The Hydrolysis of Monolayers of Phosphatidyl[Me-¹⁴C]choline by Phospholipase D

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1. The hydrolysis of monolayers of phosphatidyl[Me-¹⁴C]choline at the air/water interface by phospholipase D (phosphatidylcholine phosphatidohydrolase) was investigated by a surface-radioactivity technique by using a flow counter. 2. Phosphatidylcholine of high specific radioactivity was prepared biosynthetically in good yield from [Me-¹⁴C]choline by using *Saccharomyces cerevisiae*. 3. At initial monolayer pressures between 12 and 25 dynes/cm. the hydrolysis occurred in two stages, an initial slow hydrolysis followed by a rapid hydrolysis. Below 3 dynes/cm. and above 28 dynes/cm. no enzymic hydrolysis of pure phosphatidylcholine monolayers could be detected. 4. The rapid hydrolysis was proportional to the enzyme concentration in the subphase, its pH optimum was 6.6, and 0.2 mM-Ca²⁺ was required for maximal activity. 5. Hydrolysis of the film was accompanied by a pronounced fall in the surface pressure even though the phosphatidic acid formed did not leave the film. When the pressure fell to low values the hydrolysis ceased even if the film was only partially hydrolysed. 6. Above monolayer pressures of 28 dynes/cm. enzymic hydrolysis could be initiated by inclusion of phosphatidic acid (and less effectively stearyl hydrogen sulphate) in the film, although the rates were not appreciably higher than those observed at 25 dynes/cm. with a pure phosphatidylcholine film. 7. The initiation of the hydrolysis by phosphatidic acid was facilitated by the inclusion of high Ca²⁺ concentrations and certain carboxylic acid buffer anions in the subphase, although these did not activate by themselves. 8. The initiation of the hydrolysis at high pressures could not be related to any change in the surface potential brought about by the addition of the long-chain anions to the film, nor could it be ascribed to a surface dilution effect. 9. The results are discussed in relation to previous studies on the hydrolysis of phosphatidylcholine particles by the enzyme and also similar investigations on phosphatidylcholine monolayers with other phospholipases.

Recent studies have emphasized that the activity of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is greatly influenced by the physical nature of the substrate. Thus large phosphatidylcholine particles are not appreciably hydrolysed, whereas ultrasonically treated particles (Dawson & Hemington, 1967) and phosphatidylcholine in lipoprotein combination (Condrea, Fabian & de Vries, 1964) are readily broken down.

The reaction rate is greatly accelerated by the inclusion in the incubation media of certain organic solvents and anionic amphipathic substances (Kates, 1957; Dawson & Hemington, 1967), both of which are likely to influence predominantly conditions at the substrate/water interface rather than the enzyme itself. The stimulatory effect of anionic amphipathic substances has been shown not to

depend on any change in the ζ -potential or size of the substrate particles (Dawson & Hemington, 1967), factors that can completely control the activity of certain other phospholipases (Dawson, 1965).

To obtain further insight into the way in which the physicochemical nature of the substrate affects the enzymic reaction we have now studied the hydrolysis of monolayers of phosphatidylcholine by purified soluble phospholipase D. Although the monolayer technique for studying lipolytic enzymes was introduced as early as 1935 (Hughes, 1935) it has been comparatively little used. This is probably because of the difficulties in interpreting changes in the surface pressure and potential of the monolayer during the enzymic hydrolysis, especially when metal ion cofactors are present and when lipoidal

hydrolytic products leave the film. In the present experiments we have followed the reaction more directly by a surface-radioactivity method with films of phosphatidyl[*Me*-¹⁴C]choline of very high specific radioactivity prepared biosynthetically with baker's yeast, while observing the surface pressure and potential of the monolayer.

The monolayer technique has certain advantages over bulk-phase methods: the phospholipid molecules are orientated in a precise and comparatively well understood manner compared with those in particulate substrates. Moreover, the area occupied by each substrate molecule can be accurately controlled and measured and variable factors such as the dispersion of substrate particles and the influence of buffers etc. can be eliminated from the interpretation of the results (Dawson, 1968).

EXPERIMENTAL

Preparation of Me-¹⁴C- and ³²P-labelled yeast phosphatidylcholine. Phosphatidyl[*Me*-¹⁴C]choline of high specific radioactivity was prepared biosynthetically from cultures of *Saccharomyces cerevisiae*. The composition of the basal culture medium (11.) was as described by Dawson & Bangham (1959). [*Me*-¹⁴C]Choline (200 μ C; 3.9 μ moles) was added and, after inoculation with the yeast, the culture was grown aerobically at 30°. When the E_{620}^{cm} value was 1.2 the yeast was centrifuged and washed with water. The lipids were extracted from the pellet, and the labelled phosphatidylcholine was isolated and purified by the methods described by Hauser & Dawson (1967a). With the counting apparatus described a monolayer of phosphatidylcholine at 20 dynes/cm. gave a counting rate of 60–80 counts/sec. whereas the background of the instrument was less than 5 counts/sec.

