LIPID ASYMMETRY, CLUSTERING AND MOLECULAR MOTION IN BIOLOGICAL MEMBRANES AND THEIR MODELS

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The term 'biological membrane' is often used in a sense that implies considerable uniformity among different types of membranes. Such uniformity of course, can only refer to some common principles in organisation (structure) but not in function. There is after all no reason to suppose that say carrier mediated transport, protein synthesis, energy coupling or signal transmission all operate by a similar mechanism. It is perhaps the large diversity of membrane functions based on structural similarities that makes research in this area of such current interest.

In that all membranes are made up of lipids and proteins they are similar. (Carbohydrates, also constituents, are often considered in a separate class, being on the membrane surface). Yet the differences in fine detail (lipid-protein ratio, lipid and protein composition and structure) must be responsible for the functional variations. Combining spectroscopic studies (providing some of the fine structural detail) with biochemical ones (describing functions) we can determine some structure-function relations. Present spectroscopic techniques alone cannot provide an overall structural view while diffraction and electron microscopic measurements are likely to produce sufficient detail only in specialised circumstances (1). We must therefore combine the results of different types of measurements. In this paper we shall examine some of the properties of the chromaffin-granule membrane and describe observations on model membranes that are relevant to our understanding of the former system.

I. THE BIOCHEMISTRY OF CHROMAFFIN GRANULES

Chromaffin granules are membrane limited vesicles of about 2000 A diameter contained within the adrenal medullary chromaffin

cells. They are the major storage vesicles for catecholamines which they concentrate to 0.55M together with ATP (0.l25M) inside the vesicles. Their two major biochemical functions are the release and uptake of catecholamines. The former process is thought to occur by excocytosis, i.e. the fusion of the membrane of the granule with that of the cell resulting in the release of the total content of the storage system. The trigger for this event is Ca (2), although the exact form of the triggering is not known. The required energy for amine uptake is derived from the hydrolysis of ATP. The ATP-ase seems to be located on the outside of the vesicles and in a Mg+2 dependent reaction drives the accummulation of adrenaline via a proton-linked mechanism (3,4,5). It is likely that the granule membrane contains a catecholamine carrier since uptake can be competitively inhibited by reserpine (6) and also shows saturation kinetics.

The role of the other membrane proteins (notably an NADH-oxidase, cytochrome b and DOPAmine hydroxylase) is not well understood.

The composition of the membrane lipids is unusual (Table 1) with perhaps the two most emphasized features being the relatively high proportion of lysolecithin and cholesterol.

TABLE 1. PHOSPHOLIPID COMPOSITION OF BOVINE CHROMAFFIN GRANULES

Phospholipids	
Lysolecithin	16.8
Sphingomyelin	12.8
Phosphatidylcholine	27.5
Phosphatidylinositol	8.2
Phosphatidylserine	2.5
Phosphatidylethanolaine	31.8
Phosphatidic acid	
Cardiolipin	1.03

Taken from Winkler, H., Schneider, N. Ziegler, E. Naunyn-Schmiedebergs Arch.Exp.Path.Pharmsk. 1967,256, 407-415.

As in most studies on biological membranes so far we can most readily ask questions about the structure and motions of the phospho-

lipid components and to attempt to define their role in modulating biological activities. We shall therefore first summarise our work on phospholipid model membranes and then examine the functional relevance of our observations.

II. MODEL MEMBRANE SYSTEMS

Two model membrane systems are commonly employed to study the bilayer properties of phospholipids. Unsonicated aqueous dispersions (liposomes) of many phospholipids consist of layers of concentric bilayers arranged in an onion skin configuration. The much smaller **(250A)** diameter) "vesicles" obtained on sonication of liposomes consist of a single bilayer separating inner end outer aqueous phases. It should be noted that vesicles are "good" model systems as the lipid packing and the local motion available to the phospholipid do not appear to be unduly perturbed by the high curvature of the vesicle entity. In this regard it has been shown that the gel-liquid crystalline phase transition for dipalmitoyl lecithin occurs at the same temperature and has the same heat content in both vesicle and liposome preparations **(7),** strongly suggesting that the lipid packing is not significantly perturbed in the vesicle membrane. Further, ^{31}P NMR results show that the local motion in the phosphate region of the polar headgroup is similar in both vesicles and liposomes **(8).**

Both systems have advantages in certain situations for studying motional and structural details of the constituent phospholipids. In particular the small vesicles tumble rapidly, resulting in high resolution NMR spectra, anionic shift reagents such as ferricyanide may then be used to obtain details of the outside-inside distribution of choline containing lipids such as sphingomyelin and phosphatidylcholine, thus giving information on the vesicle size. In the case of ^{31}P NMR, different classes of phospholipid present in the vesicle may be resolved separately. Cationic broadening and shift reagents may then be employed to obtain information on both the vesicle size and possible assymetric distributions of the constituent phospholipids across the vesicle membrane. These points will be elaborated in the next section.

