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THE EFFECT OF FATTY ACIDS AND OF ALBUMIN ON THE ACTION OF A PURIFIED PHOSPHOLIPASE A_2 FROM COBRA VENOM ON SYNTHETIC LECITHINS

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SUMMARY

1. A study was made of the inhibition of a purified preparation of *Naja naja* phospholipase A_2 by fatty acids, and of the effect of albumin on the enzyme in the presence and absence of fatty acids.

2. It was confirmed that long chain fatty acids (palmitate, oleate, linoleate) cause inhibition at low concentrations (less than 0.1 mM). The degree of inhibition was dependent on the concentration of substrate. A relationship was found between the percentage inhibition and the inhibitor/substrate ratio. It was concluded that inhibition resulted, at least in part, from interaction of the fatty acid with substrate micelles.

3. The enzyme hydrolysed dipalmitoyllecithin faster than dioleoyllecithin or dilinoleoyllecithin. Palmitate, oleate and linoleate inhibited in an order of effectiveness which was dependent on the substrate used.

4. Bovine plasma albumin activated the enzyme in the absence of added fatty acid. At high concentrations it prevented inhibition by low concentrations of fatty acid. In the absence of added fatty acid it activated at concentrations so low (5 μg/ml) that it is unlikely that the activation resulted solely from the binding of fatty acid released by the enzyme action.

INTRODUCTION

In recent years considerable interest has centred round the action of phospholipase A_2 on mitochondrial, microsomal and plasma membranes[1–4], that is to say, on its action on phospholipid–protein complexes as they occur in cell membranes.

It has been shown previously[5,6] that this enzyme hydrolyses lipoprotein-bound phospholipids faster than free phospholipid. The rate curves observed with sonicated suspensions of substrates suggest the possibility that the enzyme might be inhibited by one of the reaction products, and added fatty acid has already been shown to be inhibitory[7]; it was of interest therefore to investigate this inhibition, and to observe

whether the addition of a protein such as bovine serum albumin, which binds fatty acids, would relieve it, since it seemed possible that the more rapid rate observed with lipoprotein complexes could be due to such an action by the apoprotein.

We have therefore carried out an investigation of the inhibitory effects of fatty acids and of the activating effect of albumin on the action of phospholipase A_2 on synthetic phospholipids using a purified phospholipase A_2 preparation from *Naja Naja* venom obtained by the method of Braganca *et al.*[8].

MATERIALS AND METHODS

Substrates

Dipalmitoyllecithin [*O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)choline] was purchased from Fluka (Ralph Emmanuel Ltd, Middlesex, England) and was purified on a silicic acid column to remove contaminating free fatty acid and lysolecithin. Thin-layer chromatography showed that the earlier 'lecithin' fractions contained an unknown compound running slightly ahead of lecithin, but showed only a single spot from the later lecithin fractions which were pooled, taken to dryness and redissolved in a small volume of chloroform. After 2 precipitations with ether a solid residue was obtained.

Dioleoyllecithin [*O*-(1,2-dioleoyl-*sn*-glycero-3-phosphoryl)choline] was synthesised essentially according to the method of Cubero Robles and De Iongh[9]. It was stored in hexane at −15 °C, and gave a single spot on thin-layer chromatography. On analysis the fatty acid:phosphorus ratio was found to be 1.99.

Dilinoleoyllecithin [*O*-(1,2-dilinoleoyl-*sn*-glycero-3-phosphoryl)choline] was obtained from Lipid Products, Epsom, England, and stored in chloroform. A fatty acid:phosphorus ratio of 2.17 was obtained and only one spot was revealed by thin-layer chromatography. Ovolecithin of a high purity was obtained from Lipid Products, Epsom.

Enzyme preparation

Lyophilised cobra venom was purchased from Sigma Chemical Co., U.S.A. and the phospholipase was purified by the method of Braganca *et al.*[8], except that instead of precipitating the enzyme from the carboxymethyl-cellulose column fractions with $(NH_4)_2SO_4$ the fractions were lyophilised before pH adjustment, heat treatment and chromatography on Sephadex G-50. The product gave a single protein band on cellulose acetate electrophoresis, but 2 major components and 3 trace components were seen on acrylamide gel electrophoresis.

