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On the Mechanism of Action of Phospholipase A

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The phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) of snake venom catalyses the hydrolyses of diacylated nitrogen-containing phosphoglycerides with the liberation of a single fatty acid and the formation of the corresponding lysophosphoglyceride. Both phosphatidylethanolamine and phosphatidylserine are attacked as well as lecithin and with all substrates only the fatty acid esterified on the β -position is released (Marinetti, Erbland & Stotz, 1958; Tattrie, 1959; Hanahan, Brockerhoff & Barron, 1960; de Haas & van Deenen, 1961).

It has recently been shown that with two other phospholipases the rate of reaction is appreciably influenced by the electrokinetic state of the enzyme and its substrate. Thus a phospholipase preparation isolated from *Penicillium notatum* splits lecithin only when the substrate micelles have a net negative ζ potential (Bangham & Dawson, 1959). On the other hand, the phospholipase C of *Clostridium perfringens* only works when the enzyme possesses a net negative charge, and the substrate contains excess of positive groupings on its surface (Bangham & Dawson, 1962). In the present work, similar physicochemical investigations have been made on the phospholipase A of snake venom, the aqueous reaction mixture recently devised by Magee & Thompson (1960) being used. While no evidence has been obtained that the initial reaction is controlled by the net ζ potential of the substrate, it has been

shown that many anionic amphipathic molecules, including the fatty acid produced in the reaction, are inhibitory. The stimulatory effect of ether when the enzyme is attacking lecithin is probably due to a surface dilution of the closely packed molecules orientated at the lipid-water interface and a removal of inhibiting fatty acid carboxyl groups from the interface. A preliminary account of this work has been published (Dawson, 1962).

EXPERIMENTAL

Preparation of purified enzyme. Venom of the cobra (*Naja naja*) was used as a source of phospholipase A. After collection at London Zoo, it was air-dried at a slightly elevated temperature and subsequently kept for a period of 2 years in a desiccator over CaCl_2 at room temperature. The crude venom was directly fractionated in a density-gradient electrophoresis apparatus (LKB Stockholm) according to the techniques of Svensson (1960). The density gradient was prepared by using 0.01M-sodium acetate-acetic acid buffer, pH 5.1, as the light component and the same buffer containing 45% (by vol.) of glycerol as the heavy component. About 20 mg. of cobra venom was dissolved in 5 ml. of heavy buffer and the density adjusted by adding light buffer so that it just floated on the heavy buffer. This enzyme solution was layered on to the heavy buffer contained in the electrophoresis column; the gradient was then built up, starting with a 'light-heavy' buffer containing 40% of glycerol. The electrophoresis was carried out for 16-18 hr. (900V, 8 mA) at a temperature of running tap water (10-14°). Fractions from the column were obtained

by moving a funnel, connected to the exterior by a thick-walled glass tube sliding in a polythene plug, through the heavy buffer to the bottom of the gradient. Successive samples (7.5 ml.) were drawn off and assayed for enzymic activity with lecithin as substrate, and their extinctions at 280 μ were measured. The active fractions were pooled and the preparation was stored at -16° . Full activity was retained for many months.

Assay of phospholipase A. Enzyme activity was measured by observing the decrease of fatty acyl groups and the method closely followed that of Magee & Thompson (1960). A sample of phospholipid solution containing about 50–60 μ g. of P was dried *in vacuo* in a stoppered glass tube. Buffer (0.8 ml., 0.05M) and 0.1 ml. of 5 mM- CaCl_2 were added and the mixture was shaken to emulsify the lipid. Lecithin emulsified easily; phosphatidyl-ethanolamine required more vigorous shaking and slight warming. Enzyme (0.1 ml.) was added and then 0.1 ml. of ether. The tube was tightly stoppered and immediately incubated at 30° for 20 min. with continuous shaking. The reaction was stopped and the fatty acyl ester determined according to Magee & Thompson (1960). Appropriate controls were used containing substrate with no enzyme and enzyme with no substrate.

Microelectrophoresis of substrate particles. Mobilities of substrate particles in a horizontal electrophoresis cell were measured by direct microscopic observation of the stationary layer as described by Bangham & Dawson (1959). Use of a bath temperature of 30° as in the enzymic assay resulted in volatilization of ether with bad seating of the electrodes and consequent non-electrical drift of the particles. Consequently a temperature of 25° was always used for all mobility determinations. Since the mobility (μ) of a particle is given by the Helmholtz–Smoluckowski equation

$$\mu = \frac{\zeta \epsilon}{4\pi\eta}$$

(where η is the viscosity and ϵ the dielectric constant of the suspending medium, and ζ the zeta potential of the particles), the mobility at 30° would be slightly higher because of the reduction of the viscous drag on the particle due to the decreased viscosity of the suspending buffer. However, the dielectric constant of the buffer would be slightly reduced and this would partially compensate for the previous change. The net effect of this temperature difference on the mobility would therefore be small and the percentage change is likely to be constant under all circumstances.

