Stability of Unimolecular Films of ^{32}P -Labelled Lecithin

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(Received 22 February 1967)

1. The stability of monolayers of a highly unsaturated yeast lecithin labelled with ^{32}P has been investigated by a surface radioactivity technique. 2. Lecithin films on distilled water at all surface pressures between 6 and 48 dynes/cm. were completely stable on rapid perfusion of the subphase and on addition of ionic amphipathic substances to the film. 3. Ultrasonically treated lecithin added to the subphase caused a slow loss of surface radioactivity but little pressure change. 4. The addition of proteins to the subphase caused negligible changes in the film even when conditions were favourable for electrostatic heterocoagulation and penetration. 5. Lecithin films were not hydrolysed by a strongly acid subphase at room temperature. The very low rate of hydrolysis produced by alkali was proportional to the subphase OH^- ion concentration: the apparent activation energy and temperature coefficient (Q_{10}) of the reaction were 14250 cal. and 2.37 respectively. 6. Alkaline hydrolysis of lecithin monolayers was markedly stimulated by adding methanol (10–20%, v/v) to the subphase. The addition of ionic amphipaths to the monolayer had the expected type of effect on the hydrolysis rate, but its magnitude was far less than that suggested by an application of the Poisson–Boltzmann equation for ion distribution at a charged interface (Davies & Rideal, 1963).

In studying the reaction of proteins, including enzymes, at the lecithin–water interface (monolayers, bimolecular layers), it is of value to know the stability of individual lecithin molecules in the organized lipid structure. Recently, for example, evidence has been obtained (Rosenberg, 1966) that monolayers of phospholipids may break up to form clusters of molecules or surface micelles in equilibrium with free molecules.

Solubilization of monolayers of fatty acids, long-chain alcohols and amines is a fairly well studied phenomenon. The rate of solution of such monolayers depends on the ionic strength and composition of the subphase solution (Davies, 1951) and it has been suggested that, with ionized straight-chain compounds, at least 24 carbon atoms in the chain are necessary to avoid solubility effects. However, little attention has been paid to unimolecular films of naturally occurring phospholipids. In the present experiments we have measured the stability of monolayers of highly unsaturated ^{32}P -labelled yeast lecithin by using a surface radioactivity technique. Such films were found to be largely stable even when the interfacial potential was changed by the introduction of charged amphipathic molecules, or when various substances (acid, alkali, proteins,

ultrasonically treated lecithin) were added to the subphase.

EXPERIMENTAL

The ^{32}P -labelled yeast lecithin was prepared as described by Bangham & Dawson (1960) except that the phospholipids were initially extracted from the yeast with boiling ethanol–water (4:1, v/v) for 5 min. followed by chloroform–methanol (1:1, v/v). The ^{32}P lecithin peak emerging from the final silicic acid column was collected as a main fraction followed by a ‘tail’ fraction. Gas–liquid–chromatographic analysis showed that both fractions were highly unsaturated, consisting mainly of palmitoleic acid and oleic acid, as found by Shah & Schulman (1965). The ‘tail’ fraction contained substantially more of a minor C_{20} polyunsaturated fatty acid than the main fraction.

The trough used and the apparatus for recording surface pressure and radioactivity are described in the legend to Fig. 1. Arrangements are also described for heating or changing the subphase when desired.

Ultrasonically treated lecithin was prepared by subjecting a suspension of coarse ovoid lecithin particles (2 ml.) to ultrasound from a Mullard 60 W generator for 15 min. until it was virtually water-clear. Triphosphoinositide was prepared by the method of Dittmer & Dawson (1961) and phosphatidic acid by the method of Dawson & Hemington (1967). Dicycylphosphoric acid (Albright and Wilson Ltd., London, W. 1), and cetyltrimethylammonium bromide (Hopkin and

