Activation and regulation systems for venom phospholipases A_2.

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A thesis submitted to the University of Glasgow

for the degree of Doctor of Philosophy.

September, 1984

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Acknowledgements

I would like to thank Professor A.S.G. Curtis for advice and providing the facilities within the department; Mr.A.W. M^{ac} Leod for excellent technical assistance; Dr. G.R. Moores for many helpful discussions; Mr. A. Hart for introducing me to photographic processing; Dr. S.L. Ullmann for the kind gift of honey bees; The Department of Agricultural Chemistry for help with gas-liquid chromatography; the Science and Engineering Research Council for funding this work with a research studentship and to all members of the academic and technical staff of the Departmemt of Cell Biology for their friendship and help in many ways.

I am indebted to my parents and family for their unfailing support throughout my academic training.

Finally my deepest thanks go to my supervisor Dr.A.J. Lawrence for his encouragement, guidance and willingness to help at all times both during the course of this thesis and in my undergraduate years.

To Jackie

Abbreviations

cAMP	:	Cyclic adenosine mono phosphate
^{14}C	:	The radioactive isotope of carbon
DFP	:	Diisopropylfluorophosphate
DTT	:	Dithiothreitol
EDTA	:	~~Phenyl-methyl-sulphonyl-fluoride~~
GPC	:	Glycerophosphoryl choline
^{3}H	:	Tritium
HEPES	:	N-2-hydroxyethylpiperazine-N'-2-ethane
^{125}I	:	The radioactive isotope of iodine
LYSO PC	:	Lysophosphatidyl choline
^{32}P	:	The radioactive isotope of phosphorous
PBPB	:	Para-bromc phenacyl bromide
PC	:	Phosphatidylcholine
PE	:	Phosphatidyl ethanolamine
PLA_2	:	Phospholipase A_2
PMSF	:	Phenyl-methyl-sulphonyl-fluoride
SDS-PAGE	:	Sodium dodecyl Sulphate polyacrylamide gel electrophoresis
TAME	:	Na-p-tosyl-L-arginine-methyl ester
TEMED	:	N,N,N,'N,'-tetramethylethylenediamine
TLC	:	Thin-layer chromatography
TRIS	:	Tris (hydroxymethyl) aminomethane

Summary

This thesis was in part a continuation of earlier studies of PLA_2 activation by fatty acids and imidazolides (Drainas, Ph.D., 1978) and on the regulation of the enzyme by reaction products and albumin (Camero, M.Sc., 1983). It also involved an investigation into the activation of PLA_2 from another source and the consequences of activation in terms of protein modification. Gel electrophoresis and fluorographic techniques confirmed that oleoyl imidazolide covalently binds to PLA_2. The activation phenomena found in a chemically defined assay system were confirmed and shown to apply when the enzyme attacked a biological membrane substrate. Both bee and wasp venom PLA_2 could be activated by oleoyl imidazolide and fatty acids, and were strongly inhibited by lysolecithin. The role of reaction products and albumin on the modulation of PLA_2 is discussed.

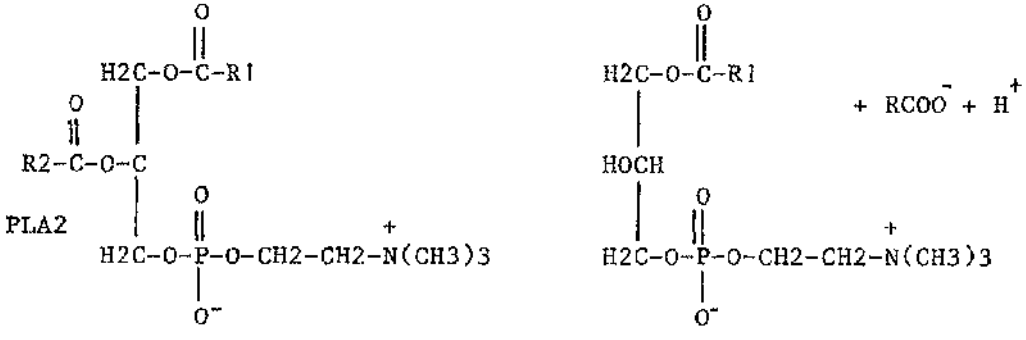
Studies on the chemistry of acyl imidazolide activation suggested that PLA_2 requires one molecule of oleoyl imidazolide for almost complete activation and that this activation is controlled by a group with a pK of about 6.5. Activated PLA_2 was protected against inactivation by thiols and trypsin, suggesting that activation of the enzyme is associated with a a conformational change in the protein. Chemical modification studies revealed that only activated PLA_2 was protected against the effects of PBPB and the implications of this are discussed.

CHAPTER ONE

INTRODUCTION

Phospholipase A_2

The enzyme phospholipase A_2 (E.C.3.1.1.4.) belongs to a specific group of esterases, the lipolytic enzymes. The enzyme catalyses the release of fatty acids esterified to the number two position of 1,2-diacyl-sn-glycerphospholipids.



Site of attack of PLA_2 on phosphatidyl choline

Phospholipases A_2 are interesting because they are involved in the control of several membrane associated processes. They release arachidonic acid, a precursor for prostaglandin synthesis, from phospholipids (Su-Chen & Levine, 1976), form part of a cyclic system for the deacylation and reacylation of phospholipids in membranes (Van den Bosch, 1980) and may also be important in membrane fusion (Lucy, 1970) and cell adhesion (Curtis et al, 1975).

Phospholipase A_2 has been isolated from a large number of snake venoms (Tu, 1977). Molecular weights of 9,000-15,000 and dimers up to 30,000 have been reported. These enzymes are not

immunologically identical and they have a variable optimum pH range but it is usually in the range 7.7-8.3.

The best characterized mammalian phospholipase A_2 is that of porcine pancreas. De Haas et al, (1968) have shown that it has a molecular weight of 14,000 and have published the primary sequence (De Haas et al, 1970). It contains 123 amino acids with six disulphide bridges and is very heat stable.

The phospholipase A_2 of bee venom is highly basic with a pI of 10.5±1. Its primary sequence, carbohydrate content and position of disulphide bridges has been determined by Shipolini et al (1971, 1974a & 1974b). The enzyme requires calcium ions for activity and is totally inhibited by excess EDTA. It has a molecular weight of 15,800, contains four disulphide bridges, has a carbohydrate group linked to the number thirteen asparagine residue and exists as a dimer in concentrated solution at high pH.

The composition of bee venom

Bee venom contains several pharmacologically and enzymatically active components, the major constituents being melittin, phospholipase A_2, mast cell degranulating (m.c.d.) peptide and hyaluronidase.The lytic peptide melittin (50% dry weight of venom) is the major component of the common European honey bee Apis mellifera (Habermann, 1972). It contains twenty six amino acids, has important biological actions for example it stimulates the action of PLA_2 (Habermann & Kowallek, 1970; Sessa et al, 1969; Rothschild, 1965) and interacts with

phosphatidylcholine to form a tight complex that can be isolated by sephadex chromatography (Mollay & Kreil, 1973). Apamin, (2% dry weight) is a neurotoxin and was first isolated by Habermann & Reiz (1965). A third major peptide, M.C.D. peptide (2% dry weight) is highly effective in degranulating mast cells (Fredholm, 1966). Other peptides include secapin (1%), tertiapin (<1%) and minimine (Tu, 1977). Bee venom also contains numerous other non-peptide components such as histamine, free amino acids, carbohydrates, lipids and other biogenic amines (Tu, 1977). Although it does not contain as many enzymes as snake venoms, it is a rich source of hyaluronidase (Franklin & Baer, 1975), esterase and acid and alkaline phosphatase (Benton, 1967) and the enzyme phospholipase A_2 (12% dry weight).

It is probable that the venom components of stinging insects like bees and wasps act synergistically to produce the pain and inflammation usually seen at the site of the sting. Melittin has noticeable effects on the activity of phospholipase A_2. Vogt *et al*, (1970) reported that melittin and phospholipase A_2 showed a marked synergism of activity when acting on cell membranes. Melittin also stimulated the hydrolysis of egg lecithin by venom phospholipase A_2 (Mollay & Kreil, 1974). The direct lytic factor from *Naja naja* venom and bacterial toxin polymyxin B can replace melittin in this respect.

Recently, it has been shown that this property of melittin is shared by the mastoparans, tetradecapeptides discovered in wasp venom and named after their ability to degranulate peritoneal mast cells (Argiolas & Pisano, 1983). Mastoporans

stimulated the hydrolysis of egg lecithin by bee venom phospholipase A_2 in a dose dependent manner. Melittin was shown to stimulate only tissue and bee phospholipase whereas mastoparans stimulated bee, rattlesnake, porcine pancreatic and tissue phospholipase A_2, but melittin was a much stronger haemolytic agent than the mastoparans. Mastoparans contain fourteen amino acids and are structurally unrelated to melittin. They bind to liposomes with a mechanism different from melittin (Higushijima et al, 1983) and thus it is interesting that both peptides exhibit similar behaviour.

Purification of phospholipases

Phospholipase A_2 is an ubiquitous enzyme and has been identified in the venoms of bees, wasps, snakes and scorpions (Tu, 1977). In mammals the enzyme occurs in highest amounts in pancreatic secretions (Nieuwenhuizen, 1974). It has been found in almost every cell that has been investigated, both prokaryotic (Albright et al, 1973) and eukaryotic (Van Den Bosch, 1973). Purification of an enzyme normally requires an assay with adequate sensitivity unless a highly specific affinity method is employed. Identification of PLA_2 in non-specialised tissues requires a much more sensitive assay method than the one needed for venom and pancreatic enzymes. Removal of small molecular weight peptides from venoms can be achieved by dialysis or molecular sieving and there is no need for extensive extraction

or homogenisation procedures required with membrane phospholipases.

The majority of purification methods for phospholipase A_2 employ a combination of gel filtration followed by ion exchange columns which allow separation of isoenzymes. Pancreatic tissue is a rich sourch of this enzyme which occurs as an enzymatically inactive precursor. The active enzyme is formed by the action of trypsin on the zymogen molecule and has an important digestive function. For the pancreatic (pro)phospholipases the purification includes homogenisation of the tissue, heat treatment at low pH, precipitation by ammonium sulphate and then chromatography on both D.E.A.E. and C.M. cellulose (Nieuwenhuizen et al, 1974 & Evenberg et al , 1977). The pro-enzyme is relatively sensitive to proteolysis and activation to phospholipase or even proteolytic breakdown of the enzyme may occur during purification (De Haas et al, 1968) unless high concentrations of DFP are used. Phospholipase A has also been purified from human panreatic juice by a two step process (Grataroli et al, 1981). The active enzyme was isolated by low pH and heat treatment followed by absorption on Octyl-Sepharose. The zymogen was purified by gel filtration, low pH and heat treatment and a salt precipitation followed by filtration on Sephadex G-25 and then chromatography on CM-Sepharose.

Phospholipase A_2 has been purified from bee venom by Shipolini et al, (1971) by five main steps which involved force dialysis of the lyophilized venom to remove small proteins and further purification on Sephadex G-50 and SE-Sephadex G-25. The

enzyme was reported to run as a single component on both starch and polyacrylamide gels although, as in many reports, no photographs of the gels were shown.

The elution patterns of phospholipases from venoms usually contain more peaks than those observed with pancreatic enzymes. This could be explained by the presence of isoenzymes or the aggregation of the enzyme leading to different molecular weight values. Both phenomena occur with most venoms and only a few exceptions, for example with Crotalus atrox are known. The enzyme might combine with a non enzymatic component in the venom (complexes have been found in several venoms (Fohlman et al, 1979)) or changes in the charge of the phospholipase may occur by desamidation and/or proteolytic breakdown. Evidence for desamidation has been presented for the venom of Vipera palestinae (Shiloah et al, 1973) where the appearance of two separate enzyme fractions could have been due to spontaneous hydrolysis (during the purification procedure) of a side chain amide group of glutamine or asparagine in about half of the enzyme molecules.

In addition to these aspecific methods, a number of more specific methods have been described. Louw & Carlsson, (1979) used hydrophobic chromatography on Phenyl Sepharose CL-4B in the preparation of phospholipase A_2 -free cardiotoxin from the venom of Naja mossambica mossambica. A simple and high yield purification of Crotalus adamanteus phospholipase A_2 by Wells, (1975) involved the precipitation of the bulk of the venom proteins with 50% isopropanol, precipitation of the enzymes from the isopropanol soluble material with neodynium chloride and

final purification on D.E.A.E. cellulose, resulting in an 80% yield of the pure enzyme.

One of the first attempts to purify phospholipase A_2 by affinity chromatography was reported by Rock and Snyder, (1975). They used alkyl ether analogs of ethanolamine and choline phospholipids which contain a carboxylate group at the end of the acyl chain thus permitting direct covalent binding to AH-Sepharose 4B. Only the enzyme calcium complex bound to the column and this was eluted with EDTA. This one step process resulted in >90% yield. Tahir & Hider (1983) have synthesised an alkyl ether analog of PC 1-(12-aminododecanoyl)2-hexadecyl-sn-glycero-3-phosoholcholine and coupled it to an affinity gel, and using this obtained a high yield purification of bovine smooth muscle PLA_2. Antibody affinity chromatography has been used by several workers including Delori & Tessier, (1980) who raised antibodies against phospholipase and made an affinity column for PLA_2 by coupling the antibody to activated Sepharose.

Finally, Gritsuk et al, (1979) have made use of the fact that bee venom phospholipase A_2 contains carbohydrate and have purified the enzyme on Concanavalin-A Sepharose 4B.

Assays for phospholipase A_2

The importance of phospholipases in lipid metabolism underscores the need for rapid and simple methods for measuring their activity. These enzymes have a broad substrate specificity and their reactivity depends on the physico-chemical properties of the aggregated phospholipids in aqueous solution. Many applications, advantages and drawbacks of methods used to determine phospholipase A_2 activity have been discussed in the

literature. Enzymatic activity can be measured by the appearance of products or the disappearance of substrate. Since under kinetic conditions, only a small proportion of the substrate will be consumed, the determination of the remaining substrate is only rarely used.

Assays for phospholipase A_2 activity are usually one of two types. The first is based on stopped reaction methods. Smith et al , (1972) have used gas-liquid chromatography to analyse reaction products. This has the advantage that it can differentiate between different fatty acids. Thin layer chromatography of reaction products (Wightman et al,(1981) is another quantitative but time consuming method. Cosentino & Ellis, (1981) used a radiochemical method in which aliquots of homogenised tissue were applied directly to columns of silica gel absorbent. Metabolites and substrates were eluted from the columns and each could be separated and quantified. The use of ^{31}P nuclear magnetic resonance spectroscopy to study hydrolysis was introduced by Henderson et al , (1975). This method was based on the difference in chemical shifts of phosphatidyl choline and lysophosphatidyl choline. It has been used to analyse simultaneously the hydrolysis of individual phosholipid species in phospholipid mixtures (Roberts et al, (1979).

Egg yolk lecithin incorporated into agar or agarose gels has been used to assay phospholipase A_2 activity (Habermann & Hardt, (1972). This method detects about 10pg of bee venom phospholipase A2 and about 50ng of pancreatic and Crotalus enzyme. Specificity can be further improved by incorporation of human erythrocytes and observing clearing of the agar gel. One possible limitation

of this method is that the ability of a given phospholipase to diffuse into the gel may vary and affect comparison between two enzymes. Marinetti, (1965) measured the change in absorbancy of egg yolk suspensions. The disadvantage of this method is that the clearing of an egg yolk suspension can be affected by factors such as pH and the presence of certain metal ions. Shier & Trotter, (1978) produced a modification of the method of Habermann but used acrylamide gels. Lecithin liposomes were trapped in the gel matrix and enzyme activity detected by clearing of the gel. Sensitivity of the method was greatly increased by staining the lipids in the gel with rhodamine 6-G.

A haemolytic method has been described by Vogel et al, (1981) which uses cardiotoxin to stimulate phospholipase A_2 against guinea pig erythrocytes. Cell leakage was measured spectrophotometrically.

Continuous methods for assaying phospholipase have the advantage that they have higher resolution for detection of rates often missed by stopped reaction methods.

One of the most widely used methods for assaying phospholipase A_2 uses the continuous titration of fatty acids in a pH stat. Both purified lecithin and whole egg yolk have been used either without or in the presence of detergents. On addition of the enzyme to the substrate, the fatty acid released causes a drop in pH that is automatically corrected by the addition of the alkaline titrant (Cottrell, (1981). One of the disadvantages of the method is that it cannot respond effectively to sudden large increases in rates which are common with PLA_2 catalysed

reactions. Thus this method would be most useful when substrate hydrolysis rates were known to be changing relatively slowly.

The bacterium Beneckea harvey emits light when either exogenous aldehydes or myristic acid is added to it. Ulitzur & Heller, (1978) have used this property as the basis of their assay. Although this is a sensitive and rapid method, bacteria cannot be used in a continuous assay where extremes in pH and temperature, or solvents are used.

A specific quantitative assay which measures enzyme mediated phospholipid hydrolysis as lipoxidase catalysed incorporation of oxygen into the released unsaturated fatty acids has been described by Gale & Egan, (1980). Released fatty acids containing the cis,cis-1,4-pentadiene system are subsequently oxidised to hydroperoxy acids. This method can detect about 20ng/ml of Naja naja venom phospholipase A_2.

$$\text{PHOSPHOLIPID} + PLA_2 \longrightarrow \text{FATTY ACID}$$

$$\text{FATTY ACID} + O_2 \xrightarrow{\text{lipoxydase}} \text{HYDROPEROXY FATTY ACID}$$

Hendrickson & Rauk, (1981) have developed a fluorimetric assay using an excimer emitting pyrene- labelled lecithin which gives monomer fluorescence when hydrolysed. The assay is sensitive to about 10ng of pancreatic phospholipase A_2 although it and the method of Gale and Egan, (1980) have the disadvantage that they use a synthetic substrate.