Other materials

Methanol was B.D.H. Analar Grade and was used undistilled. Hexane, light petroleum (80–100 °C) and diethyl ether were May and Baker reagent grade. Chloroform was distilled and methanol added to 2% (v/v). Free fatty acids were from Sigma Ltd (>99% pure). Crystallised bovine plasma albumin (Armour Pharmaceutical Co. Ltd) contained 0.006 μmole fatty acid/mg. Silicic acid (Mallinckrodt AR 100 mesh) was washed with 4 vol. methanol, 4 vol. chloroform–methanol (1:1, v/v), 2 vol. chloroform, and again with 2 vol. methanol and activated at 100 °C.

Reaction system for phospholipase A

The choice of a reaction system and assay method for phospholipase A_2 has always posed a problem[10,11]. Both for rate studies and for quantitative assay a sensitive system is needed, which permits short reaction times of 1–2 min, thereby enabling reaction rate measurements to be made which closely correspond to the initial velocity of the steady state. The simplest possible system is desirable so that the effect of various factors can be studied in the absence of detergents and similar compounds that have been used in the past. We have found that an assay based on gas–liquid chromatography is highly sensitive, does not apparently suffer from the lag referred to by Roholt and Schlamowitz[12], and also has the advantage that different fatty acids can be estimated in the presence of each other. It is therefore of particular use in studying fatty acid inhibition of this enzyme.

The majority of experiments were carried out using a suitable amount of substrate in 0.045 M glycylglycine–NaOH buffer, pH 7.3. All concentrations of substrate used were above the critical micelle concentration[13]. In order that all substrates should have been treated as far as possible identically before sonication a standard procedure was adopted, in which they all passed through a stage in which they were in solution in chloroform–hexane (2:1, v/v) and were then taken to dryness under N_2. The residue was sonicated at 2–3 mg/ml in water for 10 min at 30 °C using a Mullard Sonicator at maximum cavitation. The sonicated phospholipid suspension was then diluted with water as required for each run; in most cases 0.45 ml of this suspension was mixed with 0.45 ml 0.1 M glycylglycine–NaOH buffer (pH 7.3) in a stoppered tube, and the reaction was started by the addition of 0.1 ml enzyme (0.5 μg protein) in $CaCl_2$ solution of the concentration described for each experiment. Solutions were equilibrated at 30 °C before starting the reaction, and a shaking rate of 160 strokes/min was used.

Fatty acid estimation

The reaction was stopped by adding 4 ml chloroform–methanol (2:1, v/v) with vibration on a Vortex mixer. In later experiments the chloroform–methanol contained 0.5 mM disodium EDTA, and this reduced the slight discrepancy sometimes found between duplicates. 0.1 ml (21.6 μg) margaric acid (17:0) in light petroleum (b.p. 80–100 °C) was then added as an internal standard and the solutions mixed. The tubes were centrifuged at 5 °C to separate the layers, the upper aqueous layer was removed, the lower chloroform layer was taken to dryness under N_2 at 40 °C and redissolved in 2 ml of a solvent system containing chloroform–hexane–diethyl ether (2:1:1, by vol.)[14]. 400 mg silicic acid were added and mixed for 30 s on a Vortex mixer, the tubes centrifuged and the silicic acid given a second wash with 2 ml of the same solvent. Both washes were pooled and the solvent, containing the fatty acid, evaporated to small volume under N_2. Methylation was carried out using diazomethane, the methyl esters were taken to dryness, redissolved in a small volume of hexane and run on a Perkin Elmer F.11 gas chromatograph. The amounts of fatty acid could then be calculated by relating the area of the fatty acid peak to that of the internal standard.

RESULTS

Inhibition studies using oleic acid as inhibitor and dipalmitoyllecithin as substrate

Since Dawson's[7] original observation of the inhibition of an isolated *Naja naja* phospholipase A fraction by fatty acid, no further study of this effect has been reported. We therefore incubated the enzyme with varying amounts of dipalmitoyllecithin in the presence of several fixed amounts of oleate. We found that, although in the absence of inhibitor the expected plots were obtained, the plots of activity against substrate concentration in the presence of oleate were extremely complex (see Fig. 1).