Preparation of lecithin and phosphatidylethanolamine from egg yolk. The yolks of 12 eggs were homogenized with 300 ml. of acetone. After standing for 30 min. the mixture was centrifuged and the supernatant discarded. The residue was extracted twice for 5 min. with portions (500 ml.) of chloroform–methanol (1:1, v/v), and the combined extracts were evaporated to dryness at 50° in a rotary drier. The residue was dissolved in 100 ml. of light petroleum (b.p. 60 – 80°) and 4 vol. of acetone was added. The precipitate of crude egg-yolk phospholipids was washed three times with 50 ml. of acetone, and stored at -15° until required. The lecithin and phosphatidylethanolamine were then separated from the phospholipids by the chromatographic methods of Lea, Rhodes & Stoll (1955) and Rhodes & Lea (1957).

(a) *Lecithin.* The crude egg-yolk phospholipid (2.5 g.) was dissolved in 20 ml. of chloroform–methanol (1:1, v/v) and chromatographed on an 80 g. column of alumina (Al_2O_3 , M.F.C., Hopkin and Williams Ltd.). The column was

eluted with chloroform–methanol (1:1, v/v) and the first 100 ml. of eluate collected and evaporated to dryness in a rotary drier. The residue of crude lecithin was dissolved in chloroform–methanol (17:8, v/v) and a portion containing 20 mg. of P was chromatographed on a silicic acid (Mallinckrodt) column (20 g.). Elution was continued with the same solvent and the first phosphorus-containing peak emerging from the column was recovered and stored in chloroform solution at -15° . Analysis by alkaline degradation (Dawson, Hemington & Davenport, 1962) and determination of the fatty acyl ester/P ratio indicated that it was pure lecithin.

(b) *Phosphatidylethanolamine.* Crude egg-yolk phospholipid (2 g.) was dissolved in 20 ml. of chloroform and added to the top of a silicic acid column (100 g.) poured in chloroform. Elution was commenced with 300 ml. of chloroform–methanol (19:1, v/v) and the solvent changed to chloroform–methanol (4:1, v/v). The phosphorus-containing peak that appeared immediately on changing to this latter solvent was collected, dried *in vacuo* and stored in chloroform solution at -15° . On analysis (Dawson *et al.* 1962) a typical preparation contained 91% of phosphatidylethanolamine. The remainder was ethanolamine plasmalogen together with some alkali- and acid-stable phospholipid, which was probably the saturated ether analogue of phosphatidylethanolamine. This was reported to be present in egg yolk by Carter, Smith & Jones (1958). The fatty acyl ester/P ratio was 1.86.

Preparation of lysolecithin and mixed β -fatty acids. Lysolecithin was prepared enzymically from 0.5 g. of ovolecithin as described by Dawson (1956). The combined ethereal supernatants from the precipitation of lysolecithin containing the liberated fatty acids and unchanged lecithin were dried *in vacuo*. The residue was dissolved in chloroform and applied to a silicic acid column (20 cm. $\times$ 1 cm. diam.), which was eluted with chloroform. The first 200 ml. emerging was dried *in vacuo* and dissolved in 50 ml. of light petroleum (b.p. 40 – 60°). The fatty acid content of this solution was ascertained by titration (Dawson, 1956). It contained no P. The yield of mixed β -fatty acids was 69% and of lysolecithin 71%.

RESULTS

Purification of phospholipase A. Initial experiments were carried out in which the cobra venom was heated at 90° in 0.02M-sodium acetate–acetic acid buffer, pH 5.8, for 3–18 min. No worthwhile increase in specific activity was obtained. Density-gradient electrophoresis of crude cobra venom at pH 5.1 produced a resolution into at least seven peaks which absorbed at 280 μ (Fig. 1). Only the peak moving slowest towards the cathode possessed appreciable phospholipase A activity when incubated with ovolecithin. The material in the peak also produced haemolysis of human erythrocytes, but this was a fairly insensitive method of locating the activity. Determination of the insertion point on the column with uniformly labelled [^3H]-inositol (Bangham & Dawson, 1962) indicated that the enzyme is very slightly positively charged at pH 5.1 and its isoelectric point is probably in the region of pH 5.2. The purification based on protein

determinations by extinctions at 260 and 280 $m\mu$ was about two- to three-fold.

General properties of the enzyme. In general the properties of the phospholipase A preparation resemble those of the unfractionated enzyme in cottonmouth-moccasin (*Agkistrodon piscivorus piscivorus*) venom studied by Magee & Thompson (1960). One interesting difference, however, appears to be in the rate of reaction in various buffers; whereas the cottonmouth-moccasin venom hydrolysed lecithin appreciably better in collidine buffer than in phosphate, the present cobra-venom preparation worked equally well in both.