Aarsman et al, (1976) developed a sensitive continuous spectrophotometric assay for phospholipase A_2 and

lysophospholipase using thioester substrates in which the fatty acid was linked in thioester linkage linkage rather than in oxyester linkage to the backbone of the substrate molecule. During hydrolysis, thiol groups are released which can be detected spectrophotometrically using Ellmann's reagent.

Finally, phospholipase A_2 activity has been followed by conductimetric methods developed by Lawrence, (1971, 1972 & 1974). The apparatus consists of 6x2 ml reaction cells each with a magnetic stirrer and maintained at constant temperature by a thermostatically controlled water bath. Full-scale pen deflection can be obtained for a conductance change of about 4%. The assay is based on the fact that hydrolysis of an ester by phospholipase A yields a fatty acid anion and a proton. The conductance change depends on the fate of the proton as it is absorbed by the buffer with one of two results:

If the proton is absorbed by a cationic buffer e.g. triethanolamine the result is :

$$\text{LECITHIN} + \text{PLA}_2 \longrightarrow \text{RCOO}^- + \text{H}^+ + \text{LYSOLECITHIN}$$

$$\underset{\text{(triethanolamine)}}{(\text{CH}_2\text{CH}_2\text{OH})_3\text{N}} + \text{RCOO}^- + \text{LYSOLECITHIN} \longrightarrow (\text{CH}_2\text{CH}_2\text{OH})_3\text{NH}^+ + \underset{\text{LYSOLECITHIN}}{\text{RCOO}^-} +$$

Thus there are two new ions added to the solution and there is an increase in the conductance of the solution. Long chain fatty acids (C >12) interact with calcium and this can be overcome by using 20% n-propanol in the buffer. If the proton is absorbed by an anionic buffer, for example bicine, the result is :

$$(CH_2CH_2OH)_2NCH_2COO^- + RCOOH \longrightarrow (CH_2CH_2OH)_2NCH_2COOH + RCOO^-$$
(bicine)

and thus there is no change in conductance during the assay. The aqueous-buffer could be used to follow hydrolysis of long acyl chain lecithins (R>C12) by recording the decrease in conductance when long chain acids interact with excess calcium. The small volume of the cells means that they reach a stable temperature very quickly and events which occur within ten seconds can be recorded accurately. The multi-channel system allows a large number of assays to be carried out in a very short time and hence is extremely useful during purification of the enzyme where a large number of fractions have to be assayed. The assays are performed in low (usually 10-20mM) salt solutions, as higher salt concentrations result in reduced sensitivity. The hydrolysis curves of many substrates by phospholipase A_2 are not linear and this assay method has the advantage of being able to follow, sudden changes in the rate of reaction. A large range of substrates can be used with this method including synthetic substrates and intact membranes.

An alternative assay for PLA_2 involves conductimetric determination of electrolyte release from erythrocytes (Lawrence et al, 1974 & Lawrence, 1975). It has the advantage over lysis assays that ion release from cells is an early event which occurs at low enzyme concentrations where lysis may not take place. It has the disadvantage that it is not possible to make an explicit relationship between ion leakage and enzyme attack.

Regulatory aspects of Phospholipase A_2

(a) Regulation of Phospholipase activity by zymogen-active enzyme conversion.

The basis of this model comes from the work of De Haas et al, (1968) on the inactive zymogen of pancreatic phospholipase A_2. Active enzyme is formed by removal of a heptapeptide from the N-terminus of the polypeptide chain. It has also been reported that there was factor in crude trypsin that could activate rat plasma phospholipase A (Duchesne et al, (1972) and the phospholipase A_2 in human erythrocte ghosts and lysates (Paysant et al, 1970) but when pure trypsin was used on red cell lysates, the activation of the enzyme could not be repeated (Zwaal et al, (1974).

The activation of platelet phospholipase A_2 has been shown by Feinstein et al, (1974) to be inhibited by the serine protease inhibitor phenylmethanesulphonyl fluoride, however if exogenous arachidonic acid was added, the formation of malondiaaldehyde, which is dependent on the activation of phospholipase A_2 to supply free arachidonic acid, was not inhibited. This suggested the involvement of a serine-protease in phospholipase A_2 activation. EGTA augmented the effect of thrombin suggesting that the active site of the enzyme is located in the inner membrane although it is still possible that part of a phospholipase could be reached by a proteolytic enzyme on the outside. This irreversible zymogen activation would seem plausible for digestive enzymes but it is difficult to imagine this as a

mechanism of control for membrane associated phospholipases A_2.

(b) Interaction with regulatory proteins

The work of Gryglewski et al, (1975) and Hong and Levine, (1976) first showed that the main effect of glucocorticoids on eicosanoid metabolism in responsive tissues was to lower the availability of free arachidonate. This has been confirmed in many papers and is now generally accepted (Blackwell et al, (1980). The effect was shown to require RNA and protein synthesis and it seemed that the steroid was probably acting through its cytosolic receptor.

Flower & Blackwell, (1979) have shown that corticosteroids induce a 15,000 dalton protein (macrocortin) in macrophages, that can inhibit phospholipase A_2 activity and arachidonate release. Later, Hirata et al , (1980) showed that when rabbit peritoneal neutrophils were treated with flucinolene acetonide (a synthetic glucocorticoid), their chemotactic response to fmet-leu phe was reduced to less than half of the control level and the chemoattractant-stimulated release of arachidonic acid from the steroid-treated cells was one third that of untreated cells. The inhibitory effect was reduced by antinomycin D, an inhibitor of RNA synthesis and cyclohexamide, an inhibitor of protein synthesis. They isolated a 40,000 molecular weight glycoprotein (lipomodulin) from cell extracts. Its synthesis was induced by the addition of glucocorticoids to the neutrophils and the isolated protein was shown to inhibit phospholipase A_2.

In macrophages, corticosteroids induced a protein synthesis

dependent release of macrocortin (Blackwell et al, 1980) & (Carnuccio et al, 1981). Neutrophil lysates posessed high PLA_2 inhibitory activity suggesting that at least some macrocortin was stored in the cells. After release of macrocortin the macrophages could no longer respond to dexamethasone until a further phase of synthesis had occured, however they were responsive to added macrocortin.

Hirata, (1981) has investigated regulation of phospholipid metabolism by lipomodulin in vivo and in vitro. When purified lipomodulin was incubated with cAMP dependent protein kinase, ATP and cAMP, a time dependent inactivation of lipomodulin was observed and this inactivation was blocked by an inhibitor of the protein kinase. When rabbit peritoneal neutrophils were incubated with ^{32}P and lipomodulin and lipomodulin was then isolated by immunoprecipitation, a band labelled with ^{32}P co-migrated with a lipomodulin standard on SDS gels. When fmet-leu phe was added to the cells there was an increase in ^{32}P incorporation into lipomodulin which gradually returned to the control level with a corresponding cessation of arachidonate release. This system required calcium entry into the cells. Lipomodulin inhibited phospholipase A_2 from pig pancreas, cobra and bee venom. The results suggested that phosphorylation and dephosphorylation of lipomodulin appear to have an important role in the short term regulation of phospholipid metabolism and liberation of arachidonic acid in intact cells which respond to external stimuli.

Hirata et al, (1978) have shown that sera from patients with rheumatic diseases contain antibodies against lipomodulin. They

have developed a radioimmunoassay of lipomodulin and shown that a monoclonal antibody (derived from sera of these patients) against lipomodulin, reacted with three fractions purified from the culture media of rat macrophages, which had been treated with the glucorticoid, dexamethasone. The molecular weights of the fractions were 16,000, 30,000 & 40,000 daltons. It was postulated that the 40,000 species was cleaved to the smaller species by proteases and in keeping with this, have shown that omission of protease inhibitors during the purification resulted in poor recovery of the 40,000 species. These results suggest that macrocortin is simply a fragment of lipomodulin.

Arachidonic acid serves as a substrate for two important enzymes, cycloxygenase, which is responsible for prostaglandin synthesis and lipoxygenase, which is resonsible for 12-H.E.T.E. formation and it is these products are partly responsible for inflammation and pain. Asprin-like drugs block the cycloxygenase enzyme only. Steroids differ in that they block formation of all products by restricting substrate availability and Blackwell, (1980) has proposed that macrocortin is the probable effector of steroid action on the arachidonate cascade.

(c) The regulation of phospholipase A_2 activity by the availability of calcium ions.

Phospholipases A_2 associated with membranes require calcium for their activity and it has been suggested that in platelets at least, the enzyme could be regulated by calcium. Pickett et al, (1977) have shown that phospholipase A_2 activity in a suspension of intact platelets was induced by the addition of the calcium ionophore A23187 and their findings suggested that A23187 is able to mobilise intracellular calcium which then regulates phospholipase A_2 activity.

Thrombin induced release of arachidonic acid from human platelets was inhibited by dibutyryl cAMP and external calcium did not counteract this. When A23187 was used as an activating agent much less inhibition was produced, and it was suggested that dibutyryl cAMP may reverse the release of calcium promoted by A23187, by stimulating the active storage of calcium (Rittenhouse-Simmons & Deykin, 1978). The simple model for these results is that addition of ionophore or thrombin would result in increased cytoplasmic calcium levels by the release of calcium from an internal store and that this calcium would then activate phospholipase A_2. CAMP, which stimulates the storage of calcium, could inhibit ionophore or thrombin induced phospholipase A_2 activity.

The activation of phospholipase A_2 cannot conform to a general model since (Feinstein, et al, (1977) have shown that collagen or thrombin not only increase cytoplasmic free calcium but could also cause the conversion of an inactive phospholipase to an active form. Frei and Zahler, (1979) have shown from

experiments involving chymotrypsin treatment of whole or leaky ghosts that the enzyme is orientated towards the exterior of the sheep eryhrocyte. The enzyme requires calcium, with a Kd of 5×10^{-5} M. Since the plasma concentration of calcium is 1.5mM their data suggests that in sheep erythrocytes the phospholipase A is not regulated by calcium.

Wang & Cheung, (1979) have shown that the presence of exogenous calmodulin caused a small increase in arachidonic acid released from human platelet membranes . The relatively small effect is probably due to the fact that the membranes already contain endogenous calmodulin. Trifluoperazine, which specifically blocks the biological activity of calmodulin, suppressed the calmodulin stimulated activity to basal level. Calmodulin has also been shown to decrease the Km and Vmax of phospholipase A in synaptic plasma membrane preparations from brain tissue, the former being more significantly decreased resulting in an apparent overall six fold stimulation of the substrate turnover rate (Moskowitz et al, (1984). Prostaglandin E almost completely inhibited the stimulating effect of calmodulin and they suggested that arachidonate release from synaptic plasma membranes by endogenous phospholipase A may be metabolized to Prostaglandin E to give feed-back inhibition, while lysolecithin may enhance membrane permeability and facilitate synaptic vesicle-synaptic plasma membrane fusion. Thus calmodulin by controlling the metabolism of both cAMP and prostaglandins, provides a molecular link between these two classes of cellular regulators.

(d) Phospholipid methylation as a regulator of phospholipase A_2.

Finally phospholipid methylation has been proposed to be another regulator of phospholipase activity (Hirata & Axelrod, (1980). A close correlation was found between phospholipid methylation, arachidonic acid release and liberation of histamine from basophils. However some criticisms of this have been described by Vance & De Kruiff, (1980) and Randon, (1981).

Alteration of PLA_2 activity by reaction products.

The rate of hydrolysis of phospholipids by phospholipase A_2 depends on the physical state of the substrate. Kinetic data has been obtained from studies on monomeric substrates, micelles of short chain lecithins, mixed micelles of phospholipids and detergents, monomolecular surface films of medium-chain phospholipids and phospholipids present in bilayer structures. It is therefore not suprising that fatty acids and lysophosphatides have been described as being both efficacious and poor activators or inhibitors of this enzyme.

Epstein and Shapiro, (1959) reported that the breakdown of lecithin by intestinal mucosa lecithinase (Phospholipase A) was preceeded by a lag phase which was shortened by the addition of fatty acids, with linoleic and oleic acid being the most effective activators. The fact that the amount of fatty acid required for optimum activity depended upon the amount of substrate and was roughly equimolar to it, led them to propose a substrate- activator complex as a basis of activation.

By studying the action of Penicillium notatum phospholipase on (^{32}P) lecithin films, Dawson and Bangham, (1965) showed that the enzyme had no action on lecithin but would attack lecithin mixed with dicetylphosphoric acid. From these results they concluded that a positively charged region of the enzyme was attracted to the negative film and facilitated the absorption and penatration of the enzyme into the film. They also showed that phospholipase C from Cl.perfringens only attacks micelle-water interfaces when the micelle has a net positive charge (Bangham & Dawson, 1962).

The action of rat mitochondria phospholipase on exogenous and endogenous phospholipid has been shown to be stimulated by free fatty acids (Waite et al, 1969). lauric, myristic, oleic and linoleic were the most effective fatty acids tested. This was a similar pattern to that found by Epstein and Shapiro, (1959). Dawson, (1963) found however, that the hydrolysis of lecithin by cobra venom phospholipase A_2 was inhibited by the addition of many anionic amphipathic molecules to the substrate but this was not related to the net d-potential (the potential produced by an excess of negative groupings on the surface, measured by microelectrophoresis which determines the sign and magnitude of the charge of lecithin particles) on the lipid since the inhibition could not be reversed when cetyl trimethyl ammonium hydroxide was added to restore the isoelectric state. These results suggested that not all phospholipases exhibited a substrate-mediated mechanism of action. Porcine pancreatic phospholipase A_2 has been shown to hydrolyse lipid aggregates which are positively charged and this argues against the ideas of Van Deenen et al, (1963) that the pancreatic phospholipase A_2 requires a negative charge on the lecithin (Verger et al, 1973). In addition they showed that incorporation of short chain lecithin molecules into long chain lecithin structures had the same effect as a negative d-potential.

Kinetic studies of phospholipase A_2 from Bitis gabonica hydrolysing dipalmitoyl lecithin have shown that at low concentrations, palmitic acid is inhibitory but at higher concentrations of fatty acid the inhibition becomes progressively

less (Viljoen et al, (1974). This was explained by the fact that at low concentrations of substrate most of the substrate molecules are present in monomeric form. As the concentration of inhibitor was increased, a stage was reached where mixed micelles were formed which favoured enzyme action. A similar effect was noted by Bonsen et al, (1972) who found that in certain instances some inhibitors displayed an activating as well as an inhibiting behaviour.

Lawrence and Moores, (1975) have shown that the hydrolysis curve for phospholipase A_2 from bee venom acting on purified egg lecithin, was sigmoidal in nature, suggesting that product activation was taking place. They showed that activation was primarily due to long chain fatty acids although lysolecithin produced a very small contribution. This work showed, for the first time, that the fatty acid interacted with the enzyme and not the substrate because the amount of fatty acid required was independent of the substrate concentration, and activation could be obtained by covalent addition of an acyl chain to the protein. Initial studies with anhydrides and ethoxy formates as acylating agents gave very high activity but also a degree of non-specific inactivation. Subsequently they showed that incubation of the enzyme in the presence of gluteraldehyde did not affect the initial hydrolysis, but prevented product activation, however treatment of the enzyme with gluteraldehyde in the presence of oleic acid had a large activating effect.These results suggested that the latter treatment could stabilise an active conformation of the enzyme. From their their results they have postulated that the protein has an allosteric activating site operated by free

long-chain fatty acids (R>C12) or by acylation (R>C6). Short chain acyl residues block this site and do not activate. A second site is inhibitory, and overrides the former site when occupied by short-chain acyl groups (R<C6) but recovery of the activity of the enzyme treated with butyric anhydride and oleic acid showed that this inhibition is unstable.

Drainas & Lawrence, (1978) have shown that imidazolide derivatives of long chain fatty acids are potent irreversible activators of bee venom phospholipase A_2, with the rate of activation increasing with the chain length of the activator. Oleoyl and linoleoyl residues were the most effective activators. Activation was stimulated 100 fold at low calcium concentrations. Radiolabelling experiments using (^{14}C)-labelled oleoyl imidazolide confirmed that the activator modified the enzyme and not the sustrate (Drainas, 1978) and that activation was achieved by the addition of a single acyl residue to the protein molecule. The same authors developed an assay which was absolutely specific for covalent modification of the enzyme (Drainas & Lawrence, 1980). The erythrocyte leakage assay used fatty acid free albumin to absorb fatty acids, thus full activation could be demonstrated even when all traces of free fatty acids had been removed from the system.

Kanaho and Fujii, (1982) have shown that addition of lysophosphatidyl choline to rabbit platelets cosiderably inhibited the thrombin stimulated release of (1-^{14}C) labelled arachidonic acid from the platelet mambrane phospholipids. Lysophosphatidyl choline transformed normal platelets into spiny

discs and the authors suggested that the perturbation of the membrane had the effect of blocking access of phospholipase A_2 to the membrane phospholipids.

Lysophosphatidyl choline added to vesicles of diacylphosphatidyl choline facilitated incorporation of pig pancreatic phospholipase A_2 and the hydrolysis of the substrate (Jain et al, (1983). The effect appeared to be due to organizational defects created by asymmetrical incorporation of lysophospholipid molecules into the outer monolayer of the vesicles.

