SOME PHYSICO-CHEMICAL STUDIES OF LYSOPHOSPHATIDYLETHANOLAMINE SOLS

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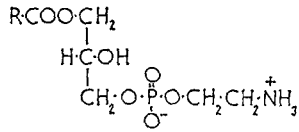
Received February 25, 1963

The solubilities of lysophosphatidylethanolamine in some organic solvents have been determined. Surface tension measurements of aqueous sols showed it to have pronounced surface-active properties. There was a surface ageing effect which was followed by a rise in the surface tension. Possible explanations for these effects are given. The effects of concentration, pH, mono- and divalent salts on the surface activity have been investigated. The critical micelle concentration was in the range 0·001 to 0·002 per cent w/v. The isoelectric point was at pH 3·25. The stability of sols of the phosphatide in the presence of mono- and divalent salts has also been examined.

Lysophosphatidylethanolamine (lysoPE) is obtained from phosphatidylethanolamine (PE) by the hydrolysis of one fatty acid ester linkage which can be effected by the enzyme phospholipase A, an enzyme found in the venom of vipers. The specific site of action has been established as the β ester position of PE (Tattrie, 1959; Hanahan, Brockerhoff and Barron, 1960, de Haas and van Deenen, 1961a). The zwitterionic structure of Lα-lysoPE is shown by (I)



I

—where R is a predominantly saturated hydrocarbon chain containing mainly 15 or 17 carbon atoms.

The molecule is amphipathic since it contains a hydrophobic non-polar hydrocarbon chain, and a hydrophilic, polar, phosphate-ethanolamine grouping. Therefore, it would be expected to show surface-active properties.

Experimental

Preparation of LysoPE

The method of preparation was based on that of Long and Penny (1957).

PE was first prepared by the method of Robins and Thomas (1963). PE (1·65 g.) was dissolved in ether (165 ml.) and Russell Viper venom (10 mg. in 4 ml. water) added. The pH of the solution was adjusted to 7·0 with ammonium hydroxide. The precipitation of lysoPE from the solution commenced after about 24 hr. and was complete after 3 days.

The ether was decanted from the pale buff precipitate, and the water removed by shaking with successive small volumes of acetone.

The sample was purified by dissolving in the minimum quantity of methanol at 55°, centrifuging to remove the venom, and precipitating by adding ether, in which residual PE is soluble. This was repeated 4 times to yield 0·6 g. of a pure white, microcrystalline, non-hygroscopic powder, having nitrogen: phosphorous ratio 1:0·96, and an iodine value of 2. The product was dissolved in methanol, and stored under nitrogen at −20°.

Solubility Studies

The solubility of lysoPE in some organic solvents was determined over the temperature range 25–50°. The method was that of Robins and Thomas (1963). The results are in Table I.

TABLE I

SOLUBILITY OF LYSOPE IN SOME ORGANIC SOLVENTS

Solvent	Solubility in g./100 ml. solution at:					
	25°	30°	35°	40°	45°	50°
Methanol	0·365	0·415	0·445	0·510	0·530	0·565
Ethanol	0·060	0·180	0·250	0·280	0·365	0·415
Chloroform	0·085	0·095	0·110	0·135	0·140	0·155
Methyl ethyl ketone	0·010	0·010	0·010	0·020	0·020	0·030
Acetone	0	0·010	0·010	0·015	0·015	0·020
Ether	0·005	—	—	—	—	—

Surface Tension Studies

Preparation of aqueous sols. A weighed amount of lysoPE was dissolved in a small volume of purified water by vigorous shaking. The sol was passed through an ion-exchange column (Robins and Thomas, 1963) and made up to volume.

Slight variations occurred in the values of the surface tension with sols of the same concentration, owing to the inherent errors in the method of preparation. To overcome these, sufficient sol was prepared for a series of experiments and portions used as required. Since the surface tension varied with the age of a lysoPE sol, the bulk sol was divided into portions and frozen at −20°. Portions were melted and used as required; freezing and melting did not affect the surface tension.