Fig. 1. Apparatus for investigating the stability of lecithin monolayers. The trough (a) (20 cm. long $\times$ 4.5 cm. wide $\times$ 0.8 cm. deep) was milled from a block of polytetrafluoroethylene (Fluon; Imperial Chemical Industries Ltd., Welwyn Garden City, Herts.) and fixed to a rigid metal framework to prevent distortion. The subphase (72 ml.) was stirred with a reciprocating magnet (b) moving a glass-sheathed steel stirrer (1 mm. diam.) (c) within the trough. Sufficient [32 P]lecithin dissolved in ether or CHCl_3 -ether was added to the subphase surface from a micrometer syringe (Agla) to form a film. The surface pressure was adjusted with a Fluon barrier (d) weighted on the top with a heavy metal strip. The surface pressure was measured, as the vertical force exerted on a glass dipping plate (e) made from half a microscope cover slip and suspended from the arm of a torsion balance (f). In some experiments this was replaced by a thin glass rod (0.5 mm. diam.) suspended from the arm of an electronic force balance (Mark 2; C. I. Electronics Ltd., Staines, Middlesex) and the pressure was continuously measured on a pen recorder. When necessary the subphase was perfused through a 2 l. separating funnel and constant-pressure device (g). The level of the fluid in the constant-pressure device (g) and of the trough outlet tube were adjusted in relation to the surface film so that the level of the subphase was maintained with a suitable flow rate of fluid through the trough. When heating of the subphase was required it was circulated over a heated glass tube by means of a simple centrifugal pump (h). This was constructed from a small glass T-piece rotated by a variable-speed laboratory stirrer. Two heaters were used; the current to the first heater (i) was adjusted with a rheostat so that it did not quite supply sufficient heat to balance the heat loss at the desired temperature. The second heater (j) was thermostatically controlled from the contact thermometer (k). To obtain uniform flow and temperature ($\pm 0.3^\circ$) within the trough, the inlet tube (l) was arranged with a series of inward-facing holes which increased in diameter towards the exit end of the trough. The radioactivity emanating from the [32 P]lecithin film was recorded with a mica-window Geiger-Müller counter (m) shielded in a lead case (n) and connected to an integrating ratemeter (Ekco) (o); the output was continuously recorded with a pen recorder (p).

Williams Ltd., Chadwell Heath, Essex) were commercial samples.

RESULTS

Perfusion of subphase

Films of [32 P]lecithin at various pressures on a subphase of distilled water (pH 5.5) or 0.9% sodium chloride were remarkably stable and no loss of radioactivity or surface pressure was observed over many hours. This stability persisted when the subphase was stirred and changed at the rate of 7–10 ml./min. (Table 1).

The addition of an anionic amphipathic substance (triphosphoinositide; molar ratio to lecithin 1:20) or cationic amphipathic substance (cetyltrimethyl-

ammonium bromide; molar ratio to lecithin 3:20) to the film in no way affected its stability (Table 1).

Addition of ultrasonically treated lecithin to subphase

A loss of radioactivity from a [32 P]lecithin monolayer was observed when a water-clear suspension of ultrasonically treated ovollecithin (about 1000 times the quantity in the film) was added to the subphase (Table 1). However, this loss was at the limit of detection (1% in 6–8 min.) and so exchange between molecules of lecithin in the film and subphase colloid was very slow (Table 1). Adding triphosphoinositide to both the film and the lecithin colloid did not change the rate detectably.

Table 1. *Stability of yeast lecithin monolayers on distilled water*

All observations were at room temperature. The main part of the [^{32}P]lecithin peak was used.

Composition of film	Surface pressure (dynes/cm.)	Treatment	Duration of experiment (min.)	Rate of loss of surface radioactivity (%/min.)
[^{32}P]Lecithin	30	Subphase changed at 6.9 ml./min.	106	0
[^{32}P]Lecithin	46	Subphase changed at 8.7 ml./min.	70	0
[^{32}P]Lecithin + triphosphoinositide (molar ratio 20:1)	46	Subphase changed at 7 ml./min.	78	0
[^{32}P]Lecithin + triphosphoinositide (molar ratio 20:1)	16	Subphase changed at 7 ml./min.	90	0
[^{32}P]Lecithin + cetyltrimethylammonium bromide (molar ratio 20:3)	6	Subphase changed at 14.5 ml./min.	74	0
[^{32}P]Lecithin + cetyltrimethylammonium bromide (molar ratio 20:3)	40	Subphase changed at 10 ml./min.	76	0
[^{32}P]Lecithin	16	Ultrasonically treated ovoidlecithin (500 μg . of P) added	142	0.12
[^{32}P]Lecithin	35	Ultrasonically treated ovoidlecithin (500 μg . of P) added	102	0.18
[^{32}P]Lecithin	18	Ultrasonically treated mixture of ovoidlecithin + triphosphoinositide (molar ratio 20:1) (500 μg . of P) added	76	0.13