Effect of calcium ions. The phospholipase A activity of crude cobra venom was only slightly stimulated by Ca^{2+} ions, but the addition of EDTA (0.5 mM) completely inhibited it. The hydrolysis of lecithin by the purified enzyme was almost totally dependent on the presence of Ca^{2+} ions (Fig. 2): Mg^{2+} ions were completely ineffective. With incubation for 20 min., 5 mM-calcium chloride produced maximal deacylation. In contrast the hydrolysis of a phosphatidylethanolamine substrate by the purified enzyme was only slightly stimulated by added Ca^{2+} ions (0.5 mM). However, EDTA (1 mM) completely inhibited the system and consequently the lack of response to added Ca^{2+} ions can prob-

ably be attributed to the presence of calcium in the phosphatidylethanolamine preparation.

EDTA added in quantities sufficient to chelate the added Ca^{2+} ions produced complete inhibition of the hydrolysis of lecithin. However, a puzzling but consistent finding has been an enhanced activity in the presence of EDTA when excess of Ca^{2+} ions is added to saturate the chelator (Fig. 2). Probably this might be associated with contamination of the system with heavy metals, which are known to inhibit phospholipase A activity (Long & Penny, 1957; Magee & Thompson, 1960). Saito & Hanahan (1962) have described a 'protective' effect of EDTA on the phospholipase A from *Crotalus adamanteus* venom.

Solvent activation. In agreement with the results of Magee & Thompson (1960) it was found that the hydrolysis of a lecithin substrate occurred to only a small extent in the absence of diethyl ether. Fig. 3 shows the effect of adding various amounts of ether to the enzyme system acting in both phosphate and collidine buffers. It is seen that the hydrolysis was stimulated well before the buffer became saturated with ether and that maximal stimulation occurred at approximately the point of complete saturation. Stimulation was still observed when excess of ether was present, although a slight fall in activity was consistently found when the ether slightly exceeded the amount required for saturation; with increasing amounts of ether the activity recovered and then gradually declined.

To confirm that a two-phase system was not essential for activation, experiments were conducted in which the buffer containing calcium

Fig. 1. Density-gradient electrophoresis of cobra venom. The point of insertion of the venom is between fractions 3 and 4, and components moving towards the cathode have been collected and their extinctions at 280 $m\mu$ recorded. The histogram represents the ability of the peaks to deacylate lecithin expressed in arbitrary units. Other details are given in the Experimental section.

Fig. 2. Effect of Ca^{2+} ions on the hydrolysis of a lecithin substrate by purified phospholipase A. Collidine buffer, 0.04M, pH 6.5. ●, 1 mM-EDTA was added to hydrolysis mixtures.

chloride was saturated with ether and incubated with lecithin and enzyme. In spite of the final solution being less than 90 % saturated with ether good activation of the enzyme was observed.

Methyl isobutyl ketone, which was shown by Kates (1957) to activate phospholipase D, also stimulated the system when added at a concentration of 1–2 % by volume, but not so effectively as ether. Larger amounts of the ketone were inhibitory. Ethanol, butanol and propane-1,2-diol had no stimulatory effect.

In contrast, the hydrolysis of a phosphatidylethanolamine substrate occurred at an appreciable rate in the absence of ether, and the addition of ether either had no effect or it produced a very slight inhibition of the rate. This applied both at pH 6·5 and at pH 9·4.

Saturation of lecithin emulsions with ether had a distinct clearing effect on the turbidity of the emulsion, even though a two-phase system was not produced. This clearing could be arbitrarily measured by determining the light-absorption of the emulsion at 660 μ . Butanol (8 %, v/v) also produced some clearing but methyl isobutyl ketone (1 %, v/v) and ethanol were ineffective. Amphipathic substances such as sodium dodecyl sulphate, cetyltrimethylammonium bromide, monophosphoinositide and dicetylphosphoric acid also produced clearing of the lecithin emulsions (Table 1), but saponin had no effect.

In contrast with this clearing action of ether on lecithin emulsions, the solvent appears to have little effect on phosphatidylethanolamine emulsions in sodium hydroxide–glycine buffer, pH 9·3. In collidine buffer, pH 6·5, a precipitation of the emulsion particles occurs.

Fig. 3. Stimulation of phospholipase A on adding various amounts of ether to lecithin emulsions. ●, 0·04M-Collidine-HCl buffer, pH 6·5; ○, 0·04M- NaH_2PO_4 - Na_2HPO_4 buffer, pH 6·5. The arrow denotes the formation of a two-phase system.

Effect of pH. The hydrolysis of both substrates was measured in a series of four buffers in an attempt to cover the range pH 6–10 (Fig. 4). The hydrolysis of lecithin at a given pH varied from buffer to buffer, which is in agreement with the results described by Magee & Thompson (1960) for cottonmouth-moccasin venom, although in contrast it was found that in phosphate buffers between pH 6 and 7 the hydrolysis was as effective as in collidine

Table 1. *Clearing action of various solvents and amphipathic substances on lecithin emulsions*

Lecithin (1·74 μ moles/ml.) in 0·04M-collidine-HCl buffer, pH 6·5, containing 0·5 mM- CaCl_2 .