The much larger liposomes on the other hand do not have such rapid tumbling rates as vesicles. Thus the ^{31}P NMR signals observed in such systems have a "solid state" lineshape which only reflects the local anisotropic motion available to the phospholipid in a bilayer configuration. Biological membrane preparations obtained by osmotic lysis also consist of relatively large membrane fragments. The observation of similar solid state ^{31}P NMR signals in such biological membrane preparations thus strongly indicates regions of bilayer phospholipid structure. In the case of the chromaffin granule membrane a large percentage of the con-

stituent phospholipids must experience a relatively fluid bilayer environment as indicated by the observation of ^{31}P NMR spectra characteristic of liquid crystalline phospholipids in a bilayer configuration (9).

1. Asymmetry Of Lipids In Vesicles

It is well known that in biological membranes not only the protein components but also phospholipids are asymmetrically distributed across the membrane.

Recently on the basis of chemical studies (10), nuclear magnetic resonance (NMR) measurements (11,12) and theoretical considerations (13) asymmetric distribution of phospholipids across vesicle membranes has been reported.

We have examined in detail the factors that are important in determining such asymmetric lipid distributions. In this we have used three methods: (i> The proton NMR signals from the choline head groups of lecithin or sphingomyelin located on the outside of a vesicle can be shifted by the addition of ferricyanide (14,15). (ii) most classes of phospholipids can be observed separately by ^{31}PNMR. Here we have used a non-penetrating broadening reagent (Co^{2+}) to measure lipid distributions (15). (iii) Finally, the ^{31}P NMR spectra of different lipids are easily resolved into outside/inside components by the addition of a non-permeating shift reagent like Nd^{3+} (7). Since the technical details of these measurements have been described elsewhere (7,12,15) only the conclusion will be summarised here.

The ratio of the molecules in the outer and inner monolayers ($R_{o/i}$) in phosphatidylcholine vesicles clearly depends on the sizes of the vesicles. This can be demonstrated experimentally in two ways. Sonicated egg lecithin vesicles can be fractionated by gel-filtration according to their size (15), or the size can be varied in pure phosphatidylcholines by changing the nature of hydrocarbon chains (7). In both cases the $R_{o/i}$ approaches unity for the larger vesicles and increases above 2.0 for the smaller ones (Table 2). (We should mention here that recently we have demonstrated that the sizes of phospholipid vesicles can be calculated from the widths of the ^{31}P NMR lines as a result of the fact that the linewidths are dependent on the tumbling rate of the vesicles, which depends on the vesicle size through Stokes law (16)).

In vesicles containing mixtures of phospholipids with different head groups both charge and the packing properties of the head group are important in determining phospholipid distribution (12,15). Thus in mixtures of lecithin with phosphatidylethenolamine, phosphatidic acid, phosphatidylserine and phosphatidylinositol, phosphatidyl-

Table 2. DISTRIBUTION OF PHOSPHATIDYLCHOLINE (PC) ON THE OUTSIDE AND INSIDE LAYERS OF PHOSPHATIDYLCHOLINE VESICLES

$$R_{o/i} = \frac{\text{amount of phosphatidylcholine outside monolayer}}{\text{amount of phosphatidylcholine inside monolayer}}$$

All $R_{o/i}$ measurements at 30°C, except for the 16:0/16:0-phosphatidylcholine and 18:0/18:0-phosphatidylcholine vesicles where $R_{o/i}$ was measured at 50 and 60°C, respectively. Error in $R_{o/i}$ is 0.05 (from ref.71

Vesicle composition	$R_{o/i}$	Membrane thickness (Å)	Calculated vesicle outer radius (Å)
14:0/14:0-PC	2.65	32	84
16:0/16:0-PC	2.2	37	112
18:0/18:0-PC	1.7	42	180
16:1c/16:1c-PC	1.8	28	110
18:1c/18:1c-PC	1.75	32	131
18:1t/18:1t-PC	2.0	36	123
Egg PC	2.0	35	120