Lawrence, (1975) & Drainas et al , (1981) have shown that lysolecithin inhibited the lysis of erythrocytes by bee venom phospholipase A_2. They showed that the initial leakage response of erythrocytes treated with oleic acid and Phospholipase A_2 was greatly reduced when low levels of lysolecithin were added.

Phospholipase A_2 does not attack bilayers very well but its activity increases about 100 fold when attacking micelles. It has been proposed that membranes contain regions of high curvature and in these regions phospholipids are packed in a similar way to micelles, and in these regions the phospholipase A_2 would work well (Israelachvili, 1977). Drainas et al, (1981) proposed that lysolecithin would accumulate in these areas and inhibit the enzyme by a type of "site-competitive inhibition".

The lytic action of phospholipases is usually thought to be due to the production of lysophospholipids in the membrane, e.g. Lankisch et al, (1972) have shown that lysolecithin potentiates the action of phospholipase A_2 from bee venom. They found that an amount of enzyme which alone caused <10% lysis of erythrocytes

added with an amount of lysolecithin which alone caused about 20% lysis, together resulted in total lysis of the cells.

The role of albumin on the activity of phospholipase A_2

Albumin has been used both to protect cells from the lytic effects of free fatty acids and lysophosphatides, which are produced by phospholipases A_2, and also to enhance the damage produced by these enzymes. When isolated membranes of Mycolpasma gallisepticum were treated with phospholipase A_2 unesterified fatty acids accumulated, but due to lysophospholipase activity, lysophospholipids did not. Extensive phospholipase A_2 treatment of cells resulted in a decrease in cell viability and increase in cell leakiness except when bovine serum albumin was added (Markowitz, 1982).

Williams, (1973) showed that bovine serum albumin increased the resistance of fresh human erythrocytes to lysis by hydrodynamic shear. Molecules which had been adsorbed were not easily removed by washing with saline. The work of Takeda et al, (1982) suggests that albumin is protective against the cytotoxic effects of phospholipase A_2 on the basis that in the presence of albumin, the uptake of (^{3}H) labelled uridine by phospholipase A_2 treated cells is increased compared to when albumin is absent. In addition, cells treated with lysophosphatidyl choline showed reduced levels of the uridine uptake compared with when albumin was present. The authors proposed that their results were in

disagreement with those of Gul & Smith, (1972,1974)

Gul & Smith, (1972) have shown that albumin potentiates the hydrolysis of erythrocytes by phospholipase A_2 but has no effect on phospholipid hydrolysis. This indicated that the albumin did not exert its effect by activating the enzyme and this was supported by the observation that addition of albumin after the reaction had proceeded for five minutes, would cause the cells to lyse without any additional release of fatty acid. This occured much more rapidly than when albumin was added with the enzyme from the start. In the absence of albumin, despite appreciable phospholipid hydrolysis no significant lysis occured. The same authors (Gul & Smith, 1974) later proposed that the action of albumin could also have been due to removal of lysoderivatives from the membrane. Prolonged pre-incubation of red cells with albumin, prior to enzyme addition resulted in less phospholipase A_2 -induced haemolysis but no explanation was offered for this.

The work of Drainas & Lawrence, (1980) and Drainas et al, (1981) confirmed the main ideas of Gul & Smith but their results differed in detail in several ways. Albumin should be able to protect cells against limited enzyme attack by removing fatty acids and lysophosphatides. However a point should be reached where the outside of the membrane is damaged to an extent at which it will infold and result in cell lysis. However their results showed that albumin potentiated leakage of the cells almost from the beginning of phospholipase attack. The authors hypothesised that the main action of albumin in their assay, was to remove the inhibitory reaction product, lysophosphatide from

the membrane.

The effect of the physical state of the substrate on phospholipase A_2 activity.

The activity of phospholipases strongly depends on the state of the state of aggregation of the substrate. Short chain phospholipid molecules exist singly in aqueous solution up to a certain concentration, the critical micelle concentration, where they form micelles. Long chain phospholipids form bilayers and vesicles in aqueous solution. An increase in enzyme activity is seen upon substrate aggregation and this has been suggested to be due to the substrate being more susceptible to enzyme attack, a conformational change in the adsorbed enzyme or the rate limiting step of product release being increased in the hydrophobic lipid-water interface.

The first detailed kinetic study of Crotalus adamenteus phospholipase was carried out by Wells, (1972). Initial velocity patterns as a function of Ca^{2+} and dibutyryl lecithin concentration were consistent with Ca^{2+} added first and dibutyryl lecithin second. The results also suggested an ordered release of products with the fatty acid being released first.

The results of experiments using short chain lecithins containing thioester bonds, have shown that in contrast to the venom enzymes the initial velocity patterns of the pancreatic phospholipase A_2 are consistent with the random addition of substrate and Ca^{2+} to the protein (Volwerk et al, 1979). The

Vmax-pH profiles showed that the activity of this enzyme is controlled by a group with an apparent pK of 5.5, probably histidine-48. De Haas et al, (1971) have studied the action of porcine pancreatic phospholipase A_2 on short chain lecithins (C6-C9) .Large increases in rates were seen above the critical micelle concentration and dioctanoyl lecithin was the best subsrate.

For the reaction:

$$E + Ca^{2+} \longrightarrow E.Ca^{2+} \xrightarrow{S} E.Ca^{2+} + S.$$

E= enzyme
S= substrate

The expected reaction velocity for infinite substrate concentration would be independent on the Ca^{2+} concentration, i.e. Lineweaver-Burke plots (V v 1/S or V v $1/Ca^{2+}$) should give a common intersection on the ordinate.

For :

$$E + S \longrightarrow E.S. \xrightarrow{Ca^{2+}} E.Ca^{2+}.S$$

The reaction velocity should be independent of substrate concentration at infinite Ca^{2+} concentrations. Again a common intersection at the ordinate would be expected.

De Haas (1971) found that both showed common intersection points on the abscissa which are consistent with a random addition of Ca^{2+} and substrate to the enzyme.

Kinetic analysis of phospholipase A_2 from Crotalus adamanteus venom acting on dibutyryl, dihexanoyl and dioctanoyl lecithin have shown that above the critical micelle concentration, a dramatic increase in catalytic attack occurs and in this case Ca^{2+} added first to the enzyme followed by the substrate. The

Vmax of phospholipase acting on monomeric dibutyryl lecithin was 3000 fold less than on dioctanoyl lecithin micelles, however dibutyryl lecithin (near the Km of the substrate) inhibited the action on micellar dioctanoyl lecithin (Wells, 1974). These results were used to support a mechanism where the enzyme, after each single encounter with the micellar interface and catalytic cycle, returns to the aqueous phase. This arguement assumes that the dibutyryl lecithin is not present in the dioctanoyl lecithin micelle which could change the quality of the lipid-water interface.

Pieterson et al, (1974) showed that the pancreatic enzyme and its zymogen both hydrolyse monomeric substrates with a low efficiency however the micellar form of the substrate is only hydrolysed by the active enzyme.

Verger et al, (1973) has proposed a model to describe kinetically the action of soluble enzymes at interfaces. The first stage is the reversible penetration of a water-soluble enzyme into an interface. Secondly is the combination of a single substrate molecule with the penetrated enzyme, which is the equivilent in two-dimensions of the classical Michaelis-Menten equilibrium. Once this has occured the catalytic steps take place regenerating the enzyme in the penetrated state, along with the liberation of the products. They used a "zero order trough" which gives linear kinetics after injection of the enzyme under the monolayer (dinonanoyl lecithin) with snake or bee venom phospholipase A_2. The pancreatic enzyme showed an initial lag phase and to explain the unusual time course they have presumed

that the pancreatic enzyme penetrates slowly into the film. The ionisation of a few amino acid residues influenced considerably the rate of the penetration process and this pointed to the existance of a localised site on the enzyme involved in the penetration process. Van dam Mieras et al, (1975) showed that the hydrophobic N-terminal sequence of the enzyme (ALA-LEU-TRP-GLN-PHE-ARG) is directly involved in the interaction with the lipid-water interface.

Allgyer & Wells, (1979) analysed the kinetics of Crotalus adamanteus phospholipase A_2on monomeric and micellar lecithins. The abnormal parabolic rather than hyperbolic velocity dependence on substrate concentration was explained by a thermodynamic model where the micelle properties changed with substrate concentration.

Detergent solutions with a low critical micelle concentration solubilise phospholipids by incorporation into mixed micelles. Dennis et al, (1975) have analysed the kinetic behaviour of phospholipase A_2 from Naja naja naja acting on lecithins (C6-C10) solubilised in the non-ionic detergent Triton X-100. The substrate was most susceptible to enzyme attack when a molar ratio of Triton:lecithin of 2:1 was used. Higher amounts of detergent resulted in inhibition and this was explained by a surface dilution of the substrate.

Roberts, (1977) proposed a model for the interaction of Naja naja phospholipase A_2 and mixed micelles of Triton and phospholipid. The "dual-phospholipid model" required two phospholipid molecules, one to sequester the enzyme to the interface and the other for subsequent catalysis. This model was

heavily based on the presumed "half site reactivity" of the enzyme which is now known to be incorrect (Roberts et al, 1977 & Darke et al, 1980). Adamich et al, (1979) compared the action of Naja naja phospholipase A_2 on mixed micelles of Triton and long-chain phosphatidylethanolamine and found that the lecithin-containing micelles wre hydrolysed at a much higher rate. However when phosphatidylethanolamine and phosphatidylcholine were added in equal amounts in mixed micelles then phosphatidylethanolamine was preferred as a substrate. The fact that this specificity reversal could be achieved using short chain phospholipids e.g. dibutyryl lecithin suggested a direct lipid-enzyme interaction as a cause of the activation.

The action of Bitis gabonica phospholipase A_2 acting on dipalmitoyl phosphatidylcholine has been studied by Viloen, (1974). The substrate concentrations used (10^{-10} M) were considerably above the critical micelle concentration so, although the authors thought they were working with lipid aggregates, they were actually studying monomer catalysis. Initial rate measurements where calcium and substrate concentrations were varied confirmed the mechanism proposed by Wells, (1972) for the Crotalus adamanteus phospholipase whereby Ca^{2+} adds first to the enzyme before the substrate. Product inhibition experiments suggested that fatty acid is released first and lysolecithin second.

Long-chain diacylphospholipids e.g. phosphatidylcholine which forms bilayer structures in water, are poor substrates for pancreatic phospholipase A_2 (Van Deenen, (1963). At the

thermotropic phase transition several fully saturated long-chain lecithins have been reported to become very susceptible to attack by porcine pancreatic phospholipase A_2. This was thought to be due to defects in the surface structure which then allowed penetration of the enzyme. This has only been found for enzymes with weak penetrating power such as the pancreatic phospholipase (Op Den Kamp et al, 1975 & Goormaghtigh et al, 1981), beta-bungarotoxin (Strong & Kelly, 1977) and platelet phospholipase A_2 (Goormaghtigh et al, 1981).

Tinker et al, (1978) have worked with dispersions of dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine and using Crotalus atrox phospholipase A_2 have studied the kinetics of hydrolysis of these substrates at different temperatures both above and below their phase transition temperatures. For hydrolysis of gel phase phosphatides the steady state initial velocities were hyperbolic functions of bulk lipid concentrations. However, for hydrolysis of liquid crystalline phosphatides, the reaction consisted of a short initial burst, a long lag period, then a large increase in reaction rate. The lag phase was abolished by the addition of lysolecithin and fatty acids. From these results they proposed a kinetic model of hydrolysis which was different from the model proposed by Verger et al, (1973). In Verger's model the rate limiting step was adsorption to the surface. In Tinker's model the enzyme binds to a single substrate molecule . This complex formation results in a conformational change in the enzyme which results in the exposure of hydrophobic sites capable of interacting with the lipid surface. Tinker et al later showed

that incorporation of hydrolysis products in the bilayer weakened the binding of the enzyme. They concluded that the rate limiting step in catalysis was desorption from the lipid surface and the increase in hydrolysis rate at later stages was due to a product facilitated desorption fron the surface.

The kinetics of bee venom phospholipase A_2 acting on phosphatidylcholine bilayers which had been altered by osmotic shock, temperature change and sonication has been reported by Upreti & Jain, (1980). The curves showed an initial fast phase, a lag phase and after production of a certain amount of reaction products, fast hydrolysis. The "cracks" in the surface were thought to enhance enzyme activity by allowing more enzyme to penetrate.

The Acheoleplasma laidlawii membrane contains 70% glycolipids and 30% phosphatidylglycerol. The membrane can be altered by growing the organism on different fatty acids. Treatment of these cells with pancreatic phospholipase A_2 when the lipids were in the liquid crystalline- state resulted in a rapid breakdown of the phosphatidylglycerol. Under conditions where the lipid was in the solid state, no hydrolysis occured (Bevers et al, 1979). In the solid state there was less chance of irregularities in the bilayer and thus it was less susceptible to enzyme attack.

Bovier et al, (1981) used Acheoleplasma laidlawii which had been grown on branched-chain fatty acids. These lipids undergo a phase change at the transition temperature which does not result in a tight packing of the lipids in the bilayer and hence were

still susceptible to enzyme attack. It has also been shown using this organism that the pancreatic phospholipase A_2 only had access to the phosphatidylglycerol molecules which were present in the fluid protein-containing areas of the lipid bilayer while those in the highly organised gel state remained unhydrolysed (Bovers, 1978).

Phospholipase A_2 dimerisation

One of the questions concerning phospholipase A_2 activity is whether the enzyme acts as a monomer or a dimer. P-bromo-phenacyl-bromide treatment of Naja naja naja venom phospholipase A_2 has been shown to result in the complete loss of enzyme activity (Roberts et al, 1970). Their results suggested that the activating agent altered 0.5 moles of histidine per enzyme molecule. They also showed that ethoxyformic anhydride modified phospholipase A_2 by acylation of two amino groups, a tyrosine and 0.5 moles of histidine. Ethoxyformylated enzyme could be further inactivated by treatment with 0.5 moles of p-bromo-phenacyl-bromide. From these results they proposed a model of "half site reactivity" where the active species of enzyme interacted with mixed micelles as a dimer. The dimer was thought to be asymmetric with the histidine of one subunit being accesible to ethoxyformic acid while the other was reacive towards p-bromo-phenacyl-bromide. However it was later shown that in contrast to most other Naja phospholipases, Naja naja naja did not contain histidine residues at positions 10 and 20 (Darke et al, 1980). Roberts' group had based their calculations on this assumption and

thus there was no longer any evidence to support half-site reactivity of the enzyme.

At concentrations below 50ug/ml the Naja naja naja phospholipase A_2 exists mainly as a monomer however there is evidence that the phosphatidylcholine can activate the enzyme towards phosphatidylethanolamine by causing a conformational change and dimerisation of the enzyme (Adamich et al, 1979). Smith and Wells, (1981) used active enzyme ultracentrifugation to show that the dimeric form of the enzyme catalyses hydrolysis of monomeric substrate and Shipolini et al reported that bee venom phospholipase A_2 exists in concentrated solutions at high pH as a dimer. The tendency of phospholipase A_2 to dimerise depends on the amount of hydrophobic and hydrophilic amino acid side chains. The enzymes from the Crotalidae have a strong tendancy to aggregate (Meneshe et al, 1981) while the pancreatic phospholipase exists as a monomer up to concentrations of several mg/ml and appears to catalytically active as monomers (Wolf et al, 1981).

CHAPTER TWO

Materials and Methods

Preparation of substrates

(1a) Preparation of lecithin :

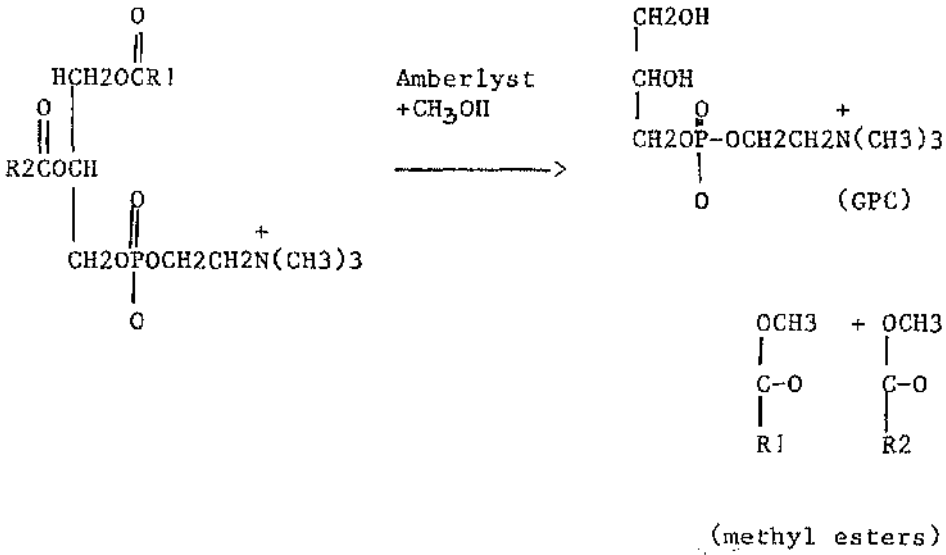
Lecithin, (1,2-acyl-3n-glycerophosphatidylcholine) was prepared by the method of (Brokerhof & Yurkowski,1965) as follows: Four dozen egg yolks were separated, stirred and repeatedly extracted in acetone to remove the yellow pigment and oils and then rotary evaporated until dry. It was further purified by adding more acetone, heating to 70°C and cooling on ice to reprecipitate the lecithin. Aproximately 60g of lecithin was recovered by this method. (stage 1)

(1b) Purification of lecithin :

The purity of the product was checked by thin-layer chromatography (TLC plastic sheets, silica gel 60, Merck AG, Darmstadt, West Germany). Phospholipids were stained with molybdemum blue reagent and the chromatogram developed in a solution of chloroform:methanol:acetic acid:water (25:15:4:2). The lecithin was further purified on an alumina column (neutral alluminium oxide, Woel grade 1, in chloroform). The main phospholipids of lecithin are phosphatidylcholine and phosphatidylethanolamine in a ratio of about 6:1 respectively. Phosphatidylethanolamine remains bound to the column and the phosphatidylcholine can be eluted with chloroform:methanol (4:1v/v). The presence of amino groups (PE) was detected by spraying with Ninhydrin (BDH Chemicals, Poole, England). Solvent was removed by evaporation and the purified lecithin stored at 4°C. (stage 2)

(1c) Preparation of GPC :

GPC was prepared by the method of Brokerhof & Yurkowski (1965). 200g of lecithin (stage 1) was dissolved in 200ml of methanol and mixed with 100g of the strongly basic anion exchange resin, Amberlyst A-26 (BDH Chemicals, Poole, Dorset). The mixture was stirred at 50°C and the breakdown of lecithin was followed at intervals by TLC (GPE is absorbed by the resin). The methanol was removed by evaporation and the solid, which contains GPC and methyl esters, was heated with chloroform. GPC is insoluble in chloroform and can be removed by filtration. It was than dissolved in methanol and deionized with a mixed bed resin (Dowex MR-3, Sigma, Dorset). Finally the resin was removed by filtration, the GPC dried and stored at 4°C.