Yeast [³²P]phosphatidylcholine was prepared as described by Hauser & Dawson (1967a).

Apparatus and monolayer techniques. The basic apparatus and techniques for measuring surface pressure, radioactivity and potential were as described by Dawson (1968). Both Fluon and Perspex troughs were used, the latter having its edges waxed with paraffin wax to avoid leakage of the monolayer past the barrier. The bulk phase in the trough was stirred by a reciprocating magnetic stirrer (2.5 cm. long $\times$ 1 mm. diam.) activated from the underside of the trough.

The surface radioactivity was measured with a flow-counter arrangement (Dawson, 1968) with a thin (6 μ m.) window of Melinex (Imperial Chemical Industries Ltd., Plastics Division, Welwyn Garden City, Herts.), which allowed approx. 70% of the impinging β -rays from the phosphatidyl[*Me*-¹⁴C]choline to enter the counting chamber. The counter had a window area of 7.8 cm.² and was mounted 5 mm. away from the monolayer. The background recorded was variable, but always less than 5 counts/sec. unless the air-ionizing electrode used to measure surface potential was also mounted over the trough, when it was as high as 20 counts/sec. but very constant. The current from the rate-meter integrated over 20 sec. was continuously recorded by a pen recorder.

The surface pressure was determined with a dipping

plate made from a microscope cover slip (12 mm. wide). The force necessary to cause the plate to leave the surface was measured on a torsion balance with the trough stirring temporarily stopped. Successive surface-pressure determinations on a phosphatidylcholine monolayer in the presence of enzyme were consistent provided that no hydrolysis occurred. When considerable hydrolysis had taken place or when phosphatidic acid was present in the film from the start, the surface-pressure readings, although initially consistent, became erratic after the dipping plate had been inserted for 5–10 min. Presumably the plate was becoming contaminated with calcium phosphatide and there was no longer a zero contact angle. Readings were therefore only taken immediately after the cleaned plate had been dipped into the surface, and continuous recordings were not practicable.

The surface potential was recorded with a ²⁴¹Am air electrode mounted 0.8 cm. from the liquid surface. The ²⁴¹Am was contained in a foil consisting of the element dispersed in a silver matrix with an outer covering of gold (The Radiochemical Centre, Amersham, Bucks.). A rectangular piece of foil (1.5 cm. $\times$ 1 cm.) was mounted above the trough and surrounded by an earthed screen consisting of a cylinder of aluminium foil, one flat end of which had been replaced by a disc of Perspex (2 mm. thick) containing a hole 1 cm. in diameter to collimate the α -emission towards the liquid surface in the trough. The ²⁴¹Am electrode was connected to a millivoltmeter (model 46A Vibret pH-meter; Electronic Instruments Ltd., Richmond, Surrey) with high input impedance, and the output from this was continuously recorded by a pen recorder. A cleaned water surface gave potential readings varying from 80 to 150 mv (positive). The change in surface potential (ΔV) on spreading a phosphatidylcholine monolayer at a given pressure was more constant (varying by no more than 10 mv in a series of runs on the same day).

The whole trough was surrounded by an earthed box with a replaceable Perspex access window at the front. This not only protected the equipment from dust particles and air currents but it also gave more consistent and reproducible surface-potential measurements.

In a typical experiment a suitable quantity of phosphatidyl[*Me*-¹⁴C]choline either alone or mixed with an anionic amphiphile was spread in light petroleum (b.p. 60–80°)–chloroform (4:1, v/v) on the aqueous surface. With some long-chain anions it was necessary to use methanol–chloroform (4:1, v/v) to solubilize the lipid mixture. After the solvent had evaporated, the magnetic stirrer was started and after 1 min. it was stopped and the pressure adjusted to that desired by moving the barrier. When the hydrolysis of mixed films of phosphatidyl[*Me*-¹⁴C]choline and phosphatidic acid at high pressures was investigated it was found absolutely essential to add a small enough amount of the lipid to form a film at low pressure (< 25 dynes/cm.) and then to compress this to the higher pressure. Otherwise inconsistent results were obtained, presumably owing to inadequate spreading of the monolayer. The stirring was restarted and, if the pressure and surface radioactivity remained constant for about 10 min., the enzyme solution was injected with a hypodermic syringe through the film at three different positions in the path of the stirrer.

