We have studied the effects of a.c. fields for two cases: first, on ready-formed liposomes and second, on liposome formation. We have observed that liposomes and other structures formed during lipid swelling in water vibrate with the same frequency as the applied a.c. field. On increasing the amplitude of the field the amplitude of the vibrations increases. Unfortunately, we do not have appropriate equipment to record the amplitude as a function of the voltage and the frequency. Instead, we have used the observations that at fixed voltage the amplitude decreases with increasing frequency and that above certain frequency visually one cannot see any change in the position of the liposome, *i.e.*, above this frequency the vibrational amplitude is very small, at least smaller than the optical resolution of the microscope (in the worst case say 1 $\mu$m). This characteristic frequency depends on the applied voltage. Increasing voltage leads to an increase of this characteristic frequency.

Fig. 2(*a*)–(*d*) shows the dependence of the characteristic frequency on the applied voltage for different conditions. The main conclusion is that this dependence does not depend on the lipid and the solution osmolarity. Probably this is a pure electrokinetic effect. Fig. 3 shows the patterns of the liquid motion.

The most important [plate 1(*a*)] observation is that without field there is no liposome formation from egg lecithin. The application of an a.c. field (frequency 10 Hz, voltage 2 V) leads to formation of giant thin-walled liposomes and many small vesicles [plate 1(*b*) and (*c*)].

The neutral lipid DMPC forms just a few giant thin-walled liposomes [plate 2(*a*)]. Application of an a.c. field leads to an increase of the number of these liposomes by at least ten times [plates 2(*b*) and (*c*)].

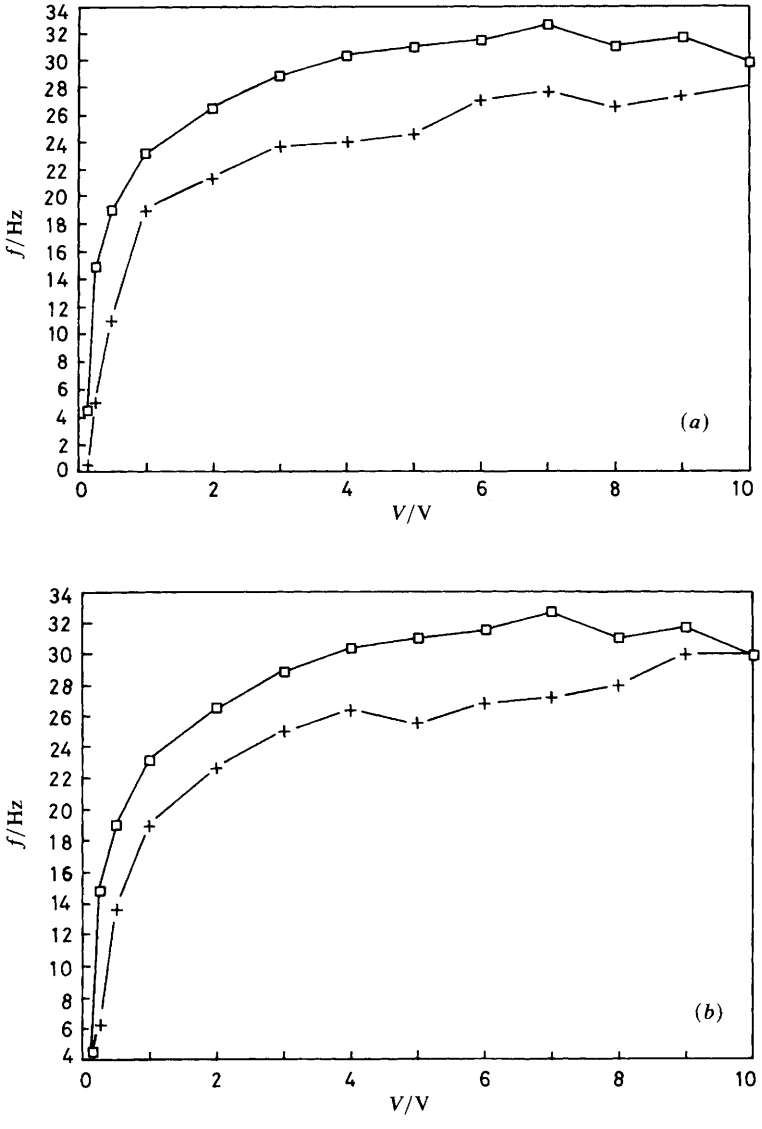


**Fig. 2.** (*a*) Egg lecithin-water, $T = 30$ °C $V = 2$ V, $f = 10$ Hz, $n = 80$, $t = 1$ h, 2% Ficoll, □, inner side; +, outer side. (*b*) Egg lecithin-DMPC-water, $T = 30$ °C, $V = 2$ V, $f = 10$ Hz, $n = 80$, 24, $t = 1$ h, 2% Ficoll; □, egg lecithin; +, DMPC. (*c*) egg lecithin-water, $n = 80$, $T = 30$ °C, $t = 1$ h, 2% Ficoll, □, $V = 0$, water; +, $f = 10$ Hz $V = 2$ V, water; ○, $V = 0$ V, 0.5 mol dm$^{-3}$ sucrose (*d*) DMPC-water, $n = 24$, $T = 30$ °C, $V = 2$ V, $f = 10$ Hz, 2% Ficoll, □, 60 min swelling; +, 30 min swelling.

The negatively charged lipid PS does not form liposomes by swelling either in the presence or in the absence of an electric field. The lipid layer breaks into flakes and detaches from the electrode surface (plate 3). (The bar denotes 50 $\mu$m.) It seems that there is a certain similarity between the action of the a.c. field and the effects of sonication when producing small vesicles. In both cases mechanical stresses may play a role in separating and destabilizing the membranes to form liposomes.

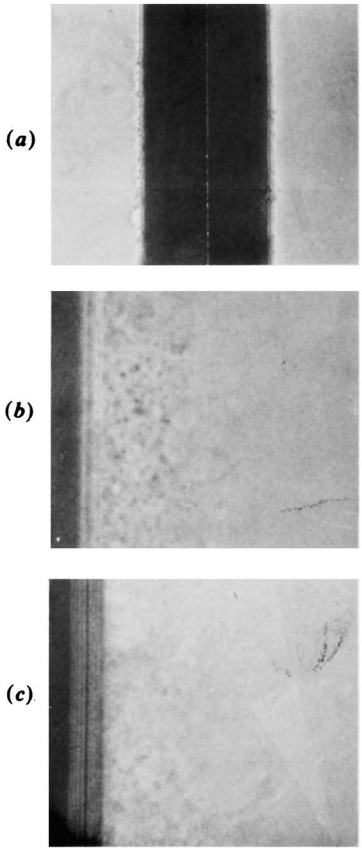


**Plate 1.** Egg lecithin swelling: (*a*) without external field; (*b*) and (*c*) in an a.c. field of $f = 10$ Hz, $V = 2$ V.

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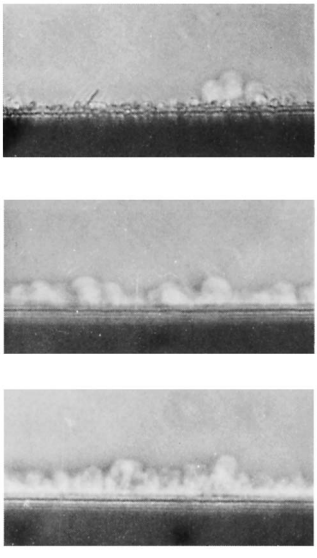