(1d) Preparation of lysolecithin

Aproximately 100g of purified lecithin (1) was dissolved in 1 litre of an n-propanol/water mixture (1:4 v/v respectively) containing 1ml of 100mM calcium chloride and the pH was adjusted to 8 with 1N NaOH. 2mg of activated bee venom PLA_2 was added to the mixture and the hydrolysis of lecithin was followed with a pH-stat by continuous titration with NaOH. When the reaction was complete the lysolecithin was extracted by addition of chloroform/methanol (1:1 v/v) and the organic solvent phase which contained the lysolecithin was separated and dried in a rotary evaporator. The solid was then dissolved in hot ethyl acetate and, upon cooling, the lysolecithin precipitated leaving most impurities in the aqueus phase. The product was further purified on an alumina column in chloroform, and eluted with chloroform/methanol (2:1 v/v) respectively. Purity was checked by TLC. The solvent was then evaporated and the lysolecithin stored at 4^{o}C.

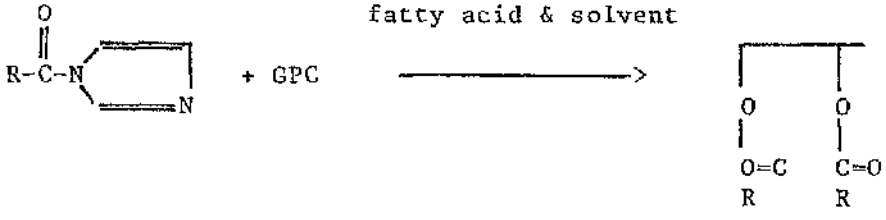
(1e): Preparation of dioleoyl phosphatidyl choline

Dioleoyl lecithin was prepared from GPC and oleoyl anhydride in the presence of potassium oleate (Cubero & van der Berg, 1969). GPC, oleoyl anhydride and potassium oleate were mixed in a proportion of 1:4:0.2 w/w/w respectively. The mixture was heated to 100^{o}C and when the GPC was totally dissolved, the temperature was kept at 90^{o}C. The product was taken up with petroleum ether, which leaves behind the potassium salt) dried, amd purified on an

alumina column in chloroform. Dioleoyl lecithin was eluted with chloroform/methanol 4/1 w/w and deionised with a mixed-bed ion exchange resin. The final product was dissolved in n-propanol and stored at 0° C.

(1f) Preparation of dinonanoyl phosphatidyl choline

This was prepared either by the method used in section 1e, or by mixing 1 part GPC, 6 parts n-nonanoic acid and 2 parts 1, 1-carbonyl diimidazole (Sigma, Poole, Dorset) at 90° C for 1 hour. Purity of the product was checked by TLC. When all the GPC had been converted to the di-acyl derivitive, excess acyl imidazolide was destroyed by adding 1ml of water. The final product was dissolved in methanol and deionized with a mixed bed resin (Dowex MR-3, Sigma, Poole, Dorset).



double ester

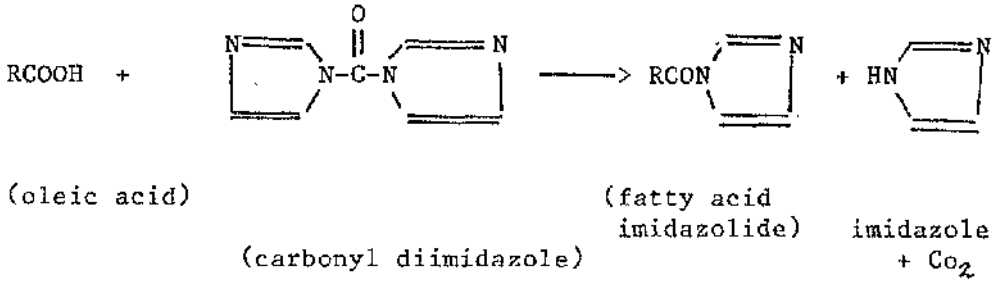
(1g): Preparation of 2-nonanoyl phosphatidyl choline.

The two nonanoyl derivative of egg-yolk phosphatidyl choline was synthesised from lysolecithin by mixing 1 part nonanoic anhydride and 0.5 parts potassium nonanoate at 90° C. The product was extracted with petroleum ether, dried, and then purified on an

alumina column. It was then eluted with chloroform:methanol (2:1 v/v).

(2) Preparation of oleoyl imidazolide :

Oleoyl imidazolide was prepared by the method of Drainas, Moores & Lawrence (1978). 1 part oleic acid (Sigma, Poole, Dorset) and 2 parts 1,1 -carbonyl diimidazole were mixed together for 15' in methylene chloride. The solvent was then evaporated, the sample dissolved in petroleum ether and kept at 4°C until a white precipitate, imidazole, formed. Oleoyl imidazolide dissolves in petroleum ether and the precipitate was removed by centrifugation at 2000 rpm for 5'. The supernatant was removed, evaporated to dryness and the final product dissolved in acetone to make a 2% stock solution. Since carbonyl diimidazole is moisture sensitive, all solvents were dried before use by passage through a column of alumina (BDH Chemicals, Poole, Dorset). The reaction for formation of oleoyl imidazolide is :



(3) Preparation of [3H]-oleoyl imidazolide:

(9,10 (n)-3H) oleic acid (5mCi/ml; 5.7Ci/mM; Amersham, Buckinghamshire) was diluted ten fold with cold oleic acid and then converted to oleoyl imidazolide by the above method with the exception that solvents were removed by freeze drying rather than rotary evaporation. The specific activity of the product was 0.57 Ci/mM.

(4)Activation of PLA_2 with oleoyl imidazolide:

To produce PLA_2 activated in a 1:1 stoichiometry with oleoyl imidazolide, 100µl of PLA_2 (1mg/ml) was incubated at 37°C for 1 hour with 1µl of 0.2% oleoyl imidazolide in acetone. 2µl samples were taken at intervals and tested for activation using the standard erythrocyte assay (8).

(5) Detection of radioactivity in acid-urea gels:

Radioactivity in acid-urea gels was detected by fluorography using the water-soluble fluor, sodium salicylate (Chamberlain, J.P., (1979). Stained gels were washed in water for 30' prior to treatment to prevent precipitation of salicylic acid, soaked in 250ml of 1M sodium salicylate (Sigma, Poole, Dorest), pH 5-7 at room temperature for 30', then dried with a gel drier (Bio Rad, Watford, England) for about two hours. When the gel was dry, it was exposed to X-ray film (Kodak X-Omat, Kodak, Glasgow) at -70°C for 24 hours-1 week.

Development of autoradiographs :

Autoradiographs were developed at 20°C by the following procedure:

a) 100ml developer (DX-80, May & Baker, Glasgow) + 400ml water. The film was checked every 3' until spots appeared .

b) Wash in 2% acetic acid for 30 seconds.

c) Fix in a solution containing 100ml amfix (May & Baker, Glasgow), 400ml distilled water and 10ml hardner (May & Baker, Glasgow) for 3'.

(6) Preparation of buffers

(6a) Isotonic sucrose hepes buffer :

Isotonic sucrose solution was used in assays involving erythrocytes and was buffered with 10mM HEPES/Na+. 95g of sucrose (0.28M moles, May & Baker, Dagenham) was added to 900ml of water containing 10ml of normal NaOH. The pH was adjusted to 7.4 with HEPES (Cambridge Research Biochemicals, Cambridge) and the final solution was made up to one litre with distilled water.

(6b) Triethanolamine buffer :

This assay was used on substrates which employ synthetic substrates. 10ml of normal HCl was added to 900 ml of distilled water and the pH was adjusted to 8 with triethanolamine (Sigma, Poole, Dorset). The final solution was made up to one litre with distilled water. When calcium was included in the buffer, 1ml of calcium chloride solution (100mM) was added before making the solution up to one litre. For assays which used long chain acyl chain containing lecithins, 20% n-propanol was included in the buffer.

(6c) Tris buffer :

This buffer was used for equilibrating Concanavalin-A Sepharose columns. 9.0g tris and 4.4g NaCl were dissolved in 900ml distilled water and the pH was adjusted to 8.1 with 5N HCl. The final solution was then made up to one litre. Bound protein was eluted with the same buffer containing 20mg/ml α-methyl-D-mannoside (Sigma, Poole, Dorset).

(6d) Bicine buffer:

Bicine buffer was used for equilibrating cation-exchange columns and was prepared by adding 10ml of N NaOH to 900 ml of distilled water and adjusting the pH to 8 with solid bicine. The final solution was then made up to one litre.

(7) Preparation of blood :

About 20ml of blood was taken from an ear vein of New Zealand white rabbits and collected into a universal bottle containing 1ml of heparin (Sigma, London; 100 units/ml). It was centrifuged at 3000 rpm, the serum and buffy coat were discarded and the cells were resuspended in isotonic saline (0.9% NaCl in distilled water). The washing procedure was repeated three times and the final haematocrit was adjusted to 30%. The erythrocytes were stored in the fridge and used within five days.

(8) Assays for PLA_2 :

Assays for PLA_2 were performed either on intact erythrocytes or using synthetic substrates. Although experiments differ in detail the general procedure was as follows :

(8a) Synthetic substrates :

2ml of triethanolamine buffer (6b) and 10µl of dinonanoyl lecithin, 40mg/ml, were added to each conductivity cell. When base lines were steady, the appropriate quantity of enzyme was added with a Hamilton syringe (range 1-5µl). When long acyl chain containing lecithins were used the buffer contained 20% n-propanol. Buffers were degassed each day before use.

(8b) Erythrocytes as substrates :

2ml of isotonic sucrose HEPES/Na+ buffer (6a) and 20µl of rabbit erythrocytes (7) were added to each conductivity cell. 20µl of a standard stock solution of bovine serum albumin (66mg/ml in distilled water) was added and when base lines had stabilised, the appropriate amount of enzyme was added or the enzyme was added after addition of erythrocytes, incubated for the required length of time and then 10µl of 100mM EDTA was added. When base lines had restabilised, 20µl of the standard albumin solution was added. Conductivity was recorded until reactions were complete.

(9) Acid-urea gels :

These gels were originally developed for analysis of histones (Riggs et al, 1977) which have similar charge properties to PLA_2. They give similar results to SDS gels but have the advantages that they are simple to prepare, destain quickly and the bands tend to be sharper.

Preparartion of acid urea gels :

A stock 15% gel solution contained 15% acrylamide (Koch-Light laboratories, Colnbrook, England), 6M urea (Koch-Light laboratories), 0.4% N,N'-methylenebisacrylamide (BDH Chemicals, Poole, England) and 5% acetic acid. The gel was polymerised by the addition of 210µl of TEMED (Sigma, Poole, England) and 210µl of ammonium persulphate (Bio Rad, Richmond, California) to 60 ml of stock solution. 10%

Sample preparation :

Samples containing 1-50ug of protein were mixed with neutral red in a concentrated sucrose solution, loaded onto the gel and run towards the cathode at a constant current of 40mA until the dye front reached the bottom of the gel.

Buffer:

Both upper and lower tanks contained 5% acetic acid.

Staining :

Gels were stained for about 20' in a solution containing kenacid blue, 0.5g; acetic acid, 35ml; distilled water, 250ml and

methanol, 250ml.

Destaining :

Gels were destained in a solution of methanol, acetic acid and distilled water in the proportions 50:70:800 respectively until the background was clear.

(10) Protein determination :

Protein concentrations were determined by the method of Bradford, M.M. (1976). This method is based on the observation that Coomassie brilliant blue G-250 exists in two colours, red and blue. The red colour changes to blue upon binding of protein to the dye. Binding is very rapid (about 2') and is stable for 1 hour.

Preparation of protein reagent :

100 mg of Coomassie brilliant blue G-250 was dissolved in 50ml of 95% ethanol. To this solution 100ml of 85% (v/v) phosphoric acid was added and the resulting solution was made up to one litre with distilled water. Final concentrations were 0.001% (w/v) Coomassie blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.

Protein assays :

Protein solutions containing 10-100ug of protein in 0.1ml of water were mixed with 5 ml of reagent. Absorbance was measured at 595nm after 2' and before 1 hour. A standard curve was made using bovine serum albumin (Sigma, Poole, Dorset).

(11) Preparation of Concanavalin A-Sepharose :

Activation of Sepharose

Sepharose was activated by the method of Parath et al, (1967). A slurry of Sepharose-4B (Pharmacia, Uppsala, Sweeden) was washed with 2-3 volumes of distilled water, 1g per 15mg of protein to be bound was weighed out and dissolved in distilled water. 1g of solid CNBr per 10g of wet sepharose was dissolved in water and than added to the Sepharose. The pH was maintained at 11.3-11.4 with 2M NaOH using a pH stat. When the pH was stable, the Sepharose was washed with H_2O then 0.1M $NaCO_3$ to stop the reaction. The activated Sepharose was then added to a 5mg/ml solution of Concanavalin A (Sigma, Poole, Dorset) and gently agitated overnight in the cold room. Any remaining active groups were blocked by washing with 1M ethanolamine, pH8 for 1-2 hours. To remove non-covalently absorbed protein 3 washing cycles of low and high pH buffer in 1M NaCl were used :

Low pH buffer : 0.1M sodium acetate; 1M NaCl; pH to 4 with HCl.

High pH buffer : 0.1M boric acid; 1M NaCl; pH to 8 with NaOH.

(12) Wasp venom :

Wasps, (plate 1) were chilled in the refrigerator. By carefully grasping and pulling the stinger with forceps, it was possible to remove the stinging apparatus together with the attached venom sac. Each part was assayed for pLA_2 activity using dinonanoyl lecithin substrate (5). The richest source of the enzyme was

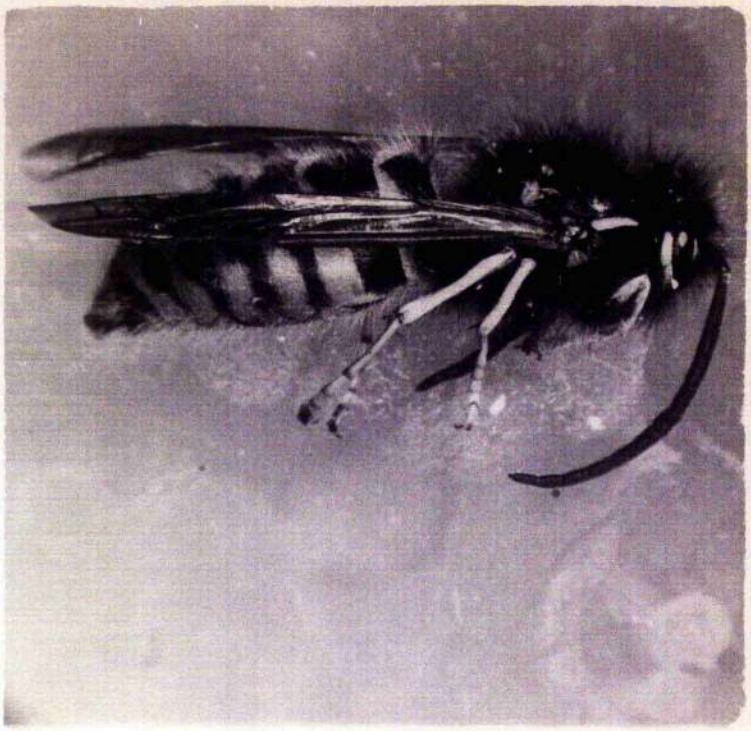


PLATE 1. The common wasp (Vespa)

PLATE 2. The stinging apparatus of Vespa.

found in a translucent sac behind the sting (plate 2). The sac was ground in 100µl of triethanolamine buffer containing 1µl of an anti-protease cocktail (2mM PMSF, Sigma, Poole, Dorset, & 1mg/ml TAME, Sigma). A morphological investigation of the different parts of the venom apparatus of vespa (Barr-Nea et al, 1976) has revealed that it contains two acid glands (pH 6) and an alkaline gland (pH7.5) both of which probably have a role in the transport of secreted substances to the stinging apparatus.