The surface pressure of the lecithin monolayer showed no marked change, although sometimes a slight increase was observed, suggesting that molecules from the ultrasonically treated lecithin were entering the film.

Addition of proteins to subphase

Proteins were added to the subphase under [^{32}P]lecithin monolayers at pressures below 30 dynes/cm. to encourage penetration as well as electrostatic adsorption (Matalon & Schulman, 1949). No significant loss of film material or pressure change occurred on adding sheep albumin (equivalent to 1 ml. of serum), sheep globulin (equivalent to 1 ml. of serum), γ -globulin (4 mg.), haemoglobin (10 mg.), cytochrome *c* (11 mg.) or experimental allergic encephalomyelitis protein (1 mg.). A barely detectable loss of surface radioactivity (1% in 12–14 min.) and a slight pressure loss (1–1.5 dynes/cm./hr.) occurred when protamine (6 mg.) and histone (3.5 mg.) were added to the subphase under a film of [^{32}P]lecithin + triphosphoinositide (molar ratio 20:1).

Variation of subphase pH

Acid hydrolysis. No loss of surface radioactivity occurred from unimolecular films of [^{32}P]lecithin

when the subphase was made strongly acid (1.6 N) with hydrochloric acid. When the subphase temperature was raised to 50°, hydrolysis commenced (1.03%/min.).

Alkaline hydrolysis. The rate of loss of surface radioactivity on adding alkali to the subphase was slow at room temperature. The rate was linearly dependent on the bulk OH^- ion concentration (Fig. 2), which suggests that the equation of Davies & Rideal (1963) for reactions at liquid surfaces is applicable:

$$-\frac{dnr}{dt} = knr[\text{OH}^-]_s$$

where nr is the number of molecules/cm.² remaining in the film after time t , k is the velocity constant of the reaction and $[\text{OH}^-]_s$ is the OH^- ion concentration in the plane of the interface, which in this case must presumably be equal or directly proportional to the bulk OH^- ion concentration ($[\text{OH}^-]_b$):

$$[\text{OH}^-]_s = k_1[\text{OH}^-]_b$$

$$-\frac{dnr}{dt} = k_1 knr[\text{OH}^-]_b = k_2 nr[\text{OH}^-]_b$$

In practice no hydrolysis was detected below an alkali concentration of 0.1 N-sodium hydroxide, but since the lines in Fig. 2 pass through the origin this is presumably because any slight loss in the surface

Fig. 2. Relation between the rate of the alkaline hydrolysis and the OH^- ion concentration. O, Main part of lecithin peak from silicic acid column (fractions 18-33); ●, 'tail' part of lecithin peak from silicic acid column (fractions 34-50).

radioactivity at lower alkali concentrations is masked by the experimental fluctuation of radioactivity emanating from the film. The velocity constant also varied slightly with the fatty acid composition of the ^{32}P lecithin used to form the film (Fig. 2).

The variation of the apparent velocity constant (k_2) with temperature (20-45°) followed the Arrhenius equation:

$$\ln k_2 = -\frac{E}{RT} + \text{constant}$$

(where E is the activation energy and R is 1.986 cal./deg./mole) so that when $\log k_2$ was plotted against $1/T$ a straight line resulted (Fig. 3). The apparent temperature coefficient (Q_{10}) of the reaction obtained from the slope is 2.37 and the activation energy is 14250 cal.

The hydrolysis of a lecithin monolayer by alkali was not appreciably affected by changing the initial surface pressure of the lecithin film between 6 dynes/cm. and collapse pressure (48 dynes/cm.) (Table 2), nor was the rate systematically affected by changing the ionic diameter of the hydrated cations of the base. Thus substituting Li^+ (7.3 Å) or K^+ (3.8 Å) for Na^+ (5.6 Å) both slightly depressed the reaction rate (Table 2).