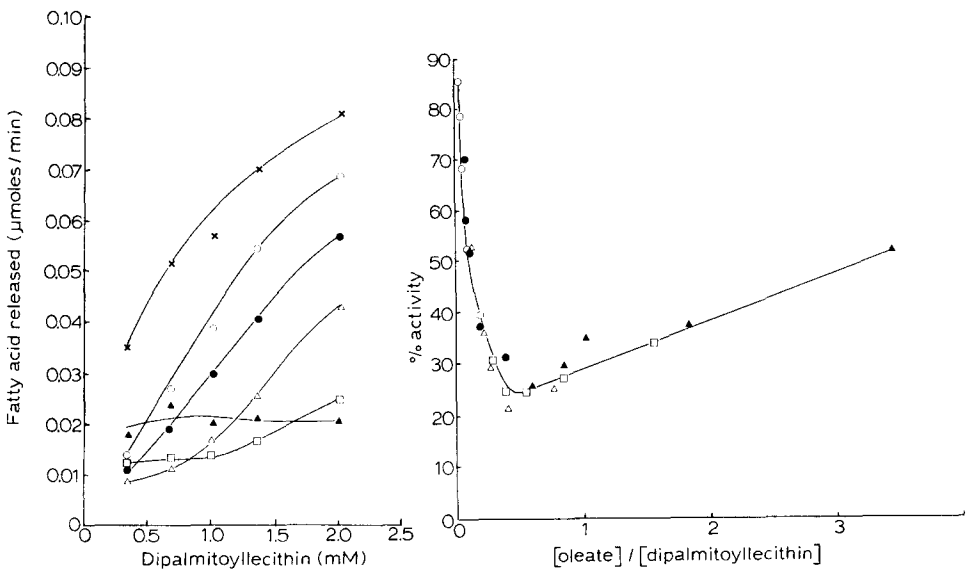

Fig. 1. Inhibition of hydrolysis of dipalmitoyllecithin by oleate. Plots of initial velocity (1 min reaction) against substrate concentration (mM). Oleic acid was sonicated for 20 min in 0.1 M glycylglycine–NaOH buffer (pH 7.3) to give the final oleate concentrations indicated below. 0.45 ml of the oleate suspension was mixed with 0.45 ml dipalmitoyllecithin suspension, sonicated for a further 1 min and equilibrated at 30 °C for about 30 min before the reaction was started. Ca^{2+} 10 mM final concentration. ×—×, no oleate; ○—○, 0.0625 mM oleate; ●—●, 0.125 mM oleate; △—△, 0.25 mM oleate; □—□, 0.55 mM oleate; ▲—▲, 1.15 mM oleate.

Fig. 2. Relationship between % activity and the ratio (on a molar basis) between oleate present in the reaction mixture and dipalmitoyllecithin. Symbols and conditions as in Fig. 1. % activity is calculated as described in the text.

As revealed in Fig. 1, non-linearity is much more pronounced using the higher concentrations of fatty acid, particularly so at low substrate concentrations. In an endeavour to clarify these effects plots were made of percent activity (defined as below) against the molar ratio of oleate to dipalmitoyllecithin (Fig. 2).

$$\% \text{ activity} = \frac{\text{Velocity for a given substrate concentration in the presence of inhibitor}}{\text{Velocity for the same substrate concentration in the absence of inhibitor}} \times 100$$

The points obtained from a wide range of substrate and inhibitor concentrations then all fall on a single discontinuous curve.

As the fatty acid concentration increases relative to the phospholipid concentration, inhibition increases until the ratio of fatty acid to phospholipid is about 0.4–0.6 (*i.e.* about 1:2), at which point there is a sharp inflexion in the curve. From this point, with increasing fatty acid:phospholipid ratios, the inhibition becomes progressively less. It is this which causes the more severe distortions in the plots in Fig. 1.