Table 3. DISTRIBUTION OF PHOSPHOLIPIDS IN MIXED VESICLES

Lipid mixture (1 :1)	lipid component	$R_{o/i}$
PC-Ps(pH 7.2)	total	2.3
	PC	2.45
	PS	2.06
PC-PS(pH 4.9)	total	2.3
	PC	3.7
	PS	1.15
PC-PE(pH 7.2)	total	1.41
	PC	1.76
	PE	7.17
PC-PA(pH6)	PC	2.8
14:0/14:0-PC-cholesterol	PC	1.9
	cholesterol	

Continuation of Table 3.

Lipid mixture (1:1)	Lipid component	$R_{o/i}$
18:lc/l8:c-PC-cholesterol	PC	2.95
	cholesterol	0.46

PC: phosphatidylcholine; PS: phosphatidylserine
PE: phosphatidylethanolamine; PA: phosphatidic acid

(From refs. 7,15,16)

choline prefer to be located at the outside of the bilayer (Table 3). The last four lipids all have a smaller head group than lecithin and this is clearly an important factor in determining the observed asymmetry. In contrast the slight preference of sphingomyelin for the outside layer in mixtures with lecithin (Table 3) may well be due to a difference in geometry of the headgroup compared to that of lecithin, requiring a slightly larger area.

The effect of charge on phospholipid asymmetry has been demonstrated by Michaelson et al (11) and by our work on the effect o the distribution of phosphatidylserine in mixed vesicles (15). As expected (13) the effect of increased charge is to decrease the preference of the charged phospholipid for the inside of the bilayer. In the case of phosphatidylserine this effect is in competition with the opposite preference due to head group size (15).

The presence of cholesterol in membranes raises special problems. The outside/inside ratio ($R_{o/i}$) of both saturated and unsaturated phosphatidylcholine species is not much affected by the incorporation of up to 3Cmol% cholesterol.

Above this level of cholesterol the outside /inside ratio of the phospholipid is markedly increased for phosphatidylcholines with cis-unsaturated fatty acid chains. In contrast this effect was either absent or in the opposite direction when the fatty acid chains were fully saturated or contained trans-unsaturation (16). However, since in all instances above 30mol% cholesterol the vesicle sizes also increased (but not below 30%> the distribution of the lipid across the bilayer became asymmetric with a disproportionately larger amount of cholesterol on the inside. We have suggested that under these conditions cholesterol-cholesterol (as opposed to cholesterol-phospholipid) interactions are mainly responsible for the increased vesicle sizes end also for the pre-

ferential location of often over 50mol% cholesterol on the inside of the bilayer. The dynamic shape of the cholesterol frame apparently favours placing of adjacent molecules on the inner monolayer, i.e. by locating the hydroxyl group on the concave surface,

It is now appropriate to enquire as to the biological significance, if any, of these observations on small lipid vesicles.

Phospholipids and cholesterol have been shown to be asymmetrically distributed in a variety of membranes. If this asymmetry is entirely biosynthetic in origin one may argue that studies on small vesicles bear no relevance to the real situation. If this is so at worst we have learned how to produce asymmetric vesicles which may allow one to determine how such lipid distributions affect transport and coulombic interaction with some proteins in situations that are analogous to the known lipid orientations in biological membranes. (The fact that in erythrocytes lecithin and sphingomyelin are largely on the outside while phosphatidylserine and phosphatidylethanolamine are mainly present in the inner layer (17) may then be no more than fortunate.)

The distribution experiments on vesicles also give us some insight into the packing requirements of different phospholipid head groups.

At the other extreme, one may wish to consider the possibility that biological membranes in vivo do possess large areas of high curvature and that the packing of the lipids in these regions are governed by the same kinds of interactions that we have discussed. Or an interesting possibility might be that during the biosynthesis of the membrane the asymmetric phospholipid distribution is initially set up in such curved regions. Transporting epithelial cells certainly have highly folded cell surfaces (18) and it has been suggested that the membranes at the tips and bases of microvilli and the deeper parts of basal infolds could be sites of high water permeability. Other examples include mitochondrial cristae and the ends of cisternae of endoplasmic reticulum. Intuitively, regions of high curvature would be expected to occur during processes like exocytosis and pinocytosis. From the functional point of view such regions therefore could have special significance.

