RESEARCH PAPERS

PHYSICO-CHEMICAL EXPERIMENTS WITH PHOSPHATIDYL ETHANOLAMINE SOLS

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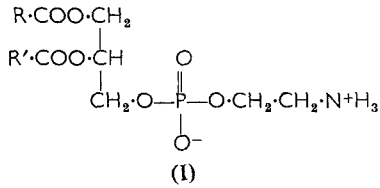
The solubilities of phosphatidylethanolamine (PE) in some organic solvents have been determined. The results of surface tension studies of aqueous sols of PE show that it has marked surface-active properties and surface ageing occurs. The effect of pH, concentration, mono- and divalent salts on surface activity has been investigated, and discussed in the light of previous theories. The critical micelle concentration is in the range 0·002 to 0·01 per cent w/v. The isoelectric point is at pH 3·1. The effect of mono- and divalent salts on the stability of PE sols has been studied.

Phosphatidylethanolamine is a member of the group of phosphatides called cephalins, which occur in nearly all living cells. The phosphatides form an important part of cell walls (Danielli and Stein, 1956), and a knowledge of their physical chemistry will assist in elucidating the structure of cell membranes and the physico-chemical aspects of drug action.

The structure of naturally occurring PE is shown by I which is the zwitterionic structure of L-α-phosphatidylethanolamine.



(I)

Where R and R′ are fatty acid chains

The fatty acid chains are of variable length and degree of unsaturation. The fatty acids attached to the α-carbon of the glycerol moiety contain mainly 16 or 18 carbon atoms and are predominantly saturated. The fatty acids attached to the β-carbon atom contain between 18 and 22 carbon atoms and are predominantly unsaturated.

The molecule is amphipathic since it contains long non-polar hydrocarbon chains which are lipophilic and a polar phosphate-ethanolamine grouping which is hydrophilic. It might be expected therefore, to be surface-active and its surface activity at the air-water interface has been studied.

Experimental and Results

Preparation of Phosphatidylethanolamine

The yolks of 60 eggs were separated and extracted repeatedly with acetone until a white powder was obtained. The powder was then

extracted with 5 litres (2 × 2 × 1 litre quantities) of absolute ethanol at 55°. The ethanolic solution was evaporated to dryness under vacuum and the residue dissolved in the minimum quantity of ether and reprecipitated by pouring the solution into 3 litres of acetone. The mixture of crude phosphatides obtained (105 g.) was passed down a cellulose column (4 cm. in diameter), containing 100 g. of cellulose, using a mixture of chloroform : methanol : water (800 : 200 : 25 v/v) as eluent. This procedure was necessary to remove amino-acids (Lea and Rhodes, 1953). The mixed phosphatides were then subjected to a chromatographic separation

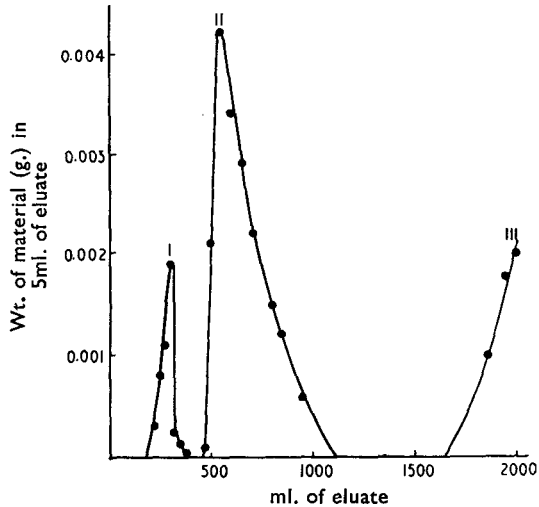


FIG. 1. The separation of mixed egg phospholipids on silicic acid. I, Yellow pigment. II, Phosphatidylethanolamine fraction. III, Lecithin fraction (commencement).

using silicic acid as the stationary phase and a mixture of chloroform : methanol (4 : 1 v/v) as eluent. A column 7·5 cm. in diameter was used into which 350 g. of silicic acid (Mallinkrodt 100 mesh) was slurried and allowed to pack under a slight pressure of nitrogen (2 lb./in.^{2}). The loading of the column was 14 g. No Celite was required as a filter aid because, with that pressure of nitrogen applied to the column, the flow-rate was satisfactory. The phosphatidylethanolamine travelled as a well-defined band immediately behind the pigment band and was well separated from the lecithin fraction (see Fig. 1). The yield of PE was 3·3 g.