These observations clearly demonstrate the ability of the fatty acid product to inhibit the reaction at low concentrations although it is apparent that the inhibition is not of a simple type.

Differential action on dipalmitoyl-, dioleoyl- and dilinoleoyllecithin

As shown in Fig. 3, the activity at a substrate concentration of 0.68 mM was in the order dipalmitoyllecithin > dilinoleoyllecithin > dioleoyllecithin.

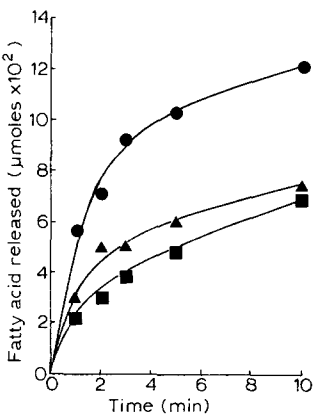

Fig. 3. Differential action on various synthetic substrates. 0.68 μmole of each synthetic lecithin was incubated in the standard reaction mixture for the times indicated. Ca^{2+} 0.5 mM final concentration. ●—●, dipalmitoyllecithin; ■—■, dioleoyllecithin; ▲—▲, dilinoleoyllecithin.

The rate curve shows a rate decreasing rapidly with time, and becoming minimal after 10 min, at which stage less than 20% of the substrate has been hydrolysed. The curves are similar to those reported by Dawson[15] for sonicated ovolecithin substrates, although in our hands the hydrolysis slowed more rapidly than that reported by him, possibly due to differences in enzyme preparation, buffer or substrate. This falling off in rate has been observed with other phospholipase A_2 enzymes[16], and it has been suggested that it is due to changes brought about in the configuration of the substrate as a result of the changed composition of the micelles.

Inhibition of the hydrolysis of three synthetic lecithins by different fatty acids

Our observations on the importance of the fatty acid product as an inhibitor suggested further the possibility that the differences in fall-off observed in the rate curves obtained with different substrates might be explained if the fatty acid released as product by the lecithin giving a lower rate were more inhibitory than that released by the lecithin giving a higher rate. The inhibitory effect of a number of different fatty

acids was therefore tested on each lecithin. It was found that with dipalmitoyllecithin as substrate the order of the inhibition of equimolar concentrations of different acids was linoleic > oleic > stearic (which did not inhibit); with dioleoyllecithin, palmitic = stearic, which is slightly greater than linoleic; with dilinoleoyllecithin, palmitic = stearic = oleic. The fatty acids were not, therefore, found to inhibit in an order related to the rates of hydrolysis of the synthetic lecithins.

These observations suggest that the different rates observed with the different synthetic lecithins are a true reflection of the enzyme action on the substrate rather than attributable to the inhibitory effects of the different products.

Effect of albumin

As shown in Fig. 4, albumin at a concentration of 5 μg/ml of reaction mixture gave a pronounced activation with dipalmitoyllecithin as substrate. Even in the presence of albumin, however, the rate falls off markedly in a few minutes even though only 20–30% of the substrate has been hydrolysed (Fig. 5).

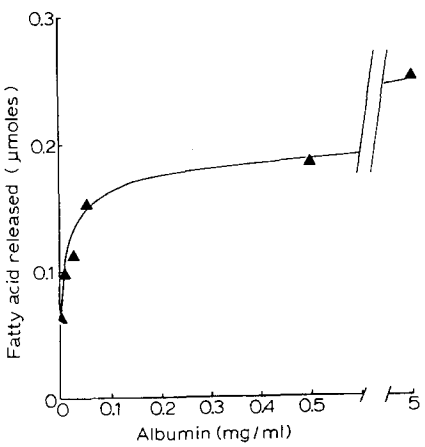

Fig. 4. Activation by bovine plasma albumin of enzymic hydrolysis of dipalmitoyllecithin. Albumin was dissolved to the appropriate concentration in 0.1 M glycylglycine–NaOH buffer (pH 7.3) and the reaction then carried out as described under Methods using 0.68 μmole dipalmitoyllecithin and a final Ca^{2+} concentration of 10 mM. Reaction time 2 min.