Materials. Phosphatidic acid (egg) was prepared as the sodium salt by the procedure of Dawson & Hemington (1967). Phosphatidylinositol was prepared from frozen peas

by a method evolved in this laboratory by Dr A. Sheltawy and described by Palmer & Dawson (1969).

The preparation and purification of phospholipase D from cabbages was based on the method described by Davidson & Long (1958) to the acetone precipitation stage. Preparations from different cabbages showed different activities per $\mu\text{g.}$ of protein, presumably because of differences in factors such as the maturity of the cabbage etc. Although prolonged efforts to purify the enzyme further were partially successful, the preparation became so unstable that it was judged unsuitable for routine use with monolayers. No differences in the behaviour of enzyme preparations of widely differing specific activities were noticed.

RESULTS

Phospholipase D hydrolysis of phosphatidyl-[Me- ^{14}C]choline monolayers at pressures below 28 dynes/cm. When enzyme was introduced below a phosphatidyl[Me- ^{14}C]choline monolayer at 12 dynes/cm. on a subphase containing 10 mM-calcium chloride in distilled water (pH 5.6), there occurred almost immediately a loss of surface radioactivity, implying that the labelled choline had been released from the phosphatidylcholine and had been stirred into the bulk phase (Fig. 1*b*). The initial loss was fairly slow (slow hydrolysis), but after a short period the rate accelerated and became approximately linear with time (fast hydrolysis). Eventually the rate tailed off and the hydrolysis virtually ceased even though 50% of the phosphatidylcholine substrate still remained in the monolayer. Determination of the surface pressure at this point showed that it had fallen to approx. 7 dynes/cm. Presumably the cessation of hydrolysis was due largely to the fall in pressure, since on compression of the film to 20 dynes/cm. the hydrolysis recommenced and the hydrolysis continued almost to completion. The resistance of films at very low pressure to hydrolysis is also shown in Fig. 1(*a*), where a phosphatidyl[Me- ^{14}C]choline monolayer at 2 dynes/cm. was not attacked by the enzyme until it was compressed to 12 dynes/cm., when hydrolysis commenced.

In experiments with monolayers at higher starting pressures (> 12 dynes/cm.) it was found that on adding the enzyme a longer phase of slow hydrolysis occurred, followed by a more rapid reaction that hydrolysed most of the phosphatidylcholine in the film (Fig. 1*c*). At film pressures above 25 dynes/cm. no reaction could be detected with amounts of enzyme that would cause rapid hydrolysis at lower pressures. On increasing the amount of enzyme manyfold a hydrolysis was occasionally observed up to 28 dynes/cm. but never at any higher surface pressure.

During the hydrolysis of phosphatidylcholine monolayers there was always a fall in surface potential. The change in surface potential coincided

Fig. 1. Recordings of the surface radioactivity and potential during the hydrolysis of phosphatidyl[Me- ^{14}C]choline monolayers at various pressures by phospholipase D. The curves run from right to left. Surface pressures of the monolayers in dynes/cm. are indicated by the numbers in parentheses. Monolayers were on 10 mM- CaCl_2 in water (pH 5.6); enzyme protein was added to the trough as shown by the arrows as follows: (a) 4.1 $\mu\text{g.}$; (b) 3.1 $\mu\text{g.}$; (c) 3.1 $\mu\text{g.}$ (a) and (b) show surface radioactivity; (c) shows surface radioactivity (upper curve) and surface potential (lower curve).

closely with the loss of surface radioactivity (Fig. 1*c*) and could be used as another index of the enzyme reaction.

In further experiments it was shown that the rate and extent of the hydrolysis of phosphatidylcholine films at 12 dynes/cm. by phospholipase D was not influenced by including 10 mM- $\beta\beta'$ -dimethylglutaric acid-sodium hydroxide buffer at the same pH (5.6) in the subphase. On varying the pH of the buffer it was found that the maximum rate of hydrolysis occurred at about pH 6.5 for the fast hydrolysis and slightly less for the initial slow hydrolysis (Fig. 2).

The hydrolytic action of phospholipase D against phosphatidylcholine monolayers (12 dynes/cm.) was completely prevented by leaving Ca^{2+} out of the subphase, but only low concentrations (0.2 mM- Ca^{2+}) were required to produce maximum activation

Fig. 2. Influence of bulk-phase pH on the slow and fast hydrolysis of phosphatidyl[Me-¹⁴C]choline monolayers by phospholipase D. Monolayers were at an initial pressure of 13 dynes/cm. Enzyme (5.8 μ g. of protein) was added to the trough, which contained 10 mM- β β '-dimethylglutaric acid-NaOH buffer at appropriate pH and 1 mM-CaCl₂ in the bulk phase. $\circ$, Fast hydrolysis; $\bullet$, slow hydrolysis.