Amino-containing compounds were identified by Lea and Rhodes' (1954) modification of the ninhydrin method of Moore and Stein (1948). Choline-containing compounds were identified by Lea, Rhodes and Stoll's modification (1955) of the phosphomolybdic acid test of Chargaff Levine and Green (1948).

The phosphatidylethanolamine was purified by dissolving in the minimum quantity of methyl ethyl ketone at room temperature and then adding acetone and warming the solution until the precipitate would just

dissolve at 50°. The solution was then allowed to stand at 0°, when the PE crystallised out. This recrystallisation procedure was repeated four times. The product was a very pale buff powder (yield, 3 g.). The compound had the following properties: iodine value = 75; N:P ratio = 1:1·02. The product was dissolved in chloroform, and stored under nitrogen at −20° in a desiccator.

Solubility Studies

The solubility of PE in some organic solvents was determined at 25° and 45° (Table I). Excess solute was placed in 25 ml. quickfit tubes together with 5 ml. of the solvent and the tubes shaken at an elevated temperature for 1 hr. and then allowed to cool to the required temperature. The tubes were then allowed to stand in a thermostat (±0·1°) for 8 hr. to reach

TABLE I

SOLUBILITY OF PE IN SOME ORGANIC SOLVENTS

Solvent	Solubility in g./100 ml. solution	
	at 25° C.	at 45° C.
Diethyl ether	18·88	—
Ethanol	5·05	6·06
Methanol	3·87	5·26
Methyl ethyl ketone	20·60	—
Acetone	0·43	1·06
Chloroform	36·23	—

equilibrium. 2 ml. of the supernatant solution was removed and evaporated to dryness overnight under vacuum at 50°. The residue was then weighed.

All organic solvents used were of Analar quality and were redistilled before use.

Surface Tension Studies

Preparation of aqueous sols. Aqueous sols could not be obtained by direct solution of PE in water, so the solute was dissolved initially in 5 ml. of ether. Successive small quantities (2 ml.) of distilled water were added with intermittent shaking. The sol became progressively thicker until it became a gel, but on further addition of water, it became fluid again. The ether was removed by gentle warming and finally by bubbling nitrogen through the sol. The sol was then passed down an ion-exchange column containing a mixture of 1·5 g. of Amberlite IR 120(H) and 1·3 g. of Amberlite IR 45(OH) resins to remove traces of electrolytes. The resins were washed with small successive quantities of distilled water and the sol was finally made up to volume.

It was found during preliminary studies that 5 per cent of the PE was lost on the resins, so a corresponding adjustment was made to the final volume of the sol. A 0·01 per cent w/v sol had a specific resistance of 100,000 ohm cm.

On preparing a number of sols of PE of the same concentration, small variations in the equilibrium values of the surface tension were observed,

because of inherent errors in the method of preparation. To overcome these, in a series of experiments, a large volume of sol was prepared and portions of this used as required.

Apparatus for surface tension measurements. A static method (Wilhelmy plate) was used for measuring the surface tension of the sols. The method involved using a chainomatic balance, reading to 10^{-4} g., as described by Harkins and Jordan (1930). It stood on a platform which could be raised or lowered by a screw thread mechanism so that zero contact angle could be obtained between the plate and the surface of the