**Plate 2.** DMPC swelling: (*a*) without external field; (*b*) and (*c*) in an a.c. field of $f = 10$ Hz, $V = 2$ V.

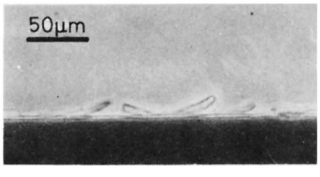


**Plate 3.** PS swelling: no liposome formation either without or in the presence of an external electric field.

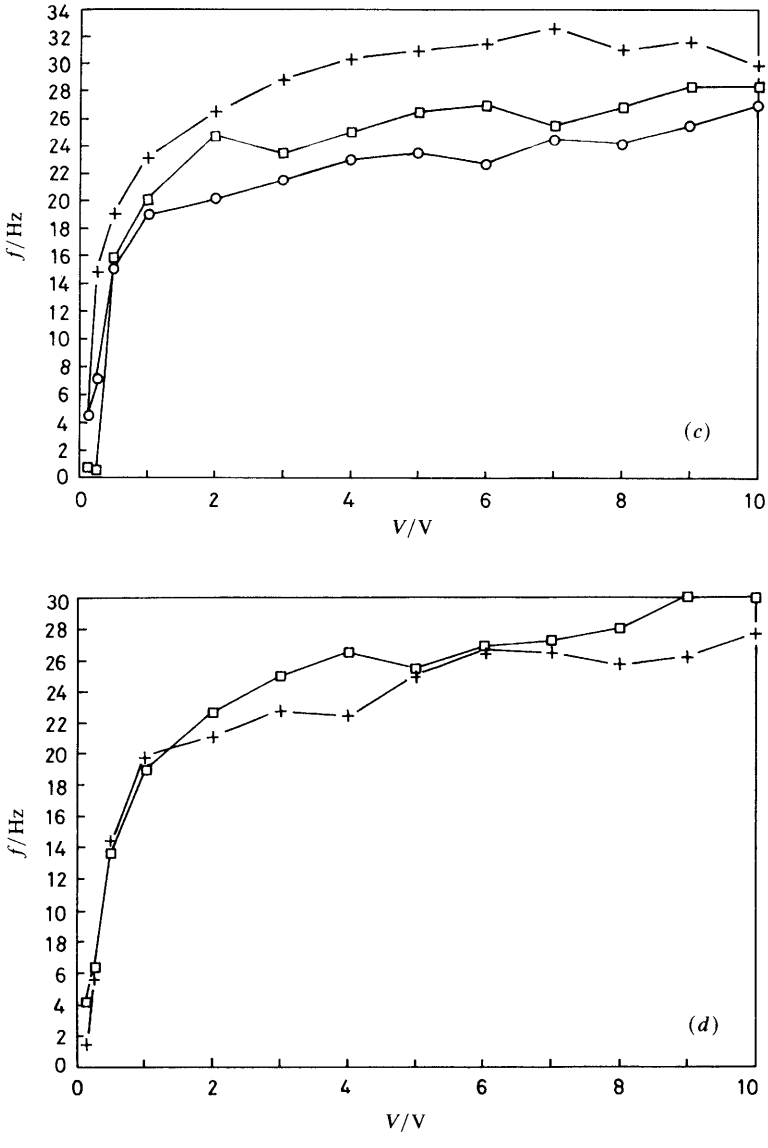


**Fig. 2.** (*continued*)

**Prof. P. Fromherz** (*University of Ulm, West Germany*) said: You have mentioned that cholate facilities the formation of vesicles by electrical fields. As cholate is a typical edge-active agent lowering the edge-tension[1] I suggest that the electrical field strength (the drop of the applied voltage in the lipid multilayer) is effective through its interaction with the edge.[2] According to Gibbs' equation the edge-tension is lowered with an applied electrical field if there exists a change of dipole moment across the membrane with increasing length of edge. Considering the dipolar structure of the headgroup of lipids and a simple model of the edge[3] Gibbs equation may be integrated for simultaneous presence of electrical field and edge-actant.[2] A mutual reinforcement of the two protagonists is predicted. The description would be consistent with your hypothetical

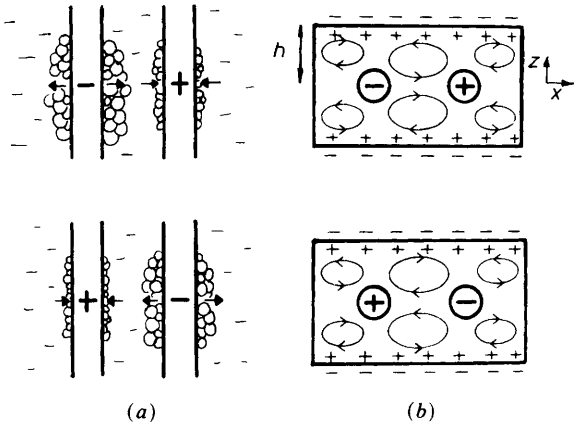


**Fig. 3.** (*a*) Vibrations of the lipid structure follow the electric field alternations; (*b*) patterns of the electro-osmotic liquid motion in a.c. fields.

sketch of primary formation of open edges with subsequent closure of the fragments to vesicles.

1 P. Fromherz, *Faraday Discuss. Chem. Soc.*, 1986, **81**, 39.
2 P. Fromherz, *Chem. Phys. Lett.*, submitted for publication.
3 P. Fromherz, in *Reverse Micelles*, ed. P. L. Luisi and B. E. Straub (Plenum Press, New York, 1984), p. 55.

**Dr D. S. Dimitrov** (*Bulgarian Academy of Sciences, Sofia, Bulgaria*) said: It is a very interesting suggestion which should be checked thoroughly. Presently, we are performing a number of experiments to understand the relationships between cholate and electrical fields.

**Prof. E. Sackmann** (*University of Munich, West Germany*) said to Dr Dimitrov:

(1) I am intrigued by your electroformation experiment. Since you apply a d.c. electric field I wonder whether the formation of vesicles is not caused by local hydrodynamic flows due to electrohydrodynamic effects in the liquid-crystalline layer on the electrode?

(2) In your model you assume that the multilayer system is composed of platelets of bilayers which implies a high edge energy. On the other side, in freeze-fracture experiments we always find that multilayers are made up of closed (compressed) vesicles, so that only edge dislocations are formed. Do you have other experimental evidence for your stack model?

**Dr D. S. Dimitrov** (*Bulgarian Academy of Sciences, Sofia, Bulgaria*) replied: (1) Hydrodynamic flows exist and have been observed when applying d.c. and a.c. fields. For d.c. fields the flow velocity is high in the beginning and sharply decreases after a couple of seconds, probably due to charging effects. For a.c. fields the liquid motion depends on the frequency. The liposome yield is different at different frequencies. Certainly, hydrodynamic effects contribute to liposome formation. It seems, however, that electrostatic and double layer effects layer effects dominate. We are not able to check for local electrohydrodynamic effects in the very liquid-crystalline layer. Such effects could cause or facilitate liposome formation. (2) We do not have any experimental evidence for the stack model. It is just a hypothesis. This model is for the structure of the dried lipid layer and at the very beginning of the hydration.

**Dr A. Nelson** (*IMER, Plymouth*) said to Dr Dimitrov: I was interested to read your paper since it addresses itself to the behaviour of phospholipids in an electric field. We have also studied the effect of electrical potential on phospholipids so our work has some relevance to yours. We perform our experiments on phospholipid monolayers adsorbed on mercury electrodic surfaces and examine the effects of both d.c. and a.c. fields. Two significant transitions are observed in the cathodic potential domain. The first of these is an increase in permeability of the layer to ions and the second at more negative potentials represents a major but reversible disruption of the membrane.[1] Both transitions are sensitive to the presence of counter-ions. The method of adsorbing phospholipid layers on to mercury electrode surfaces was first developed by Miller.[2]

It seems to be that the electric field induced transitions relate in some way to the formation of liposomes which you observe since they occur in the negative potential domain. First I must ask you what is the electrolyte composition which you use in your experiments? You are not very clear about this in your paper. Indeed, you mention that you use distilled water and that you found that high ionic strength inhibits liposome formation. However, the use of distilled water will create a large resistance between your electrodes and lower the magnitude of electric field across the phospholipid layers. On the other hand, you speak of the redistribution of counter-ions as being a contributory factor. Have you investigated this experimentally?

1 A. Nelson and A. Benton, *J. Electroanal. Chem.* 1986, **202**, 253.
2 I. R. Miller, J. Rishpou and A. Tenenbaum, *Bioelectrochem. Bioenerg.*, 1976, **3**, 528.

**Dr Nelson** then added: In this instance one immediately asks why liposomes do not form in the presence of significant ionic strength electrolytes although you suggest that this is due to osmotic forces.

I would therefore like to suggest that your liposome formation experiments could be conducted under more precise conditions of electrolyte concentration, pH buffering and potential control. In this way, electrochemical measurements of current and capacitance would aid in the elucidating the mechanism of liposome formation which you are still not clear about in your paper.

**Dr D. S. Dimitrov** (*Bulgarian Academy of Sciences, Sofia, Bulgaria*) replied to Dr Nelson: Your results about effects of electrical fields applied across phospholipid monolayers will undoubtedly be helpful in understanding mechanisms of liposome electroformation. One of the problems is that in our experiments the potential across the lipid layers is practically zero because the lipid is deposited as separate spots on the electrodes and its thickness is very small.

We have used distilled water with conductivity 4 $\mu$S cm$^{-1}$ and pH 6.8, as well as PBS (0.3 mol dm$^{-3}$). As I mentioned above, the magnitude of the electric field across the lipid layers is very small, independent of the resistance of the medium.

We have not investigated the redistribution of the counter-ions. We think that the double layers around the electrode and between the membranes are changed due to the external electrical field. At the negative electrode the positive counter-ions are attracted and the double-layer thickness is decreased. This leads to an increase of the intermembrane repulsive forces and therefore to membrane separation and liposome formation. It is very difficult to develop a theory for electrostatic intermembrane interactions in external electrical field and near to the electrodes. Presently, we are making some estimates. Liposomes do not form in solutions of high ionic strength because of the decrease of the repulsive intermembrane electrostatic forces. We have stated that the increased osmolarity can decrease the rate of swelling because of the decreased osmotic forces.

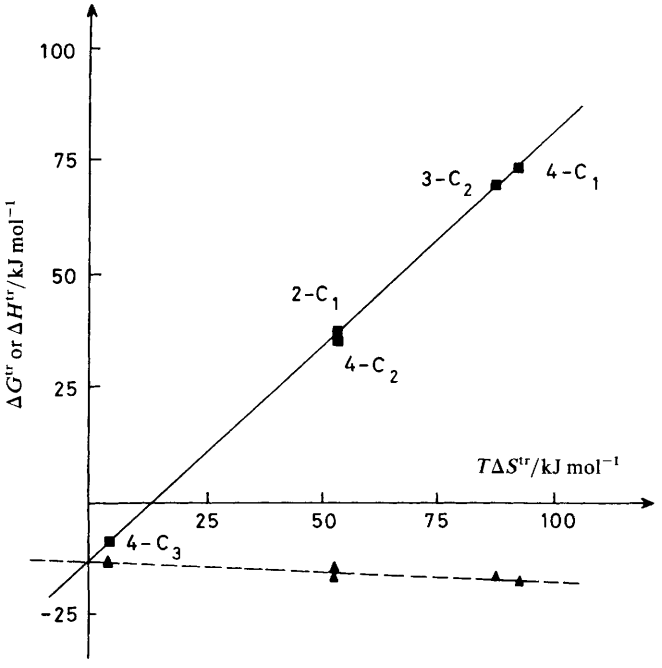


**Fig. 4.** The relation between $\Delta G^{tr}$ (▲), $\Delta H^{tr}$ (■) and $T\Delta S^{tr}$ for the transfer of alkylphenols ($m$-$C_n$) from cyclohexane to liposomes.

**Dr B. Kronberg** (*Institute for Surface Chemistry, Stockholm, Sweden*) addressed Dr Anderson: When studying the ordering in liquids through transfer functions it is customary to use a non-structured liquid as a reference liquid. In this work the results of transferring probes from water to lipid membrane are discussed in terms of the destruction of order in the lipid membrane. The choice of water as a reference liquid in this work is, however, inappropriate, since water itself is structured in different ways by the presence of different probes. Thus the transfer functions in table 3 also reflect the influence on the structuring of the water by the probes.