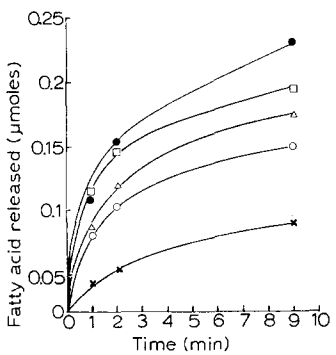

Fig. 5. Time course of activation by bovine plasma albumin of enzymic hydrolysis of dipalmitoyllecithin. Albumin was dissolved to the appropriate concentration in 0.1 M glycylglycine–NaOH buffer (pH 7.3) and the reaction then carried out as described under Materials and Methods using 0.68 μmole dipalmitoyllecithin and a final Ca^{2+} concentration of 10 mM. ×—×, no albumin; ○—○, 5 μg/ml albumin; △—△, 10 μg/ml albumin, □—□, 25 μg/ml albumin; ●—●, 50 μg/ml albumin.

Condrea *et al.*[5] had earlier reported that albumin did not activate phospholipase A when acting on purified ovolecithin. Although it is not clear that this finding related to *Naja naja* venom the possibility of a discrepancy between their results and ours merited investigation. In their experiments Condrea *et al.*[5] studied the albumin action in a reaction mixture containing 10% ether. When we incubated our enzyme preparation under the standard conditions used in the present work, using either ovolecithin or dipalmitoyllecithin, we found that the presence of 10% ether

abolished the activating effect of the albumin (see Table I). A similar abolition of the activating effect of albumin was given by the presence of 10% ether in collidine buffer. The activating effect of ether had earlier been shown to be much less marked using sonicated substrates[15], and in the present work although activation, in the presence of ether, of ovolecithin hydrolysis in glycylglycine buffer was observed, no activation was seen using dipalmitoyllecithin in glycylglycine buffer, or ovolecithin in collidine buffer.

TABLE I

EFFECT OF ETHER ON ALBUMIN ACTIVATION

The incubation mixture contained the additions shown in either 0.045 M 2,3,6-collidine–HCl buffer, pH 7.3, or 0.045 M glycylglycine–NaOH buffer, pH 7.3, containing 0.68 μmole substrate and 10 mM Ca^{2+}. The fatty acid released during 5 min incubation was measured.

Additions	*Fatty acid released (μmoles)*		
	Ovolecithin substrate in collidine buffer	*Ovolecithin substrate in glycylglycine buffer*	*Dipalmitoyllecithin substrate in glycylglycine buffer*
None	0.082	0.080	0.080
0.1 ml ether	0.077	0.157	0.095
2 mg albumin	0.140	0.174	0.322
0.1 ml ether *plus* 2 mg albumin	0.098	0.141	0.100

TABLE II

EFFECT OF SIMULTANEOUS PRESENCE OF ALBUMIN AND OLEATE

The reaction mixture contained the above additions to 0.45 M glycylglycine–NaOH buffer (pH 7.3), 10 mM Ca^{2+}, enzyme and 0.68 μmole dipalmitoyllecithin. The fatty acid released from the substrate was measured at 2 min.

Additions	*Fatty acid (16:0) released (μmoles)*
None	0.053
2 mg albumin	0.191
0.1 mg albumin	0.154
0.1 μmole oleate	0.036
0.4 μmole oleate	0.019
2 mg albumin *plus* 0.1 μmole oleate	0.172
0.1 mg albumin *plus* 0.4 μmole oleate	0.018

When albumin and added fatty acid were both present in the reaction medium, the results shown in Table II were obtained. With 2 mg albumin and 0.1 μmole oleate, the activating effect of the albumin was almost the same as that seen with 2 mg albumin alone. If only 0.1 mg albumin were present with 0.4 μmole oleate, the effect was the same as with the fatty acid alone. The effects of the albumin and fatty acid are mutually opposed and the results show that if the appropriate compound is present in excess it will nullify the effect of the other.

pH optimum

Salach *et al.*[10] and Dawson[7] obtained somewhat different types of optimum pH