Fig. 3. Influence of enzyme concentration on the slow and fast hydrolysis of a phosphatidyl[Me-¹⁴C]choline monolayer. Monolayers were at an initial pressure of 12 dynes/cm. Enzyme solution (containing 15.4 μ g. of protein/ml.) was added as indicated to the trough, which contained 10 mM- β β '-dimethylglutaric acid-NaOH buffer, pH 6.4, and 10 mM-CaCl₂ in the bulk phase. $\circ$, Fast hydrolysis; $\bullet$, slow hydrolysis.

of the reaction at the optimum pH (Table 1). Mg²⁺ was unable to replace Ca²⁺.

The rate of the fast hydrolysis of phosphatidylcholine films at 12 dynes/cm. on β β '-dimethylglutarate buffer, pH 6.4, was directly proportional to the amount of enzyme added, although at higher

Table 1. Effect of Ca²⁺ concentration on the rate of fast hydrolysis of phosphatidyl[Me-¹⁴C]choline monolayers by phospholipase D

Monolayers were at an initial pressure of 12 dynes/cm. Enzyme (7.7 μ g. of protein) was added to the trough, which contained 10 mM- β β '-dimethylglutaric acid-NaOH buffer, pH 6.4, in the bulk phase.

Conc. of CaCl ₂ (mM)	Hydrolysis (%/min.)
0	No reaction
0.1	No reaction
0.2	3.1
0.5	2.7
1.0	2.8
5.0	2.6
10.0	2.7

enzyme concentration this relationship was lost (Fig. 3). The rate of the slow hydrolysis seemed to reach a maximum at much lower concentrations of enzyme. The rate of the fast hydrolysis at 12 dynes/cm. was independent of the total area of the monolayer spread on the trough when this was varied between 40.5 cm.² and 81 cm.².

Fig. 4 shows a series of experiments in which the rate of the fast hydrolysis and the extent of the reaction were correlated with the starting pressure of the phosphatidylcholine monolayer. The determinations were carried out on β β '-dimethylglutarate buffer, pH 6.4, containing 10 mM-calcium chloride. The rate of the reaction is expressed both as an absolute rate denoted as the loss of surface radioactivity/min., which represents the number of phosphatidylcholine molecules disappearing from the area under the counter/min. (Fig. 4a), and as a relative rate representing the loss as a percentage of the original radioactivity present (Fig. 4b). In both instances the rate increased with increasing initial film pressures, but whereas the absolute rate increased up to the limits of measurement (24 dynes/cm.) the relative rate levelled off at pressures of about 12 dynes/cm. The extent of the film's hydrolysis (Fig. 4c) increased steadily from 25% hydrolysis at 5 dynes/cm. to approx. 75% hydrolysis at 24 dynes/cm.

Phospholipase D hydrolysis of phosphatidyl[Me-¹⁴C]choline monolayers at pressures above 28 dynes/cm. Although pure phosphatidylcholine monolayers at pressures above 28 dynes/cm. were not hydrolysed by phospholipase D, mixed monolayers of phosphatidylcholine and phosphatidic acid were readily hydrolysed. Thus in the experiments denoted by Δ in Fig. 4, mixed monolayers of phosphatidyl[Me-¹⁴C]choline + phosphatidic acid (2:1 molar ratio) were hydrolysed at pressures up in the region of the collapse pressure of the film. However, the rate/ μ g. of protein and extent of the

Fig. 4. Effect of the initial starting pressure of phosphatidyl-[$Me-^{14}C$]choline monolayers on the surface radioactivity lost (a) and on the rate (b) and extent (c) of the hydrolysis by phospholipase D. The bulk phase contained 10mM- $\beta\beta'$ -dimethylglutaric acid-NaOH buffer, pH 6.4, and 10mM- $CaCl_2$. ●, 0.41 μ g. of enzyme protein; phosphatidyl-[$Me-^{14}C$]choline monolayer; Δ, 0.3 μ g. of enzyme protein; phosphatidyl-[$Me-^{14}C$]choline + phosphatidic acid (2:1 molar ratio) monolayer.

hydrolysis of such mixed monolayers at pressures both above and below 28 dynes/cm. were not markedly greater than those of pure phosphatidylcholine films (Fig. 4).