Substance added to emulsion	Concn.	Light-transmission (% of original)
None	—	100
Diethyl ether	8% (by vol.)	189
Butanol	6% (by vol.)	180
Ethanol	10% (by vol.)	99
Methyl isobutyl ketone	1% (by vol.)	100
Dodecyl sulphate (sodium)	0·1% (w/v)	322
Cetyltrimethylammonium bromide	0·04% (w/v)	194
	0·66 (μ mole/ml.)	331
Dicetylphosphoric acid	0·33 (μ mole/ml.)	294
Phosphatidylinositol	0·33 (μ mole/ml.)	227

Fig. 4. Effect of pH and buffer composition on the hydrolysis of (a) lecithin and (b) phosphatidylethanolamine. ▲, NaH_2PO_4 - Na_2HPO_4 buffers (0·04M); □, collidine-HCl buffers (0·04M); ○, tris-HCl buffers (0·04M); ●, NaOH -glycine buffers (0·04M).

buffer. In spite of these variations a marked difference can be seen between the two substrates. Whereas the rate of hydrolysis of lecithin shows little evidence of change between pH 6 and 9, the extent of hydrolysis of phosphatidylethanolamine appears to increase substantially with increasing pH. Magee & Thompson (1960) reported a constancy of hydrolysis of a lecithin substrate by cottonmouth-moccasin venom between pH 6.5 and 8.5, and Long & Penny (1957) reported that the same venom attacked phosphatidylethanolamine in ether only when ether-soluble bases such as ammonia and ethanolamine were added to increase the alkalinity.

In Fig. 5 the rates of hydrolysis of lecithin and phosphatidylethanolamine in a series of tris-hydrochloride buffers are compared with the electrophoretic mobility of the emulsion particles. Whereas the lecithin micelles remain virtually isoelectric between pH 6.9 and 8.9 the phosphatidylethanolamine particles show a steadily increasing negative ζ potential.

Effect of amphipathic substances on the hydrolysis. When lecithin was emulsified in collidine buffer, pH 6.5, and examined electrophoretically the particles

had a slight negative charge ($0.6 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$). The addition of 0.5 mM-calcium chloride hardly affected this but when the buffer was saturated with ether the net charge was reduced almost to zero. Attempts to replace the Ca^{2+} ions or ether as activators of the reaction by adding a long-chain cation (cetyltrimethylammonium hydroxide) or long-chain anion (dicetylphosphoric acid) were not successful.

In further experiments amphipathic substances were added to the lecithin in a complete system containing Ca^{2+} ions and ether. The addition of cetyltrimethylammonium hydroxide did not appreciably affect the reaction even though the substrate possessed a high positive ζ potential (Fig. 6). On the other hand, the addition of dicetylphosphoric acid inhibited the reaction progressively with increasing negative ζ potential (Fig. 6). Since the dicetylphosphoric acid would decrease the surface pH of lecithin (pH s) according to the equation $\text{pH } s = \text{pH bulk} + 0.325 \mu$ (where μ is the mobility), experiments were also conducted in buffers at pH 7 and 7.5. These showed a similar inhibition to that at

Fig. 5. A comparison of (a) electrophoretic mobilities of lecithin and phosphatidylethanolamine micelles with (b) the rate of their hydrolysis by phospholipase A at various pH values. Tris-HCl buffers (0.04M): ●, lecithin (ether added); ○, phosphatidylethanolamine (no ether present).

Fig. 6. Effect of the addition of cetyltrimethylammonium hydroxide and dicetylphosphoric acid on (a) the electrophoretic mobility of lecithin and on (b) its hydrolysis. 0.04M-Collidine-HCl buffer, pH 6.5. ○, Dicetylphosphoric acid; ●, cetyltrimethylammonium hydroxide.

pH 6.5 and indicated that the mechanism of inhibition was not merely due to a reduction in surface pH. The inhibition was not overcome by adding sufficient cetyltrimethylammonium hydroxide to a lecithin-dicetylphosphoric acid mixed particle to reduce the ζ potential to zero or reverse the charge. It was shown that the concentration of cetyltrimethylammonium hydroxide required did not inhibit the hydrolysis on its own. No significant relief of the inhibition was obtained by increasing the calcium concentration of the bulk solution tenfold.

The hydrolysis of lecithin was also inhibited by the presence of monocetylphosphoric acid and sodium dodecyl sulphate but with the latter substance a greater negative ζ potential was required for an equivalent inhibition to that produced by dicetylphosphoric acid. Phosphatidic acid (7 moles/100 moles) in the lecithin inhibited the hydrolysis but on the further addition of this substance to 25% the activity was restored to its former value even though the ζ potential of the particles had become more negative. Phosphatidylinositol added to the lecithin produced no inhibition of the hydrolysis even when the mixed particles had a mobility of $-4.3 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$.