2. Phase Behaviour of Phospholipids

In recent years many reports have dealt with existence of phospholipid "phase transitions" in biological membranes. Generally non-linear Arrhenius plots for membrane linked functions and "discontinuities" in probe (i.e. spin and fluorescent labels) behaviour have been used is support of such conclusions. It is unlikely that temperature dependent phase changes have any direct biological rele-

vance. but their observation provides a valid and valuable method or understanding the way the physical state of the membrane lipids modu_ lates biochemical functions.

In pure phospholipid bilayers (or their mixtures) two types of well documented phase changes may take place: (i) gel-liquid crystalline transitions and (ii) lateral phase separations.

(i) Co-operative gel-liquid crystalline transitions are a result of the tight crystal like packing of the hydrocarbon chains of phospholipids being expanded to produce a less ordered and expanded structure.

Here we only wish to emphasize two sets of observations.

(a) The first is that ^{31}P NMR can be used to follow such changes in vesicles (7), liposomes (8) and in some special biological membranes (19). This is because below the phase transition temperature the rotational motion of phospholipids is hindered (9) resulting in large NMR line widths, as the temperature is raised towards the transition temperature the spectra undergo motional narrowing owing to the onset of rapid axial rotation of the phospholipid. Perhaps an important feature of such observations is that line narrowing takes place over a relatively broad temperature range ($\sim 15^{\circ}C$) and is essentially complete several degrees below the calorimetrically observed phase transition temperatures.

(b) The second set of observations relies on an entirely different type of measurement. Here we rely on the introduction of probe molecules (in our case various fluorescent molecules (20))into the phospholipid or membrane system. The apparent rotational motion of such molecules (derived from measurements of the polarisation and life-time of fluorescence) reflects the expected phase changes in single lipid systems (21). In those measurements too the observed temperature range for the transition is somewhat broader than that obtained from calorimetric data. The most likely reason for this is the progressive exclusion of the probe molecule from the crystalline regions of the phospholipids (see below and (22)).

From the biological point of view, where one often relies on correlations between functions and physical (structural) measurements, it is therefore important to understand the precise contributions to the observed changes in the physical measurements if the observations are to be interpreted in terms of the effect of structure and motion on the functional behaviour.

(ii) When the difference between the transition temperatures of two iipids is too large to allow co-crystallization of the fatty acid chains in their mixture lateral phase separation takes place.

Thus in an equimolar mixture of 18:lc/l8:lc-phosphatidylcholine (dioleoyl lecithin) and 16:0/16:0-phosphatidylcholine (dipalmitoyl lecithin) two phase transitions are observed. The phase transition of 16:0/16:0-phosphatidylcholine, in the mixture, is broadened and shifted to lower temperatures because of interactions with 18:lc/18:1c - phosphatidylcholine which remains in the liquid-crystalline state down to -20°C (7). Effects corresponding to lateral phase separation in vesicles composed of mixed phospholipid species may also be observed with ^{31}P NMR . Fig. 1 shows the ^{31}P NMR spectra

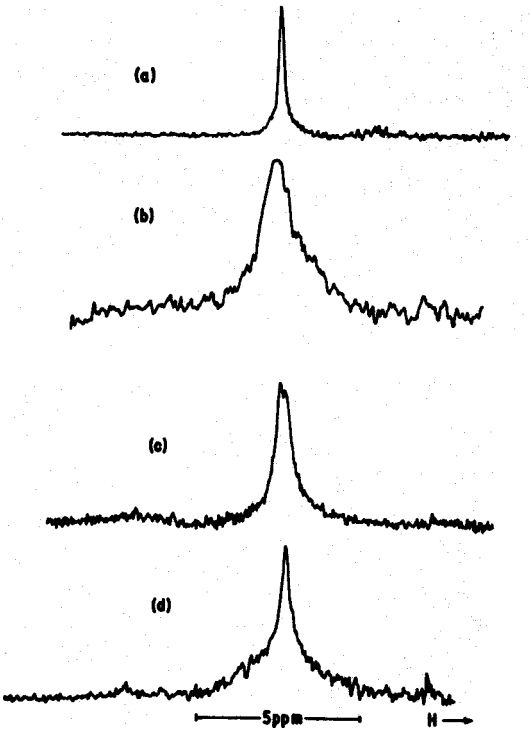

Fig.1. 36.4 MHz ^{31}P NMR spectra at 10°C of (a) 18:1c/18:1c-phosphatidylcholine vesicles, (b) 16:0/16:0-phosphatidylcholine vesicles, (c) 16:0/l6:O-phosphatidylcholine-l8:1c/l8:lc-phosphatidylcholine (1:1) vesicles and (d) 16:0/16:0-phosphatidylcholine-l~:lc/l8:Ic-Phosphatidylcholine vesicles in the presence of 6 mM Co^{2+}.