The effect of substances that might be expected to change the surface concentration of OH^- ions is shown in Table 2. Anionic amphipathic substances (dicetylphosphoric acid, phosphatidic acid), when added to the monolayer, decreased the rate of radioactivity loss from the film, whereas a large amount of cetyltrimethylammonium bromide had a slight stimulatory effect. The addition of sodium chloride at high concentration (4M) did not appreciably affect the rate.

The addition of methanol (10-20%, v/v) to the subphase produced a marked stimulation of the loss

Fig. 3. Plot of the log of the apparent velocity constant (k_2) against the reciprocal of absolute temperature.

of radioactivity from a ^{32}P lecithin monolayer on adding alkali (Table 2). Such films in the absence of alkali were stable on subphases containing methanol up to 30%, (v/v), whereas complete solubilization occurred on increasing the methanol concentration to 35% (v/v). The addition of ethanol (10%, v/v) to the subphase caused a less-marked stimulation, as did octadecanol (molar ratio to lecithin 3:10) added to the lecithin monolayer (Table 2).

DISCUSSION

Present evidence suggests that the molecular associations of many amphipathic substances in water (e.g. micelles) should not be necessarily regarded as persistent fixed structures (Pethica, 1960). Rather they may be in dynamic equilibrium with single amphipathic ions or simple associations of two or three ions in the bulk aqueous phase. Indications exist, however, that micro-structures formed by naturally occurring lecithin in the presence of water do not dissociate into free phospholipid molecules. Thus lecithin in aqueous dispersion does not pass through a dialysis membrane, and when very low concentrations (0.5 mg./l.) are examined by light-scattering lecithin is still found to exist as particles of molecular weight about 2×10^7 (Robinson, 1960).

The present results suggest that an organized monolayer of lecithin at the air-water interface is extremely stable, and that if any exchange or loss to the bulk phase occurs it must be extremely slow and not detected by the present techniques. Thus no loss of lecithin molecules or micelles was observed when the subphase under ^{32}P -labelled yeast lecithin

Table 2. *Digestion of [^{32}P]lecithin films with alkali*All observations were at room temperature. The main part of the [^{32}P]lecithin peak was used.

Composition of monolayer	Initial surface pressure (dynes/cm.)	Composition of subphase	Rate of loss of surface radioactivity (%/min.)
[^{32}P]Lecithin	6	NaOH (0.13N)	0.45
[^{32}P]Lecithin	25	NaOH (0.13N)	0.40
[^{32}P]Lecithin	48	NaOH (0.13N)	0.45, 0.43
	(collapse)		
[^{32}P]Lecithin	Collapse	LiOH (0.14N)	0.37
[^{32}P]Lecithin	Collapse	KOH (0.14N)	0.33
[^{32}P]Lecithin	Collapse	NaOH (0.13N) + NaCl (4M)	0.47
[^{32}P]Lecithin	23	NaOH (0.13N) + methanol (10%, v/v)	1.0
[^{32}P]Lecithin	Collapse	NaOH (0.13N) + methanol (10%, v/v)	1.0
[^{32}P]Lecithin	34	NaOH (0.13N) + methanol (20%, v/v)	1.8
[^{32}P]Lecithin	Collapse	NaOH (0.14N) + ethanol (10%, v/v)	0.70
[^{32}P]Lecithin + octadecanol (molar ratio 10:3)	Collapse	NaOH (0.14N)	0.72
[^{32}P]Lecithin + cetyltrimethylammonium bromide (molar ratio 20:3)	Collapse	NaOH (0.14N)	0.43
[^{32}P]Lecithin + cetyltrimethylammonium bromide (molar ratio 2:1)	Collapse	NaOH (0.14N)	0.53
[^{32}P]Lecithin + dicetylphosphoric acid (molar ratio 2:1)	Collapse	NaOH (0.14N)	0.29
[^{32}P]Lecithin + phosphatidic acid (molar ratio 1:1)	Collapse	NaOH (0.14N)	0.18