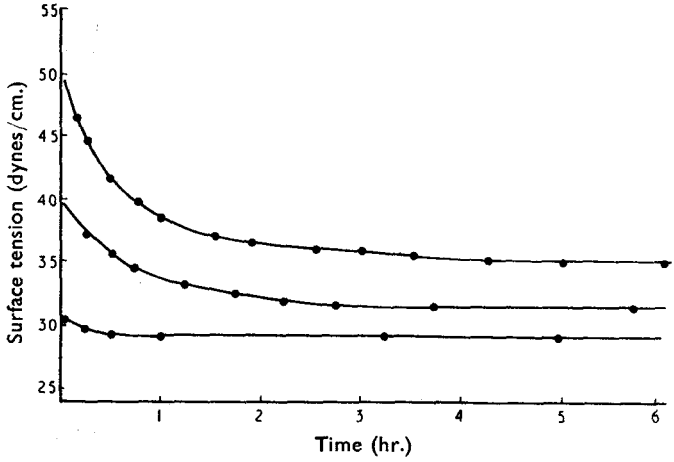


FIG. 2. Variation of surface tension of phosphatidylethanolamine sols with time. Upper curve, 0·005 per cent. Middle curve, 0·0075 per cent. Lower curve, 0·01 per cent.

sol. The platinum plate was suspended from one arm of the balance by a thin chrome-nickel wire, which had a levelling device on it to ensure that the plate hung horizontally to the surface of the sol. The sol was in a large Pyrex glass beaker immersed in a thermostat ($\pm$0·05°) which was fitted with a lid to ensure that the atmosphere above the sol was saturated with water vapour. The wire on which the platinum plate hung, passed through a small hole in the lid.

Variation of surface tension of PE *sols with time.* The surface tensions of a series of sols of PE were measured over a period of time. The results (see Fig. 2) show an ageing effect. There was an initial rapid fall of the surface tension with time, then the rate of the fall gradually decreased until an equilibrium value was reached. As the concentration of the sol increased, the time taken to reach equilibrium was reduced.

Effect of concentration of PE *on the surface tension of aqueous sols.* Since the surface tension varied with time, it was evident that sufficient time was needed for an equilibrium value to be reached. Hence, the surface tension was studied for 6 hr. for each concentration and the equilibrium values obtained plotted against concentrations (see Fig. 3).

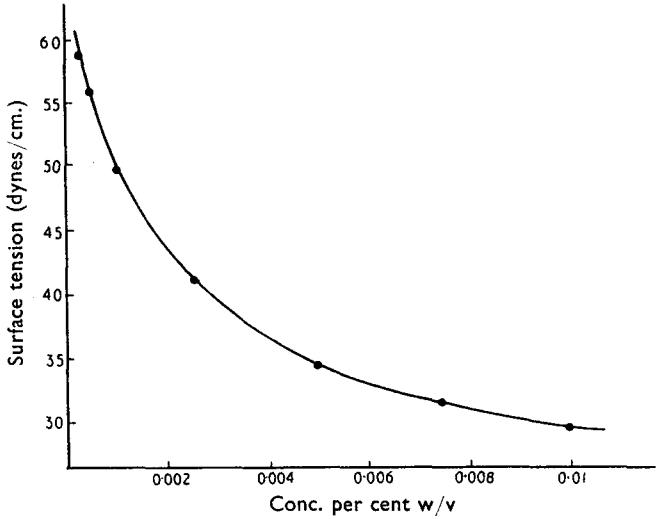


FIG. 3. Variation of surface tension of phosphatidylethanolamine sols with concentration.

Effect of pH on the surface tension of PE *sols.* The surface tensions of PE sols were investigated over the pH range of 1·65 to 6·1 which should include the isoelectric point of PE. The pH values were obtained using a Dynacap pH meter after the addition of small volumes of hydrochloric acid to the sols. The surface ageing effect was still present even in acid solution (see Table II). The equilibrium values obtained for the surface tensions at various pH values of the sols are also given in Table II.

TABLE II

VARIATION OF THE SURFACE AGEING EFFECT WITH pH (0·005 PER CENT W/V PE)

pH	Time taken to reach equilibrium value (in hr.)	Equilibrium value of S.T.
6·1	6	38·10
5·7	5	35·57
4·9	4¼	32·55
4·0	2¼	28·50
3·28	½	27·28
2·60	½	27·00
1·75	2¾	28·40

Effect of monovalent and divalent cations on the surface tensions of PE *sols.* The effects of potassium chloride and calcium chloride on the surface tension of the sols were investigated. The equilibrium values, and the time taken to reach them are given in Table III.