Apparatus. A static method (Wilhelmy plate) was used for surface tension measurements, as described by Robins and Thomas (1963).

Variation of surface tension with time. A 0·005 per cent w/v lysoPE sol was prepared, and the variation of its surface tension with time was investigated. There was initially a rapid fall in the surface tension, the rate of fall progressively decreasing, until eventually a steady value was reached in 3 to 6 hr. (Fig. 1). On subsequent days, the minimum surface tension value of the same sol was higher on each successive day.

The variation of surface tension with time for sols of different concentrations is given in Fig. 2. With the most dilute sols, the surface tension was still falling slightly after 5 hr., but with increase in concentration

the time required to reach a minimum value decreased. The values of the surface tension after 6 hr. for these sols are given in Fig. 3.

Variation of surface tension of lysoPE sols with pH. 0·005 per cent w/v lysoPE sols were prepared, various amounts of hydrochloric acid added, and the pH measured with a Dynacap pH meter. The equilibrium values of the surface tension of these sols, and the times required to reach them are given in Table II.

TABLE II

VARIATION OF THE SURFACE TENSION OF 0·005 PER CENT W/V LYSOPE SOLS WITH pH

pH	Time to reach equilibrium (hr.)	Equilibrium value of surface tension (dynes/cm.)
6·1	5·25	34·27
5·42	2·75	32·75
4·83	2	31·71
4·30	1·5	30·11
3·82	0·75	29·70
3·1	0·5	28·80
2·3	1	30·40
2·02	1·75	31·51
1·7	3	32·00

The surface tension of the sol at pH 2·3 was measured on successive days and, within the limits of experimental error, the values were found to be identical on each day (Table III).

TABLE III

VARIATION OF THE SURFACE TENSION OF A 0·005 PER CENT W/V LYSOPE SOL WITH TIME AT pH 2·3

Time (hr.)	Surface tension (dynes/cm.)		
	1st day	2nd day	3rd day
0·5	30·41	30·21	29·78
1	30·10	29·27	29·01
2	29·44	28·80	28·53
3	29·23	28·76	28·40
4	29·00	28·74	28·46
5	29·04	28·70	28·40
6	28·98	28·70	28·40

Sol agitated at the beginning of each day.

With a 0·005 per cent w/v sol, the pH of which had been adjusted to 10·5 with sodium hydroxide, the surface tension fell on the first day, reaching a minimum in 1 hr.; it then rose, reaching a constant value after 4½ hr. On subsequent days, no minimum was obtained, but there was a prolonged surface-ageing effect; the surface tension after 6 hr. being lower on each successive day (Fig. 4).

Variation of surface tension of lysolecithin sols with pH. The effect of time on the surface tension of a 0·0075 per cent w/v lysolecithin sol at pH values of 6·5, 1·6 and 10·1 was also examined.

At pH 6·5 there was a small surface-ageing effect, not followed by a rise in the surface tension, which confirmed Robinson and Saunders' (1958) findings. At pH 1·6, the surface tension fell about 0·75 dyne/cm.

over 2 hr., after which it remained constant. At pH 10·1, the surface tension rose 1–2 dynes/cm. to a maximum after 3 hr., and then fell slightly. On the following day, on the same sol, no maximum was exhibited, and the surface tension was lower and fell continuously (Table IV).

TABLE IV

VARIATION OF THE SURFACE TENSION WITH TIME OF A 0·0075 PER CENT W/V LYSOLECITHIN SOL AT PH 10·1

Time (hr.)	Surface tension (dynes/cm.)	
	1st day	2nd day
0·25	38·50	34·70
0·5	39·27	34·07
1	39·82	33·40
2	40·21	32·65
3	40·29	32·46
4	40·04	32·08
5	39·93	32·02

Effect of salts on surface tension. The effects of potassium and calcium chlorides on the surface tension of 0·005 per cent w/v lysoPE sols were investigated. The minimum values and the times taken to reach them are given in Table V.