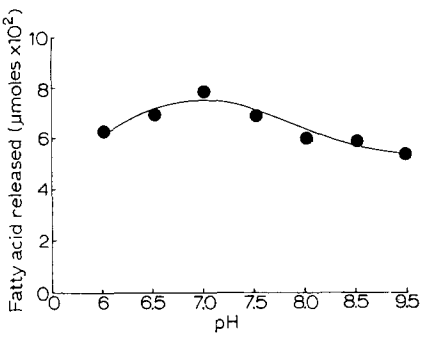

Fig. 6. pH–activity curve. 0.68 μmole dipalmitoyllecithin was incubated for 2 min in 1 ml 0.045 M glycylglycine–NaOH buffer adjusted to appropriate pH, containing 0.6 μg enzyme protein and a final Ca^{2+} concentration of 2 mM.

curves for their enzyme preparations. Salach *et al.*[10] find for all of their iso-enzymes a pronounced bell-shaped curve, whilst Dawson[7] found for his preparation a rather flat curve over a relatively wide pH range. It therefore seemed of interest to examine the pH–activity curve of our preparation, and the result is shown in Fig. 6. It shows a curve resembling that obtained by Dawson.

DISCUSSION

Roholt and Schlamowitz[12], and more recently De Haas *et al.*[17] and Wells[18], have commented on the problems encountered when phospholipids in the micellar form are used as the substrate in kinetic studies. For this reason, water-soluble lecithins have been used in kinetic studies of phospholipase A_2[12,17,18]. It is true that where the aim of the work is to establish rate constants, or to investigate the sequence of events at the active site, it is desirable to avoid the complications introduced by the use of phospholipid aggregates. Nevertheless, the substrates of these enzymes in the cellular environment are phospholipids built into highly complex aggregates of lipid and protein, and ultimately studies of the action of this type of enzyme must be aimed at explaining its action on substrates in their natural surroundings.

Our results here confirm that, using long chain fatty acids and lecithins containing long chain acids, inhibition of the *Naja naja* phospholipase A_2 is obtained at very low fatty acid concentrations. Attempts to analyse this inhibition kinetically are frustrated by the theoretical problems encountered when using the aggregates which such substrates form in aqueous solution. It is evident however that the plots shown in Fig. 1 will not give linear reciprocal plots. Despite the theoretical complications of a kinetic analysis of these lamellar structures, it seems likely that if the inhibition were of a simple competitive type, linear reciprocal plots meeting on the ordinate would be obtained. (It may be pointed out that linear reciprocal plots are obtained with all the substrates in the absence of inhibitor, and also in the presence of albumin.) The indications are, therefore, that the inhibition is of a complex type, and the form of the curve shown in Fig. 2 strongly suggests that inhibition partly results from an interaction of the fatty acid with the substrate micelles.

This conclusion is in accord with that of Dawson[7], which was based on studies of the electrophoretic mobility of the substrate micelles, and agrees also with the

general view that the activity of phospholipase A_2 is highly sensitive to micellar change. However, this further evidence gives added support to the conclusions based on physicochemical experiments.

The inflexion (Fig. 2) which occurs at a ratio of oleate: dipalmitoyllecithin of about 0.4–0.6 may indicate that at this point a transition occurs in the micellar arrangement. Dawson[7] has speculated that fatty acid orientated at the lipid–water interface will have a more adverse effect on enzyme action than fatty acid contained within the micelle. It may be that as the fatty acid content of the micelle increases and reaches the point at which the inflexion occurs a relatively greater proportion of the fatty acid now accumulates in the centre of the micelles, with proportionally more phospholipid on the outside.

The possibility that some inhibition occurs through interaction of the fatty acid with the enzyme protein is not excluded by the present results. The finding that the order in which different fatty acids inhibit the enzyme varies with the substrate used argues somewhat against this, but in no way discounts it. It is possible that the fatty acid binds to the enzyme protein (not necessarily at the active site) and thereby inhibits it. Indeed, we have some evidence (S. Gul, unpublished) that the enzyme does bind to precipitates of fatty acid soaps, and is lost from the solution with these precipitates on filtration. It is possible that direct binding studies between fatty acid and enzyme in solution might provide useful information on this point.

