J. Pharm. Pharmac., 1969, **21**, 137–144 *Received August* 13, 1968

Kinetics of the hydrolysis of phosphatidylcholine and lysophosphatidylcholine

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An experimental technique capable of estimating as little as 10 nano-equivalents of long-chain carboxylic acids (fatty acids) has been used to study the decomposition of phospholipids. The rate of release of acids varied with the monovalent cation which was associated with the hydroxyl ion in the hydroxide used. Because the concentration of hydroxide was much greater than the concentration of phospholipid, the rate of hydrolysis was first order with respect to the phospholipid. The pseudo-first order reaction velocity constant varied as the first power of the concentration of lithium hydroxide, but as the second power of the concentration of potassium, sodium, tetramethylammonium, or tetraethylammonium, hydroxides. It is postulated that (i) lecithin and lysolecithin, in strong alkaline solution, bind lithium ions much more strongly than potassium, sodium, tetramethylammonium, or tetraethylammonium ions, and (ii) it is only this cation-phospholipid complex which is broken down by hydroxyl ions.

Phosphatidylcholine (lecithin) contains two ester groups per molecule, each of which, in theory, should be hydrolysed both by hydrogen ions and hydroxyl ions.

Many workers, who have analysed hydrolysates of lecithin, have observed a migration of the phosphate group. In explaining the resulting isomerization reaction schemes have been put forward in which it has been assumed that the fatty acids were rapidly removed from the lecithin before any other action could take place, either in acid or alkaline conditions. This is certainly a correct assumption in alkaline solutions which contain a high concentration of hydroxyl ions, but the results with acid solutions and other salt solutions are not so easy to interpret, except under extreme conditions, for example, N HCl at 120° for 7 h (De Koning & McMullan, 1965; Long & Maguire, 1953).

Certain enzymes act specifically on either the 1- or 2-linked fatty acid ester bond only, of lecithin. On the other hand, at 0°, both the 1- and 2-fatty acyl groups are removed by methoxyl ions in chloroform–methanol solution to produce lysolecithins, although there may be "a small preference" for the 1-group (Marinetti, 1962).

Both in the saponification of esters and in acid-catalysed hydrolysis of esters, cleavage is in the $R_1CO–OR_2$ link and not in the $R_1COO–R_2$ link (Polanyi & Szabo, 1934; Roberts & Urey, 1938) and hydroxides break down lecithin and lysolecithin at a much faster rate than H^+ ions, to release the fatty acids.

EXPERIMENTAL

Method

Into a long-necked 100 ml flask, which was immersed (up to an inch below the stopper) in a water bath at a fixed temperature, was added methanol (96%), aqueous hydrolysing agent, and water, to a total volume of 24 ml. The volume of water, kept as low as possible, varied from 1 to 7 ml depending on the hydrolysing agent

used. The quantity of aqueous hydroxide solution was measured in ml, taken from a solution of known strength. To this mixture was added 1 ml of a chloroform solution of phospholipid after the previous mixture had equilibrated in the water bath. 1 ml aliquots of this final mixture were withdrawn at stated time intervals and run into an aqueous solution of 5N HCl, which was above a chloroform layer in a separating funnel. Liberated fatty acids were initially precipitated but, on shaking, they dissolved in the chloroform layer. The lower chloroform layer and washings were run off into a 10 ml beaker and the solvent removed by a vacuum pump, then each sample was titrated separately, as follows.

The acids were dissolved in about 5 ml (a fixed quantity measured by a device delivering a constant volume) of dimethylformamide (DMF), under nitrogen, together with four drops of a methanolic solution of quinaldine red (0·1% w/v), and titrated, under nitrogen, with standardized lithium methoxide which was in a mixed solvent of benzene and methanol. The end point of the titration was a distinct change of indicator colour from pink to colourless. The complete titration was made in an atmosphere free from water and carbon dioxide.

The burette was an Agla micrometer-syringe type readable to 0·0001 ml.

The titrant was standardized using recrystallized benzoic acid as a solid standard, dissolved in DMF as above. A typical series of titrations consisted of two blank titrations against DMF only, followed by two standardizations against accurately weighed samples of benzoic acid. Then another blank titration, followed by the samples in random order, another standardization against benzoic acid and a final blank titration against DMF only.

Materials used

Lecithin was prepared from a sample of purified egg lecithin (Merck A.G.) by chromatography and crystallization.

Lysolecithin was prepared from lecithin by the action of phospholipase A of Russell viper venom and was purified as described by Perrin & Saunders (1960).