TABLE V

EFFECT OF SALTS ON THE SURFACE TENSION OF 0·005 PER CENT W/V LYSOPE SOLS

Molar conc. of KCl	Minimum surface tension values (dynes/cm.)	Time taken to reach the minimum value (hr.)
—	36·07	3·25
1×10^{-5}	33·13	2·5
1×10^{-4}	32·94	2·75
1×10^{-3}	33·23	2·75
1×10^{-1}	33·69	2·75
Molar conc. of $CaCl_2$		
1×10^{-6}	35·96	0·5
1×10^{-5}	36·80	0·75
1×10^{-4}	37·28	1
1×10^{-3}	38·23	1·75
1×10^{-2}	39·63	1·75

Effect of salts on the stability of lysoPE sols. One ml. of a 0·05 per cent w/v lysoPE sol was placed in each of a series of sample tubes. Potassium chloride 5×10^{-1} to 1×10^{-5}M or calcium chloride 1×10^{-2} to 1×10^{-6}M solutions were added by Agla micrometer syringe, and the volume adjusted with water to a final concentration of 0·04 per cent w/v lysoPE in each tube.

A heavy precipitate occurred with 5×10^{-1} and 2×10^{-1}M potassium chloride and with 1×10^{-2}M calcium chloride and a weak precipitate with 5×10^{-3}M calcium chloride. There was no precipitation at the other concentrations.

DISCUSSION

LysoPE has been prepared by the direct action of venom, as reported by Long and Penny (1957) and de Haas and van Deenen (1961a, 1961b). No difficulty was encountered in obtaining lysoPE in this way, provided

that 72 hr. were allowed for complete reaction at room temperature, despite references to the impossibility of the method (Chargaff and Cohen, 1939; Lea, Rhodes and Stoll, 1955). A slow degradation of the phosphatide prefaced by a lag period of 24 hr. noticed by Davidson, Long and Penny (1955) and the need to adjust the reaction mixture to pH 7 as found by Long and Penny (1957) may be the reasons for the differing reports.

The solubility of lysoPE in organic solvents showed a similar pattern to the figures quoted for lysolecithin (Robinson and Saunders, 1958), but the values were lower. This was probably because of the larger net charge on the lysoPE molecule. The results are in accord with previous indications (Levene, Rolf and Simms, 1924). The solubility of lysoPE in water was low. A 0·05 per cent w/v sol was turbid, and was obtained only after prolonged shaking at 40°.

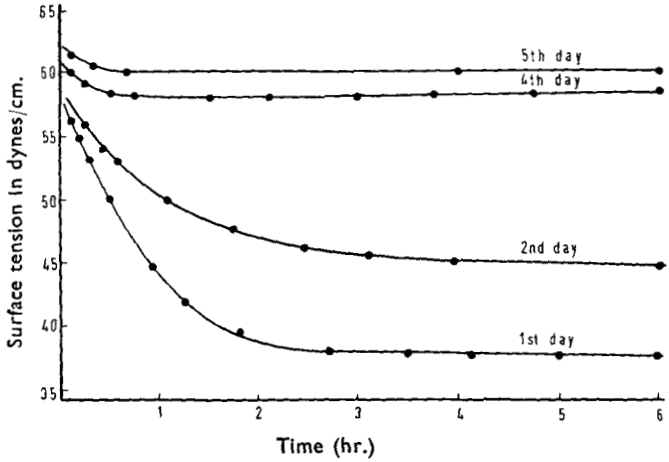


FIG. 1. Variation of surface tension of a 0·005 per cent w/v lysoPE sol with time.

LysoPE sols showed a surface-ageing effect, but this was not so pronounced as that shown by PE sols. As the concentration of the sol was increased, the surface-ageing effect decreased, and was very small above the critical micelle concentration. The possible explanations for the surface ageing in PE sols have already been discussed (Robins and Thomas, 1963). In lysoPE sols we consider the main factor is the existence of an electrical double layer at the surface. Evidence supporting this view is, firstly, that the ageing effect in lysoPE, where the surface-active ion has a distinct net negative charge, is much greater than in lysolecithin which only has a very small net negative charge. Secondly, the surface tension studies on lysoPE sols at various acidic pH values have shown (Table II) that as the net charge on the molecule is reduced, the surface-ageing effect is reduced, reaching a minimum at the isoelectric point.

