547. *Diffusion Studies with Phosphatide Sols.*

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Integral and semi-differential diffusion studies have been performed with mixed sols of lecithin and lysolecithin. The diffusion of the phosphatides followed the theory for the diffusion of a single solute. The value of the diffusion coefficient decreased rapidly as the weight fraction of lecithin was increased. Calcium chloride had some effect on the diffusion rate.

Phosphatides are important constituents of cell membranes. Lecithin is present in most cell membranes, but the presence of lysolecithin has not been proved. However lysolecithin is very likely to be present since lecithinase A enzyme, which specifically hydrolyses lecithin to lysolecithin, is widely distributed in the body. Studies of the physical properties of mixed aqueous sols of these materials should help in the elucidation of the structure and mechanism of formation of cell membranes.

We showed previously[1] that lysolecithin existed in aqueous sols as large, fairly uniform micelles and that the diffusion rate of the lysolecithin was unaffected by calcium chloride. Details are here given of the diffusion of mixed sols of lecithin and lysolecithin, and the effect of calcium chloride on the diffusion rate.

Experimental

The diffusion rate was measured by using a Gouy diffusiometer.[2,3] The photographing of the Gouy patterns was made wholly automatic. The photographic plate, in a carrier, was moved by an electric motor *via* a rack and a one-tooth pinion, so that one revolution (½ hr.) of the pinion moved the rack ¼ in. The shutter, at one side of the thermostat bath containing the diffusion cell, was controlled by a time switch so adjusted that a photograph was taken every ½ hr., and the exposure times could be varied. Photographs were taken only whilst the plate was stationary.

Materials.—Lysolecithin was prepared as described previously.[1] Lecithin was prepared as described by Saunders.[4] "AnalaR" calcium chloride was used.

Preparation of the Sols.—Small quantities of the alcoholic stock solutions of lecithin and lysolecithin were evaporated to dryness under vacuum, and weighed quantities of each substance were placed in the same sample flask. A small quantity of ethyl alcohol was added and upon gentle warming a clear solution was obtained. This alcoholic solution was evaporated to dryness under vacuum, leaving a residue of intimately mixed phosphatides. A small volume of distilled water was added and the flask was attached to an automatic shaking machine and placed in a water-bath whose temperature was kept below 55°. The flask was shaken until a clear sol was obtained. In order to remove any electrolytes present as impurities the sol was then passed over a bed of mixed ion-exchange resins consisting of 0·25 g. of Amberlite Resin IR-120(H) and 0·4 g. of Amberlite Resin IRA-400(OH). Small successive quantities of distilled water were passed over the ion-exchange resins to remove any adherent phosphatide. The sol was finally made up to volume with distilled water.

Preparation of sols containing calcium chloride. A mixed phosphatide sol was prepared as above, except that its concentration was twice that required finally. To this was added an equal volume of calcium chloride solution of twice the final concentration required. All concentrations (C) are expressed as percentage (w/v).

Integral Diffusion.—With all the integral diffusion experiments with mixed phosphatide sols, the C_t values obtained on analysing any Gouy pattern were constant, but the apparent coefficient (D) decreased with increased time of diffusion. On plotting D against $1/t$ a straight line was obtained, and the true value of D was obtained by extrapolation to $1/t = 0$.[2] The values so obtained for D were not very consistent; its value decreased rapidly as the proportion of lecithin in the mixture was increased.

[1] Saunders and Thomas, *J.*, 1958, 483.
[2] Saunders, *J.*, 1953, 519.
[3] Brudney and Saunders, *J.*, 1955, 2916.
[4] Saunders, *J. Pharm. Pharmacol.*, 1957, **9**, 834.

Good boundaries were formed quite readily with mixed sols having a total phosphatide concentration of 2% and a weight fraction of lecithin of 0·2 or less. With sols having a weight fraction of lecithin between 0·2 and 0·3 much greater difficulty was experienced, whilst with sols having a weight fraction between 0·3 and 0·6 it was impossible to form good boundaries. These difficulties are explained in the Discussion. With a sol having a weight fraction of lecithin of 0·6 light passed through the sol initially, but within 2 hr. of the commencement of diffusion, the sol close to the boundary started to become opaque, and this opacity spread rapidly throughout the sol. The boundary remained perfectly sharp even after four days, and no Gouy patterns were obtained on the photographic plate. On examining the sol after the experiment was completed, the opacity was found to be due to a white gelatinous coagulate. A similar result was obtained with a sol having a weight fraction of lecithin of 0·65, but in this case much longer elapsed before it became opaque.