This problem can, however, be circumvented by using cyclohexane as the reference liquid. Thus the transfer function of interest is

$$\Delta X^{tr}_{\text{cyclohexane} \rightarrow \text{liposome}} = \Delta X^{tr}_{\text{water} \rightarrow \text{liposome}} - \Delta X^{tr}_{\text{water} \rightarrow \text{cyclohexane}}$$

where $X$ is either $G$, $H$ or $S$.

These transfer functions are, of course, sensitive to the chemical dissimilarity between the probes, and hence only probes in a homologous series can be compared. From tables 2, 3 and 4 we obtain the relation between $\Delta G^{tr}$, $\Delta H^{tr}$ and $T\Delta S^{tr}$ for alkylphenol probes as shown in fig. 4. A large $T\Delta S^{tr}$, or $\Delta H^{tr}$, is associated with the destruction of order in the lipid membrane. The figure reveals the typical enthalpy–entropy compensation, which is inherent in the process of destruction, or build-up, of order. The intercept, at $T\Delta S^{tr} = 0$, is due to the chemical difference the probe experiences when transferred. Thus it can be attributed to hydrogen bonding of the phenyl group in the lipid bilayer. It should be interesting to investigate larger n-alkyl groups in the $p$-position on the phenol group, in order to obtain an ordering upon transfer from cyclohexane, *i.e.* $\Delta S^{tr} < 0$. Such a net ordering should be experienced by the probe in the lipid bilayer, since in the reference liquid the probe is completely disordered.

**Dr N. H. Anderson** (*Long Ashton Research Station, Bristol*) replied: The objective of our work was to compare the partitioning of solutes between water and liposomes with that between water and organic solvents, as indicated in the paper. Thus the thermodynamic analysis was based on the comparison of water–organic phase transfer, but Dr Kronberg is correct in pointing out that the thermodynamic transfer functions for cyclohexane–liposome can be derived from our data and these functions are of interest. In the paper we explain that it is unwise to use plots of enthalpy against entropy to test for enthalpy–entropy compensation, but for the alkylphenols I have confirmed that compensation does exist for the transfer from cyclohexane to liposomes. Because the liposome structure is complex the intercept is likely to represent the sum of several interactions including that due to hydrogen bonding.

**Prof. J. F. Nagle** (*Carnegie-Mellon University, Pittsburgh, PA*) said: I was pleased to see that your $\Delta H$ of *ca.* 5.7 kcal mol$^{-1}$ for pure DMPC, obtained with a Perkin Elmer DSC-2 instrument, agrees with the values obtained with the more sensitive, slower scanning rate Privalov and Microcal calorimeters. In contrast, DSC-2 measurements over the years have tended to give 6.6 kcal mol$^{-1}$. I am curious if you also measured $\Delta H$ for other lipids such as DPPC and if your values agree with the commonly obtained value of *ca.* 8.7 kcal mol$^{-1}$.

**Dr N. H. Anderson** (*Long Ashton Research Station, Bristol*) replied as follows: The reported $\Delta H$ of 5.7 kcal mol$^{-1}$ for the DMPC liposomes (prepared by hand-shaking) was obtained using a scanning rate of 5 K min$^{-1}$. However, a previous determination using DMPC liposomes prepared by mechanical agitation gave $\Delta H = 7.5$ kcal mol$^{-1}$.[1] $\Delta H$ values for DPPC and DSPC were 9.51 and 12.31 kcal mol$^{-1}$, respectively,[1] supporting the view that the Perkin Elmer DSC-2 instrument normally gives a higher $\Delta H$ than slower scanning, more sensitive instruments. Possibly the precise method of preparing the liposomes and hence their size distribution affects the enthalpy of transition.

1 M. Ahmed, J. Hadgraft, J. S. Burton and I. W. Kellaway. *Chem. Phys. Lipids*, 1980, **27**, 251.

**Dr G. J. T. Tiddy** (*Unilever Research, Port Sunlight*) commented: A simple calculation based on the Clausius–Clapeyron equation suggests that a solute mole fraction of 0.1 in the lipid should reduce the $L_\alpha$/gel transition temperature by *ca.* 3 K. In fact the dispersion without solute (two components) is a two-phase sample which must have a sharp transition according to the phase rule. With the solute present there are three components, hence three phases (dilute aqueous phase, $L_\alpha$, gel) can coexist over a range of temperatures. In this case the compositions are expected to vary with temperature. I would expect the solute to be preferentially solubilised in $L_\alpha$, hence stabilising this phase as more and more gel phase (with a much lower concentration of solute) is formed. Thus the $L_\alpha$ phase would become richer in solute at lower temperatures. It could result in the partition being strongly temperature dependent. The three phase co-existence would cause a reduction of the observed transition heat and increased hysteresis between the heating/cooling d.s.c. curves. The changes in temperature would be most difficult to detect in heating curves. Can you rule out the presence of $L_\alpha$ in your systems?

**Dr N. H. Anderson** (*Long Ashton Research Station, Bristol*) replied: The Clausius–Clapeyron equation is only applicable to ideal systems and the strong solute–phospholipid interactions believed to be present in the liposomes of the system we studied limit its usefulness. Using a mole fraction of 0.1 4-methylphenol in the DMPC liposomes, no change was detected from the normal transition temperature of 23.5 °C, using a differential scanning calorimeter (d.s.c.) of limited sensitivity.

Partitioning experiments involved a maximum mole fraction of 0.02 solute in liposome and d.s.c. showed a reduction in enthalpy of transition from 23.9 to 19.5 kJ $mol^{-1}$ with this mole fraction of 4-methylphenol. The half-height width of the transition peak was increased by 1.56 times under these conditions. Even at higher solute mole fraction than those used by us, a plot of log $K$ against reciprocal temperature showed a clear discontinuity at the normal phase transition temperature.[1] Taken together, these data suggest that the solute concer trations used did not cause a major perturbation to the phospholipid gel phase or phase transition. However, as stated in the paper, the enthalpies of solute partitioning are exceptionally large and this is interpreted as due to a local perturbation of the phospholipid by the solute; the proportion of phospholipid molecules affected would depend on the nature and concentration of the solute in the liposome. It is not possible to test this model without spectroscopic (*e.g.* n.m.r.) data, which would show whether the phospholipid adjacent to the solute could be regarded as a localised domain and thus at present we cannot say whether such domains are present or not.

1 Ref. (16) of our paper at this Discussion.

**Prof. J. K. Thomas** (*University of Notre Dame, IN*) said: You indicate that $T_c$ values were measured in your work, I cannot find them in the paper. Could you give me some values?

**Dr N. H. Anderson** replied: The transition temperature of the liposomes was measured by d.s.c. and is recorded in the paper as 23.5 °C.

**Dr A. Nelson** (*IMER, Plymouth, Devon*) said: (1) As I understand it from your paper, you did not measure the rate of establishment of equilibrium partition of the solute between the liposome and aqueous phase. Is this correct? (2) In terms of toxicological studies the rate of permeation of a xenobiotic into the membrane is highly significant. We measure the rate of penetration of hydrocarbons into mercury adsorbed lipid membranes. Preliminary findings show the rate of permeation to correlate with the *in vivo* toxicity of these compounds.

**Dr N. H. Anderson** (*Long Ashton Research Station, Bristol*) said: I take Dr Nelson's questions in order. (1) Preliminary experiments (not reported) showed that complete equilibration of solute between the aqueous phase and the liposomes was achieved within the 48 h time allowed. (2) We agree that rates of membrane permeation as well as partitioning equilibria are important in interpreting biological effects caused by xenobiotics.

**Prof. E. Sackmann** (*University of Munich, West Germany*) said: You report an astonishingly high solubility of the substituted phenols in bilayers of DMPC at 22 °C. This is quite surprising since the lipid is in the $P_{\beta'}$ phase at this temperature. Moreover, you find only minor changes of the heat of transition. In lateral diffusion measurements we found[1] that the solubility limit of fatty acids in the $P_{\beta'}$ phase is *ca.* 1 mol %, whereas higher concentrations lead to lateral phase separation. Do you know whether the phenol is really incorporated into the bilayer or could it also be intercalated between the stacks of bilayers? Did you do lateral diffusion measurements to see whether the lipid is fluidized by the incorporation of the phenols?

1 H. G. Kapitza, D. A. Rüppel, H-J. Golla and E. Sackmann, *Biophys. J.*, 1984, **45**, 577.

**Dr N. H. Anderson** (*Long Ashton Research Station, Bristol*) said: The fatty acid referred to by Prof. Sackmann is glycophorin, a protein of molecular weight *ca.* 30 000

with three parts: a hydrophobic aminoacid core, a hydrophilic carboxylic end and an $NH_2$-terminal segment to which 16 oligosaccharides are linked. We find it difficult to relate the effects of this protein on DMPC bilayers to those observed by us, since the solutes we used were in the 100–150 molecular weight range. We would expect such low-molecular-weight solutes to be considerably more soluble than glycophorin in the DMPC bilayer $P_{\beta'}$ phase and the effect of 1–2 mol % solute on the enthalpy of transition to be much less than that of 1 mol % glycophorin.

It has been reported that alcohols such as octan-1-ol are relatively insoluble in the phospholipid gel phase and reduce the phase transition temperature. In contrast, we found that 4-methylphenol has little effect on the transition at 1–2 mol %, and this may be related to its ability to hydrogen-bond to the phospholipid head groups.

Our results are consistent with the view that the solutes we used did partition into the bilayer, rather than intercalating as Prof. Sackmann suggests, but we do not have any direct evidence on this point. We did not make any measurements of lateral diffusion.