In contrast the hydrolysis of phosphatidylethanolamine was not affected by the addition of dicetylphosphoric acid in amounts (15 moles/100 moles) that produced a large inhibition of the hydrolysis of a lecithin substrate.

Effect of the fatty acids released on the enzymic activity and ζ potential. When lecithin micelles in collidine buffer were incubated with enzyme and Ca^{2+} ions in the absence of ether, their electrophoretic mobility of about $-0.6 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$ was increased to $-1.7 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$. This is presumably due to the liberation of a small amount of fatty acid which orientates at the lipid-water interface. When ether was present in the system, the mobility remained virtually constant at the initial value of $-0.2 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$ on incubation with enzyme even when over half of the substrate had been hydrolysed. Presumably therefore the much larger numbers of fatty acid molecules liberated were no longer orientating at the lipid-water interface in the presence of ether, or, alternatively, they were being removed from the lecithin particles entirely.

This effect of ether was investigated by preparing a series of emulsions of lecithin in which the lecithin was progressively replaced by an equimolar mixture of lysolecithin and fatty acids, as occurs in the enzymic reaction. In the absence of ether this replacement caused the particles to develop a pronounced negative ζ potential (Fig. 7). When ether was added there was no such progressive increase in negative ζ potential (Fig. 7). Addition of

2% (v/v) of methyl isobutyl ketone also produced a reduction of the negative ζ potential although this was not so marked as that produced by ether. Mixtures of lecithin and fatty acid with no lysolecithin present showed the same dramatic reduction of the negative ζ potential on the addition of ether.

Emulsions of phosphatidylethanolamine in collidine buffer, pH 6.5, possessed a higher negative potential (mobility $-2.3 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$) than lecithin. As the pH was increased this became more pronounced so that at pH 9.34 (sodium hydroxide-glycine buffer) the mobility of phosphatidylethanolamine was $-6.6 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$. A similar effect was observed in a series of tris-hydrochloride buffers, pH 6.9–8.9 (Fig. 5). It is presumably due to the discharge of the amino groups as the pH increases. The addition of ether to phosphatidylethanolamine emulsions did not appreciably alter the mobility of the particles.

When phosphatidylethanolamine particles were incubated with enzyme at pH 9.3 for 20 min. there was a small increase in their mobility (e.g. in two experiments the mobility changed from -7.4 to -8.7 and from -6.5 to $-7.6 \mu\text{sec.}^{-1} \text{v}^{-1} \text{cm.}^{-1}$ after roughly 50% of the substrate had been hydrolysed). This increase was hardly affected by the presence of ether in the system.

When mixed emulsions of phosphatidylethanolamine and fatty acids were prepared it was observed that the electrophoretic mobility increased appreciably, with increasing fatty acid concentration (Fig. 8). In marked contrast with lecithin, the presence of ether only reduced the ζ potential of the mixed phosphatidylethanolamine fatty acid particles very slightly (Fig. 8).

Fig. 7. Electrophoretic mobility of mixed particles of lecithin, fatty acid and lysolecithin. ○, Ether present; ●, ether absent. The fatty acid was the mixed fatty acid preparation described in the Experimental section. The mixed dry lipids were emulsified in 0.04M-collidine-HCl buffer, pH 6.5.

The addition of fatty acid to both lecithin in the presence of ether and phosphatidylethanolamine in the absence of ether produced an inhibition of their hydrolysis by phospholipase A (Fig. 9). The inhibi-

tion of the phosphatidylethanolamine hydrolysis was not overcome by adding ether to the system, but increasing the calcium content tenfold did partially relieve the inhibition.

DISCUSSION

The present results indicate that the initiation of cobra-venom phospholipase A action is not so directly dependent on the ζ potential of the substrate as was found with the lecithinase from *Penicillium notatum* and the phospholipase C of *Cl. perfringens* (Bangham & Dawson, 1959, 1962). Although the hydrolysis of lecithin by phospholipase A is inhibited by many anionic amphipathic substances, this is a progressive effect and there is no evidence of a sharp ζ potential threshold when the enzyme becomes inactive as was seen with the other enzymes. Furthermore, the extent of the inhibition depends on the nature of the anionic amphipathic molecule and is not directly related to the ζ potential. Thus the inhibition due to dicetylphosphoric acid cannot be relieved by adjusting the ζ potential to zero by adding cetyltrimethylammonium hydroxide. Moreover, phosphatidylinositol added to the lecithin produced a negative ζ potential but did not inhibit the hydrolysis.

The enzyme system requires Ca^{2+} ions as an obligatory cofactor. Unlike the finding with the phospholipase C of *Cl. perfringens*, the Ca^{2+} ions cannot be replaced by adding either cetyltrimethylammonium hydroxide to the lipid or Mg^{2+} ions to the buffer. Consequently Ca^{2+} ions must be playing a direct and specific part in the reaction between enzyme and substrate rather than merely adjusting the ζ potential of the substrate to a favourable value.