No experiments were carried out with sols having a higher lecithin weight fraction than 0·65, since they were not optically clear.

Semi-differential Diffusion.—The boundaries formed between mixed phosphatide sols of different concentrations were so sharp that the diffusion coefficient varied only randomly with the time of diffusion, and so no extrapolation was necessary to obtain the true value of D. An analysis of three Gouy patterns obtained on the semi-differential diffusion of a 1·5% mixed phosphatide sol into a 0·3% mixed sol, each sol containing a weight fraction of lecithin of 0·15, is in Table 1. In Table 2 are the values of D obtained from five Gouy patterns on repeating the experiment twice.

TABLE 1.

	t = 13,500 sec.		t = 22,500 sec.		t = 27,900 sec.	
j	Y	C_t	Y	C_t	Y	C_t
1	1·7360	1·916	1·3423	1·482	1·2047	1·330
2	1·6714	1·910	1·2940	1·479	1·1623	1·328
3	1·6179	1·912	1·2514	1·479	1·1252	1·330
4	1·5692	1·911	1·2158	1·481	1·0915	1·329
5	1·5219	1·912	1·1773	1·479	1·0593	1·331
6	1·4798	1·912	1·1448	1·479	1·0287	1·329
7	1·4396	1·914	1·1137	1·481	1·0015	1·332
8	1·3999	1·912	1·0834	1·480	0·9741	1·331
9	1·3610	1·914	1·0540	1·482	0·9472	1·332
10	1·3258	1·913	1·0268	1·482	0·9232	1·332
S	0·0014		0·0014		0·0017	
10^7D	$6·13_1$		$6·14_0$		$6·13_2$	

S = standard deviation of the C_t values.
Definitions of the terms used in the above Table have been given in an earlier paper.[2]

TABLE 2.

Number of expt.	10^7D				
1	$5·91_2$	$5·91_9$	$5·92_9$	$5·93_1$	$5·92_9$
2	$6·13_1$	$6·14_0$	$6·14_6$	$6·13_2$	$6·02_7$
3	$5·92_0$	$5·90_4$	$5·90_3$	$5·89_2$	$5·94_6$

When semi-differential diffusion experiments were performed with sols containing varying weight fractions of lecithin, it was found that good boundaries were formed with all sols containing a weight fraction of lecithin of 0·3 or less, but with sols having a weight fraction greater than 0·3 it was impossible to form good boundaries. The diffusion coefficients for the mixed sols with weight fractions of lecithin below 0·3 are given in Table 3.

TABLE 3.

Wt. frac. of lecithin	0	0·100	0·150	0·200	0·250	0·300
10^7D	$6·50_2$	$6·32_2$	$5·98_5$	$3·90_2$	$2·12_2$	$1·30_4$

Effect of calcium chloride on the semi-differential diffusion rate of lecithin–lysolecithin sols. In Table 4 is given an analysis of three Gouy patterns obtained when a 1·5% mixed phosphatide sol was allowed to diffuse into a 0·3% mixed sol. The weight fraction of lecithin in each sol was 0·15, and the concentration of calcium chloride in both sols was 10^{-6}M. In Table 5 are given the semi-differential diffusion coefficients obtained when 1·5% mixed phosphatide sols were allowed to diffuse into 0·3% mixed sols in the presence of various concentrations of calcium chloride.

TABLE 4.

	t = 18,000 sec.		t = 23,400 sec.		t = 28,800 sec.	
j	Y	C_t	Y	C_t	Y	C_t
1	1·6424	1·819	1·4394	1·594	1·2997	1·439
2	1·5803	1·816	1·3882	1·596	1·2524	1·440
3	1·5293	1·812	1·3426	1·591	1·2088	1·432
4	1·4818	1·814	1·2978	1·588	1·1723	1·435
5	1·4376	1·815	1·2609	1·592	1·1361	1·434
6	1·3966	1·816	1·2219	1·589	1·1040	1·436
7	1·3549	1·814	1·1870	1·589	1·0729	1·436
8	1·3169	1·814	1·1550	1·591	1·0433	1·437
9	1·2799	1·815	1·1223	1·592	1·0158	1·441
10	1·2460	1·816	1·0940	1·595	0·9869	1·439
S	0·0017		0·0028		0·0028	
10^7D	$4·77_1$		$4·77_3$		$4·75_9$	

TABLE 5.