RESULTS

Both lecithin and lysolecithin were hydrolysed in the same way by all the hydroxides used. There was no apparent difference in the graphs obtained. The rate of hydrolysis of phospholipid depended both on the concentration of phospholipid and the concentration of hydroxyl ions.

$$-d(L)/dt = k[L]\,[OH^-]^n \quad .. \quad .. \quad .. \quad .. \quad (1)$$

L is either lecithin or lysolecithin, k is the reaction velocity constant, n is an integer.

If $[OH^-] \gg [L]$ then equation (1) becomes—

$$-d(L)/dt = k^1[L] \quad .. \quad .. \quad .. \quad .. \quad (2)$$

In the experiments reported $[OH^-]$ was at least $100 \times [L]$.

The following quantities are defined: a = the maximum amount of acid released from a 1 ml sample of solution (total volume 25 ml), i.e. it represents the initial concentration of phospholipid in 1 ml of the solution before any hydrolysis occurred. x = the acid present at time t, in a 1 ml sample of solution. (a − x) is, therefore, a measure of the intact phospholipid in the reaction mixture at time t.

Substituting these quantities into equation (2),

$$-d(L)/dt = dx/dt = k^1(a - x)$$

which, on integration, becomes

$$\log (a - x) = \log (a) - k^1t/2{\cdot}303 \quad .. \quad .. \quad (3)$$

Equation (3) represents a first order reaction with respect to phospholipid. Hence on plotting a graph of log (a — x) against t, a straight line (with slope $-k^1/2{\cdot}303$), would indicate such a reaction, which is evident in Fig. 1a and b. The concentration

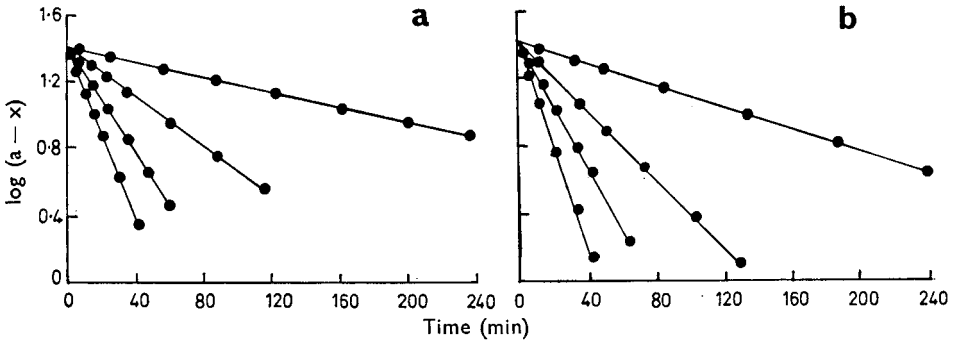

FIG. 1. The breakdown of lecithin (a) and lysolecithin (b) by NaOH under conditions of constant phospholipid concentration, constant temperature and four concentrations of OH^- for each phospholipid.

of hydroxyl ions was constant for each of the straight lines and the slopes of the straight lines depended on the hydroxyl ion concentration. Similar sets of straight lines were obtained for the hydrolysis of either lecithin or lysolecithin using LiOH, NaOH, Me_4NOH or Et_4NOH, at temperatures of 25°, 30° or 35°. Neither sodium borate, sodium bicarbonate, disodium hydrogen phosphate, nor dilute hydrochloric acid released any detectable amounts of carboxylic acids from lecithin in aqueous methanolic solution under the conditions of the experiments. It therefore seems that hydroxyl ions are necessary for the hydrolysis of the phospholipids.

In Fig. 1a it can be seen that the reaction constant, k^1, which is measured as $k^1/2{\cdot}303$ by simply calculating the slope of the straight line, varies with the concentration of hydroxyl ions for a constant lecithin concentration.

Substituting $t = \tau$ and $x = a/2$ into equation (3), we have, on rearranging

$$k^1\tau/2{\cdot}303 = \log 2 \quad .. \quad .. \quad .. \quad .. \quad (4)$$

Equation (4) is seen to hold approximately for the experimental results given in Tables 1 and 2. These results indicate that τ is independent of the initial concentration of phospholipid and confirms that the reaction is first order with respect to the phospholipid.

Assay of the reaction mixtures showed that, ultimately, all the acids were released from lecithin and lysolecithin.