films was changed at a rapid rate. Yeast lecithin is highly unsaturated and this hinders it from forming the close intermolecular associations found with more saturated lecithins (Shah & Schulman, 1965). This might be expected to favour dissociation, but even with expanded films at surface pressures of only 6 dynes/cm. when the area per molecule of lecithin is 100–110 Å², depending on the preparation, no loss to the bulk phase was apparent. Clearly lecithin molecules in a monolayer form an extremely stable association with each other. This is probably due to the zwitterionic nature of the hydrophilic 'head' group, minimizing electrostatic repulsion, and the roughly cylindrical shape of the molecule, which allows symmetrical packing and binding by non-ionic forces (Kavanau, 1965). Coulombic interactions between the zwitterionic 'head' groups, which are arranged tangentially coplanar, would also help to stabilize a lecithin monolayer (Pethica, 1965). If the molecule is made more wedge-shaped by the enzymic removal of a single fatty acid, the resulting lysolecithin rapidly leaves the film (Dawson, 1966). However, even when the surface potential of lecithin monolayers was increased by the introduction of cationic or anionic amphipathic substances they were still completely stable. In physicochemical terms, either the lecithin has no solubility in the bulk phase or the energy barrier

preventing solution of molecules from the film is very large so that the metastable film approaches absolute stable equilibrium at an infinitely slow rate.

The very slow loss of radioactivity occurring when ultrasonically treated lecithin was added to the subphase is probably not due to any solubilization of individual molecules, but may occur when a colloidal particle of the lecithin collides with the film. Possibly exchange of phospholipid can occur within the collision complex without free phospholipid molecules being released into the bulk phase. The observation that the film pressure did not rise appreciably in the presence of ultrasonically treated lecithin again indicates that the bimolecular lamella structure of these lecithin particles (Bangham, 1963) does not readily dissociate into free molecules or spherical micelles.

The observation that the addition of protein to the subphase causes no loss of radioactivity or change in the surface pressure emphasizes the general lack of reactivity of the lecithin–water interface to protein (Matalon & Schulman, 1949). Even when the interfacial potential was made more negative by the introduction of triphosphoinositide into the monolayer, the solubilization and pressure changes produced by highly positively charged basic proteins were minimal. Such potential

differences would be expected to encourage the adsorption of protein on the monolayer (Matalon & Schulman, 1949), although of course this need not necessarily lead to a solubilization of the film as a lipid-protein complex.

The high stability of lecithin monolayers to an acid subphase might be explained by the difference between the interfacial H^+ ion concentration (pH_s) and the bulk (pH_b):

$$pH_s = pH_b + \frac{e\psi}{2.3kT}$$

where ψ is the interfacial potential, k is Boltzmann's constant, e is the electronic charge and T is absolute temperature. Now at low pH values the lecithin polar 'head' group will acquire a positive charge owing to suppression of ionization of the phosphate group. This is seen experimentally in the sudden increase in the ζ -potential of lecithin particles on lowering the pH below 3 (Bangham & Dawson, 1959). As a result of this positive surface potential H^+ ions would be repelled away from the film and the surface pH would be below that of the bulk. Assuming complete suppression of ionization to derive ψ from the Gouy equation, the surface pH of lecithin in 1.6N-hydrochloric acid can be calculated to be about 2.3. However, apart from this electrostatic effect, it is likely that H^+ ions are much less powerful catalysts than OH^- ions for the hydrolysis of long-chain esters in films as they are for these esters in the bulk phase. Thus Alexander & Rideal (1937) found that monolayers of simple glycerol esters of fatty acids were hydrolysed extremely slowly on strongly acid subphases.