The second possible factor causing the ageing effect is the time required for the molecules to orientate themselves at the surface. The lysoPE

molecules are less bulky than the PE molecules, and the fatty acids are more saturated, thus enabling them to orientate more readily at the surface. This would account for the shorter surface-ageing effect.

In Fig. 3, the surface tensions of sols after 6 hr. are plotted against the various concentrations. There is a sharp change in the slope of the graph between 0·001 and 0·002 per cent w/v indicating that the critical micelle concentration for lysoPE occurs within this concentration range.

Upon plotting the values of surface tension against pH, a minimum in the curve occurred at pH 3·25, indicating this pH value to be the isoelectric point for lysoPE. At the isoelectric point, the net charge on the molecule would be at a minimum, and consequently the molecules would pack more closely in the surface, causing the greatest lowering of the surface tension. As would have been expected, the isoelectric point of lysoPE is very similar to that of PE.

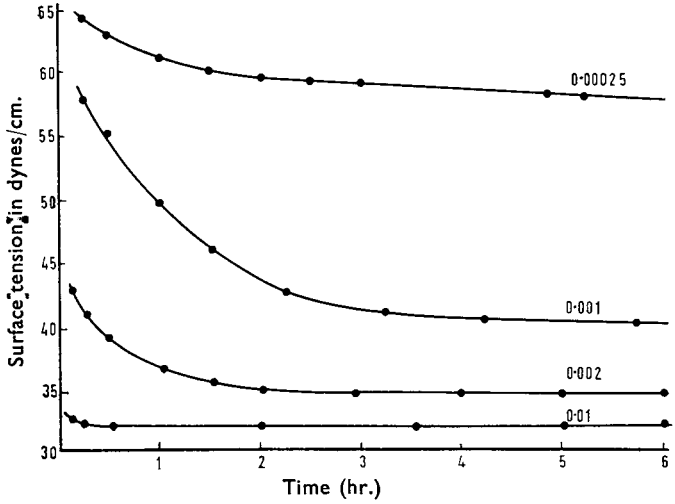


FIG. 2. Variation of surface tension with time of lysoPE sols of varying concentration. Figures on the curves are concentrations in per cent w/v.

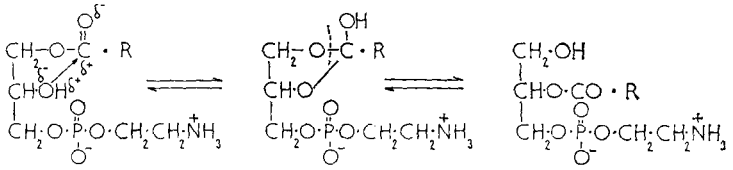
There are a number of possible explanations for the changes in surface tension of lysoPE sols with time at various pH values. Firstly, they may be due to hydrolysis of one or more of the ester linkages present in the molecule. Secondly, they may result from an intramolecular rearrangement, whereby the fatty acid chain migrates from the α- to the β-position. Thirdly, they may be due to migration of the phosphate-ethanolamine moiety from the γ- to the β-position.

Whilst hydrolysis of the ester linkages probably occurs in alkaline conditions it is doubtful whether this is the cause of the change in surface tension at neutral pH, since were it so, PE, lysoPE and lysolecithin would behave similarly, and no such change occurs with PE or lysolecithin sols.

An intramolecular migration is likely, since there is a free hydroxyl group on the β-carbon atom. In PE, this hydroxyl group is esterified

with another fatty acid, thus rendering migration impossible. It has been found that after an initial surface-ageing effect, the surface tension of PE sols remained constant (Robins and Thomas, 1963).