Using the Arrhenius equation in the form

$$k^1 = A \exp(-E/RT) \quad .. \quad .. \quad .. \quad .. \quad (5)$$

the values of E and log A, which were the same for both lecithin and lysolecithin, were calculated (Table 3). When $\log (k^1/2{\cdot}303)$ is plotted against 1/T, the slopes of the lines are $-E/2{\cdot}303$ and the intercept on the $\log (k^1/2{\cdot}303)$ axis is $\log (A/2{\cdot}303)$.

Table 1. *Rate of hydrolysis of lecithin at* 35°

Initial concn of lecithin mM $ml^{-1} \times 10^3$	Cation	[OH^-] g ions $litre^{-1} \times 10^2$	τ min	$k^1/2{\cdot}303 \times 10^3$	$k^1\tau/2{\cdot}303$ (= log 2)
1·119	K	7·6	156	2·04	0·32
0·772			147	2·16	0·32
1·121	K	18·9	25	12·33	0·31
0·768			25	12·63	0·32
1·080	K	23·7	16	17·66	0·28
0·780			18	18·39	0·33
1·077	Na	18·2	44	6·71	0·30
0·785			45	7·93	0·36
1·097	Na	24·2	20	15·05	0·30
0·771			14	22·39	0·31
1·110	Na	30·3	10	27·34	0·27
0·752			10	27·05	0·27
1·078	Li	5·9	46	6·45	0·30
0·749			46	6·39	0·29
1·102	Li	11·8	18	17·27	0·31
0·749			17	17·70	0·30
1·108	Li	17·7	15	21·44	0·32
0·795			17	20·99	0·36

τ is the time for half the total acid to be released from the phospholipid.

Table 2. *Rate of hydrolysis of lysolecithin at* 35°

Initial concn of lysolecithin mM $ml^{-1} \times 10^3$	Cation	[OH^-] g ions $litre^{-1} \times 10^2$	τ min	$k^1/2{\cdot}303 \times 10^3$	$k^1\tau/2{\cdot}303$ (= log 2)
1·799	K	18·9	27	10·61	0·28
1·016			20	15·67	0·31
1·831	K	23·7	13	24·39	0·32
1·014			14	22·81	0·32
1·846	Na	9·7	120	2·65	0·32
1·099			123	2·47	0·30
1·775	Na	18·2	34	9·10	0·31
1·030			36	8·49	0·31
1·753	Na	24·2	17	16·23	0·28
1·123			16	19·62	0·31
1·890	Na	30·3	11	32·29	0·36
1·056			9	38·89	0·35
1·813	Li	5·9	35	8·78	0·31
1·126			36	8·79	0·32
1·856	Li	11·8	14	23·96	0·34
1·078			16	20·30	0·32
1·840	Li	17·7	11	27·79	0·31
1·069			11	23·82	0·26

Table 3. *Arrhenius constants for the hydrolysis reactions*

Hydroxide	E kcal $mole^{-1}$	log A*
LiOH	21·0 ± 2·3	11 to 15
NaOH	18·2 ± 3·6	8 to 14
KOH	14·8 ± 2·3	6 to 9

* Range of log A which varied with hydroxyl ion concentration.

From equations (1) and (2) we have the relation

$$k^1 = k[OH^-]^n \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Taking logarithms of equation 6 we have

$$\log k^1 = \log k + n \log[OH] \quad .. \quad .. \quad .. \quad (7)$$

Table 4. *Effect of hydroxide concentration on rates of hydrolysis*

° C	Cation and phospholipid	$[OH^-]$ g ions litre^{-1} $\times 10^2$	τ min	$[OH^-]^2\tau$
30	K	23·7	27	1·52
	Lecithin	18·9	38	1·40
		14·2	73	1·47
35	K	23·7	16	0·90
	Lecithin	18·9	25	0·89
		14·2	48	0·96
35	Na	30·3	11	1·01
	Lecithin	24·2	19	1·08
		18·2	44	1·46
25	Me_4N	12·2	76	1·13
	Lecithin	8·5	166	1·19
		15·8	44	1·10
25	Et_4N	8·3	105	0·72
	Lecithin	5·0	291	0·73
		11·6	58	0·78
25	K	23·7	45	2·52
	Lysolecithin	18·9	70	2·50
		14·2	123	2·48
30	Na	36·4	16	2·12
	Lysolecithin	30·3	27	2·48
		24·2	38	2·22
35	Na	30·3	11	1·01
	Lysolecithin	24·2	17	1·00
		18·2	34	1·12