of 18:1c/18:lc-phosphatidylcholine (Fig.la) 16:0/16:0 phosphatidylcholine (Fig.lb) and 16:0/16:0-phosphatidylcholing-18:lc/l8:lc-phosphatidylcholine (1:l) vesicles (Fig.lc) at IO C. The spectrum of the 16:0/16:O phosphatidylcholine-18:lc/l8:lc-phosphatidylcholine (1:l) vesicles is composed of a broad line, which is ascribed to 16:0/16:0-phosphatidylcholine molecules in the gel state and two narrower components, presumably due to the liquid crystalline 18:lc/18:lc-phosphatidylcholine molecules on the inside; and outside of the vesicle. The outside resonances can be broadened beyond detection by the addition of Co2+. The resultant spectrum of the inside resonances at 10°C, as shown in Fig. la is composed of a broad and narrow line, indicating the occurrence of phase separation in the inside monolayer of this vesicle. Fig.1 also demonstrates that the chemical shift difference between the outside and inside resonances of the narrow component is much larger than the chemical shift difference between the outside and inside resonances of 18:lc/l8:lc-phosphatidylcholine vesicles.

In a similar type of experiment in mixtures of dipalmitoyl and dilauroyl phosphatidylcholines lateral phase separation occurs between the two endothermic phase transitions at 8 and 38 C (21).

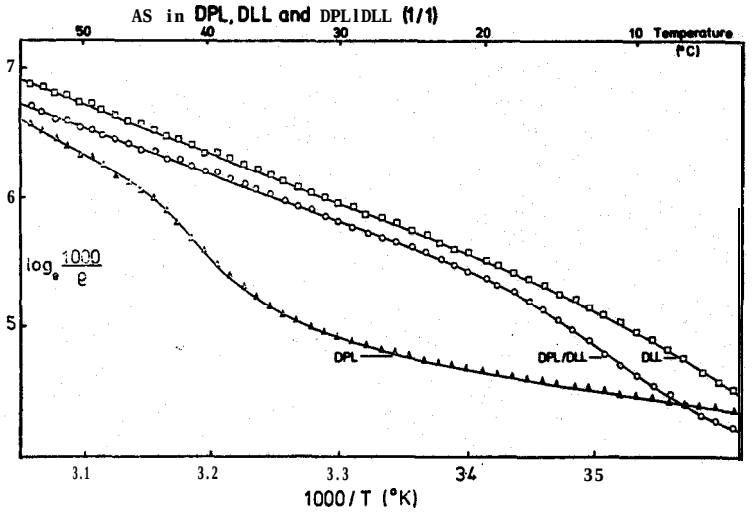

Fig.2 Arrhenius plots of the rate of rotation of 12-(9-anthroyloxy)-stearate bound to dipalmitoyl and dilauroyl phosphatidylcholines and in equimolar mixture of the two. Probe concentration 1μM, lipid concentration 0.5mM. Excitation at 385nm, emission measured above 410nm. 12-AS in dipalmitoyl phosphatidylcholine (A_r), in dilauroyl phosphatidylcholine (□), in equimolar mixture (O).

When the fluorescent probe 12-(9-anthroyloxy-stearic acid is introduced in this mixed system the probe mobility is insensitive to the higher of the two transitions although as mentioned before it detects the phase transition in pure dipalmitoyl lecithin (Fig.2). This leads to the conclusion that in regions of lateral phase separation the probe is almost exclusively localised in the fluid phase. This observation has important implications when fluorescent probes (and possibly other labels) are used in estimating the fluidity ("microviscosity") of biological membranes, The likelihood is that the fluorescent molecule, being an impurity, is always excluded from the regions of the membrane containing lipids in a more ordered form and hence will only measure the motion of the probe in the less structured domains of the membrane.