The experiments in Fig. 4 were performed with a subphase containing $\beta\beta'$ -dimethylglutarate buffer, pH 6.4, and it was observed that in the absence of buffer the hydrolysis of high-pressure films was very different. Thus with a subphase containing no buffer, a film of phosphatidyl[$Me-^{14}C$]choline + phosphatidic acid (2:1 molar ratio) at 40 dynes/cm. showed a long slow hydrolysis after the introduction of the enzyme into the trough, followed by a rapid hydrolysis (Fig. 5a). During the slow hydrolysis the pressure of the film fell to 34 dynes/cm. (and the surface potential fell by 9mv), but this decrease in the surface pressure is insufficient in itself to account for the commencement of the rapid-hydrolysis phase, although it may be a contributing factor. On the other hand, if 10mM- $\beta\beta'$ -dimethylglutarate buffer, pH 6.4, was added to the subphase rapid

Fig. 5. Recordings of the surface radioactivity during the hydrolysis of phosphatidyl[$Me-^{14}C$]choline + phosphatidic acid mixed monolayers at high pressures by phospholipase D. The curves run from right to left. Surface pressures of the monolayer in dynes/cm. are indicated as numbers in parentheses. Monolayers were on 50mM- $CaCl_2$ in water (pH 5.6) for (a), (b) and (d) and on 50mM- $CaCl_2$ in 10mM- $\beta\beta'$ -dimethylglutaric acid-NaOH buffer, pH 6.4, for (c). Enzyme was added to the trough as shown by the arrows as follows: (a) 10.2 μ g.; (b) 6.1 μ g.; (c) 1.2 μ g.; (d) 1.2 μ g. (a) and (c) Phosphatidyl[$Me-^{14}C$]choline + phosphatidic acid (2:1 molar ratio); (b) and (d) phosphatidyl[$Me-^{14}C$]choline + phosphatidic acid (1:1 molar ratio).

hydrolysis commenced immediately after the addition of enzyme (Fig. 5c), even though much less enzyme had been added compared with the previous experiment (Fig. 5a). This rapid response still occurred if the pH of the buffer was adjusted so that it was equivalent to that of a non-buffer-containing subphase. The $\beta\beta'$ -dimethylglutarate buffer needed to be present at a concentration of at least 3mM to have its maximal effect. Other dicarboxylic acid buffers at a concentration of 10mM and the same pH showed an ability to shorten the slow-hydrolysis phase to different extents. Thus succinate was slightly less effective than $\beta\beta'$ -dimethylglutarate, glutarate showed slight activity

and maleate, malonate, adipate and pimelate were ineffective. Among monocarboxylic acid buffers, acetate showed no effect, whereas propionate, *n*-butyrate, *n*-valerate, *n*-hexanoate, isobutyrate and isovalerate shortened the lag phase somewhat, although there was no apparent correlation between the structure of the anion and the time by which the lag phase was shortened.

The slow-hydrolysis phase could also be shortened by increasing the phosphatidic acid concentration in the monolayer so that it and the phosphatidyl-[*Me*-¹⁴C]choline were in equimolar proportions (Fig. 5*b*). However, a slow-hydrolysis phase was still observed with these films if the concentration of enzyme was fivefold lower (Fig. 5*d*).

In a number of experiments it was shown that relatively high Ca²⁺ concentrations facilitated the hydrolysis of mixed phosphatidylcholine + phosphatidic acid films at high pressures. Thus increasing the Ca²⁺ concentration in the range 1–40 mM substantially shortened the time of slow hydrolysis both on water and $\beta\beta'$ -dimethylglutarate buffer.

In general other anionic amphipathic substances mixed with the phosphatidyl[*Me*-¹⁴C]choline were much less effective, or ineffective, at inducing rapid hydrolysis of high-pressure monolayers compared with phosphatidic acid (Table 2). Phosphatidylcholine-stearyl hydrogen sulphate films (1:3 molar ratio) were hydrolysed rapidly after a long slow-hydrolysis phase, whereas with a 1:2 molar ratio no attack by the enzyme was observed. Neither phosphatidylinositol nor dicetyl hydrogen phosphate induced a significant hydrolysis of monolayers of phosphatidyl[*Me*-¹⁴C]choline at equimolar ratios. There appeared to be no correlation between the decrease of surface potential of the phosphatidyl-[*Me*-¹⁴C]choline monolayers caused by the introduction of the anionic amphipathic substance and susceptibility of the mixed monolayers to hydrolysis (Table 2). Thus phosphatidylinositol lowered the surface potential more than phosphatidic acid did, but it was not an activator, and stearyl hydrogen sulphate, which lowered the surface potential to much lower values, was not nearly so effective as phosphatidic acid. In addition, there appeared to be no evidence that the activation of hydrolysis produced by anionic amphipathic substances is produced merely by a surface dilution effect on the phosphatidylcholine molecules (Table 2). Thus the anionic amphipathic substances that are ineffective decrease the surface radioactivity just as much as the phosphatidic acid. Moreover, a pure phosphatidyl[*Me*-¹⁴C]choline film at 20 dynes/cm. with a surface radioactivity greater than those of the unhydrolysed mixed films was readily attacked.