The rate observed with dipalmitoyllecithin is markedly greater than that observed for the other synthetic substrates, dioleoyllecithin and dilinoleoyllecithin, or for ovolecithin, when a substrate concentration of 0.68 mM is used; for the dioleoyllecithin this is also true at all concentrations of substrate. The suggestion has been made[19,20] that in the case of *Crotalus adamanteus* venom differences in the rates observed between synthetic lecithins can be explained on the basis of differences in micellar shape, and especially that dioleoyllecithin gives a spheroidal micelle which is not favourable to enzyme action. It is possible that similar considerations may apply in the case of the present experiments. Saunders[21] has reported that dipalmitoyllecithin is less readily dispersed by sonication at room temperature than lecithins containing unsaturated fatty acids. At 30 °C we have obtained satisfactory dispersions of dipalmitoyllecithin. It may well be that the forms of the micelles obtained in these dispersions of dipalmitoyllecithin are of the ellipsoid, or elongated shape, thought to favour phospholipase action.

In agreement with the view that snake venom contains only phospholipases releasing fatty acids from the 2-position of lecithin substrates[22], it was found that the preparation under investigation here produced only unsaturated fatty acids from ovolecithin.

The activation by albumin could be due (a) to the binding of the fatty acid by the albumin (thus preventing the fatty acid from penetrating the lecithin micelles); (b) to penetration of the lecithin micelles by albumin in a way which renders them more susceptible to enzyme attack or (c) to an effect of the albumin directly on the enzyme protein.

The first of these possibilities appears the most probable, and this view is supported by the results in Table II in which it is shown that when the ratio of fatty acid to albumin is low, the albumin prevents the inhibition by fatty acid, but when the amount of fatty acid relative to the albumin is increased, inhibition by fatty acid is

seen. A point of difficulty in accepting this as the whole explanation of the activating effect of albumin is that at very low concentrations of albumin, the amount of albumin present would appear to be too small to bind the required amount of fatty acid. Albumin binds only 9 moles of oleic acid per mole[23] with an affinity constant lower than $1.66 \cdot 10^{-4}$, but may bind up to 63 moles with a somewhat higher affinity constant[24,25]. Even if one takes the highest suggested figure, only 6.3 nmoles of oleate would be bound at the lowest albumin concentration. The enzyme rate does not appear to slow pronouncedly until about 50 nmoles fatty acid product have been released.

If the albumin does not activate by binding fatty acid it may do so by penetrating the lecithin micelles to render them in some way more susceptible to enzyme attack. Colacicco[26], and Quinn and Dawson[27], have provided evidence that albumin will penetrate ovolecithin monolayers, and a claim has been made that 7–15 molecules of lecithin interact with one ovalbumin molecule as measured by the technique of equilibrium dialysis[28]. It therefore seems possible that the albumin could activate the enzyme in this way, but it should be pointed out that at the lowest albumin values the ratio of albumin to lecithin, on a molar basis, is 1:7000. The third possibility, that activation is due to an effect of the albumin directly on the enzyme protein, is purely conjectural, but is put forward in view of the objections to the other two hypotheses.

We conclude, therefore, that albumin can relieve the fatty acid inhibition of this phospholipase A_2 when it is present in sufficiently high amounts, but that the activation seen at low albumin concentrations cannot be due to this eflect, and may be explained on the grounds of an interaction with the enzyme protein. The present experiments suggest that the more rapid rate observed with lipoprotein substrates may not be due to an effect caused by the apoprotein in relieving inhibition by fatty acid product, at least in any simple way, but the apoprotein could possibly maintain the lipid substrates in a conformation favourable to the phospholipase even after extensive hydrolysis. Camejo[29] showed that even after the hydrolysis by *Naja naja* venom of essentially all the phosphatidylcholine and phosphatidylethanolamine present in human high density lipoprotein, the lipoprotein particles showed the same ultracentrifugal properties as untreated lipoprotein, and the conclusion was drawn that the size and shape of the high density lipoprotein remained unchanged by treatment with this phospholipase.

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