(13) DTT treatment of PLA_2:

10µl of 1mg/ml PLA_2 was mixed with 10µl of triethanolamine buffer and then 1µl of DTT (Sigma, Dorset) was added. The final concentration of DTT was 50mM.

(14) Acetylation of albumin

Albumin was acetylated with acetic anhydride. The protein has about 50 sites capable of being acylated and therefore a 50 fold molar excess of acetic anhydride was used. The pH was kept at 8.0 by the addition of NaOH, controlled by a PH-stat.

(15) The conductivity meter

The circuit of the conductivity meter described by Lawrence et al (1974), and the conductivity cell are shown on Figs (i) and (ii) respectively.

The resistance varies with the distance 'L' between the two electrodes in the conductivity cell and inversely with the area, 'A' of the electrode. In each cell L & A are constant and L/A is

called the cell constant K and with current construction methods (Glass Blowers, Glasgow University), cell constants vary by $\pm$ 20% maximum. Electrode polarisation is minimised by the AC (3000Hz) circuitry.

The six conductivity cells are in a glass water jacket which is conented to a thermostat and has excellent temperature control ($\pm$ 0.005^{o}C). The solutions in the cell are stirred continuously by a small magnetic pellet inside the conductivity cell. The most important technical detail is the response is also sensitive to small bubles nucleating on the electrodes and this was prevented by degassing buffers before use. During a response bubbles may be removed by tapping the cell holder.

$$K = L/A$$

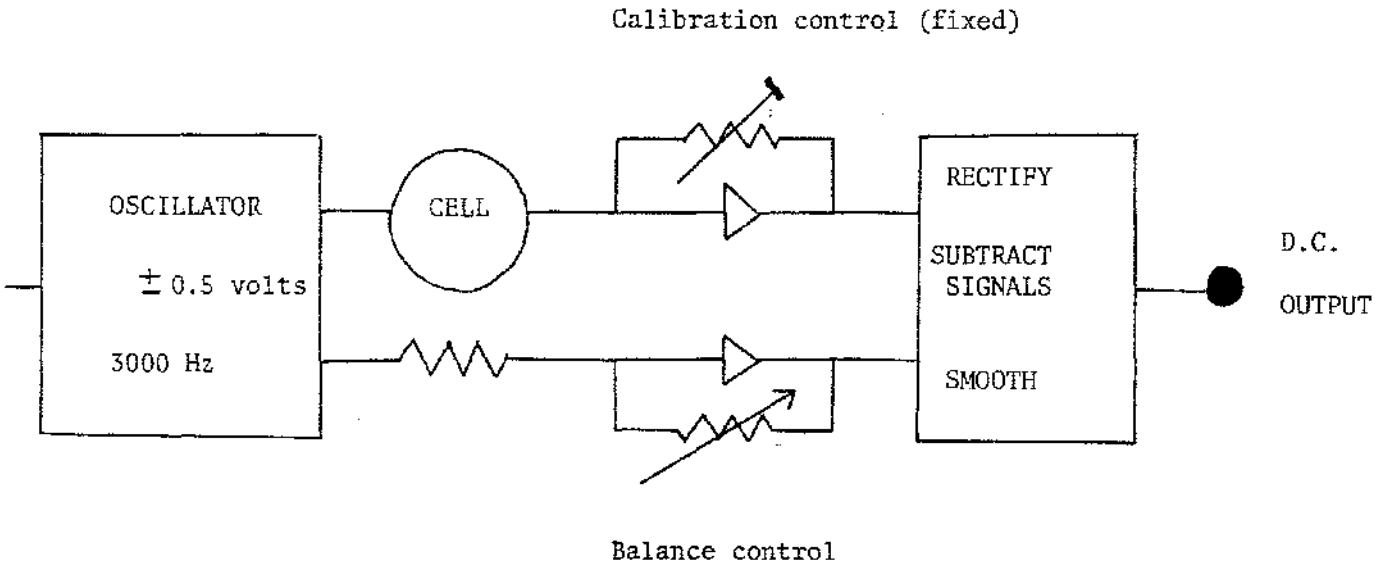


Fig. (i) The conductivity meter.

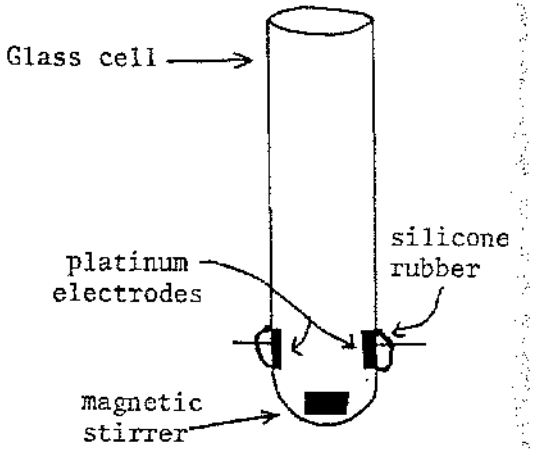


Fig. (ii) The conductivity cell

CHAPTER THREE

Results

The purification of bee venom phospholipase A_2.

Many methods have been described in the purification of PLA_2 from the venom of the common honey bee, *Apis mellifera* but most of these procedures are very time consuming and in no published method ,known to the author, are gel photographs shown. Although for the work in this thesis the main reason for purifying PLA_2 from bee venom was to further study the activation and control of the enzyme, a large amount of time was devoted to it because of some of the interesting observations which arose. This chapter contains the results and discussion of the results obtained during the various purification stages.

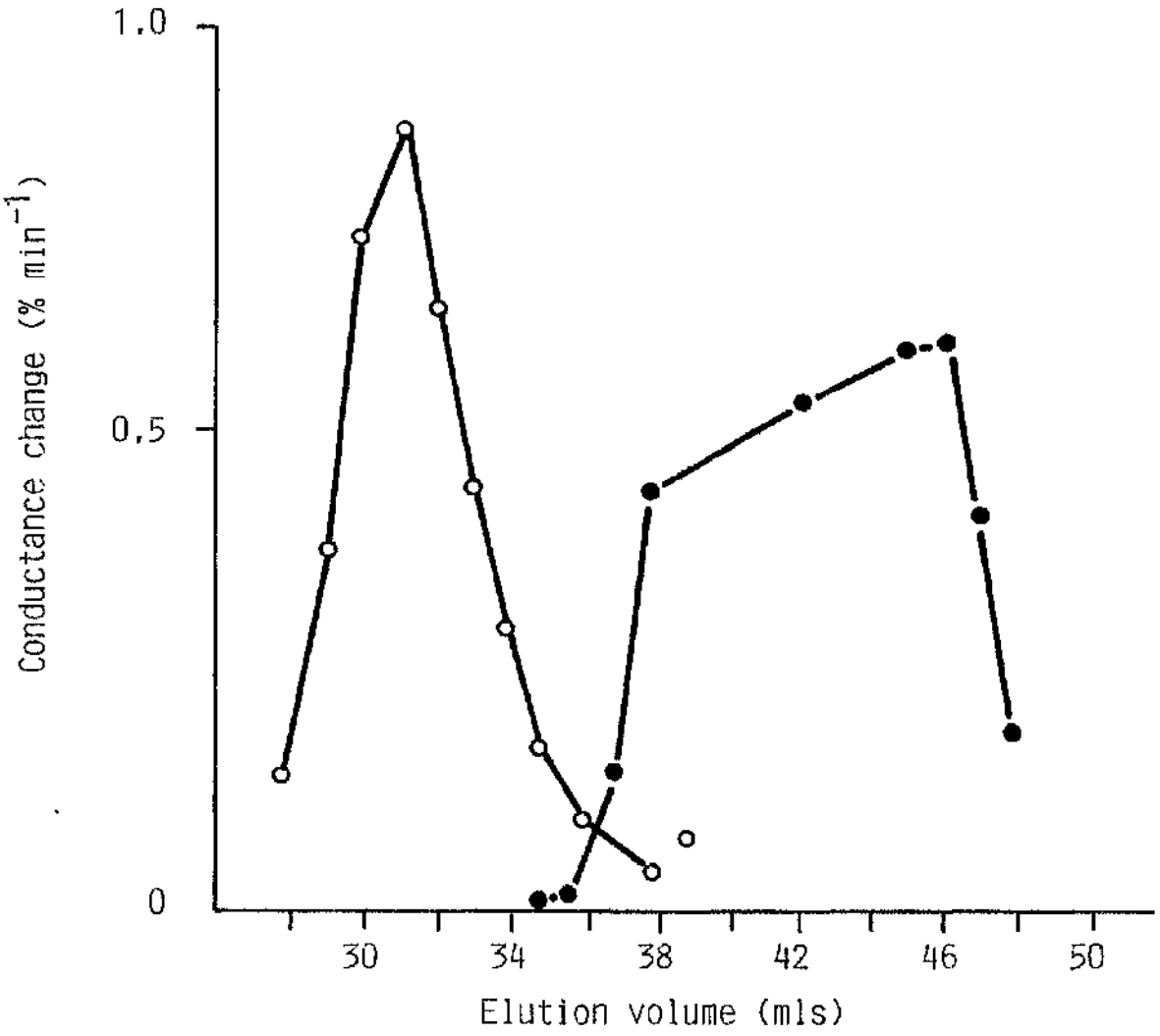
Dried bee venom was a gift from Dr. B. Banks, University College, London. The crude venom contains several high and low molecular components (plate 5, track 6) with the major component being melittin. Because of its direct lytic action on erythrocytes and its stimulatory effect on PLA_2 , it was of special importance to separate PLA_2 from this peptide. Purity of fractions collected was followed by both SDS polyacrylamide gel electrophoresis (Laemmli, U.K.,1970) and using acid urea gels (chapter 2, section 13). Although both methods could be used, acid urea gels proved to be quicker, easier to use and technically more satisfactory. Recovery of PLA_2 activity was followed using micellar dinonanoyl phosphatidyl choline as substrate. This substrate produced the most linear curves and the response was not affected by the presence of detergents.

Based on previous studies, (Drainas, Ph.D., 1978) we employed gel filtration and ion exchange chromatography. PLA_2 was

purified on columns of either Bio-Gel P-30 or Sephadex-G75. Fig.1 shows a typical elution profile on P-30 and plates 3 & 4 shows a typical acid urea gel of the results obtained. Although the separation of PLA_2 from melittin was good, the enzyme still remained associated with another component running just ahead of it. When the fractions containing high PLA_2 activity were pooled, the total yield of PLA_2 activity was always higher than the amount added to the column, with typically about 200% recovery. This was unexpected as melittin is reported to stimulate PLA_2 activity and this suggested that the venom also contains a PLA_2 inhibitor which had been removed during the purification. Complete purification of PLA_2 by this method should be possible, but would require repitition of the method many times and good separation is not compatible with good yield of activity.

Because of these problems another, hopefully more efficient method of purification was tried. Most other procedures use cation exchange steps but it was not certain that this would effect an easy separation, because both PLA_2 and melittin are very positively charged. Fig.2 shows the elution profile of crude bee venom run through a small (15x2cm) column packed with carboxymethyl cellulose. The venom was dissolved in 0.1M NaCl in bicine buffer, pH 8 (chapter 2, section 6d) and then passed through an ion exchange column using the same buffer for washing the sample through. A peak came straight through the column and nothing further was eluted until 1M NaCl in bicine buffer was added. The first peak was identified as PLA_2 and the second as melittin by gel electrophoresis and conductimetric analysis. Plate 5 shows an acid urea gel demonstrating the typical

Fig.1 Separation of PLA_2 from melittin using gel filtration.



Melittin (●—●) and PLA_2 (o—o) were detected in the erythrocyte and dinonanoyl phosphatidyl choline assays respectively.

PLATES 3 & 4.

Acid urea gels showing the main PLA_2 fractions obtained following separation of crude bee venom on Bio-Gel P-30. Tracks 12-15 are still contaminated with melittin. The later fractions show the progressive enrichmemt of another component running slightly ahead of the main PLA_2 band.