No visible precipitation was seen during the investigation in those sols containing sufficient electrolyte to cause precipitation in 24 hr.

Precipitation Studies

1 ml. of sol containing either 0·04 or 0·02 per cent of PE was placed in each of a series of sample tubes. Varying quantities of salt solution were

added to the tubes by means of an Agla micrometer syringe and the volume in each tube was adjusted so that the final concentration of phosphatide in each tube was either 0·036 per cent or 0·018 per cent. The effect of potassium and calcium chlorides on the stability of the sols was investigated and the results are given in Table IV.

TABLE III

EFFECT OF SALTS ON THE SURFACE TENSION OF A 0·005 PER CENT PE SOL

Molar conc. of potassium chloride	S.T. at equilibrium	Time taken to reach equilibrium value (in hr.)
—	33·0	7
5×10^{-5}	36·2	5
1×10^{-4}	37·30	6
5×10^{-4}	36·64	$4\frac{3}{4}$
1×10^{-3}	36·70	5
5×10^{-3}	35·10	$4\frac{1}{4}$
1×10^{-2}	28·8	3
Molar conc. of calcium chloride		
1×10^{-5}	32·1	$1\frac{1}{2}$
5×10^{-5}	32·1	2
1×10^{-4}	32·5	$3\frac{1}{2}$
5×10^{-4}	31·3	2
1×10^{-3}	30·8	$1\frac{1}{2}$
5×10^{-3}	28·9	$\frac{1}{2}$
1×10^{-2}	28·4	$\frac{1}{2}$

TABLE IV

EFFECT OF SALTS ON STABILITY OF PE SOLS

Potassium chloride moles/litre	0·036 per cent PE	0·018 per cent PE
$2{\cdot}5 \times 10^{-1}$	+++	+++
$2{\cdot}0 \times 10^{-1}$	+++	+++
$1{\cdot}5 \times 10^{-1}$	+	+
$1{\cdot}0 \times 10^{-1}$	—	—
$1{\cdot}0 \times 10^{-2}$	—	—
$1{\cdot}0 \times 10^{-3}$	—	—
Calcium chloride moles/litre		
$5{\cdot}0 \times 10^{-1}$	+++	+++
$5{\cdot}0 \times 10^{-2}$	+++	+++
$5{\cdot}0 \times 10^{-3}$	+++	+++
$6{\cdot}0 \times 10^{-4}$	+++	+++
$4{\cdot}0 \times 10^{-4}$	++	++
$2{\cdot}0 \times 10^{-4}$	—	—
$1{\cdot}0 \times 10^{-4}$	—	—
$1{\cdot}0 \times 10^{-6}$	—	—
$1{\cdot}0 \times 10^{-7}$	—	—
$1{\cdot}0 \times 10^{-11}$	—	—
Control	—	—

+++ = heavy precipitate
+ = faint precipitate
— = no precipitate

DISCUSSION

Although the cephalin group of phosphatides predominates in brain tissue (Chargaff, Ziff and Rittenberg, 1942) it was not used as the source of PE because other members of the cephalin group are present in high proportions and are not easy to separate. Instead, egg-yolk was employed, where the cephalin fraction, despite being in small concentration compared with the lecithin fraction, is almost pure PE.

Hawke (1959) has reported that, using a chromatographic column containing 50 g. of silicic acid and a loading of phosphatides of 30 mg. of phosphorus for 25 g. of adsorbent, a separation of PE and lecithin was obtained. When he tried to scale up the preparation using 200 g. of adsorbent and the same loading ratio no separation of PE and lecithin was obtained. Since we required fairly large quantities of PE we scaled up the silicic acid chromatographic separation by following a suggestion made by Rhodes (personal communication) of increasing the diameter of the column rather than its length. By doing this we obtained about 3 g. of PE which was well separated from the lecithin.