Loss of surface pressure associated with phospholipase hydrolysis of phosphatidylcholine monolayers. As stated above, the hydrolysis of pure phosphatidylcholine alone and mixed with anionic amphipathic substances and their hydrolysis by phospholipase D

Table 2. Surface potential, pressure and radioactivity of monolayers of phosphatidylcholine alone and mixed with anionic amphipathic substances and their hydrolysis by phospholipase D

Subphase	Monolayer (ratios are molar)	Surface pressure (dynes/cm.)	Surface potential (ΔV) (mv)	Surface radioactivity (counts/sec.)	Protein added (μ g.)	Duration of slow-hydrolysis period (min.)	% 'hydrolysis' at 40 min. and/or time at which 80% hydrolysis was completed
10 mM-CaCl ₂ in water (pH 5.6)	Phosphatidylcholine	40	+422	80	20	> 40	1%
	Phosphatidylcholine + phosphatidic acid (1:1)	20	+356	66	20	4	80% at 8 min.
	Phosphatidylcholine + phosphatidic acid (1:1)	40	+413	50	20	8	80% at 14 min.
	Phosphatidylcholine + phosphatidylinositol (1:1)	38	+408	49	8	12	80% at 23 min.
	Phosphatidylcholine + phosphatidylinositol (1:1)	38	+394	49	8	> 40	3%
	Phosphatidylcholine + stearyl hydrogen sulphate (1:2)	40	+298	58	20	> 40	3%
10 mM-CaCl ₂ in 10 mM-dimethylglutarate buffer, pH 6.4	Phosphatidylcholine + stearyl hydrogen sulphate (1:3)	40	+242	49	20	> 40	12% (80% at 54 min.)
	Phosphatidylcholine	40	+419	82	20	> 40	4%
	Phosphatidylcholine + phosphatidic acid (1:1)	40	+399	52	20	1	80% at 5 min.
	Phosphatidylcholine + phosphatidylinositol (1:1)	38	+382	47	8	> 40	2%
	Phosphatidylcholine + stearyl hydrogen sulphate (1:2)	40	+290	57	20	> 40	2%
	Phosphatidylcholine + stearyl hydrogen sulphate (1:3)	40	+250	49	20	35	29% (80% at 49 min.)
	Phosphatidylcholine + dicetyl hydrogen phosphate (1:1)	40	+394	58	20	> 40	10%*
	Phosphatidylcholine + dicetyl hydrogen phosphate (2:3)	40	+396	53	20	> 40	9%*

* At 35 min.

tidylcholine monolayers at low pressures was associated with a considerable fall in surface pressure, and this was also observed with mixed films of phosphatidylcholine and phosphatidic acid at high pressures (Fig. 5*d*). Experiments with monolayers of yeast [^{32}P]phosphatidylcholine and surface-radioactivity measurements gave no evidence that the phosphatidic acid formed was leaving the monolayer during the reaction, the hydrolysis being followed by the change in surface potential produced. In addition, no evidence could be obtained from test-tube incubations that the enzyme preparation contained other types of phospholipase activity, e.g. phospholipase A, that could release lysolecithin from the film.

DISCUSSION

The present results show that phospholipase D will not attack pure yeast phosphatidylcholine monolayers at pressures above 28 dynes/cm. under any of the experimental conditions tried. Similarly phospholipases B and C cease to attack yeast phosphatidylcholine monolayers at about the same limiting pressure (Bangham & Dawson, 1960, 1962), and the hydrolysis of phosphatidylcholine monolayers by venom phospholipase A shows a similar cut-off at higher pressures whose precise magnitude depends on the fatty acid composition of the phosphatidylcholine (Dawson, 1966; Colacicco & Rapport, 1966; Shah & Schulman, 1967*a*). Presumably at these higher pressures the spacing between the phospholipid molecules is insufficient to allow the penetration of the active centre of the enzyme between them to initiate enzymic hydrolysis. Thus in experiments on the penetration of protein into monolayers of phospholipid, an increase in surface pressure ($\Delta\pi$) indicating penetration is only observed on addition of the protein to the subphase below films that have an initial surface pressure below certain limits (Matalon & Schulman, 1949; Quinn & Dawson, 1969). These limits will presumably depend on such factors as the nature of the phospholipid and the protein as well as the temperature and pH at the interface.