Although alkali does hydrolyse [^{32}P]lecithin monolayers at room temperature the rate is by no means rapid. Presumably the removal of the one fatty acid from the lecithin molecule by the alkali causes a loss of radioactivity from the film as lysolecithin (Dawson, 1966) unless the solubilization of the lysolecithin is in some way prevented by the alkali present. It is likely that any lysolecithin micelles would be rapidly hydrolysed in the subphase and in fact, after the reaction, radioactive glycerylphosphorylcholine could be detected by a paper-chromatographic technique (Bangham & Dawson, 1960). In comparison with monolayers, lecithin in methanolic solution is hydrolysed extremely rapidly and is completely deacylated within 1 min. at room temperature (Marinetti, 1962). This suggests therefore that the structural organization of the orientated lecithin molecules in a unimolecular film must present an effective steric barrier to the approach of an OH^- ion to the acyl ester bond. Lowering of the packing density of the film does not remove the steric hindrance to hydrolysis. Alexander & Rideal (1937) have reported that the absolute reaction velocity for

the alkaline saponification of trilaurin monolayers was only slightly changed when the surface pressure was varied between 5 and 16 dynes/cm. However, a very marked effect of surface pressure on the alkaline saponification of monolayers of ethyl palmitate has been observed (Alexander & Schulman, 1937). In this latter case it is probable that at high pressures the ethyl groups are forced down into the subphase and protect the carbonyl links from alkali attack. Since the addition of methanol, and to a smaller extent ethanol, to the bulk phase markedly increases the alkaline hydrolysis rate of lecithin monolayers, this may mean that the methoxyl ions find it easier to penetrate into the region of comparatively low dielectric constant where the acyl ester bonds are situated. It does not seem likely that the methanol is merely providing spacer molecules, since the hydrolysis rate is not affected by varying the surface pressure of the film.

A second factor that might be expected to affect the hydrolysis rate is the sign and magnitude of the interfacial potential. The observations of Davies (1954) and E. Havinga (unpublished work, quoted by Gaines, 1965) have made it clear that the attraction and repulsion of OH^- ions, brought about by altering the interfacial potential, can have a marked effect on the saponification of monolayers containing simple fatty acid esters. Thus the incorporation of a long-chain quaternary nitrogen compound into such a monolayer accelerated the hydrolysis, presumably by attracting OH^- ions and increasing their concentration near to the surface, whereas long-chain anions inhibited the hydrolysis by the converse mechanism.

The magnitudes of these effects can be calculated from the equation:

$$[OH^-]_s = [OH^-]_b e^{-\psi e/kT}$$

where $[OH^-]_s$ and $[OH^-]_b$ are the concentrations of OH^- ions in the surface (s) and bulk (b) respectively, ψ is the surface potential, e is the electronic charge, k is Boltzmann's constant and T is the absolute temperature.

In the present instance, the introduction of cetyltrimethylammonium bromide into lecithin films produced a comparatively small increase in the hydrolysis rate (nil and 20% respectively at lecithin/cetyltrimethylammonium bromide molar ratios 20:3 and 2:1), whereas dicetylphosphoric acid (molar ratio to lecithin 1:3) inhibited the hydrolysis by 34% and phosphatidic acid (molar ratio to lecithin 1:1) by 59%. Though these results indicate that the electrostatic conditions at the interface do have an effect on the hydrolysis rate, their magnitude is very much less than that predicted from the ionic distribution equation (above). The surface potential is calculated either from the Gouy or Donnan equations (Davies &

Rideal, 1963), it being assumed that lecithin itself is a balanced zwitterion and has no surface charge.

The results suggest that, when studies are made of the way in which phospholipases attack unimolecular films of lecithin, it can be assumed that the enzyme is really catalysing the hydrolysis of the film rather than of 'solubilized' lecithin in equilibrium with the monolayer. Thus the rate of hydrolysis of yeast lecithin monolayers by phospholipases (A, B and C) is many hundreds of times faster than any 'solubilization' of such films recorded in the present investigation. The results also have some relevance to the problem of the continual dynamic exchange of lecithin molecules in the bimolecular lipid layers of natural membranes. They suggest that either the lecithin molecules may enter and leave the membrane in the form of a more soluble component, e.g. lysolecithin, or that the presence of protein must lower the energy barriers, preventing the interchange of phospholipid molecules, as it presumably does in the collision complex formed by two soluble lipoproteins (Gurd, 1960).

H. H. is grateful to the British Council for a grant, and is on leave from the University of Graz.

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