The rate of the enzymic attack against phosphatidylethanolamine increases as the pH is increased from 6 to 9.3, but there is no evidence either in the present studies or in those of Magee & Thompson (1960) of a similar increase occurring with a lecithin substrate. This suggests that the effect may be due to a discharge of the amino group on phosphatidylethanolamine rather than on the enzyme itself. The increase in the negative ζ potential of phosphatidylethanolamine particles when the bulk pH is raised from 6 to 9.3 is presumably a reflexion of this discharge. Lecithin particles, on the other hand, are isoelectric between pH 6 and 9.3 (Fig. 5) (Bangham & Dawson, 1959) because the quaternary nitrogen of the choline is not discharged until much higher pH values are reached.

It was shown by Hanahan (1952) that when snake-venom phospholipase A reacts with lecithin an ether-soluble enzyme-substrate complex is formed. When excess of ether was added to the present incubation mixtures so that a two-phase

Fig. 8. Electrophoretic mobility of mixed particles of phosphatidylethanolamine and fatty acid. ○, With ether; ●, ether absent. The fatty acid was the mixed fatty acid preparation described in the Experimental section. The mixed dry lipids were emulsified in 0.04M-NaOH-glycine buffer, pH 9.3.

Fig. 9. Inhibition of phospholipid hydrolysis by fatty acid. ●, Substrate phosphatidylethanolamine (1.62 μ moles/ml.) with oleic acid added in varying amounts; 0.04M-NaOH-glycine buffer, pH 9.3. ○, Substrate lecithin (1.69 μ moles/ml.) with mixed fatty acid preparation (see Experimental section) added in varying amounts; 0.04M-collidine-HCl buffer, pH 6.5; ether added.

system was formed it could be shown that enzyme was removed from the aqueous phase when substrate was present. This occurred both with a lecithin substrate as well as with phosphatidylethanolamine, although the hydrolysis of the latter is not stimulated by ether. In the single-phase system studied in the present investigation such a solubilization of the enzyme in an ether-rich phase might still occur if the ether molecules penetrated into the lipid particle.

With a lecithin substrate the polar head groups of the phospholipid at the lipid-water interface would be tightly packed together because their net charge would be zero. The penetration of ether into the micelle would tend to dilute the density of the polar head groups in the lipid-water interface, and might consequently allow the enzyme easier access for attacking the acyl ester bond. In this connexion it has been shown by Hughes (1935) that the rate of hydrolysis of a unimolecular surface film of lecithin by venom is much faster when the film is expanded and the surface pressure reduced. It is also likely that the whole stability of the particle would be reduced by the ether penetration so that increased dispersion would result. This is the probable explanation of the distinct clearing action that ether has on the turbidity of lecithin emulsions. Although a greater dispersion of the lecithin substrate might increase the rate of enzymic attack, it seems unlikely to be the sole explanation of the stimulatory effect of ether. Thus many amphipathic substances have been shown to have a similar clearing action on lecithin emulsions yet they do not stimulate the enzymic reaction. Moreover, 1% by volume of methyl isobutyl ketone activates the reaction yet it does not produce a clearing of the emulsion.

When lecithin particles are incubated with phospholipase A in the absence of ether their ζ potential becomes more negative. This indicates that the lecithin molecules orientated at the lipid-water interface are being attacked by the enzyme and converted into fatty acid and lysolecithin. This accumulation of enzymic products at the interface will stop the reaction by preventing effective contact between the enzyme and unhydrolysed lecithin molecules in the interior of the micelle. Furthermore it is likely that the fatty acids accumulating in the interface will tend to inhibit the reaction between the enzyme and any unhydrolysed lecithin molecules in the surface. Thus added fatty acid inhibits the hydrolysis of lecithin particles by the enzyme even in the presence of ether when only a small percentage of the added fatty acid molecules are orientating at the lipid-water interface.

When the enzymic hydrolysis of lecithin is conducted in the presence of ether the relative constancy of the electrophoretic mobility of the particles indicates that very few of the liberated

fatty acid molecules are appearing in the lipid-water interface. The addition of ether appears either to remove the fatty acid from the lecithin particle or, more probably, to cause a rearrangement of the organization of the particles so that the fatty acid is repositioned in the interior. This effect is clearly demonstrated by the action of ether in reducing the electrophoretic mobilities of mixed particles of lecithin, lysolecithin and fatty acid as well as of lecithin and fatty acid only.

With phosphatidylethanolamine particles, the polar head groups of the phospholipid in the lipid-water interface would be more widely separated than with lecithin because their net negative charge would cause mutual repulsion of the orientated molecules. Thus the enzyme might have better access to the susceptible acyl ester bond than with a lecithin substrate. Increasing the bulk pH increases the net negative charge on the polar head groups of phosphatidylethanolamine (Fig. 5) and consequently the greater repulsion would cause a wider spacing of the orientated molecules at the lipid-water interface. This situation might allow an even easier penetration of the enzyme into the phospholipid so that hydrolysis would increase with pH, as is observed experimentally.