No change in pressure caused by the penetration of the phospholipase D into the monolayer was detected experimentally, presumably because the catalytic amount of protein involved is so small and any minor increase in pressure would be masked by the fall in pressure due to the changing chemical composition of the film. The nature of this pressure fall during the enzymic hydrolysis is not clear; the experiments with [^{32}P]phosphatidylcholine indicate that the phosphatidic acid formed does not leave the film, nor do other enzymes in the enzyme preparation cause a further decomposition of the phosphatidic acid or an attack on a different site of the phosphatidylcholine molecule. It therefore

seems that the removal of the choline may allow tighter packing of the fatty acid chains in the film, with a corresponding fall in pressure. This is the reverse of the change that would be predicted from the force-area curves of pure phosphatidylcholine and phosphatidic acid monolayers in the absence of Ca^{2+} , where the more expanded nature of the latter film has been ascribed to electrostatic repulsion between the negatively charged head groups (Shah & Schulman, 1967*b*). These electrostatic factors would of course be largely eliminated by the Ca^{2+} present in the system, as these would have a high affinity for the free phosphate ions of the phosphatidic acid formed at the interface (Hauser & Dawson, 1967*b*).

On the other hand, at very low film pressures (2–3 dynes/cm.) it is clear that no hydrolysis of the phosphatidylcholine monolayer occurs. Here the film is in a very expanded state with ample space between the molecules and with the fatty acid chains flopping over rather than being vertically orientated to the surface as they are at higher pressures. Two explanations of the lack of activity are possible. First, all the enzyme molecules colliding with the surface are irreversibly denatured at the air/water interface between the phospholipid molecules; secondly, hydrophobic reactions between the more readily available fatty acid chains and the protein could prevent the correct stereochemical alignment of the active centre of the enzyme around the ester bond between the phosphate and choline moieties. Obviously the cessation of enzymic activity at low pressures limits the extent of the hydrolysis of phosphatidylcholine films at intermediate pressures. When the latter films are hydrolysed, the pressure fall occurring as the phosphatidylcholine is replaced by phosphatidic acid can be sufficient to take the pressure of the film below a certain limit so that hydrolysis only recommences when the partly hydrolysed film is compressed. This is reflected in the extent of the hydrolysis of the monolayers increasing from 25% at 5 dynes/cm. initial starting pressure to 74% at 24 dynes/cm. starting pressure (Fig. 4*c*). The fact that the stopping point for a film that is being hydrolysed is somewhat higher (7 dynes/cm. in Fig. 1*b*, but varying with the starting pressure) than the lower limit at which hydrolysis of a pure phosphatidylcholine film can be initiated (approx. 3 dynes/cm.) suggests that other factors may also be involved. For example, in the film that has been partially hydrolysed the phosphatidylcholine substrate is spaced out both by phosphatidic acid and possibly by protein side chains that have penetrated the film during the hydrolysis.

The hydrolysis of phosphatidylcholine monolayers at intermediate film pressures (5–25 dynes/cm.) shows certain similarities to, as well as distinct differences from, the reaction of the enzyme with

phosphatidylcholine particles (ultrasonically treated or activated with ether) in a bulk-phase system. In both systems Ca^{2+} is an absolute requirement for activity, unlike the situation with phospholipase C, where the necessity for bivalent metal ion co-factors disappears at monolayer pressures below 25 dynes/cm. (Bangham & Dawson, 1962). However, the Ca^{2+} concentration required in the sub-phase for maximal activity (0.2 mM) is 200-fold less than the optimum necessary in the bulk-particle system (Dawson & Hemington, 1967).