[$CaCl_2$] (M) in upper and lower sols ...	0	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
10^7D	5·98	5·92	5·70	4·76	5·66	5·76	5·50	5·04	4·07

DISCUSSION

The difficulties encountered in forming boundaries between sols containing various weight fractions of lecithin can be explained by our results of viscosity experiments.[5] With sols having a weight fraction of lecithin of 0·2 or less, the particles present are roughly spherical since the ratio of the specific viscosity to the volume fraction occupied by the phosphatides remains constant with increasing concentration. With sols having weight fractions of lecithin of 0·4 or 0·6 the particles are very asymmetric since this ratio increases rapidly with increasing concentration of phosphatides. The presence of these highly asymmetric particles in sols having weight fractions of lecithin 0·3—0·6 explains why they flowed anomalously, making it impossible to form good boundaries.

TABLE 6.

Wt. frac. of lecithin	Molar volume of micelle (10^{-5} c.c.)	Volume of one micelle (in 10^5 Å^3)	No. of molecules in each micelle
0	1·315	2·18	279
0·10	1·430	2·38	288
0·15	1·685	2·80	330
0·20	6·081	11·0	1160
0·25	37·8	62·8	7070
0·30	163	271	29,700

The results of integral diffusion experiments with sols having weight fractions of lecithin of 0·6 and 0·65 are very interesting. The white gelatinous coagulate, which does not diffuse, may be a molecular complex between lecithin and lysolecithin. (A sol having a weight fraction of lecithin of 0·6 contains an equimolecular mixture of the two phosphatides.)

The Gouy patterns from all experiments with mixed phosphatides sols, even in the presence of calcium chloride, were regular, and C_t's for any particular pattern were very constant (Tables 1 and 4). We infer that the diffusion of the mixed phosphatides alone, or in the presence of calcium chloride, follows the theory for the diffusion of a single solute. This indicates that in a particular sol the micelles are of fairly uniform size and shape.

With both the integral and semi-differential diffusion results, the diffusion rate of the mixed phosphatides decreases as the proportion of lecithin in the sol is increased, indicating that the micellar size is increasing. Assuming the micelles to be spherical, and our viscosity results with a sol having a weight fraction of 0·2 indicate this, we can calculate the size of the micelles by using the Stokes–Einstein equation. The results are in Table 6.

[5] Thomas and Saunders, *J. Pharm. Pharmocol.*, 1958, **10**, T182

With sols having a weight fraction of lecithin of 0·25 or 0·3 the particles are probably asymmetric.

Table 2 shows that on repetition of the same experiment the results showed some scatter, so they have been analysed statistically.

The variance of the values of D (Table 2) about their mean was estimated as $1{\cdot}00 \times 10^{-16}$, giving a standard deviation of $1{\cdot}00 \times 10^{-8}$ with fourteen degrees of freedom. The limits of error of a mean diffusion coefficient, calculated from five Gouy patterns, were estimated as $\pm 0{\cdot}133 \times 10^{-7}$ at the 0·99 probability level. The mean value of D was $5{\cdot}984 \times 10^{-7}$, and so these limits expressed as a percentage of the mean are $\pm 2{\cdot}22$.

To test whether various concentrations of calcium chloride were significantly reducing the diffusion rate of the phosphatides, the t-test of significance was applied to all the diffusion coefficients obtained for the phosphatides in the presence and absence of calcium chloride. Values for t are given in Table 7.

TABLE 7.

$[CaCl_2]$ (M)	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
t	1·23	5·26	24·6	5·82	4·15	9·23	18·3	36·7

The theoretical t at the 0·95 probability level and with eighteen degrees of freedom is 2·11. Therefore a significant reduction in the diffusion rate of the phosphatides occurred with all the concentrations of calcium chloride used except 10^{-8}M. The marked fall in the diffusion rate of the phosphatides in the presence of 10^{-6}M-calcium chloride is probably due to bonding between the calcium ions and the negatively charged phosphate groups of the lecithin molecules from different micelles causing a linking of the micelles. The fall in the diffusion rate with high concentrations of calcium chloride is probably due to salting out, the high concentration of salt causing dehydration of the particles, thus facilitating aggregation.

The fact that there are two regions where the diffusion rate falls fits Malquori's observation [6] that small calcium concentrations flocculated lecithin sols, intermediate ones peptised them, and still larger ones caused flocculation again. Elworthy and Saunders [7] have also reported two maxima in the surface force–calcium chloride concentration curve for lecithin sols.

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[6] Malquori, *Atti IV Congr. naz. Chim. pura applicata,* 1932, 752.
[7] Elworthy and Saunders, *J.*, 1957, 330.