**Dr A. M. Howe** (*AFRC Institute of Food Research, Norwich*) commented: You have compared the partitioning of solute molecules between liposomes and bulk water with that between organic solvents and water. Is not the surface/interfacial activity of the solute molecules likely to be an important factor in determining the partitioning of some molecules between surfactant aggregates, such as liposomes, and bulk water, particularly in the case of strongly surface-active molecules?

**Prof. E. Sackmann** (*University of Munich, West Germany*) said: Vesicles of normal diacetylene containing phospholipids are quite unstable in the crystalline state and tend to aggregate into multilayers. Do Drs Bader and Ringsdorf have any information concerning the stability of the vesicles of their bipolar diyne lipids in the solid state?

**Prof. P. Fromherz** (*University of Ulm, West Germany*) said: The stability of vesicles is a wide field. Drs Bader and Ringsdorf have mentioned the stabilization as attained by applying membrane spanning and polymerized lipids with respect to spontaneous leakage as tested by carboxy-fluorescein. What about other mechanisms of destabilization, such as the effect of detergents, fusogens, electrical fields, enzymes and biological fluids?

**Dr K. Kurihara** (*Institute for Surface Chemistry, Stockholm, Sweden*) commented: Concerning the stability of polymerized vesicles against their fusion, we have found that photopolymerization of vesicles prepared from the mixture of dipalmitoylphosphatidylcholine (DPPC) and a styrene-containing surfactant (**1**) completely obviates the growth of 300 Å diameter unilamellar vesicles (fig. 5).[1] Each vesicle contained *ca.* 40% of the polymerizable surfactant (**1**), and such partial polymerization was enough to stabilize the vesicles in this case.

$$H_2C{=}CHC_6H_4NHCOC_{10}H_{20}\overset{+}{N}(C_{16}H_{33})Me_2Br^- \qquad (\mathbf{1})$$

1 K. Kurihara and J. H. Fendler, *J. Chem. Soc., Chem. Commun.*, 1983, 1188.

**Prof. D. A. Haydon** (*Cambridge University*) asked: What proportion of diyne lipids polymerize in the systems described and what is known about the molecular weight of the polymers formed?

**Prof. J. F. Holzwarth** (*Fritz-Haber-Institut, Berlin, West Germany*) (*communicated*) I wish to make some extensive comments on the dynamics of the phase transition in phospholipid vesicles.

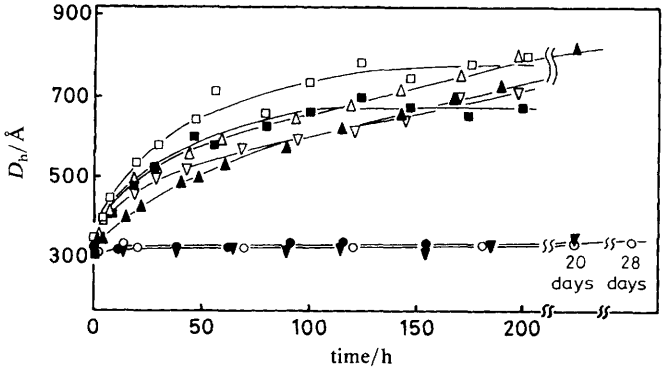


**Fig. 5.** Spontaneous growth of DPPC (□, 1.02 mmol $dm^{-3}$; ■, 0.5 mmol $dm^{-3}$) and non-polymerized DPPC-(**1**) {△, [DPPC], 0.74 mmol $dm^{-3}$ + [(**1**)], 0.44 mmol $dm^{-3}$; ▲, [DPPC], 0.47 mmol $dm^{-3}$ + [(**1**)], 0.28 mmol $dm^{-3}$; ▽, [DPPC], 0.34 mmol $dm^{-3}$ + [(**1**)], 0.23 mmol $dm^{-3}$} vesicles as a function of incubation time. Polymerized DPPC-(**1**) vesicles are seen to retain their sizes for extended periods {▼, [DPPC], 0.53 mmol $dm^{-3}$ + [(**1**)], 0.35 mmol $dm^{-3}$; ●, [DPPC], 0.36 mmol $dm^{-3}$ + [(**1**)], 0.25 mmol $dm^{-3}$; ○, [DPPC], 0.26 mmol $dm^{-3}$ + [(**1**)], 0.16 mmol $dm^{-3}$}. Plotted are the hydrodynamic diameters ($D_n$) of the vesicles, determined by dynamic light scattering, against incubation time at 23 °C.

In contrast to thermodynamic studies only a few kinetic investigations of well defined lipid bilayer systems have been carried out. E.s.r.[1] and n.m.r.[2] studies as well as fluorescence polarization lifetime measurements[3] have been favourite techniques, but these instruments only cover the nanosecond to picosecond time range. Pressure-jump techniques[4] and conventional Joule-heating temperature jump[5] have been applied but either strong field effects or the release of pressure-limited both methods from 300 μs to 100 ms. Only our iodine-laser temperature-jump (ILTJ)[6] technique covers the whole time range from $10^{-9}$ to $10^{0}$ s without producing unwanted physical or chemical effects.[7] Because of the so far unmatched time range we applied our ILTJ to well defined unilamellar vesicles (UVs) from dipalmitoylphosphatidylcholine (DPPC) or dimyristoylphosphatidylcholine (DMPC). The major targets of our investigations were the dynamic processes associated with the main phase transition (PT) which occurs in a temperature range of *ca.* 10 °C. By covering the dynamic changes from $10^{-9} \leqslant$ to $10^{-0}$ s we hoped to be able to compare the kinetic results with thermodynamic data either from turbidity/temperature or microcalorimetric measurements (d.s.c.). UVs containing Cholesterol (CHOL) synthetic peptides (PP) or channel-forming units like Gramicidin (G) or functional proteins like the light-driven proton pump bacteriorhodopsin (bR) were also investigated with respect to marked changes of the lipid dynamics caused by their incorporation into the lipid membrane.

To monitor the kinetics of the crystalline–fluid transition after a fast temperature jump in the UVs we used three different techniques: light scattering or turbidity, light absorption from especially tailored lipids and fluorescence anisotropy changes observed through special probe molecules. In this way we could rule out any specific misleading effect from one of the detection methods.

The lipids DPPC and DMPC as well as gramicidin A′ (GA′) were of the purest grade available from Fluka, Switzerland; the fluorescence probe molecules diphenylhexatriene (DPH) and trimethylaminodiphenylhexatriene (TMADPH) were supplied by Molecular Probes, Texas. The specially tailored lipids 1{3[*p*-(6-phenyl-1,3,5-hexatrienyl)-phenyl]-propionyl}-2-palmitoyl-3-phosphatidylcholine (DPHPC) was synthesized by E. Thomas, University of Salford, and {2-[3,6-bis(dimethylamino)-10-acridino]ethyl}-(2,3-dipalmitoyl-D,L-1-glyceryl)-phosphate (AOL) was a gift of Prof.

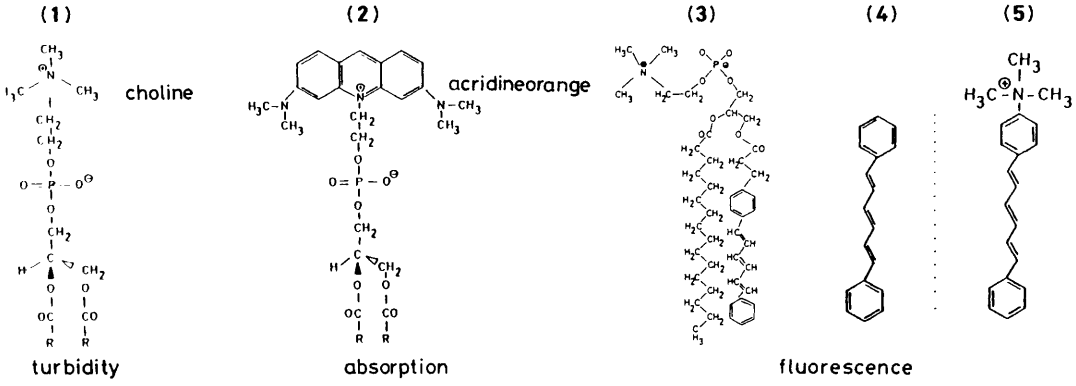


**Fig. 6.** Bond structure of (**1**) lecithin, (**2**) acridine orange lecithin, (**3**) diphenylhexatriene palmitoylphosphatidylcholine, (**4**) diphenylhexatriene and (**5**) trimethylamminodiphenylhexatriene.

Zimmermann, University of Freiburg (fig. 6). The synthetic peptide $PP_{24}$ with the amino-acid sequence $lys_2$-gly-$leu_{24}$-$lys_2$-ala-amide was a generous gift from R. S. Hodges, Edmonton and M. Bloom, Vancouver. All other chemicals were purchased from Merck, West Germany, either p.a. or Suprapure if available. Only triply distilled water from quartz instruments was used.

The vesicles were prepared by a 'modified injection method'.[8,9,11] 20–50 $\mu$mol dm$^{-3}$ of lipid were dissolved in 1 cm$^3$ of ethanol and slowly injected (10–20 min) using a Hamilton syringe into 10 cm$^3$ of pure buffer at a temperature 10 °C above the phase transition temperature ($T_m$) while the emulsion was carefully stirred. Afterwards the solution was dialized for 8 h against pure buffer to remove the alcohol.[10] The vesicle preparations were characterized by electron microscopy, laser light scattering as well as their temperature dependence of turbidity, absorption or fluorescence;[9] differential scanning calorimetry (d.s.c.) was also used.[11] Fluorescence and absorption labelling was achieved by adding a few mm$^3$ of the probe dissolved in methanol to the UVs, giving the desired probe/lipid ratio. After an incubation time of 1 h at a temperature above $T_m$ the methanol was completely removed. DPHPC and AOL were incorporated[9,12] into the UVs together with the lipid in the original ethanolic solution. Fig. 6 gives the bond structure of the lipid and probe molecules.