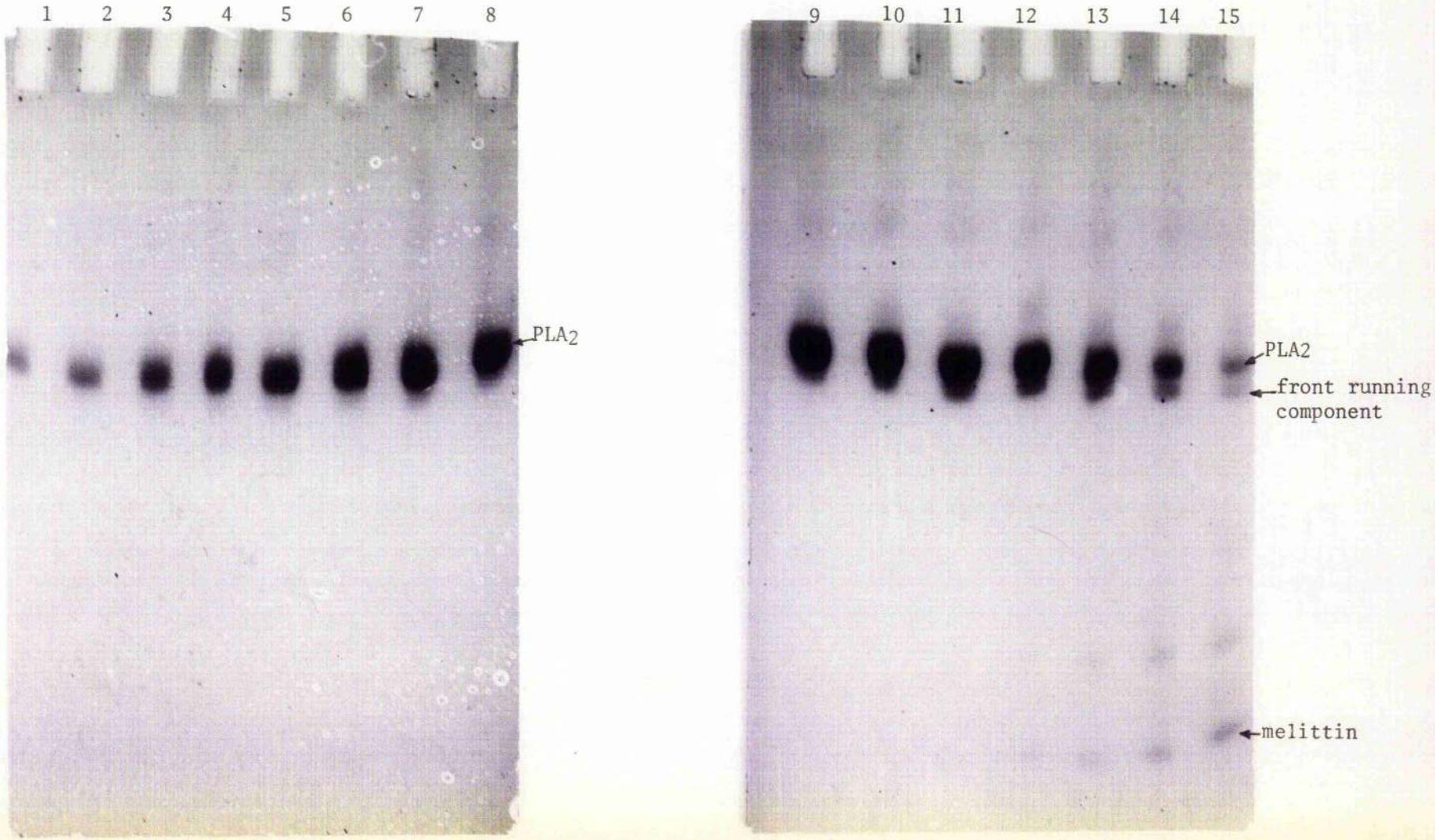

1
2
3
4
5
6
7
8
PLA2
9
10
11
12
13
14
15
PLA2
front running component
melittin

Fig.2 Separation of PLA_2 from melittin using ion exchange chromotography

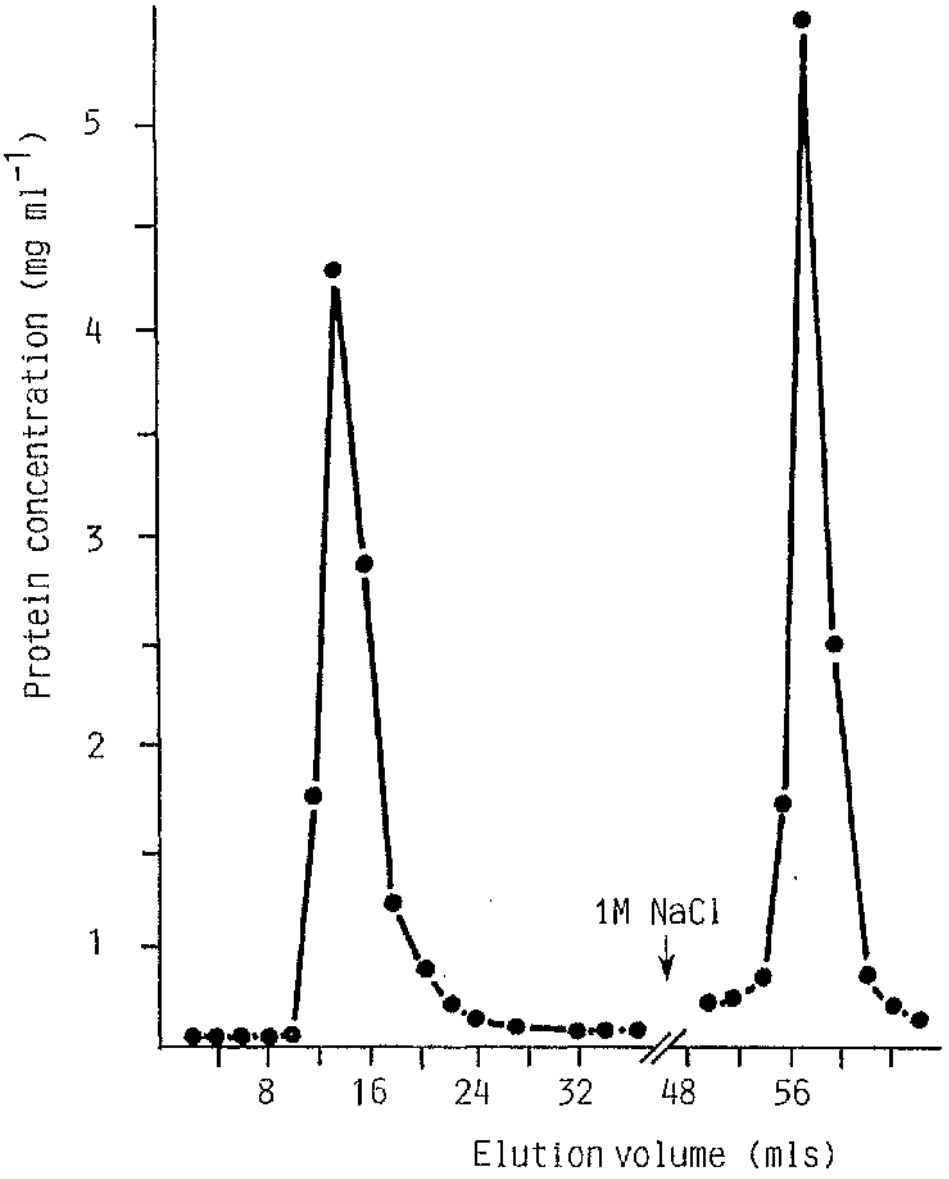
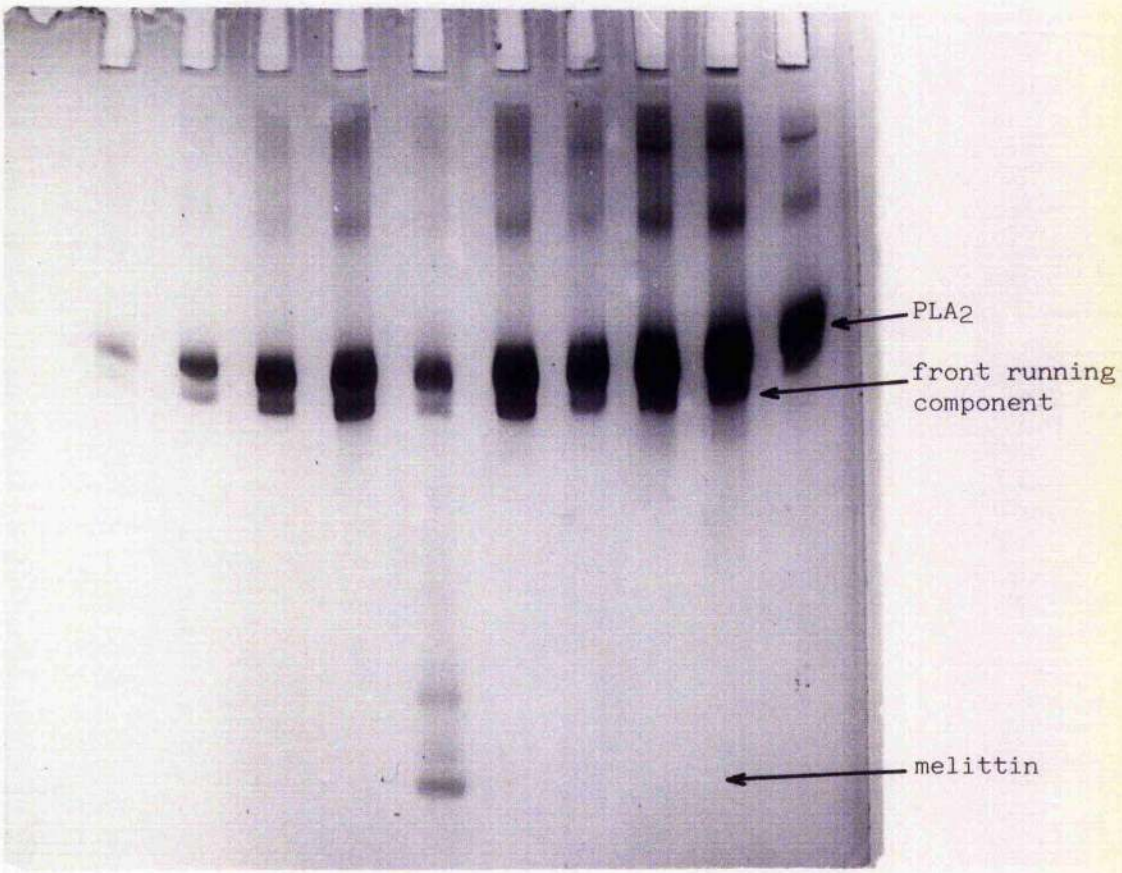


PLATE 5



Acid urea gel of PLA_2 fractions separated from melittin by ion exchange chromatography. Track 6 is the crude venom which was loaded onto the column, and all other tracks are PLA_2 fractions, free from all traces of melittin.

separation of PLA_2 from melittin obtained by this method. Fractions containing PLA_2 were totally free from any signs of melittin. Gel filtration had the disadvantage that the columns had a limited lifetime because they eventually became clogged with the pigment associated with the crude venomn. The pigment remained bound to the top of the ion exchange column and was largely, but not totally eluted with the melittin though no irreversible loss of column function was observed. This particular separation resulted in 500% recovery of activity. Although ion exchange chromatography proved to be extremely efficient in separating PLA_2 from melittin and made the ideal starting point for PLA_2 purification, both the PLA_2 and the extra front running component remained associated with each other and there was no sign of any separation by this method.

Phospholipase A_2 from bee venom has also been purified using Concanavalin A-Sepharose 4B (Gritsuk et al, 1980). Whole venom was deposited on the column and PLA_2 activity was eluted with α-D-mannopyranoside. After desalting, the fraction from this wash which contained PLA_2 activity was further purified on SE-Sephadex C-25. No evidence was presented to show whether or not the front contaminent was revealed during this procedure and it seems doubtful that the investigators were aware of its existance at all. We therefore stained gels of venom fractions for glycoprotein using both the PAS method of Parish et al, (1977), and by labelling acid urea gels with ^{125}I-Con.A and found that PLA_2 stained strongly as glycoprotein. Because the two bands were close together and the stain rather diffuse they were not

very sharply delineated making it difficult to tell if the extra component was glycosylated.

Because the component associated with PLA_2 runs only slightly ahead of it, it was possible that this was an unglycosylated form of the enzyme and might be separated from the PLA_2 by this affinity method. Fig.3 shows a typical experiment in which PLA_2 initially purified on CM cellulose, was passed through a Con.A Sepharose column (chapter 2, section II) and plate 6 shows an acid urea gel of the peaks obtained. A small peak came straight through the column and this contained both PLA_2 and the front component, however the second peak obtained by elution with α-methyl-mannoside, was totally free of the front running component. The fractions from the first peak were pooled and processed by a second passage down the column. Plate 7 shows an acid urea gel of both peaks obtained. The first peak which passed freely through the column was now free from the major PLA_2 component (tracks 1,2 &3) yet still contained PLA_2 activity. The second peak was high in PLA_2 activity and again ran as a single band slightly further behind. PLA_2 has an unusually high pI (10.5) yet attempts to separate the two proteins by isolelectric focussing failed possibly suggesting that the two polypeptides are very similar.

Finally, high molecular weight glycosylated components accompanying PLA_2, after separation on Con-A sepharose could be removed by a single run through a gel filtration column. In plate 6 track 10, an acid urea gel of highly purified PLA_2, which was used for the experiments in this thesis can be seen.

In an attempt to combine the gel filtration step with

Fig.3 The separation of PLA_2 from non-glycosylated proteins on Con.A Sepharose -4B.

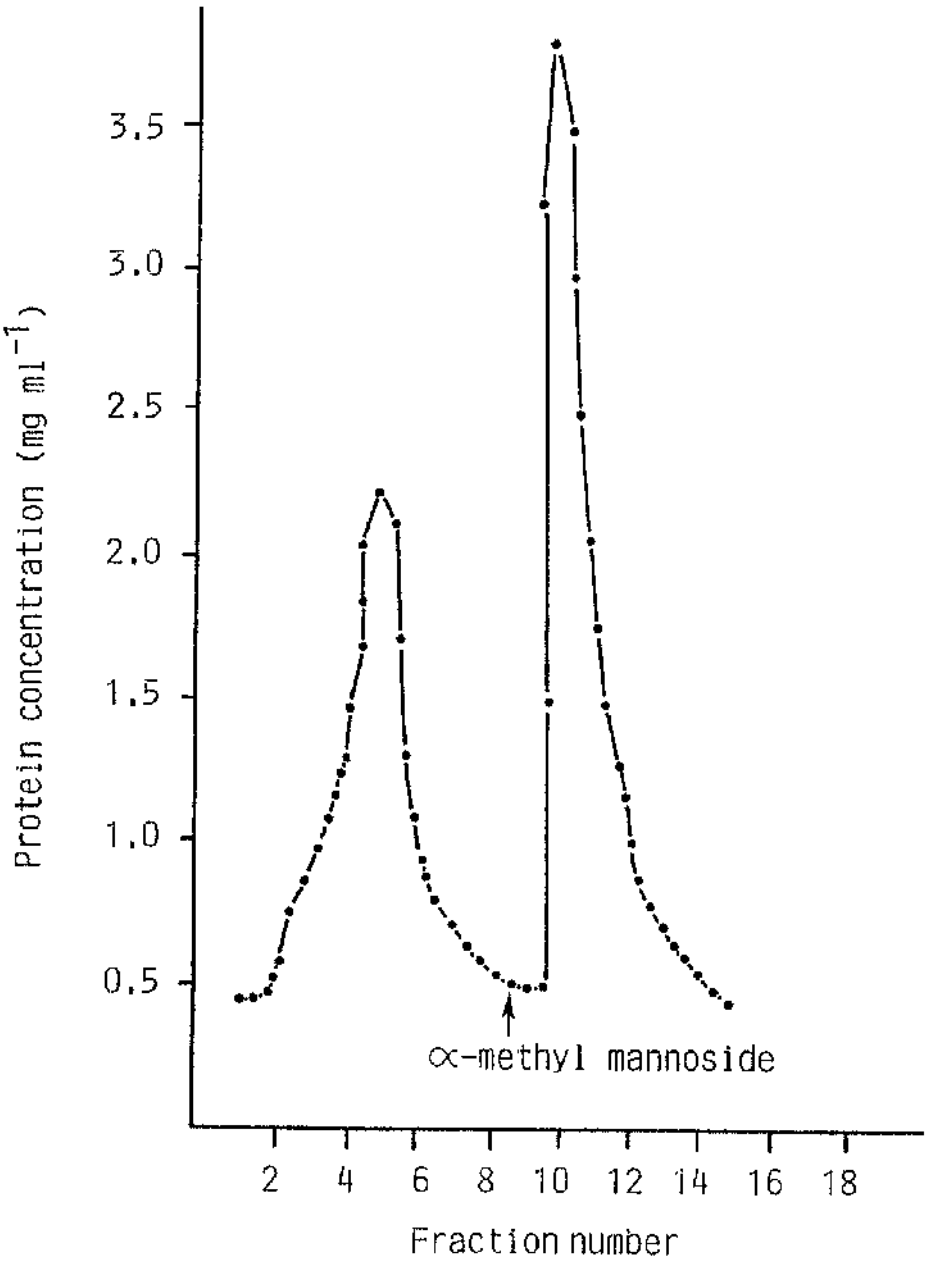


PLATE 6

Acid urea gel of fractions obtained from a Con. A Seharose-4B column. The bee venom sample loaded onto the column had been partially purified by ion exchange chromatography. Tracks 2,5,7 & 9 are the fractions which came straight through the column and contain both PLA_2 and the extra front running component. Tracks 6,8 & 10 are the fractions eluted with α-methyl-mannoside and contain PLA_2 free from the front component.

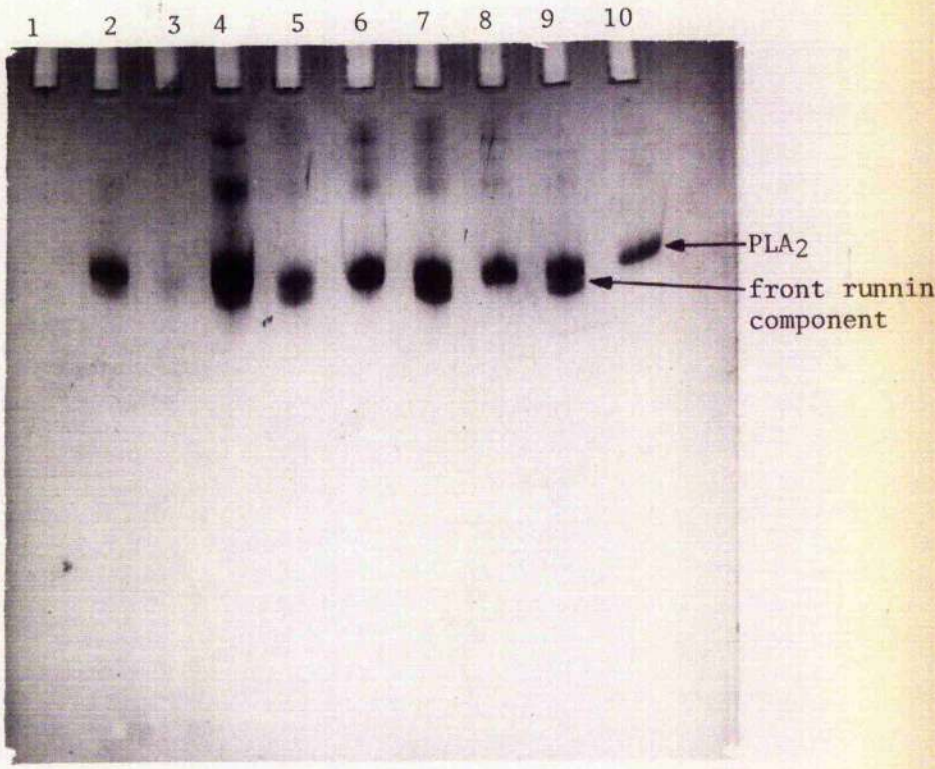
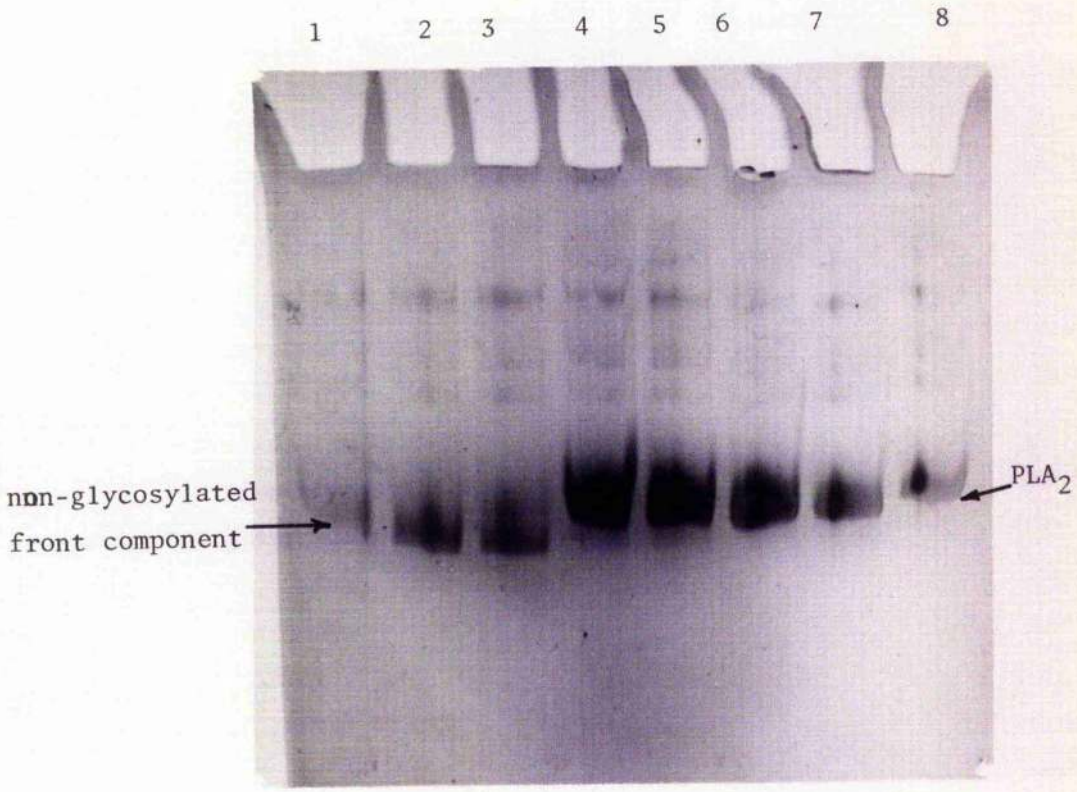


PLATE 7

Acid urea gel showing the separation of the major PLA_2 band (tracks 4,5,6,7,& 8) from the front running non glycosylated component (tracks 1,2,& 3). The separation was achieved using Con.A Sepharose-4B.



glycoprotein separation, PLA_2 was mixed with Con.A and run through a column of Bio-Gel P-30. It was hoped that the PLA_2 which binds to Con.A would now be much larger than the front running component and run well ahead. This expected separation was not obtained but an acid urea gel of the broad peak obtained is shown in plate 8 because it showed the best resolution of the two components seen in this work.