When phosphatidylethanolamine particles are incubated with phospholipase A in the absence of ether hydrolysis proceeds readily and at 50% completion the negative ζ potential has increased by only about 15%. This suggests that the fatty acids produced in the reaction are not orientating at the lipid-water interface and are being replaced by fresh substrate molecules which are attacked by the enzyme. When, however, fatty acid is added to phosphatidylethanolamine particles to form a mixed micelle there is a marked increase in the negative ζ potential (Fig. 8) and the hydrolysis is inhibited (Fig. 9). It is difficult to understand why added fatty acid, in contrast with enzymically liberated fatty acid, orientates at the lipid-water interface and increases the negative ζ potential, but this may be due to the absence of lysophosphatidylethanolamine, which is liberated in the enzymic system.

It is difficult to explain the inhibitory effect which many anionic amphipathic molecules including fatty acids have on the enzymic hydrolysis of lecithin. Since the isoelectric point of the enzyme is about 5.2 it will have a net negative charge at the H^+ ion concentrations normally used to determine activity. However, there are a number of reasons why the inhibitory effect cannot be due to a simple repulsion between the negatively charged enzyme and similarly charged substrate. Thus the inhibition caused by dicetylphosphoric acid is not reversed by adding cetyltrimethylammonium hydroxide to the lecithin to restore the isoelectric state. Moreover phosphatidylinositol added to the lecithin does not

inhibit the reaction although it does produce a pronounced negative charge on the phospholipid's surface.

It is possible that the enzyme initially attaches itself to a free negative (phosphate) site on the surface of the substrate. The surface of lecithin would contain very few free negative sites uncompensated by the positive charge of the choline moiety and it is possible that added long-chain anions such as dicetyl phosphate when orientated in the lipid-water interface could in certain circumstances compete with the substrate sites for enzyme. If this is correct, the hydrolysis of phosphatidylethanolamine should not be so readily inhibited by the addition of dicetylphosphoric acid, since this substrate contains many more free negative sites on its surface than does lecithin, and this is found experimentally. From Fig. 9 it would appear that fatty acid inhibits phosphatidylethanolamine hydrolysis as effectively as lecithin hydrolysis but it must be remembered that whereas added (as distinct from liberated) fatty acid orientates at the phosphatidylethanolamine-water interface it does not readily do so at the lecithin-water interface in the presence of ether.

The inhibition by long-chain anions cannot be due to the competition of the foreign anion for Ca^{2+} ion sites since increasing the Ca^{2+} ion content of the buffer tenfold does not relieve the inhibition. The attraction, if it does exist, must therefore be between substrate and enzyme or more likely an enzyme- Ca^{2+} ion complex.

The inability of phosphatidylinositol to inhibit lecithin hydrolysis does not fit this general picture. However, the behaviour of phosphatidic acid, which inhibits at low concentrations and does not do so at higher concentrations, indicates that with amphipathic substances with an analogous structure to that of lecithin the physicochemical situation is complicated. It might possibly happen that such substances compete in the interface for substrate positive groups, so that more free substrate negative sites become available for enzyme attachment.

SUMMARY

1. The phospholipase A of cobra (*Naja naja*) venom has been purified by density-gradient electrophoresis.

2. The hydrolysis of lecithin and phosphatidylethanolamine by the purified enzyme has been studied in the aqueous system described by Magee & Thompson (1960) and correlated with the electrophoretic mobility of the substrate particles.

3. Ca^{2+} ions are obligatory for the hydrolysis; they cannot be replaced by Mg^{2+} ions added to the buffer, nor by cetyltrimethylammonium hydroxide added to the lipid.

4. The rate of hydrolysis of phosphatidylethanolamine increases as the pH is increased from 6 to 9.3; that of lecithin is comparatively unchanged.

5. The hydrolysis of lecithin is inhibited by the addition of many anionic amphipathic molecules (e.g. dicetylphosphoric acid) to the substrate, but this is not related to the net negative ζ potential produced on the lipid since the inhibition cannot be reversed when cetyltrimethylammonium hydroxide is added to restore the isoelectric state.

6. Diethyl ether added to saturate the aqueous phase greatly stimulates the rate of hydrolysis of lecithin, whereas the breakdown of phosphatidylethanolamine is not affected.

7. Light-transmission through lecithin emulsions is increased by the addition of ether. This effect is also produced by amphipathic substances which do not stimulate phospholipase A hydrolysis. Conversely, methyl isobutyl ketone stimulates the hydrolysis but does not increase light-transmission.

8. Lecithin on incubation with enzyme in the absence of ether shows a change in mobility from -0.6 to $-1.7 \mu\text{sec.}^{-1} \text{ v}^{-1} \text{ cm.}^{-1}$. In the presence of ether the mobility remains constant in spite of a greatly increased liberation of fatty acid.