We were unable to compare in detail the solubilities of PE and lecithin in various organic solvents since solubility figures for lecithin do not appear to be in the literature. However, lecithin is very soluble in ether, chloroform and methyl ethyl ketone, fairly soluble in ethanol and methanol, and slightly soluble in acetone. PE exhibits a similar solubility pattern.

The surface ageing of PE sols is an interesting phenomenon. Adam and Shute (1935), working with paraffin chain salts, noted this slow ageing

TABLE V

THE EFFECT OF TIME ON THE SURFACE TENSION OF A 0·005 PER CENT PE SOL

Time (in hr.)	Surface tension (in dynes/cm.)		
	1st day	2nd day	3rd day
1	42·7	38·5	40·3
2	37·4	36·2	37·6
3	36·2	35·8	36·6
4	35·5	35·2	35·8
5	35·1	34·9	35·5
6	35·0	34·8	35·3

The sol was agitated at the beginning of each day.

effect and also that as the concentration of the surface-active agent was increased, the time required to reach an equilibrium surface tension value was decreased. Above the critical micelle concentration these authors found an equilibrium value was attained rapidly. We have observed similar results with PE sols (see Fig. 2). This ageing phenomenon applies only to the surface and not to the solution itself, because when surface tension measurements were made on the same sol on different days after agitation, the changes in the surface tension with time were similar (see Table V).

There is at present no adequate theory to explain this slow surface ageing effect. It can not be explained by a simple diffusion theory, because the time taken for the surface tension to reach equilibrium is much longer than it would take molecules to diffuse from the bulk of the phase into the surface layer. Doss (1939), Nutting, Long and Harkins (1940) consider that the diffuse electrical double layer which is formed by the monolayer of the surface-active molecules in the surface, acts as an electrical barrier past which molecules must pass before they can be adsorbed. This view is incompatible with McBain and Perry's (1940) observation that the effect occurs in non-ionised systems. Alexander

(1941) also disagrees with the concept of an electrical barrier because he found that the ageing effect which occurs with hydrocinnamic acid, disappeared when the sodium salt was used. McBain and Perry (1939) think that in some cases the ageing effect may be due to the formation of two or more superimposed monolayers in the surface. Alexander (1941) thinks that the main factor determining the rate of adsorption in non-micellar solutions is the rate of penetration and reorientation of the molecules in the surface layer, possibly coupled with a dehydration of the hydrophobic portion of the molecule. Sutherland (1959) considered the effect to be due to the transport of very small amounts of electrolyte impurities to the surface.

There are, therefore, many possible explanations for the slow surface ageing of a solution. It is probable that the predominant factor controlling the rate of attainment of equilibrium in the surface is different for different surface-active agents.

Robinson and Saunders (1958), using a static method for measuring surface tension, have reported that a lysolecithin sol, the concentration of which was close to its critical micelle concentration, showed only a very small surface ageing effect. We found with lysophosphatidylethanolamine (LysoPE) sols, at a concentration just below the critical micelle concentration, that there was a long surface ageing effect which was even longer with PE sols. In the light of these facts we do not think that the ageing effect exhibited by PE sols can be explained by (*a*) the time taken for the molecules to diffuse into the surface, (*b*) the formation of multilayers at the surface, or (*c*) the presence of minute amounts of electrolytes, because if any of the above explanations were correct then lysolecithin would behave similarly. In our opinion one main factor causing the ageing is the existence of an electrical double layer at the surface. Evidence in support of this is firstly that the ageing effect is much greater with lysoPE, where the surface-active ion has a strong negative charge, than with lysolecithin, where the surface active ion has only a very weak negative charge. Secondly the surface tension studies on PE sols at various pH values have shown (see Table II) that as the charge on the molecule is reduced, so the surface ageing effect is reduced, reaching a minimum value at the iso-electric point. The second main factor involved is the time required for reorientation of the molecules in the surface. That PE sols take longer to reach an equilibrium value compared with lysoPE can be explained by the fact that the PE molecules are more bulky and a high proportion of the fatty acid chains present are unsaturated.