As the crude venom is obtained from pooled venom, it was of interest to see whether PLA_2 and the front running component were present in venom taken from a single honey bee or if some bees contained one component and some the other. Plate 9 (tracks 5,6,13 &14) shows half the contents of a honey bee venom sac in each track and track 9 contains a sample of the standard crude venom. The front component was clearly visible in all samples suggesting that both components were present in all bees.

Evidence for covalent binding of oleoyl imidazolide using gel electrophoresis and fluorography.

There is no doubt that PLA_2 is covalently modified by oleoyl imidazolide and further proof of this was shown by the following experiments. ^3H labelled oleoyl imidazolide was added to PLA_2 and the sample was then run on either SDS-PAGE or acid/urea gels. After the gel was stained it was prepared for autoradiography by the fluorographic method of Chamberlain, J.P. (1979).The initial results obtained with acid urea gels often showed spots on the autoradiograph at the top of the gel wells.

PLATE 8

Acid urea gel of fractions from a Bio-Gel P-30 column, showing the best resolution of PLA_2 and the non-glycosylated front running component obtained.

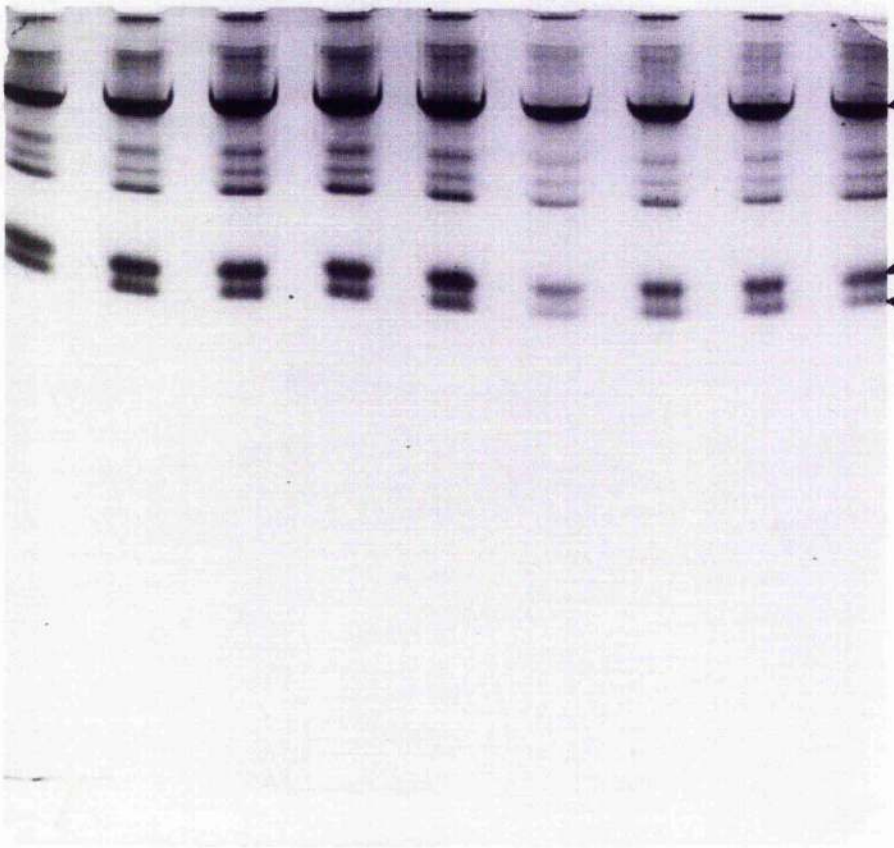
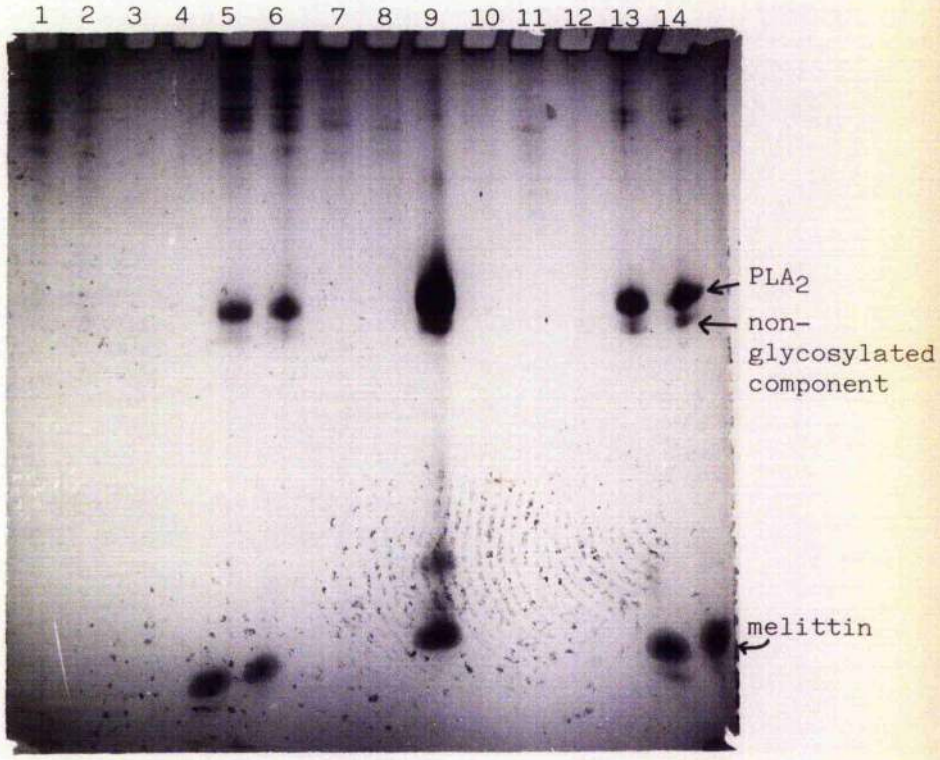


PLATE 9

Acid urea gel showing pooled crude venom (track 9) and the contents of individual honey bee sacs (tracks 5,6, 13 & 14). The front non-glycosylated component and the major PLA_2 band is clearly visible in all samples.



To check the possibility that the enzyme was not dissociated from the activator under acid conditions, activated PLA_2 was dissolved in 5% acetic acid and then assayed in the erythrocyte system. The activated enzyme still retained all of its activity showing that the activator remained bound to the enzyme. It was also possible that acylation could cause the enzyme to self-associate and then prevent it from entering the gel. To test this, PLA_2 was treated with oleoyl imidazolide and when the enzyme was fully activated, native PLA_2 was added and the sample was loaded onto a gel filtration column. No evidence for enzyme aggregation, which would have been detected by more than one peak of PLA_2 activity, was obtained by this method.

Plate 10 shows an autoradiograph of an SDS-PAGE gel developed after four weeks. Tracks 1,2 4,6,8 & 10 contained unlabelled PLA_2 containing some high molecular weight components, tracks 3,5,7 & 9 contained the same sample but treated with oleoyl imidazolide ($0.5x10^6$ counts per minute), tracks 11 & 12 contained unlabelled crude venom and finally track 13 contained crude venom labelled as above. In this gel PLA_2 was clearly labelled with the reagent but radioactive spots showed up along the bottom of the gel and this must represent free oleic acid. Plate 11 shows an autoradiograph of an SDS gel typifying this. Tracks 1,2,3,4 & 5 contain free activator ($1.5x10^6$ cpm), tracks 6,8,10,12 & 14 unlabelled PLA_2 and tracks 7,9,11 & 13 PLA_2 labelled with oleoyl imidazolide (1.5x10 6cpm). After exposure for 4 days most of the spots ran alongside the free oleic acid however in this case, some faint traces of spots were seen in the area where PLA_2 runs.

PLATE 10

Autoradiograph of SDS PAGE gel. Tracks 1,2,4,6,8 & 10 contained unlabelled PLA_2, tracks 3,5,7 & 9 contained the same, but labelled with ^{3}-H oleoyl imidazolide (0.5 x 10^6 cpm), tracks 11 & 12 contained unlabelled crude venom and finally track 13 contained crude venom labelled as above.

PLATE 11

Autoradiograph of SDS gel. Tracks 1,2,3,4 & 5 contained free oleoyl imidazolide (1.5 x 10^6 cpm), tracks 6,8,10,12 & 14 unlabelled PLA_2 and tracks 7,9,11 & 13 PLA_2 labelled with oleoyl imidazolide (1.5 x 10 cpm). The autoradiograph was exposed for 4 days.

PLATE 12

As in plate 11 but the autoradiograph was exposed for 1 week.

PLATE 11

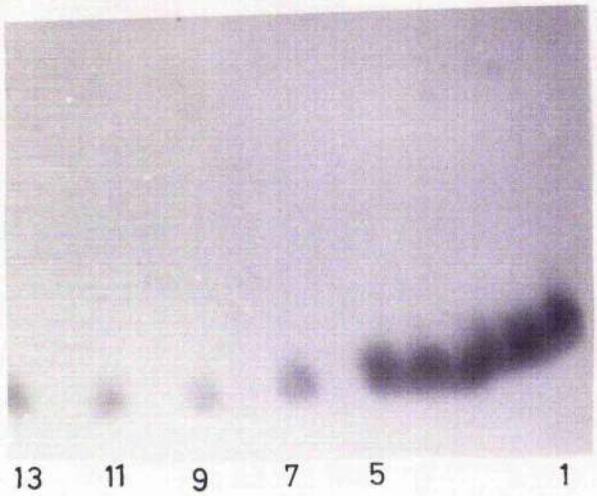


PLATE 12

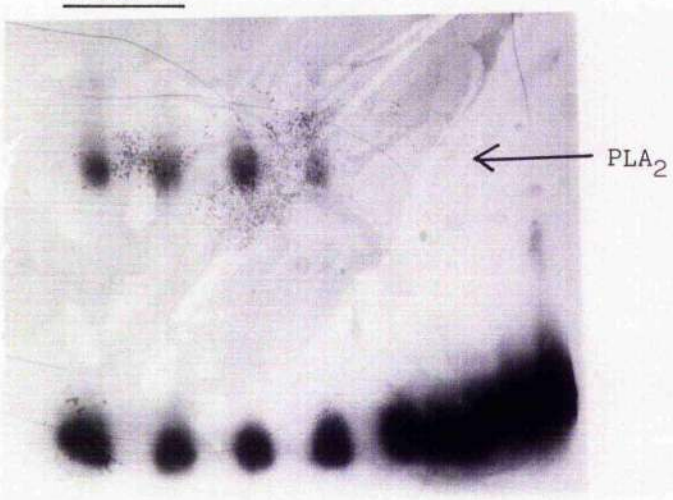
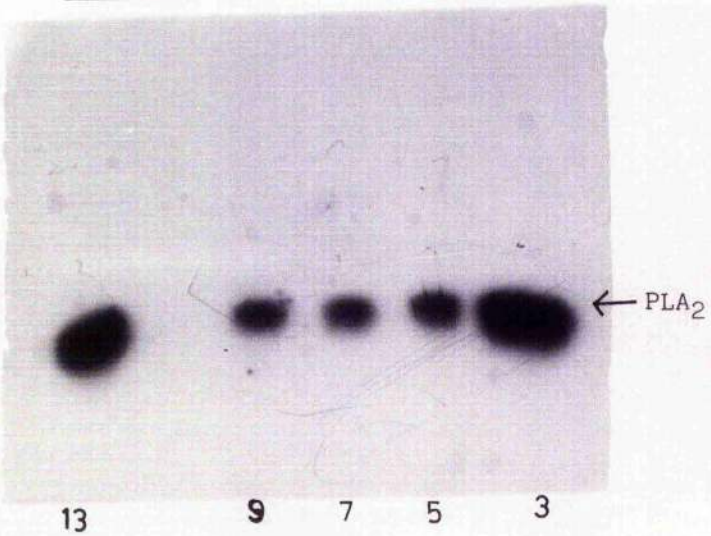


PLATE 10



The autoradiograph was re-exposed for one week and this time spots showed up where the PLA_2 runs on the gel (PLATE 12)

Fluorography is reported to be an extremely sensitive technique and the number of counts used in these experiments would have been expected to show up within 24 hours. It could be that the spots associated with the PLA_2 actually represent a fraction of the total activity layered onto the gel or that the stoichiometry of activation is not as clear as originally thought.

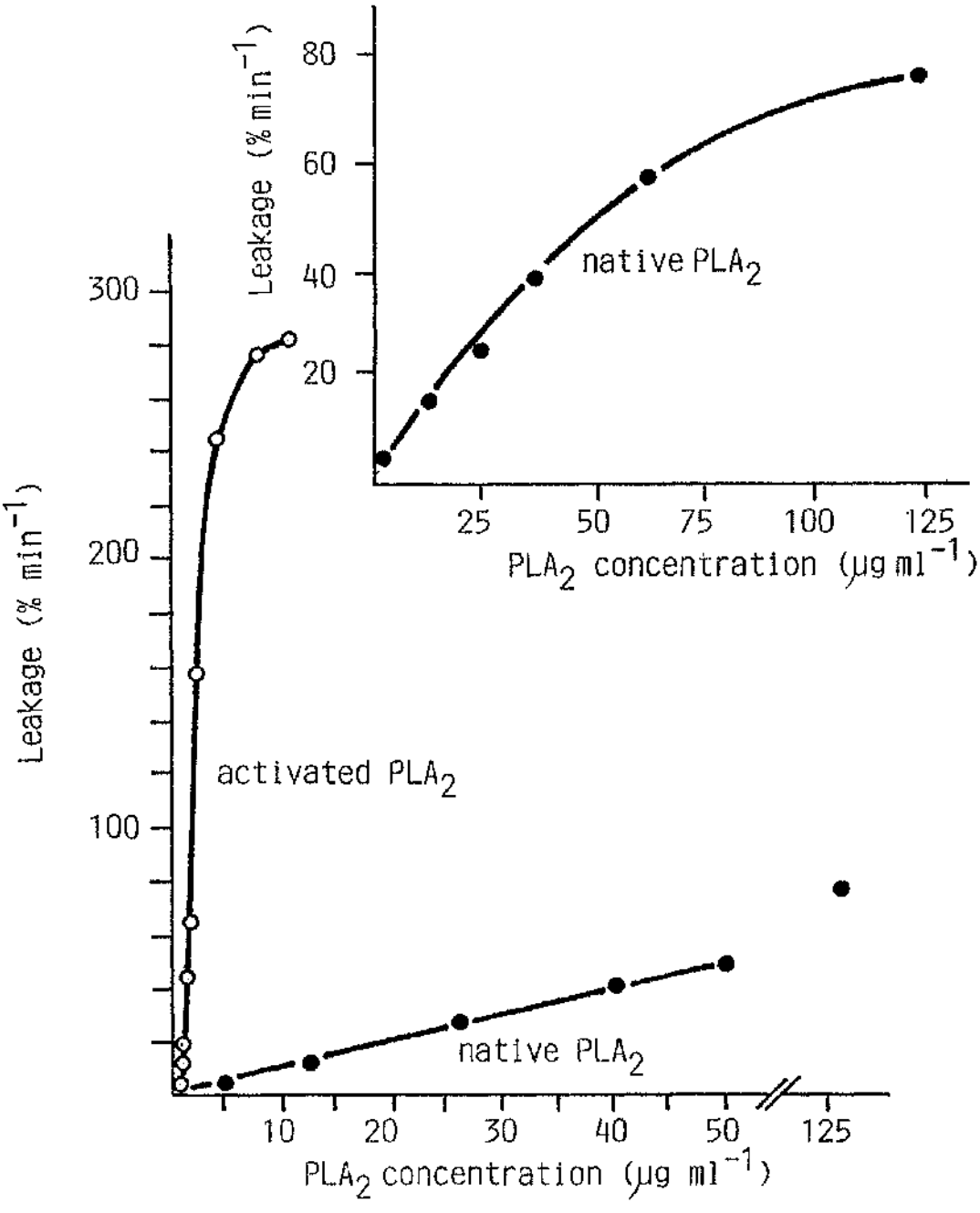
Despite some of the technical problems with this method the experiments presented here further confirms that oleoyl imidazolide can covalently bind to PLA_2. When oleic acid instead of oleoyl imidazolide was used, spots were never detected in association with the enzyme.

The measurement of activation.

The addition of either normal or activated bee venom PLA_2 to erythrocytes results in a small amount of leakage from the cells. If albumin is added to the reaction mixture before the enzyme, leakage from the cells is greatly increased, and activated, but not normal enzyme lyses the cells. Both the erythroctye and egg phosphatidyl choline assays show a slow phase which then changes to a rapid increase in conductance. With egg phosphatidyl choline the latter phase is due to product activation but it is not clear what this is due to in the erythrocyte assay, certainly not fatty acid activation since albumin is present in the reaction mixture. Activation is not a straightforward effect and can only be detected under certain assay conditions for example the activated enzyme is only slightly more active towards dihexanoyl and dinonsnoyl phosphatidyl choline (ca.1.5 fold) than the native enzyme and use of this was made throughout the work to standardise activated against native enzyme. The activated PLA_2 only seems to be more active than the native enzyme on liposomal substrates and bilayers.