It is relevant and interesting to note that in lipid-protein complexes (23) and biological membranes (24) proteins have a preference for the disordered lipid regions when phase separation presents a choice to the "protein impurity". Because of this it is important to have a method for measuring the presence of gel and fluid phases (and their amounts) in biological membranes. It is evident that both probe methods and to a lesser extent NMR tend to detect preferentially the more mobile components in mixtures of phospholipids.

Recently we have devised a new method that involves the introduction of positrons, positively charged anti-electrons, into the lipid matrix and the determination of their decay times. The properties of positrons are currently investigated in-experimental physics and theoretical chemistry (25,27). Since, however, the concepts and measurements are not familiar to most biologists we shall briefly summarise them here.

Positrons are positively charged particles with the mass of an electron. There are several sources of such particles but the most conveniently and commonly used source is the isotope 22 Na which decays to an isotope of neon by positron emission. This emission is accompanied by a gamma photon from the excited state of neon, and for most purposes the gamma emission and positron emission can be regarded as simultaneous. Once formed, the positron must dissipate most of its energy before annihilation with an electron is Possible. The positron may then either remain free, in which case it has a natural lifetime of the order of tens of picoseconds before annihilation, or else may bind an electron without being immediately annihilated. The bound state is known as 'positronium' and may be formed in either the singlet or triplet state with a yield weighted by the multiplicity. The singlet state, p-positronium, has a lifetime of the order of I25 picoseconds in free space before selfannihilation with the emission of two simultaneous 0.511 MeV gamma

photons at 180°. The triplet, o-positronium,' is much longer lived with a free space lifetime of about 140 ns, whereafter it decays by the emission of three simultaneous gamma photons at 120° to conserve momentum. Since the triplet lifetime is comparatively long, the species is susceptible to various 'quenching processes' which shorten the measured decay time by providing additional routes for annihilation. One such process is intersystem crossing with subsequent fast annihilation of the singlet positronium, while another of chemical interest is the oxidation leading to a 'bare' positronium. One other process leading to premature annihilation of the triplet state positronium is 'pick-off' annihilation. In this process, the o-positronium, after reaching thermal energy, interacts with one of the electrons from an outer orbital of a surrounding atom. The o-positronium is thus annihilated in a two-photon event. In molecular materials this 'pick-off' annihilation is the predominant route for loss of o-positronium in most circumstances. The pick-off annihilation rate is sensitive to the physical state of the material, and subsequently it is possible to distinguish different phases of a given substance by measurement of this rate.

One difficulty in the measurement is that the large amount of water in biological materials results in a significant contribution to the annihilation pick-off rate. Previously an external source of ^{22}Na sandwiched within two thin mica films and placed between the scintillation detectors of the measuring apparatus was used. We have taken advantage of the presence of water in biological samples by incorporating the $^{22}Na^{+}$ directly into the solution. In this approach a relatively small amount of isotope is needed and the effect of pick-off annihilation within the walls of the containing vessel is minimised.

Positron decay times were measured using conventional fast-slow coincidence circuitry (28). The operational time resolution was 0.7 nsec, or better, using ^{60}Co as calibration source.

Typical decay time spectra obtained for aqueous dispersions of dipalmithyl lecithin are shown in Fig.3. The 'tail' of the decay is a result of pick-off annihilation in the lipid matrix: the component due to water contributes to the earlier part of the decay (see Table 4.) The lifetime component resulting from pick-off annihilation in the lipid remains constant within the accuracy of the analysis at temperatures below the gel-liquid crystalline transition temperature. Above this temperature, the decay time becomes longer and again remains constant at high temperatures. Decay times of 2.8 and 3.3ns are found for the frozen and fluid phases respectively. For dioleoyl and dilauroyl phosphatidylcholine, which are fluid at room temperature, pick-off decay times of 3.3ns are found. In a 1:1 mixture of dioleoyl and dipalmitoyl phosphatidylcholine at a temperature where phase separation occurs, the positron pick-off

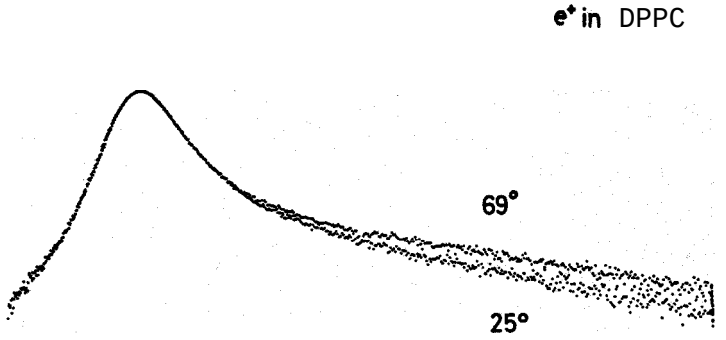

Fig.3. Decay time spectra for positrons in dipalmitoyl phosphatidylcholine at 69° and 25°. Log (intensity) is plotted against time, and least squares analysis of the decay 'tails' gives lifetimes of 2.8 and 3.3 ns at 25 and 69 respectively. Gel-liquid crystalline transition temperature for this lipid is 41.5°.