Fluorescence anisotropy and d.s.c. measurements are reported in ref. (9), (11) and (12). Phase diagrams of pure lipid water systems are shown in ref. (4) for DPPC and ref. (10) for DMPC.

Our ILTJ was used either in the mode-locked mode,[6] the free-run mode[10] or the oscillator mode,[6,7,12] producing pulses at 1.315 $\mu$m of halfwidth, either 100 ps to 3 ns or 80 ns as well as 2.4 $\mu$s containing an energy of 1 J to produce a temperature-jump of 1 K in 150 mm$^3$ of solution.[7] The relaxation processes were monitored spectroscopically. The signals from especially designed photomultiplier heads[6,12] were stored in transient recorders from Tektronix or Biomation and processed in Hewlett Packard computers for sampling and relaxation time as well as amplitude calculations. As detection light sources we used 150 W XBOs from Osram or Hannovia. Details of the optics, electronic circuits and the data registration and processing system are given elsewhere.[4,6,7,9–12] In fig. 7 the schematic experimental arrangement is shown.

Equilibrium phase-transition-temperature dependences for DPPC obtained from turbidity or fluorescence anisotropy measurements are given in the literature[4,7,9,11,12] and in our comment to the papers of Prof. Fromherz and Evans. In fig. 8 d.s.c. results are summarized for UVs of DPPC with increasing CHOL content and fig. 9 shows the turbidity and absorption changes with temperature of UVs containing AOL as a probe

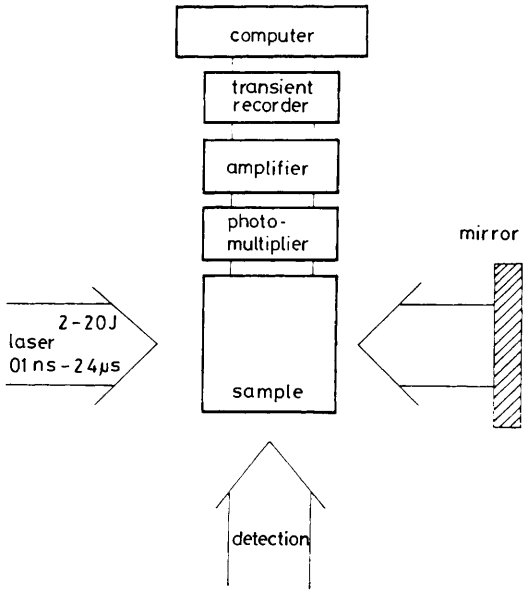


**Fig. 7.** Schematic arrangement of the iodine-laser temperature-jump (ILTJ) experiment: time resolution $10^{0}$–$10^{-10}$ s.

molecule. A size distribution from electron micrographs is included in our question to Dr Cornell's paper and in fig. 10. In fig. 11 the five relaxation processes which could be monitored after a 1 ns temperature jump are shown together with their corresponding amplitudes. We did not monitor changes faster than 4 ns and slower than 100 ms. The five relaxations are clearly separated; by changing the hydrocarbon chains and the head groups it was possible to attribute the fastest process to the formation of simple rotational isomers (kinks) in single molecules because the 4 ns signal called 1 is not sensitive to the length of the hydrocarbon chains or the nature of the head groups.[4,10] Relaxation 1 also showed only a very weak maximum of $\tau_1$ and $A_1$ near $T_m$, and its amplitude decreased steadily. Signal 2 around 300 ns showed a maximum at the midpoint of the phase transition in its relaxation times as well as the corresponding amplitudes. It also changed with the nature of the head groups.[10] We therefore believe that process 2, having only a moderate cooperativity, can be explained by the onset of the free rotation of the head groups, since they are no longer fixed with respect to the hexagonal lattice of the hydrocarbon chains of the all-*trans* conformation.

If we now proceed along the time axis we can see a strongly cooperative relaxation around 10–20 $\mu$s; this third process showed strong maxima of the relaxation times $\tau_3$ and the amplitudes $A_3$ which were due to an increase in complexity of the molecular interactions involved. By applying the absorption probe-lipid AOL we could prove that lateral diffusion has already started in this time range because we could monitor the monomer/aggregate equilibrium of AOL; this can only occur if lateral diffusion has started (fig. 12). An important condition for lateral diffusion in the plane of both monolayers forming the bilayer membrane is that complex rotational isomers such as *gauche* forms are already formed which shorten the hydrocarbon chains so that they are no longer hindered by the chains of the opposite monolayer in their lateral movement. We could not see much lateral diffusion in the nanosecond time window. At longer times processes 4 and 5 are observed. Their explanation is more difficult than for the three faster dynamic phenomena. From the time scale, the strong cooperativity, and the influence of additives such as CHOL and peptides as well as proteins, we conclude

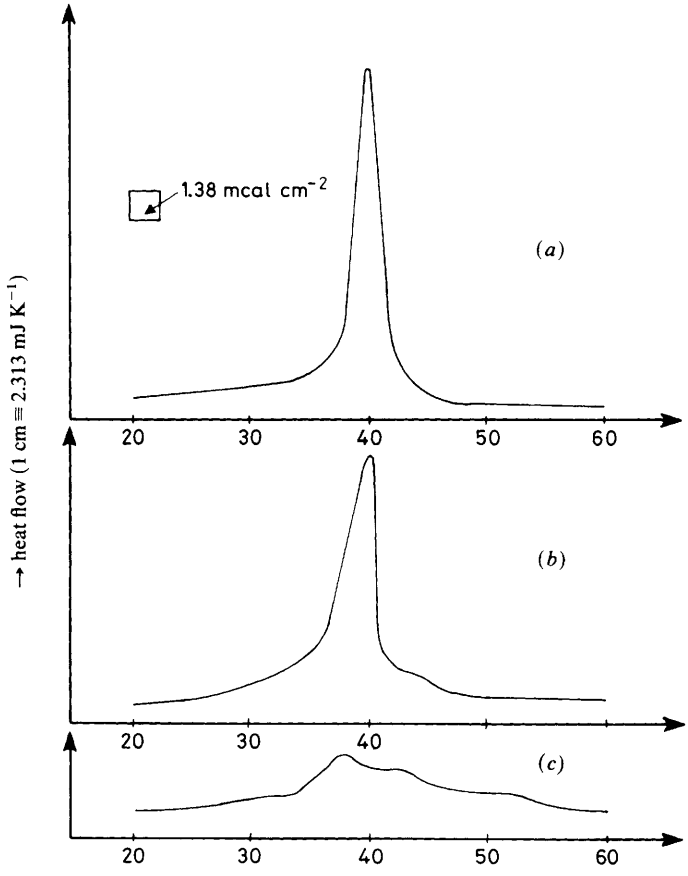


**Fig. 8.** Differential scanning calorimetric measurements of DPPC vesicles containing different amounts of cholesterol: (*a*) ○, (*b*) 7.5 and (*c*) 16.5%, $c_{DPPC-CHOL} = 2.7 \times 10^{-3}$ mol dm$^{-3}$, 0.9 cm$^3$ probe volume in MC1.

that process 4 is the formation of lipids in a more liquid state surrounding clusters of more crystalline states of order, with intermediate lipids as buffer. This discontinuity in the state of order of the lipids exists until we reach the 10–30 ms time range, where it disappears leaving the bilayer in the fluid state.Fig. 13 summarizes all five relaxations in a schematic way. Fig. 14 shows how the relaxation amplitudes are distributed over the whole time range (equilibrium sum) and proves the following: (1) The five relaxation processes represent the whole crystalline–fluid transition dynamics because they reproduce the equilibrium dependence of fig. 9 perfectly. (2) The major part (>80%) of the relaxation amplitude is monitored in the $\mu$s to ms time range; this is also true for the fluorescence anisotropy time dependence around $T_m$, as fig. 15 shows. Details of these measurements are given in the literature.[9,12] DMPC preparations of UVs show similar behaviour.[9,10] In fig. 16 we show the kinetic results of DPPC preparations containing 16.5% CHOL. The relaxation amplitudes $A_5$ are decreased by a factor of two, which also holds for process 4. Besides a slightly broader profile above $T_m$ nothing dramatic has happened. However, relaxation 3 is strongly affected; the relaxation times $\tau_3$ have lost their maximum and can still be monitored even 10 K above $T_m$. If we summarize the influence of CHOL on DPPC UVs we find that the phase-transition relaxation is shifted from $\tau_4$ and $\tau_5$ towards the 10 $\mu$s time range of $\tau_3$ and the transition is broadened,