Table 5. *Rates of hydrolysis by lithium hydroxide*

° C	Cation and phospholipid	$[OH^-]$ g ions litre^{-1} $\times 10^2$	τ min	$[OH^-]\tau$
30	Li	23·6	18	4·2
	Lecithin	17·7	23	4·1
		5·9	73	4·3
25		23·6	33	7·8
		17·7	48	8·5
		5·9	128	7·6
35		23·6	10	2·4
		17·7	15	2·7
		5·9	44	2·6
30	Li	23·6	16	3·8
	Lysolecithin	7·7	22	3·9
		11·8	42	4·9
25		23·6	23	5·4
		17·7	28	5·0
		5·9	112	6·6
35		17·7	11	2·0
		11·8	16	1·9
		5·9	37	2·2

As before, k^1 is represented as $k^1/2{\cdot}303$ from the slope of the practical curves (Fig. 1a and b), so log ($k^1/2{\cdot}303$) was plotted against log [OH^-] and the slopes of the resulting straight lines are 1 for lithium hydroxide and 2 for all the other hydroxides.

In Tables 4 and 5, $[OH^-]^n\tau$ is constant, within experimental error, with n equal to 1 for lithium, and n equal to 2 for the other hydroxides.

Results obtained for both lecithin and lysolecithin are summarized in Table 6.

Table 6. *Summary*

Symbol	Equation number	Conditions kept constant	Conclusion
k	1	Temperature and hydroxide concentration	$Et_4NOH > Me_4NOH >$ KOH > NaOH
k^1	6	As for k	$Et_4NOH > Me_4NOH >$ LOH > KOH > NaOH
E	5	Hydroxide concn	Has a value between 12·5 and 23·3 kcal KOH < NaOH < LiOH
A	5	Hydroxide concn	KOH < NaOH < LiOH
n	1		Equals 1 for lithium; equals 2 for other hydroxides

DISCUSSION

The values of the activation energy E obtained, are not significantly different from those reported in the literature for ester saponification (Moelwyn-Hughes, 1947). More significance can be attached to the variations in log A.

According to the activated complex theory of reaction rates, the fact that log A (a measure of the frequency factor of the reaction) is smaller for KOH than LiOH, may be due to modifications to the entropy of activation of the reacting molecules (or entropy of formation of the activated complex). Important effects can arise if a reactant, or the complex, has a high dipole moment. The solvent molecules in the neighbourhood of a highly polar activated complex will be acted upon by strong electrostatic forces causing them to have less freedom of motion than they would otherwise have. As a result there is a loss of entropy by the system and an abnormally low frequency factor because the activated complex tends to bind solvent molecules more strongly than the reactant molecules. The entropy of activation for the reaction between KOH and phospholipid is less positive than the entropy of activation for the reaction between LiOH and phospholipid. The KOH-phospholipid interaction can therefore be said to involve processes which are less likely to occur than the processes which occur between LiOH and phospholipid. This suggests that the KOH-phospholipid interaction produces a more polar activated complex than the LiOH-phospholipid interaction.

In the collision theory of reaction rates, the theoretical collision frequency varies but slightly from reaction to reaction, the average value being about $2{\cdot}77 \times 10^{11}$, i.e. for a normal bimolecular reaction log A is about 11·3. The approximate constancy is due to the compensating influences of two factors.

It is to be noted that log A is greater than 11·3 for LiOH, but is less than 11·3 for KOH. The variation of A with hydroxide concentration may be simply due to the relative concentration of OH^- ions. Z, the collision frequency, will vary with the concentration of hydroxyl ions.

The pK for the dissociation of metal hydroxides in aqueous solution ($MOH \rightleftharpoons M^+ + OH^-$) has been calculated as −0·08 for LiOH (Darken & Meier, 1942), and −0·70 for NaOH (Bell & Prue, 1949); no evidence for association (into ion pairs) of the K^+ and OH^- ions in KOH could be found (Davies, 1959). Both NaOH and KOH behaved, at low concentrations, as typical strong electrolytes. Lithium hydroxide is incompletely dissociated in such aqueous solutions (Darken & Meier, 1942). This indicates that the presence of OH^- ions in these aqueous solutions decreases in the order KOH > NaOH > LiOH. However, the value of k cannot be compared between LiOH and the other hydroxides because the units of k for LiOH are different from the units of k for the other hydroxides. The pseudo first-order reaction velocity constant, k^1, can be compared though, because the units of k^1 are s^{-1} for all hydroxides studied.

Because both lecithin and lysolecithin are broken down by hydroxides in a similar fashion, the difference between the action of LiOH and the other hydroxides cannot involve the structural difference between lecithin and lysolecithin.