A possible mechanism for the fatty acid migration would be as follows:



Hanahan and Uziel (1957) have reported that a migration of the fatty acid in lysolecithin occurred in the presence of either a migratase enzyme or 0·05N hydrochloric acid. However, we have found in acid solution with both lysolecithin and lysoPE that once an equilibrium value is reached after the initial surface-ageing effect, the surface tension does not change. Assuming the above mechanism to be correct, in acid conditions the slight ionisation of the β-alcoholic grouping would be suppressed and thus prevent migration. In alkaline conditions, the ionisation would be increased and would thus favour migration, with which our results agree. However, the argument against fatty acid migration is the failure of lysolecithin to show a similar rise to lysoPE in surface tension with time at neutral pH values.

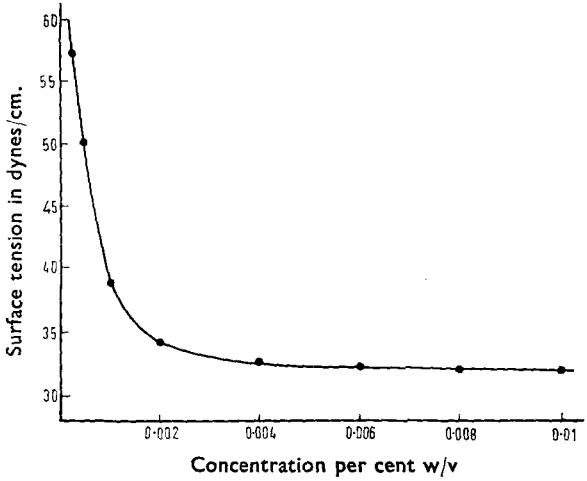
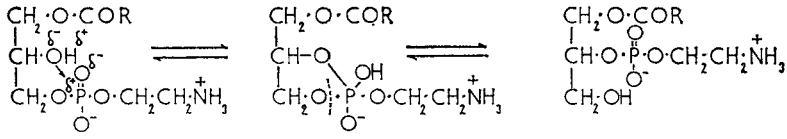


FIG. 3. Variation of surface tension of lysoPE sols with concentration.

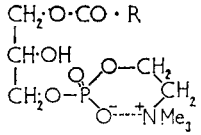
The third possibility is the migration of the phosphate-ethanolamine grouping from the γ- to the β-position. Many workers have studied the effects of acid and alkali on glycerophosphoric acid (GPA). Bailly (1938, 1939) has shown that when β-GPA is treated with acid, some is

converted into α-GPA without any liberation of phosphoric acid or glycerol, and, in the equilibrium mixture obtained, the α-form predominated. Verkade, Stoppelenburg and Cohen (1940) confirmed Bailly's findings and Chargaff (1942) showed that the conversion of β-GPA to α-GPA was an intramolecular rearrangement by treating β-GPA with acid in the presence of radioactive sodium phosphate. The isolated α-GPA contained no radioactive phosphorous. Bailly and Gaumé (1934a, 1934b) have reported that α-GPA is partially converted into β-GPA in alkaline conditions, and that the β-form predominates in the equilibrium mixture. Baer and Kates (1948) have shown that on acid or alkaline hydrolysis of glycerylphosphorylcholine, a reversible α to β migration of the phosphoric acid grouping occurs, accompanied by the liberation of choline. They also found that in N hydrochloric acid the α-form predominated (91 per cent), whilst in N sodium hydroxide the β-form predominated (56 per cent). We are not aware of any report of a migration of the phosphate-base grouping in an intact phosphatide molecule. A possible mechanism for such a migration in lysoPE would be as follows:



Here again, in acid solution the slight ionisation of the alcoholic grouping would be suppressed, thus reducing the possibility of migration. However, it has been reported that a little of the phosphoric acid in α-GPA does migrate to the β-position even in acid solution. Thus a small migration of the phosphate-base grouping may be occurring in lysoPE and lysolecithin sols, but the consequent change in surface tension may be too small to be detected. Increased ionisation of the alcoholic grouping in alkaline solution would favour migration, which the results with lysoPE and lysolecithin sols agree.