9. Phosphatidylethanolamine micelles possess an electrophoretic mobility of $-7.4 \mu\text{sec.}^{-1} \text{ v}^{-1} \text{ cm.}^{-1}$ at pH 9.3, which is changed to -8.7 when the enzymic hydrolysis has gone to 50% completion.

10. Mixed particles of lecithin and fatty acid have a negative ζ potential, which is reduced almost to zero on the addition of ether. In contrast, ether does not remove the fatty acid carboxyl groups from the surface of mixed particles of phosphatidylethanolamine and fatty acid.

11. The addition of fatty acid to either lecithin or phosphatidylethanolamine inhibits the enzymic reaction.

12. It is concluded that the stimulatory effect of ether on lecithin hydrolysis is due to a penetration of ether into the lipid particle. This will have two consequences: (i) a wider spacing of the lecithin molecules orientated at the lipid-water interface, allowing easier access of the enzyme to the susceptible acyl ester bond; (ii) the fatty acids produced in the reaction will be removed from the surface of the lecithin particles and replaced with fresh substrate molecules, thus preventing inhibition of the hydrolysis.

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A Uridine Diphosphatase from Sugar Cane

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The metabolic role of non-specific phosphatases is difficult to assess but there is some information about the function of specific phosphatases. For instance, fructose 1,6-diphosphatase of liver (Mokrasch, Davidson & McGilvery, 1956) and photosynthetic tissue (Racker & Schroeder, 1958; Smillie, 1960) is probably essential for reversal of the glycolytic pathway associated with the processes of glycogenesis and photosynthesis.

During a study of uridine diphosphate glucose-fructose transglucosylase from sugar-cane storage tissue (Hatch, Sacher & Glasziou, 1963) a phosphatase that rapidly hydrolysed UDP but not UTP or UMP was found. With extracts containing both these enzymes, UDP-glucose and fructose were converted quantitatively into sucrose, UMP being the only other detectable product. The present paper describes the partial purification and properties of the uridine diphosphatase.

EXPERIMENTAL

Preparation of uridine diphosphatase

From sugar-cane storage tissue. Tissue from the mature internodes of sugar-cane stalks (hybrid variety, Pindar) was used as a source of enzyme. Juice was obtained by passing strips of the rind-free tissue through a laboratory roller-mill. After filtration, 100 ml. of the juice was cooled to 0°, its pH was adjusted to 7.0 with 1N-KOH, and then saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.1, 0°) was added to give 70% saturation. The saturated $(\text{NH}_4)_2\text{SO}_4$ was neutralized with aq. NH_3 and its pH was measured after 1 ml. had been diluted with 10 ml. of water. After standing for 1 hr. the

precipitated protein was obtained by centrifuging at 10 000 g for 10 min. The sedimented protein was dissolved in 10 ml. of 5 mM-tris-HCl buffer, pH 7.5, and dialysed against 2 l. of the same buffer for 16 hr. at 0°. When this preparation was frozen and thawed, approx. 25% of the protein was precipitated without loss of uridine-diphosphatase activity. The precipitated protein was removed by centrifuging and the supernatant was adjusted to pH 4.8 with 0.1N-HCl. After standing for 5 min. at 3° the precipitated protein was removed by centrifuging at 10 000 g for 5 min. and the supernatant was adjusted to pH 7.5 with 0.1N-KOH. A portion (10 ml.) of this supernatant was added to the top of a column containing 0.4 g. of diethylaminoethylcellulose (DEAE-cellulose; Cellex-D, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) that had been thoroughly washed with 5 mM-tris-HCl buffer, pH 7.5, and then packed into the column under pressure. The column was washed with 10 ml. of 0.02M-KCl in 5 mM-tris-HCl buffer, pH 7.0, and then the uridine diphosphatase was eluted with 4 ml. of 0.1M-KCl in 5 mM-tris-HCl buffer, pH 7.0. This preparation is referred to below as the 'partially purified enzyme'. A unit of uridine-diphosphatase activity is defined as the amount of enzyme that will hydrolyse 1 μ mole of substrate/min. at pH 8.1 and 30°.

From other plant tissues. For a survey of the distribution of uridine diphosphatase, 10 g. of each of the various tissues was ground in a mortar and the juice obtained by squeezing through fine muslin. Each extract was cooled to 0°, adjusted to pH 5.2 with 1N-HCl and centrifuged at 10 000 g for 10 min. After readjustment of the pH to 7.5 the supernatant was added to 0.2 g. of DEAE-cellulose (previously washed with 5 mM-tris-HCl buffer, pH 7.0) in a centrifuge tube. The DEAE-cellulose was washed twice with 5 ml. of 5 mM-tris-HCl buffer, pH 7.0, then with 5 ml. of 0.2M-KCl in 5 mM-tris-HCl buffer, pH 7.0. The protein fraction eluted with 0.2M-KCl was tested for uridine-diphosphatase activity.