The monolayer hydrolysis differs from that of bulk particles in often showing a lag or slow-hydrolysis phase, which can vary in time depending on such factors as the initial pressure of the film. Owing to the smallness and variability of this slow-hydrolysis phase and the experimental difficulties involved it was not possible to measure its kinetics in precise quantitative terms, but the impression gained was that it increased in duration as the starting pressure of the phosphatidylcholine film was increased. The meaning of this slow-hydrolysis phase is not obvious, but it is possible that to initiate the fast reaction a certain accumulation of autocatalytic amounts of phosphatidic acid must occur. Once the fast reaction has commenced it continues at a constant rate until activity ceases owing to the pressure of the film falling below the value required for activity. When this rate is expressed as the number of phosphatidylcholine molecules disappearing from the area scanned by the counter the rate increases as the initial film pressure of the film is increased from 5 to 24 dynes/cm. (Fig. 4a). At pressures above 12 dynes/cm. this increase seems to be due to the increase of surface substrate concentration brought about by the compression of the film favouring the chances of a 'successful' collision between enzyme and substrate molecules, since the rate expressed as a percentage hydrolysis of the substrate under the counter remains fairly constant. At pressures below 12 dynes/cm. the percentage hydrolysis falls more rapidly than that predicted from the decrease in substrate concentration brought about by the more expanded film. Here the same factors may be operating to decrease the activity that finally cause a cessation of activity at very low pressures, namely irreversible surface denaturation or hydrophobic reactions between the fatty acid chains and the enzyme or both.

The pH optimum of the fast phase of the monolayer hydrolysis appears to be at about 6.5. This is considerably higher than that observed for the bulk-particle hydrolysis of ultrasonically treated phosphatidylcholine or large phosphatidylcholine particles activated with ether, which show maximal activity at about pH 5 (Quarles & Dawson, 1969). However, in the same investigation it was shown that this optimum shifts to pH 6.6 if added phos-

phatidic acid is used to activate the bulk-particle hydrolysis. Thus it seems possible that the pH optimum of the fast hydrolysis is high because of the phosphatidic acid formed in the slow hydrolysis. In fact the pH-activity profile of the slow hydrolysis (Fig. 2) seems to be shifted more towards an acid value and nearer to that observed in bulk-phase studies in the absence of added phosphatidic acid.

At pressures above 28 dynes/cm. hydrolysis of the film by the enzyme is observed only if the phosphatidylcholine is mixed with certain anionic amphipathic substances that also activate the bulk-particle system. As in this latter system, phosphatidic acid is the most effective activator. Stearyl hydrogen sulphate is a poor activator for the monolayer hydrolysis, whereas it is a very effective activator of the bulk-particle hydrolysis. Phosphatidylinositol and dicetyl hydrogen phosphate, which are much less effective activators of particle hydrolysis, are ineffective as activators with the monolayer. The phosphatidic acid does not appear to increase the rate of the hydrolysis markedly above that occurring at film pressures below 28 dynes/cm., but merely allows an initiation of hydrolysis at the higher pressures. In this action it seems to be helped by anionic buffer ions, although the latter cannot initiate hydrolysis on their own. Shah & Schulman (1967a) showed that the rate of enzymic digestion of phosphatidylcholine monolayers by snake-venom phospholipase A is affected by the buffer used. They suggested that the buffer anion competes with the substrate monolayer for Ca^{2+} (an essential cofactor) and keeps these bivalent cations more available for the enzyme in the bulk aqueous subphase. In the present investigation we found that high Ca^{2+} concentrations facilitate the hydrolysis of high-pressure films both in the presence and in the absence of buffer. In being activated by high Ca^{2+} concentrations the hydrolysis of mixed high-pressure films resembles bulk-particle hydrolysis more than the hydrolysis of low-pressure films, where only a very low Ca^{2+} concentration is required.

The present studies do not give any conclusive evidence as to why phosphatidic acid and other anionic amphipathic substances activate the phospholipase D reaction. Dawson & Hemington (1967) showed that the activation cannot be directly related to the electrostatic ζ -potential in the surface phase near the phosphatidylcholine/water interface, as it can when phospholipases B and C are attacking the same substrate (Dawson, 1966). Nor does the initiation of activity depend on the change in surface potential of the interface produced by the activator (Table 2). This surface potential is a summation of the permanent dipoles of the film molecules and an electrostatic factor dependent on the charge on the lipid molecule 'head groups' orientated towards the subphase and modified by counter-ions in that

subphase. It is also clear that dispersion or surface-dilution factors are not operative, e.g. dilution of the phosphatidylcholine molecules in the film by the activators so that they occupy the same average surface area as phosphatidylcholine molecules at a lower film pressure. In this respect the initiation of hydrolysis at high film pressures is rather like that observed with phospholipase B, where again surface-dilution effects or dispersion of the phosphatidylcholine do not seem to be responsible for the activation produced by anionic amphipathic substances (Dawson & Hauser, 1967). Presumably the introduction of the anion into the film must provide electrostatic and steric conditions at the interface that assist the active centre of the enzyme to penetrate between the closely packed substrate molecules, even though such an effect is not apparent from the measurements that have been made. It is perhaps of biological significance that phosphatidic acid, which is the product of the reaction, is much more effective than other anionic amphipathic substances in providing the required conditions.

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