Table 4. PICK-OFF DECAY TIMES FOR 0-POSITRONIUM IN PHOSPHOLIPIDS

System	Temperature (°C)	Positronium lifetime (ns)	State of liquid
Water		1.9	----
18:lc/18:1c-PC	18	3.3	liquid crystal
12:0/12:0-PC	18	3.3	liquid crystal
egg PC	18	3.25	liquid crystal
16:0/16:Q-PC	69	3.3	liquid crystal
16:0/16:0-PC	25	2.8	gel
18:lc/18:1c-PC 16:0/16:0-PC (1:11	18	3.05	gel and liquid phases

Samples contained 25-5OuCi of $^{22}Na^{+}$ (as NaCl) in 20mg of lipid dispersed in 0.5 ml buffer (10 mM trishydroxymethylaminomethsne, pH 8.5).

annihilation rate would be expected to resolve into components characteristic of annihilation in fluid and frozen phases. Since these rates are similar (decay times of 3.3 and 2.8ns respectively), with equal weighting of components the resultant decay would be difficult to analyse, but would visually resemble a single component of intermediate lifetime. The measured positron pick-off decay time for the mixed lipid system was 3.05nsec (Table 4).

Clearly the decay times of positrons are sensitive to the fluidity of phospholipid-water systems. Positron lifetimes characteristic of fluid and frozen lipids have been established. In a system where fluid and frozen phases co-exist at room temperature on account of lateral phase separation, an intermediate apparent life-time is found; Careful analysis of the decay times is thus likely to provide a method for estimating the extent of phase separation in biological membranes.

3. Lateral Diffusion and Cluster Formation

Rapid lateral diffusion of phospholipids and of some membrane proteins has been demonstrated by several methods in recent years. It is true to say that we do not know if such observations have any direct biological relevance. Nevertheless, their demonstration undoubtedly contributes to the dynamic view of membrane structures (29), first proposed in the 'fluid mosaic model' by Singer and Nicholson (30).

We have recently introduced a new fluorescence method for diffusion studies on lipid and membrane systems (22). The fluorescent molecule 12-(+anthroyloxy)-stearic acid that has been widely used as a lipid like probe (20) dimerises on irradiation with light of 366nm wavelength.

This dimerization proceeds by a diffusion limited second order mechanism in many solvents and in homogeneous fluid lipid dispersions and vesicles. The 'apparent diffusion coefficients' (for details see ref.221 for this probe in a variety of systems are shown in Table 5. In oriented lipid multilayers these diffusion coefficients are similar to those found by other techniques. It is, however, significant that the photodimerisation rates for the probe in fluid lipid vesicles are greater than those found for the rates in oriented multilayers of the same lipids.

There are two effects which may contribute towards the higher rates of diffusion of 12-(9-anthroyloxy)-stearic acid in vesicles. Firstly, the curvature of vesicles may cause structural differences permitting more rapid diffusion than in the multilayers. Secondly,. the Brownian motion of the bulk aqueous phase, experienced by the probe in vesicles but not in multilayers, may increase the rate of

lateral diffusion (31).

To determine which of these effects is responsible, the rates of diffusion of a probe which is located exclusively in the hydrocarbon region of phospholipids, and thus experiencing the Brownian motion of the bulk aqueous phase in neither vesicle nor oriented multilayers were investigated using excimer formation by pyrene derivatives as a means of comparing diffusion rates in the two model systems. Pyrene is located in the hydrocarbon interior of model membranes, while pyrene-butyric acid is accessible to the bulk aqueous phase in vesicles on account of its amphiphilic character. For a given lipid: probe ratio the amounts of excimer formed in vesicles and oriented multilayers were comparable for pyrene. This is consistent with the data of Cullis and Vanderkooi, who calculated a diffusion coefficient of 3.0×10^{-8} $cm^2 sec^{-1}$ for this probe in vesicles (32). For pyrene butyric acid excimer formation was considerably greater in vesicles than in oriented multilayers for a given lipid:probe ratio.