The failure of an intramolecular migration to occur in lysolecithin in a neutral aqueous sol, may be explained by a ring structure being formed by the equivalently highly ionised phosphoric acid and choline groupings, thus:



A model of such a molecule shows that it is possible for such a ring structure to sterically prevent the migration of the phosphate-choline moiety.

In a neutral aqueous lysoPE sol such a ring structure would be less

likely to be formed, since the amino-group of ethanolamine would be only slightly ionised, and hence migration could occur.

The complex time effect exhibited by the lysope sols at pH 10·2 can be explained as follows. Initially the surface-ageing effect more than offsets the rise in surface tension due to intramolecular migrations so that the surface tension falls. After 1 hr. it rises because the migration has now become the predominant factor. After 4·5 hr. an equilibrium between the α- and β-forms is reached and the surface tension remains constant. The unbroken fall found with the same sol on subsequent days is probably due to the slow hydrolysis of the ester linkages. The fatty acids released would form soaps with the sodium hydroxide present, and thus cause the surface tension to fall slowly.

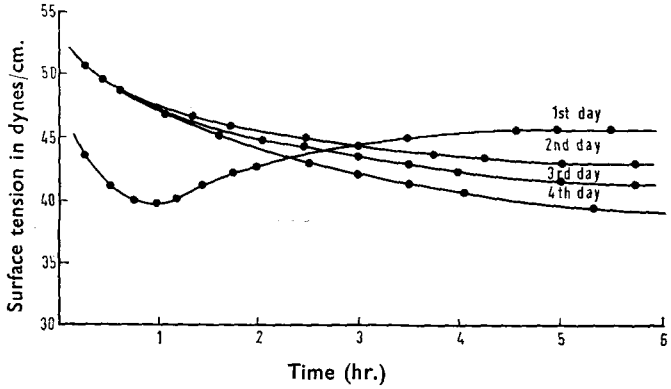


FIG. 4. Variation of surface tension of a 0·005 per cent w/v lysope sol with time at pH 10·5.

The time effect shown by a 0·0075 per cent w/v lysolecithin sol at pH 10·1 may be explained similarly, except that no initial fall in surface tension occurred, since the small surface-ageing effect is masked by the rise in surface tension due to the intramolecular migration.

Neither calcium nor potassium ions prevented the change in surface tension values of lysope sols with time. Hence the expression "minimum" values are used rather than "equilibrium" values to indicate the lowest values obtained from the first day's readings.

It has been reported (Nutting, Long and Harkins, 1940) that the presence of electrolytes reduces the surface-ageing effect, and the higher the concentration and valency of the ions, the greater is the reduction. With lysope sols the ageing effect was found to be dependent on the minimum value of the surface tension rather than the electrolyte concentration. The lower the minimum value the shorter the ageing effect. The results in Table V also show that the higher the valency of the added cation, the shorter was the surface-ageing time.

The presence of various concentrations of potassium chloride caused a slight fall in the surface tension of lysope sols. These results are as expected, since potassium ions, being univalent, are not capable of linking

two lysoPE molecules together, and thus do not improve their packing at the surface. An increase in the concentration of calcium chloride caused a slight rise in the surface tension of the sols. This is the reverse of what would have been expected, since any linkage between the calcium ion and the phospholipid molecules would have facilitated closer packing in the surface. However, Robinson and Saunders (1958) found that calcium chloride caused a similar rise in the surface tension of lysolecithin sols.

Concentrations of 5×10^{-3}M and above of calcium chloride caused flocculation of the lysoPE sols, whilst concentrations of 2×10^{-1}M and above of potassium chloride were needed. Since the surface tension results did not indicate conclusively that any interaction took place between the salts and lysoPE, it is probable that the flocculation was due to a salting-out effect. Thus, it would be expected that the divalent calcium ions would be more effective than the monovalent potassium ions. It is interesting to compare these results with those obtained by Saunders (1957), who was unable to cause flocculation of lysolecithin sols with either potassium or calcium chlorides. This difference is probably because lysolecithin is very soluble in water and consequently would require a very high concentration of salt to cause flocculation.

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