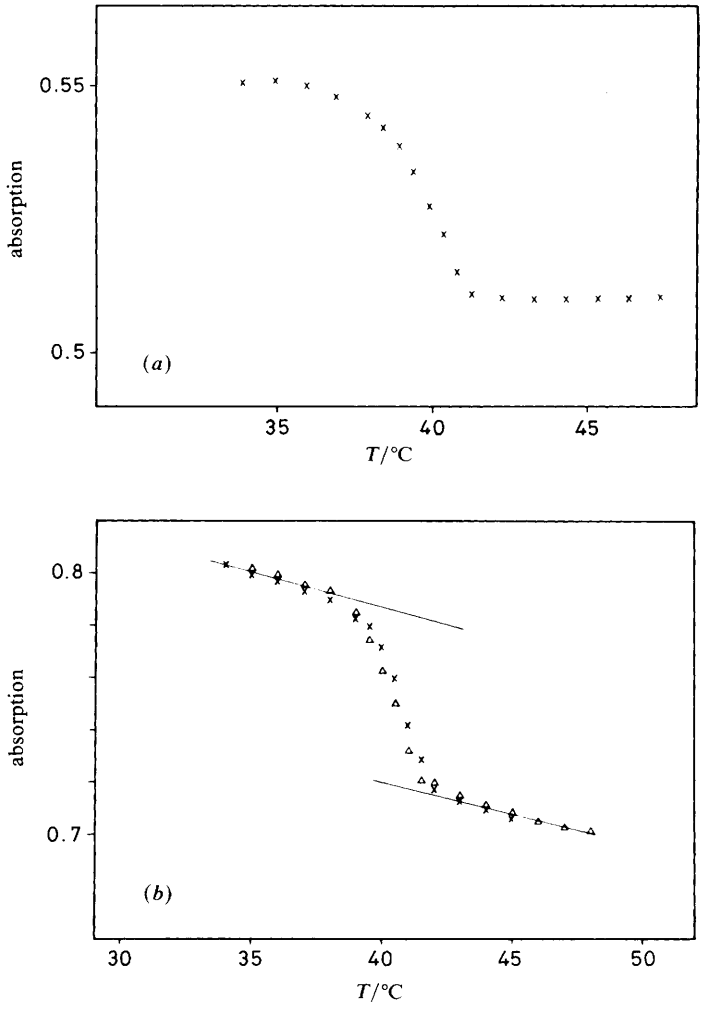


**Fig. 9.** Temperature/absorption (484 nm) (*a*) or turbidity (300 nm) (*b*) measurements representing the main phase transition of UVs from DPPC containing *ca.* 1% AOL: ×, $T$ increasing; △, $T$ decreasing. $R \approx 50$ nm; $c_{\text{lipid}} = 2.7 \times 10^{-3}$ mol dm$^{-3}$.

similar to the d.s.c. measurements in fig. 8. Further details are included in the literature.[11] If we investigate preparations containing the channel forming peptide gramicidin A (GA′, the dimer spans the bilayer) we find a similar broadening and increase in the amplitudes for signal 3, as is demonstrated in fig. 17.[13] Signal 5 has almost disappeared and signal 4 is reduced by 40%. Reconstituted UVs of DMPC containing *ca.* 1% bacteriorhodopsin (bR) showed behaviour like (GA′) with an even stronger decrease of signal 4 as well as signal 5. Fig. 18 shows the only remaining strong relaxation signal, $\tau_3$, caused by the lipids and a signal $\tau_{\text{bR}}$ which results from the change in absorption around 550 nm belonging to the photocycle of bR. The latter represents the change in the absorption of the chromophore retinal by pumping a proton through the membrane. From the Arrhenius plot in fig. 18 we learn that the activity of bR is strongly influenced by the state of the lipid bilayer. Only when the lipids have switched from the crystalline to the fluid state, bR can effectively pump protons using light as an energy source.

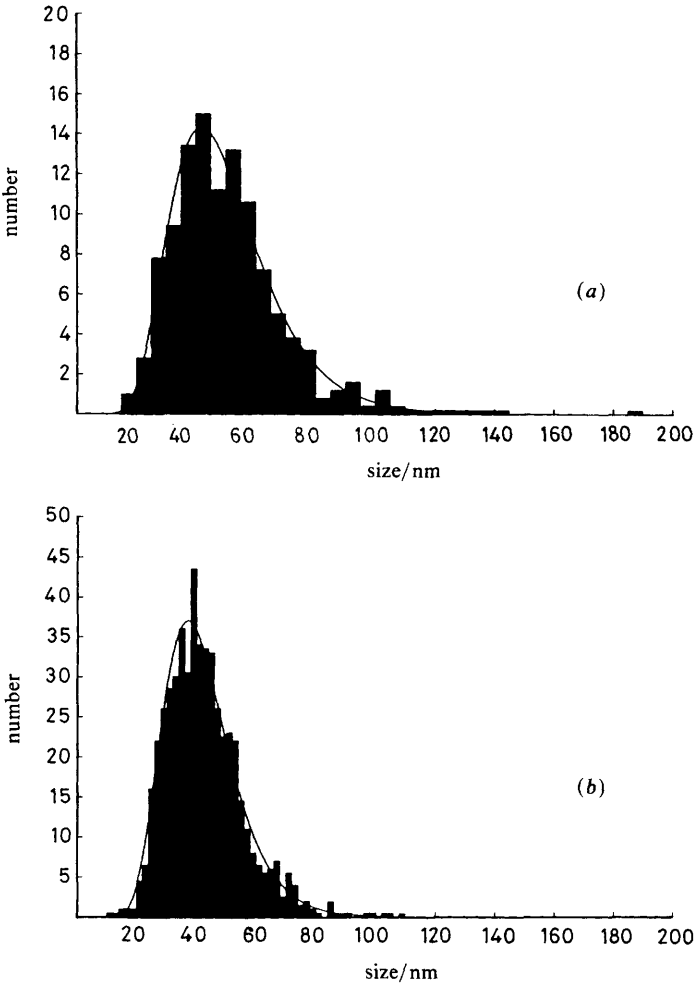


**Fig. 10.** Size distribution of vitrified DPPC UVs, taken from electron micrographs using a fast freezing method ($10^4$ K $s^{-1}$) without applying contrast chemicals. (*a*) Injection method: total no. of vesicles = 554, maximum no. = 14.3; size at maximum = 41.7 nm; direct width parameter = 1.5. (*b*) Injection method plus sonification; total no. of vesicles = 1046; maximum no. = 37; size at maximum = 37 nm; direct width parameter = 1.41.

In conclusion, we have measured five well separated relaxation processes representing the whole main crystalline-fluid transition in pure UVs of DPPC and could develop a model on the basis of molecular changes to explain the different steps. New theoretical model calculations using Monte Carlo simulations[14] have reached similar conclusions about the existence of states of different order in a bilayer system if intermediate states are acting as buffer. They could not give a timescale for their transition model so far but this could be achieved using our experimental data. The incorporation of substances like CHOL or Gramicidin shifts the relaxation from longer times into the 10 $\mu$s time range and favours lipids in an intermediate state. Measurements with UVs containing reconstituted bacteriorhodopsin show a clear preference for the relaxation in the 10 $\mu$s regime and prove that the lipids have to be rather mobile to help the protein pumping protons. Further work will probably show if the relaxation around 10 $\mu$s can be

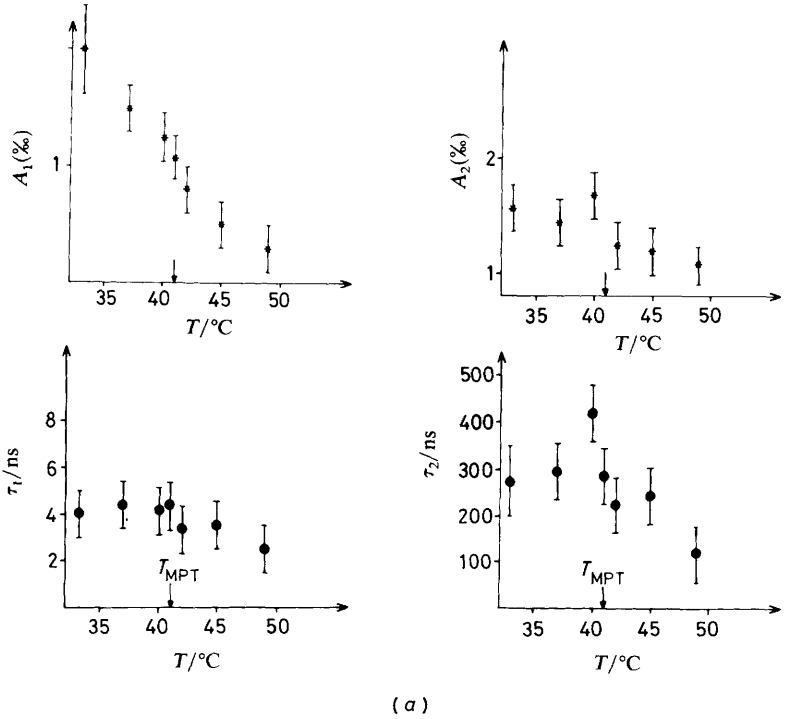


**Fig. 11.** Five relaxation times $\tau$ and their corresponding amplitudes $A$, representing the whole crystalline-fluid transition in UVs of DPPC. (*a*) $c_{DPPC} = 2.7 \times 10^{-3}$ mol dm$^{-3}$; $\lambda_{obs} = 360$ nm; $\Delta T \approx 0.8$ K. (*b*) $c_{DPPC} = 2.8 \times 10^{-3}$ mol dm$^{-3}$; $\lambda_{obs} = 360$ nm; $\Delta T \approx 0.9$ K.

connected with a functionally important movement as proposed by Frauenfelder for haemoglobin.