Table 5. INITIAL RATES OF BLEACHING OF l2-(9-ANTHHOYLOXY)-STEARIC ACID IN VARIOUS MEDIA

Medium	$k_{bleaching}$	Probe Distribution
n-Butanol	$2.9 \cdot 10^{-7}$	isotropic
Water	$2.0 \cdot 10^{-6}$	clustered
Dodecane	$3.7 \cdot 10^{-6}$	partly clustered
Lauric acid	$2.0 \cdot 10^{-6}$	clustered
Dilauroyl-PC		
vesicles	$2.8 \cdot 10^{-7}$	isotropic
multilayers	$2.0 \cdot 10^{-8}$	"
Dielaidoyl-PC		
vesicles	$2.3 \cdot 10^{-7}$	"
multilayers	$2.5 \cdot 10^{-8}$	"
Dioleoyl-PC		
vesicles	$1.9 \cdot 10^{-7}$	"
multilayers	$2.0 \cdot 10^{-8}$	"
Dipalmitoyl-PC		
vesicles	$2.0 \cdot 10^{-6}$	clustered

Bleaching rates are in arbitrary units at 20°C. Diffusion coefficients can be calculated from these as in ref. 22. These calculations involve certain assumptions, but the ratio of rates in different lipid systems gives the ratio of diffusion coefficient directly.

These observations suggest that molecules which are accessible to the bulk aqueous phase will have significantly greater diffusion coefficients in phospholipid vesicles than in oriented multilayers on account of reduction of translational drag by the surrounding water (31).

Finally we should mention that anomalously high rates of photodimerization of 12-(9-anthroyloxy)-stearic acid are seen in water, in heterogenous hydrocarbons like liquid paraffin, phospholipids below their phase transition temperatures end in heterogeneous but fluid lipids like egg lecithin (Table 5.) In all these cases we have attributed the observed anomaly to the formation of localised high concentrations (clusters) of the fluorescent molecule. While it is not surprising that below the gel-liquid crystalline transition temperature of the phospholipid the probe tends to be excluded from the gel phase of the lipid matrix (see in lateral phase separation above) clustering in fluid lipids (and in hydrocarbon mixtures) is not necessarily obvious. We have suggested before on the basis of fluorescence polarisation experiments that even in liquid systems 'short range order' or 'liquid clustering' takes place (21). This would also account for the unusually high photodimerization rates of the fluorescent molecule.

As far as the biological problems are concerned 'correlated fluid motion' (which in a sense is equivalent to the formation of short range order above the melting temperature) could have a role in the rapid opening and closing of 'channels' in the membrane that could be responsible for passive diffusion processes across the lipid bilayer.

III. THE RELATION BETWEEN THE PROPERTIES OF PHOSPHOLIPIDS AND BIOCBEMICAL FUNCTIONS OF THE CHROMAFFIN GRANULE MEMBRANE

We now return to the problem of the chromaffin-granule membrane and some of its special properties that may be important in the process of exocytosis (membrane fusion). We record that both the ATPase end NADH: acceptor oxido-reductase activities of the chromaffin-granule membrane have discontinuous Arrhenius temperature versus activity relationships with 'transitions' at **33 C (33).** The transition for the NADH : acceptor oxido-reductase is removed by treatment with the detergent triton X-100. It is important to emphasize that such anomalies in Arrhenius behaviour in themselves may have no special significance (a fact that is often overlooked in membrane studies). What is significant in the chromaffin granule system is that the fluorescence properties of four different fluorescent probes (33,34) and the behaviour of five other types of spin labels (35) all indicate that some ordering of the membrane lipids occur below 33°C. What makes this observation particularly attractive is that this transition temperature is so close to the physio-

logical operating range of the system. It is almost as if this membrane is poised to undergo some transition (responding to the triggering signal) as part of the fusion process. The cholesterol content of the membrane which is close to the region of 30% cholesterol per total lipid, where cholesterol segregation (see above) is just beginning to become a possibility, may well be significant to this. Although at present it is clear that the biological system is still too complex to draw definite conclusions, the model studies have allowed us to point to possibilities and defined the limitations and advantages of the various methods that hopefully will lead to a solution.

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