The fact that n is unity for LiOH but 2 for all the other hydroxides can be explained as follows:

(The following symbols are used: p = phospholipid, fp = free phospholipid, tp = total phospholipid, M^+p = cation/phospholipid complex.)

Since the aqueous methanolic solution has a high concentration of OH^- ions the phospholipid will have a net negative charge, due to the phosphate group in the molecule. This net negative charge will tend to repel the OH^- ions, which are needed to breakdown the ester bonds in the phospholipid. If this net negative charge on the phospholipid is neutralized by cations, we have the following equilibrium,

$$M^+ + fp \rightleftharpoons M^+p$$

let
$$K_M = [M^+p]/[M^+]\,[fp] \quad .. \quad .. \quad .. \quad .. \quad (10)$$

and
$$[tp] = [M^+p] + [fp] \quad .. \quad .. \quad .. \quad (11)$$

by experiment, the rate measured was equal to $k^1[tp]$.

If only the neutralized phospholipid molecules are attacked by OH^- ions, then

$$\begin{aligned} k^1[tp] &= k_1[OH^-]\,[M^+p] \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (12)\\ &= k_1[OH^-]\{[tp] - [fp]\}, \text{ from (11)}\\ &= k_1[OH^-]\{[tp] - [M^+p]/K_M[M^+]\}, \text{ from (10)} \end{aligned}$$

Rearranging the last equation

$$k_1[OH^-][M^+p]\,\{1 + 1/K_M[M^+]\} = k_1[OH^-][tp] \quad .. \quad .. \quad (13)$$

From equations (12) and (13) we have

$$k^1[tp] = k_1[OH^-][tp]/\{1 + 1/K_M[M^+]\}$$

Therefore
$$k^1 = k_1[OH^-]K_M[M^+]/\{K_M[M^+] + 1\} \quad .. \quad .. \quad .. \quad (14)$$

From equation (10) we see that

$$K_M[M^+] = [M^+p]/[fp] \quad .. \quad .. \quad .. \quad .. \quad (15)$$

For LiOH, assuming a strong complex is formed between Li^+ ions and phospholipid because Li^+ will be small, $[M^+p]$ will be much greater than [fp], in equation (15); so

$K_M[M^+] \gg 1$. Therefore, from equation (14), $k^1 = k_1[OH^-]$ and so n has a value of 1, for LiOH. For the other hydroxides, a weak complex is formed between cation and phospholipid (the cations are either large themselves, e.g. Et_4N^+, or hydrated); so $[M^+p]$ is not much larger than [fp], which means that equation (14) can be written as,

$$k^1 = (k_1K_M/\{K_M[M^+] + 1\})[M^+][OH^-] \simeq k_2[OH^-]^2$$

($[M^+]$ being taken as proportional to $[OH^-]$) and so n has a value of 2, for all the other hydroxides.

An assumption made in this explanation is that only the cation-phospholipid complex, M^+p, is attacked by OH^- ions. This is reasonable because of the repulsion that is likely to occur between negatively charged phospholipid and OH^- ions. This assumption can be used to explain why the entropy of activation for KOH-phospholipid interaction is less positive than that for LiOH-phospholipid interaction. The KOH-phospholipid activated complex will be more negatively charged than the LiOH-phospholipid activated complex because the lithium ion is smaller than the potassium ion and so would be more strongly held by the phospholipid. This assumes that the lithium ion is dehydrated on complex formation. The dehydration would lead to an increase in entropy, which could explain why the entropy of activation for LiOH-phospholipid interaction is more positive than for KOH-phospholipid interaction. Since the hydrolysis is brought about by OH^- ions the mechanism for KOH/phospholipid interaction involves the combination of two like charges (both negative) whereas the LiOH/phospholipid interaction involves the combination of two unlike charges (either uncharged phospholipid or positively charged phospholipid-cation complex, with OH^- ions).

The difference in charge between LiOH-phospholipid complex and KOH-phospholipid complex can also explain why k^1, for LiOH, is larger than k^1 for the other hydroxides. For the other hydroxides the OH^- ions are repelled by the negatively charged phospholipid-cation complex.

Another assumption is that $K_M[M^+]$, which is equal to $[M^+p]/[fp]$, is much greater than unity for Li^+, but not for any other monovalent cation used. This too is a reasonable assumption because Li^+ ions form the least stable association with solvent molecules, compared with the other cations. Therefore free non-hydrated Li^+ ions will be more strongly held by the negative phospholipid macromolecule than hydrated K^+, Na^+, or quaternary ammonium ions.

This work forms part of a Thesis, accepted for the degree of Ph.D., University of London in 1966.

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