Many workers (Nutting, Long and Harkins, 1940; Hartley, 1936; Adams and Shute, 1938; Nutting and Long, 1941) have noticed that at or above the critical micelle concentration the surface ageing effect is very small or non-existent. No definite theory to explain this has yet been evolved, but some possible explanations are given here. Doss (1939) thinks that the electrical barrier which impedes the diffusion of ions to the surface would be reduced if the ionic strength of the solution were increased by the formation of micelles having a high charge. Adam and

Shute (1938) consider that micelles carrying a high charge may repel any similarly charged long chain ions so much more than such ions repel each other, that ions are driven to the surface more easily if micelles are present in the solution. Another suggestion they make is that the surface layer may be regarded as a kind of two-dimensional micelle, the conditions for its formation being much the same as those for the formation of micelles in the interior of the solution, so that it is formed most easily at similar concentrations. Nutting, Long and Harkins (1940) state that an ion in a micelle can get into the surface more easily than can a single ion because the rate of diffusion per ion is larger for the ions in a micelle than for simple ions. Also as the micelle approaches the surface it will encounter the diffuse region in which the gegenions of the surface-active ions in the surface are concentrated. The high charge density at the surface of the micelle will cause a large number of the gegenions to become attached to it. Consequently the net charge of the micelle will be reduced as it approaches the surface. Therefore, the electrostatic barrier to the approach of an ion in a micelle to the surface will be considerably less than for a simple ion. They have also suggested that the effect could be explained in terms of micelles having very low charge density (as postulated by McBain). The diffusion of such micelles would be only slightly affected by the electrical double layer.

Since there is no sharp break in the surface tension-concentration curve for PE sols (see Fig. 3) it is difficult to estimate the critical micelle concentration from the graph, but the greatest change of slope of the graph occurs at a concentration of about 0·002 per cent. However, since the surface ageing effect is very small when the concentration is 0·01 per cent we conclude that the critical micelle concentration is within the concentration range of 0·002–0·01 per cent.

The results given in Table II show that a minimum occurs in the surface tension-pH curve at a pH value of about 3·1. We conclude that this is the isoelectric point for PE, because when the resultant charge on the molecules is zero the packing of the molecules in the surface will be at an optimum and consequently the surface tension will be at a minimum.

It has been reported that the presence of electrolytes reduces the ageing effect. Generally it has been found that the higher the concentration of electrolyte the bigger the reduction. It seems that with PE sols, the ageing effect is dependent upon the equilibrium value of the surface tension rather than the electrolyte concentration. The lower the equilibrium surface tension value the shorter the ageing effect (see Table III). It is interesting to recall that this statement also applies to the sols containing hydrochloric acid (see Table II). It would seem that the greater the interaction between the added electrolyte and PE the less charged the PE molecules become and consequently they are able to pack more closely into the surface causing a lower equilibrium surface tension. The ageing effect should be shorter the higher the valency of the ions of the added electrolyte. We have found this to be so.

Calcium chloride causes a slight fall in the surface tension of the sols. Generally speaking as the concentration of calcium chloride is increased

so the surface tension is decreased. A possible explanation is that the higher the concentration the greater the interaction between the calcium ions and the PE molecules. It is possible that the calcium ion forms a linkage between the phosphate groups of two molecules of the phosphatide, causing them to pack more closely in the surface. Potassium chloride in concentrations of 5×10^{-3} M and below caused a rise of between 2 and 4 dynes/cm., whilst a concentration of 10^{-2} M caused a fall of about 4 dynes. We have no explanation to offer for these results.

In the flocculation studies it was found that concentrations of calcium chloride of 4×10^{-4} M and above caused flocculation, whilst with potassium chloride, concentrations of $1{\cdot}5 \times 10^{-1}$ M and above were required. It is interesting to note that PE sols do not exhibit a peptisation zone in the presence of divalent metal ions, as has been reported for lecithin by Malquori (1932), Rona and Deutsche (1926), Saunders and Elworthy (unpublished), and Thomas (1962).

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