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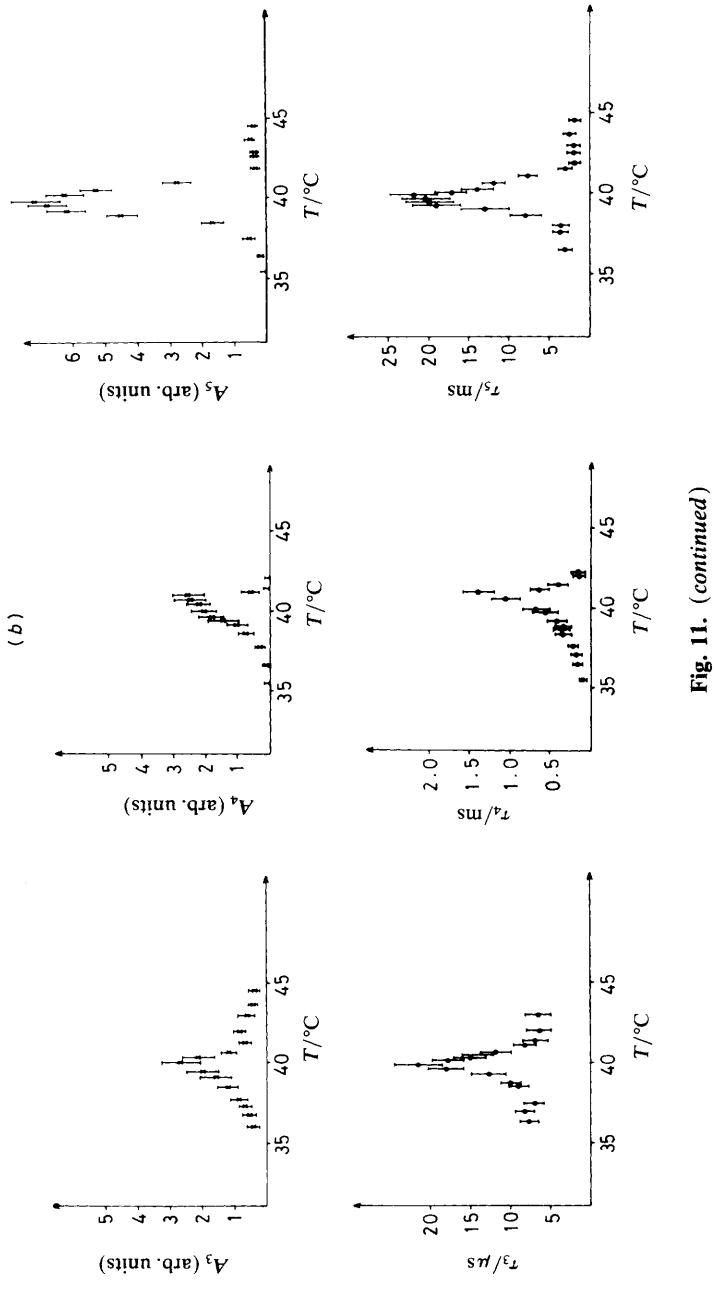


**Fig. 11.** (*continued*)

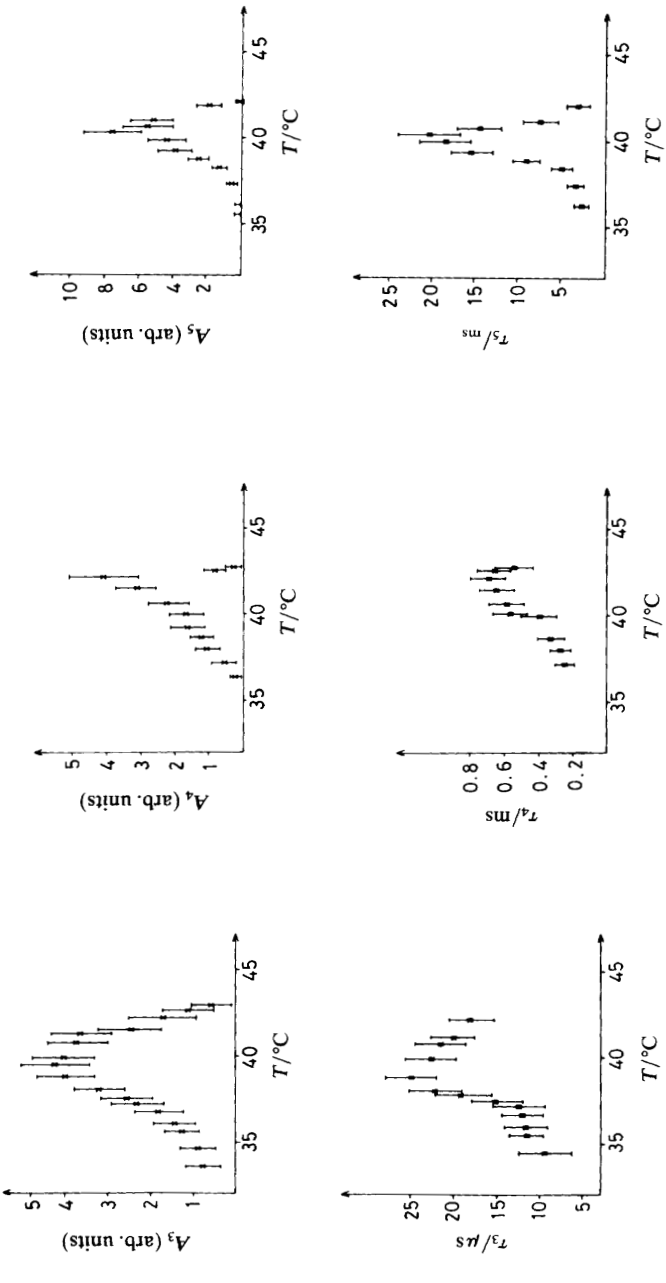


**Fig. 12.** The three slower relaxation processes between 1 $\mu$s and 100 ms monitored either by turbidity changes at 300 nm or absorption changes at 484 nm in UVs of DPPC containing 1% AOL as probe molecule. $c_{DPPC} = 2.7 \times 10^{-3}$ mol dm$^{-3}$; $\lambda = 480$ nm; $\Delta T \approx 1$ K.

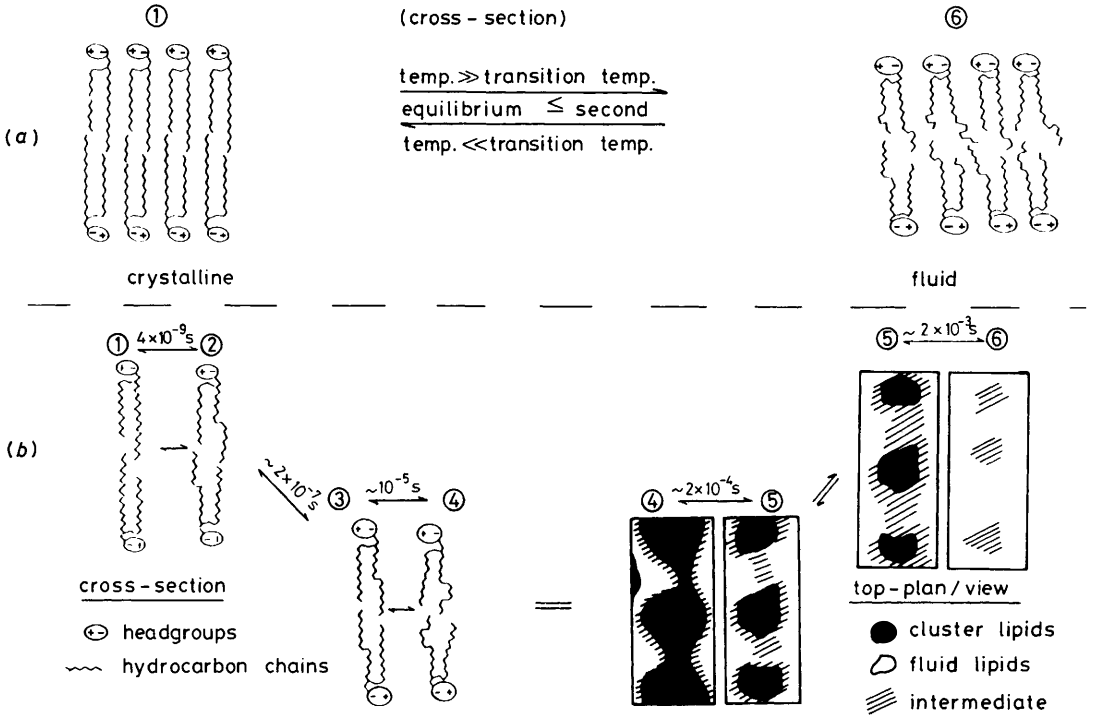


**Fig. 13.** Schematic molecular model of the main phase transition in UVs between $10^{-9}$ and $10^{0}$ s. (*a*) Initial ① and final ⑥ states. (*b*) Model of the dynamic processes.

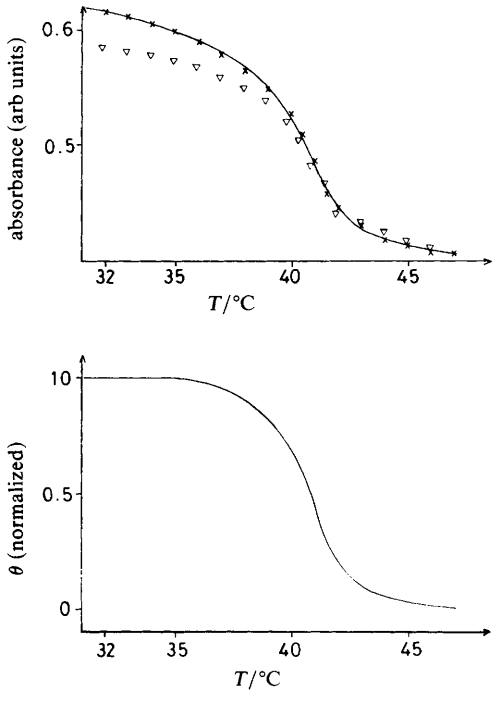


**Fig. 14.** Normalized amplitude sum of the five relaxation processes around $T_m$ of DPPC UVs and the equilibrium turbidity/temperature dependence (solid line). $R = 60$ nm; pH 7.5. ×, Σ5 relaxations ($10^{-9}$-$10^{-1}$ s); ▽, Σ3 relaxations ($10^{-6}$-$10^{-1}$ s).

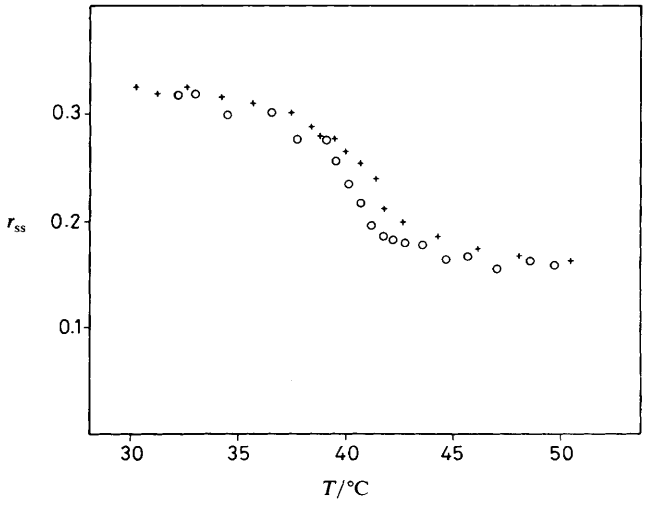


**Fig. 15.** Fluorescence anisotropy $r_{SS} = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ temperature dependence for the probe DPHPC in UVs of DPPC between 1 μs and 100 ms. $\lambda_{exc} = 360$ nm; $\lambda_{obs} = 430$ nm; $c_{DPPC} = 2.7 \times 10^{-3}$ mol dm$^{-3}$. +, $T$ increasing; ○, $T$ decreasing.

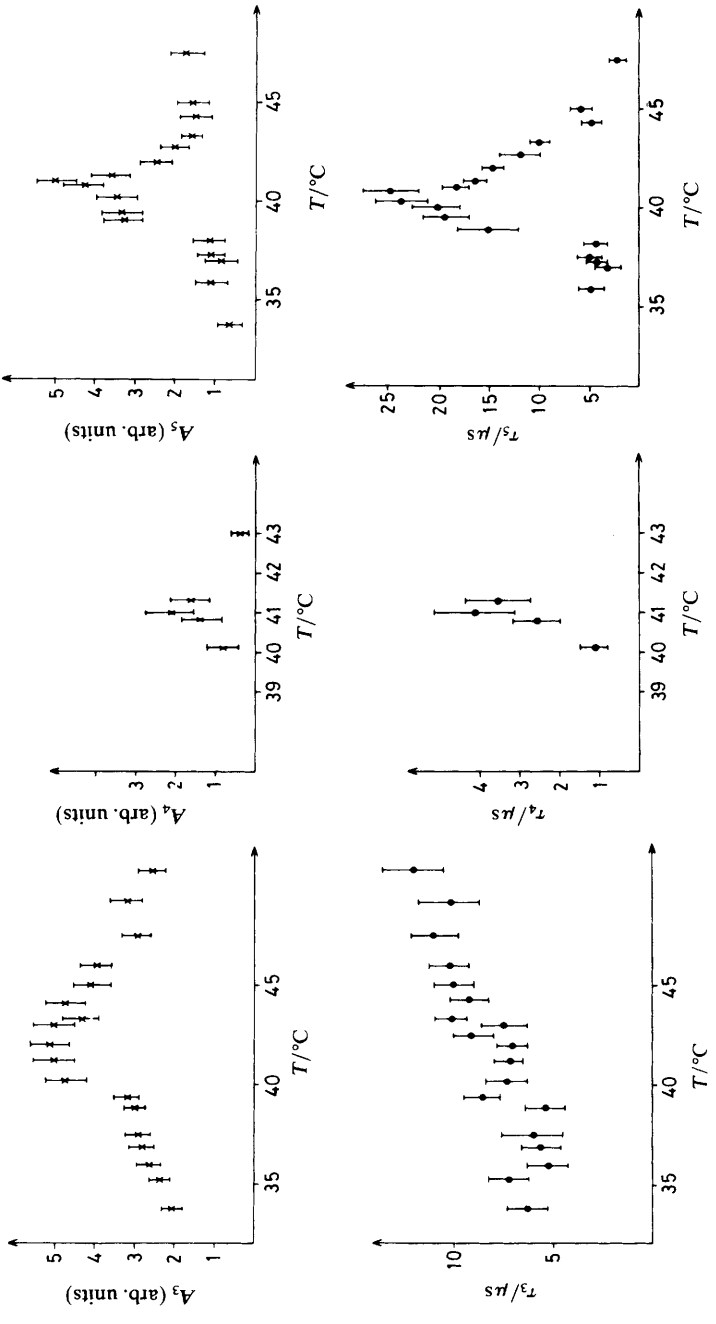


**Fig. 16.** Turbidity/temperature relaxations between $10^{-6}$ s and $10^{-1}$ s for UVs of DPPC containing 16.5% cholesterol. $c_{\mathrm{DPPC-CHOL}} = 2.8 \times 10^{-3}$ mol dm$^{-3}$; $\lambda_{\mathrm{obs}} = 360$ nm; $\Delta T \approx 1.4$ K.

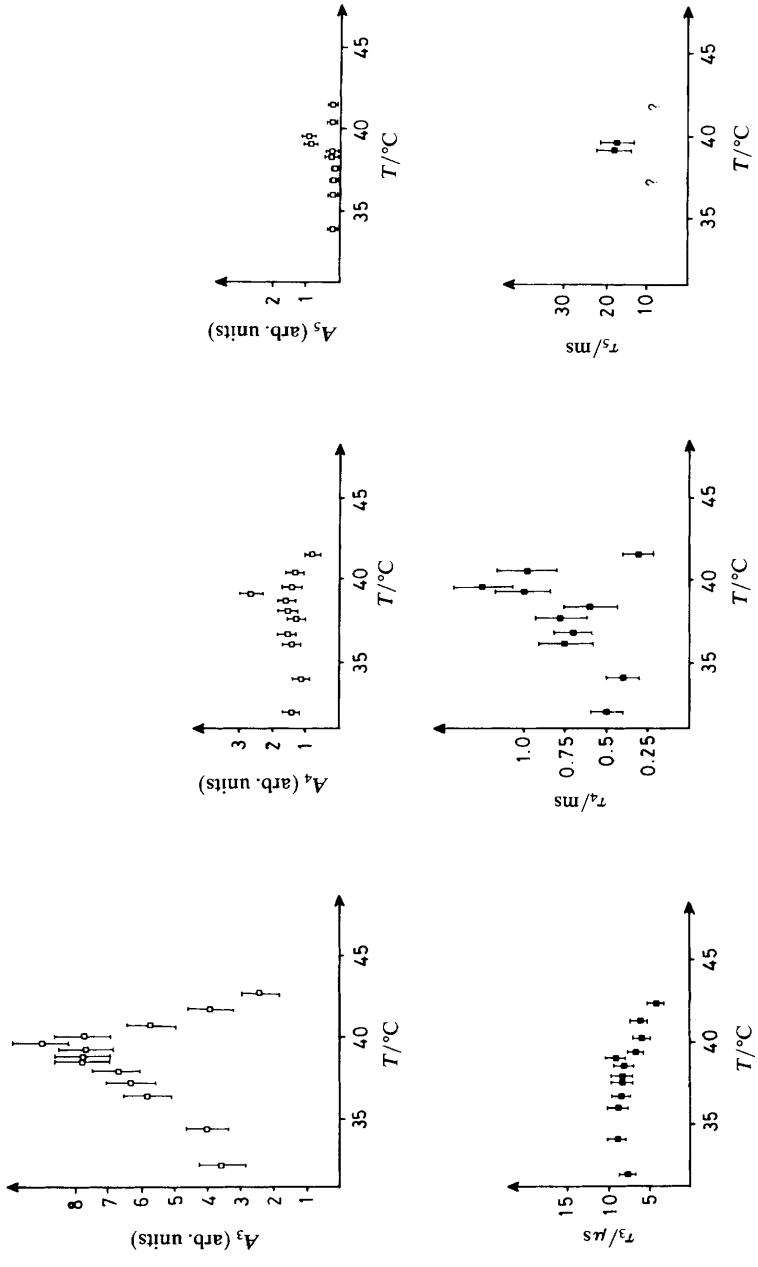


**Fig. 17.** Turbidity/temperature relaxations between $10^{-6}$ s and $10^{-1}$ s for UVs of DPPC containing the channel forming gramicidin A′ at a ratio of 15/1. $c_{DPPC} = 2.7 \times 10^{-3}$ mol dm$^{-3}$; $\lambda_{obs} = 360$ nm; $\Delta T = 1.3$ K.

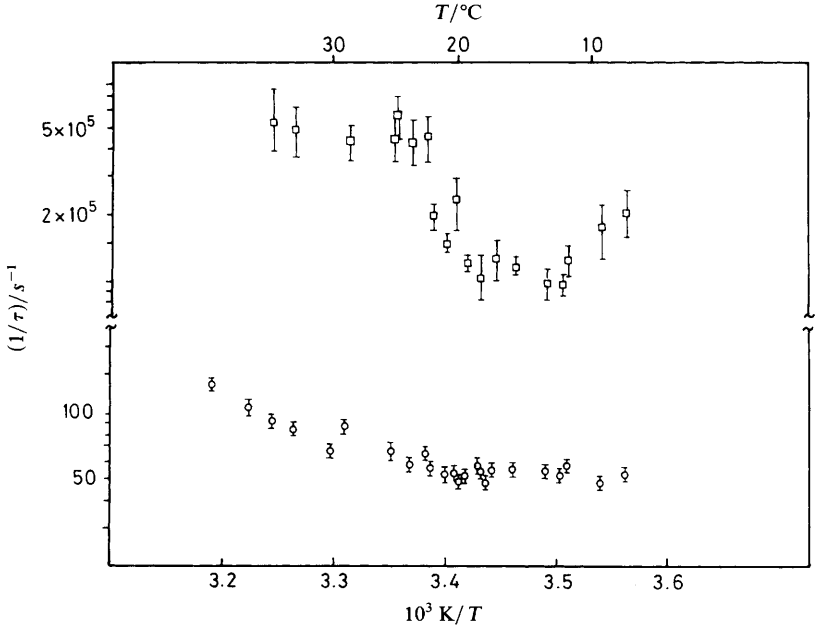


**Fig. 18.** Arrhenius dependence of the lipid relaxation $\tau_3$ and the protein relaxation $\tau_{bR}$ in the temperature range of the phase transition in DMPC UVs, containing 1.1% bacteriorhodopsin. $c_{DMPC} = 3 \times 10^{-3}$ mol dm$^{-3}$. □, lipid signal; ○, protein signal.