In all chemical assays the dose response curves for normal and activated enzyme were linear but this was not true for the erythrocyte assay. Fig.(4) shows the effect of varying the concentration of native and activated PLA_2 on the maximum rate of leakage from erythrocytes in the presence of albumin. Both curves show apparent saturation suggesting that the native enzyme could never reach the level obtained by the activated enzyme and that maximal activity of the activated enzyme was about four times

Fig.4 The effect of varying the concentration of normal and activated PLA_2 on the leakage of erythrocytes in the presence of albumin



that of the native enzyme. At low concentrations the apparent degree of activation is very much larger and activation factors in excess of thirty fold could be measured. This appears to be analogous to Vmax and Km factors and it is possible that the effect of activation is to raise the affinity of the enzyme for the cells. Kinetic analysis of enzyme activated with oleoyl imidazolide and assayed on dioleoyl phosphatidyl choline showed that activation was almost entirely determined by enhancement of Vmax (53fold) with a small favourable modification of Km (0.13m to 0.1m), Drainas & Lawrence, 1978).

The pH dependence of activation.

One of the aims of investigation with activated enzyme was to try to elucidate the nature of the group which binds the activator and to characterise the changes produced in the enzyme structure. The first approach was to study pH dependence of activation kinetics. The erythrocyte assay (chapter 2, section 8b) is the most convenient method of detecting activation, firstly because the presence of albumin eliminates any effects attributable to substrate mediated phenomena and secondly because the variable quantity, the maximum rate is the easiest to measure accurately. The enzyme (1mg/ml) was incubated in 10mM Mes buffer for the pH range 5.5 to 7.0 and in triethanolamine buffer to obtain pH 8.0. Oleoyl imidazolide was added to give a 1:1 ratio of enzyme to activator. 2 µl samples were withdrawn at various time intervals and added to the assay system. The rate of the reaction at each time point was determined and this was repeated for each pH

tested. The slope of the best line obtained for each set of times was then plotted against pH. The rate of activation was maximal around pH8 and the pH dependence followed a sigmoidal curve (fig.5) with an inflection point around pH 6.5, suggesting that activation is controlled by a group with a pK of about 6.5.

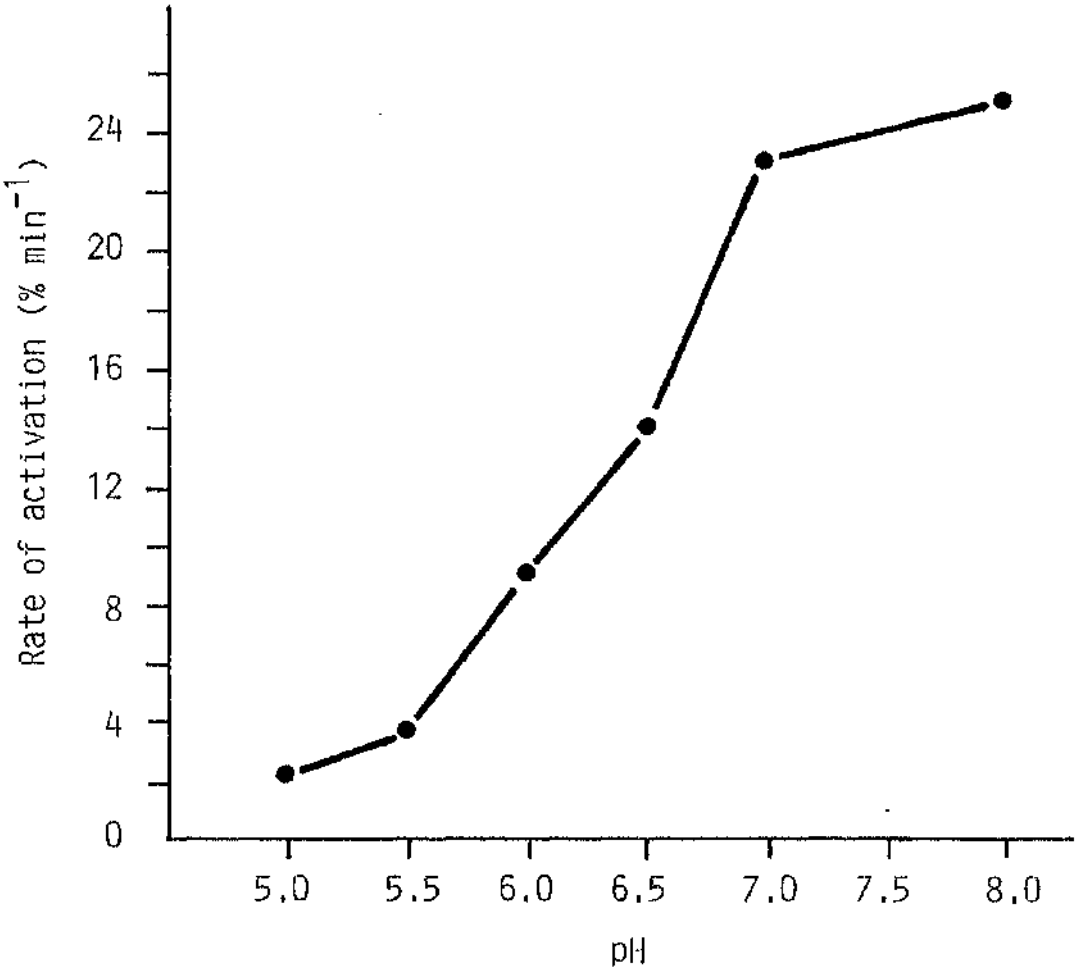
The stoichiometry of activation.

Fig.6 shows the effect of up to 5 fold molar excess of oleoyl imidazolide on the response curve of activated PLA$_2$ in the erythrocyte assay. Each point represents the the maximun rate of leakage at a particular time point when 1.75µg of enzyme was added. Thus the enzyme appears to require one molecule of oleoyl imidazolide for complete activation.

The effect of lysophosphatidyl choline on normal and activated PLA$_2$

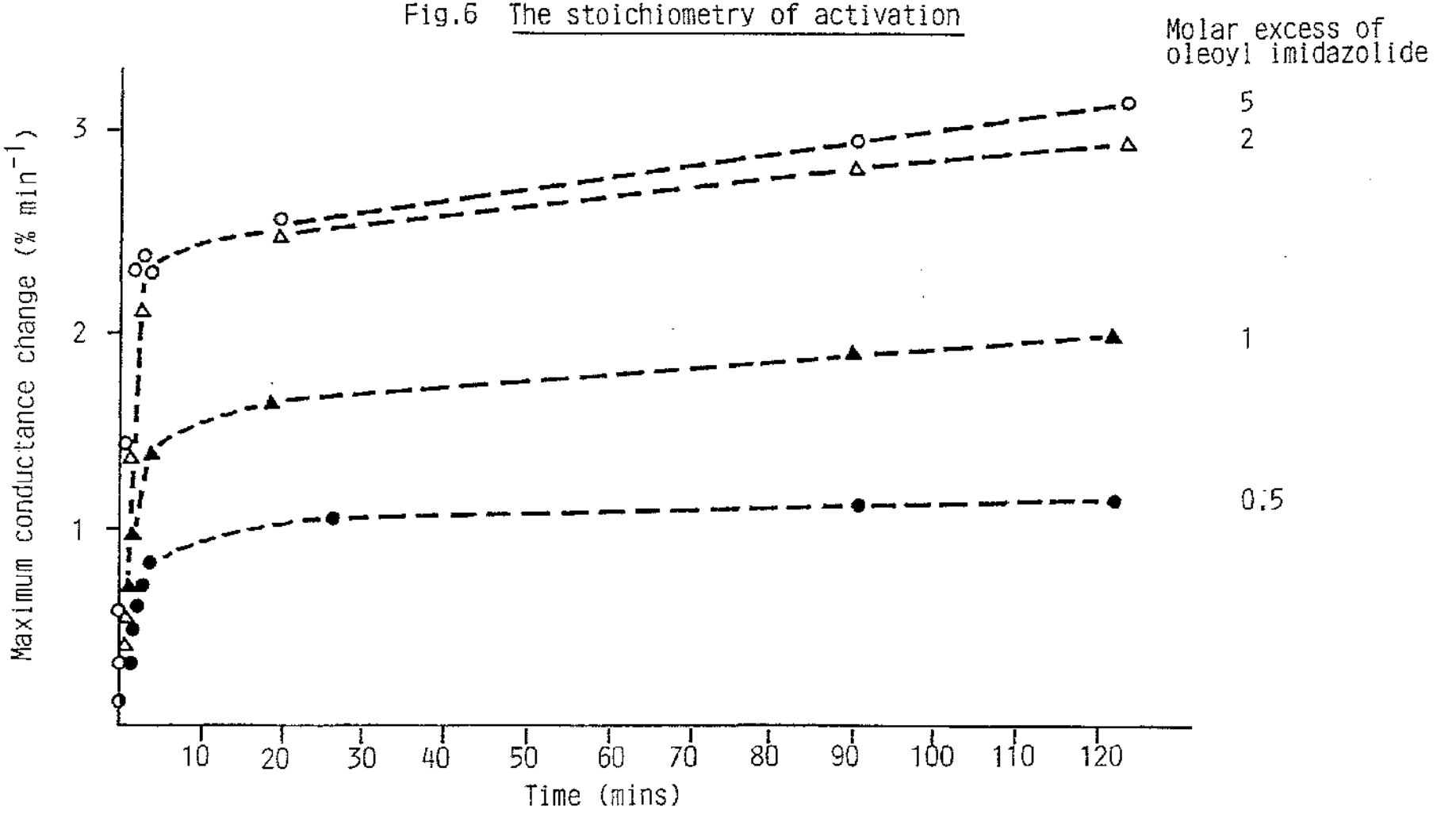
Because high levels of lysophosphatidyl choline (10^{-9} µg/cell) cause erythrocyte lysis, it has often been assumed that lysis caused by PLA$_2$ attack is due to the accumulation of lytic products in the membrane (chapter 1). In the presence of albumin both fatty acids and lysophosphatidyl choline are continuously removed from the membrane and could never accumulate in high enough levels to cause cell lysis. Normal PLA$_2$ is inhibited by lysophosphatidyl choline in the erythrocte assay. It is possible that the large effect of activated enzyme was due to it being insensitive to product inhibition. This emphasises the fact that

Fig.5 The pH dependence of activation



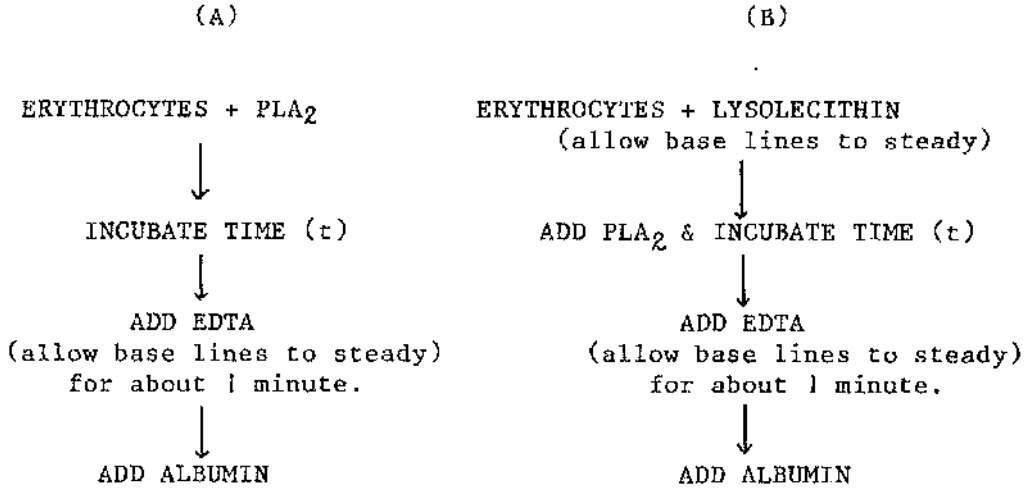
The rate of activation of PLA_2 by oleoyl imidazolide was determined by assaying incubation mixtures over a period of 3 minutes. The initial slopes of curves of activity against time, were then plotted as a function of pH

Fig.6 The stoichiometry of activation



PLA_2 was incubated with different concentrations of oleoyl imidazolide. The rate of activation of the enzyme, for each concentration of oleoyl imidazolide, was determined in the erythrocyte assay, over a period of 2 minutes

the immediate cause of PLA_2 stimulated leakage is not known though one intriguing possibility is that it is assocciated with the act of removal of fatty acids from the membrane. If sublytic doses of fatty acids are added to cells and subsequently removed with albumin, their extraction results in leakage of a certain amount of the cell contents. This leakage is dependent on the quantity of fatty acids added but in a highly non-linear fashion with a quite distinct lower concentration threshold. The ability of albumin to extract reaction products and give a response indicative of the amount of product in the membrane was employed in the following experiments. The basic outline of these experiments is shown in the scheme below.



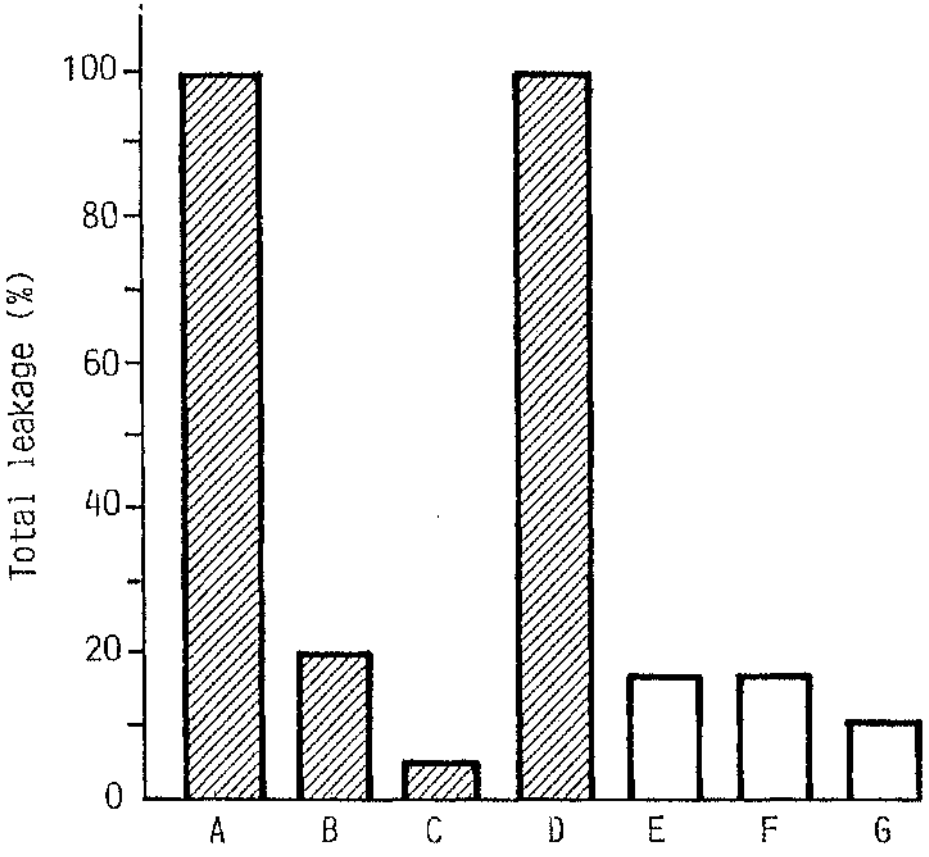
The effect of lysophosphatidyl choline on normal and activated PLA_2 was investigated in the erythrocyte assay system using 2.5µg of PLA_2 in the erythrocyte suspension. The enzyme was incubated for 2' before addition of 10µl of 100mM EDTA. When used, lysophosphatidyl choline (1µg) was added before PLA_2. A control response produced by addition of albumin added to incubated cells

alone was subtracted from all results. Fig.7 shows that activated enzyme produced a much larger response in the assay than normal enzyme but both activated and normal PLA_2 were strongly inhibited by lysophosphatidyl choline. If lysophosphatidyl choline was added after exposure to activated enzyme but before addition of albumin it had no effect on the leakage response suggesting that lysophosphatidyl choline can prevent the accumulation of fatty acids in the membrane but once they have accumulated it cannot prevent the changes that accompany their removal by albumin. An essential control experiment was carried out where activated enzyme was added to cells which had been treated with normal PLA_2 then inhibited with EDTA to show that the response was not due to any direct lytic effects of the activated enzyme. These experiments show quite conclusively that modified enzyme does not depend on the presence of albumin to show activation in the erythrocyte system.

Fig.(8) shows the leakage response when albumin was added to cells treated for increasing time lengths with native enzyme (5μg) and on cells which were preincubated with 1μg of lysophosphatidyl choline. The response curve to native enzyme suggests that initially the products accumulate very rapidly and the reaction is very slow thereafter.

Similar results were produced with activated enzyme but accumulation of the products was much faster and the level reached was higher. Using the same method (1μg of PLA_2), inhibition by lysophosphatidyl choline was studied in more detail and time courses of product accumulation in the presence of

Fig.7 The effect of lysolecithin on the leakage of erythrocytes in response to albumin, after treatment with native or activated PLA_2



2.5µg of either normal or activated PLA_2 was added to erythrocytes for 2', before the addition of EDTA. Total leakage on addition of albumin was then measured

A: Activated PLA_2

B: Activated PLA_2 (cells preincubated with 1µg lysolecithin)

C: Activated PLA_2 (EDTA added before the enzyme)

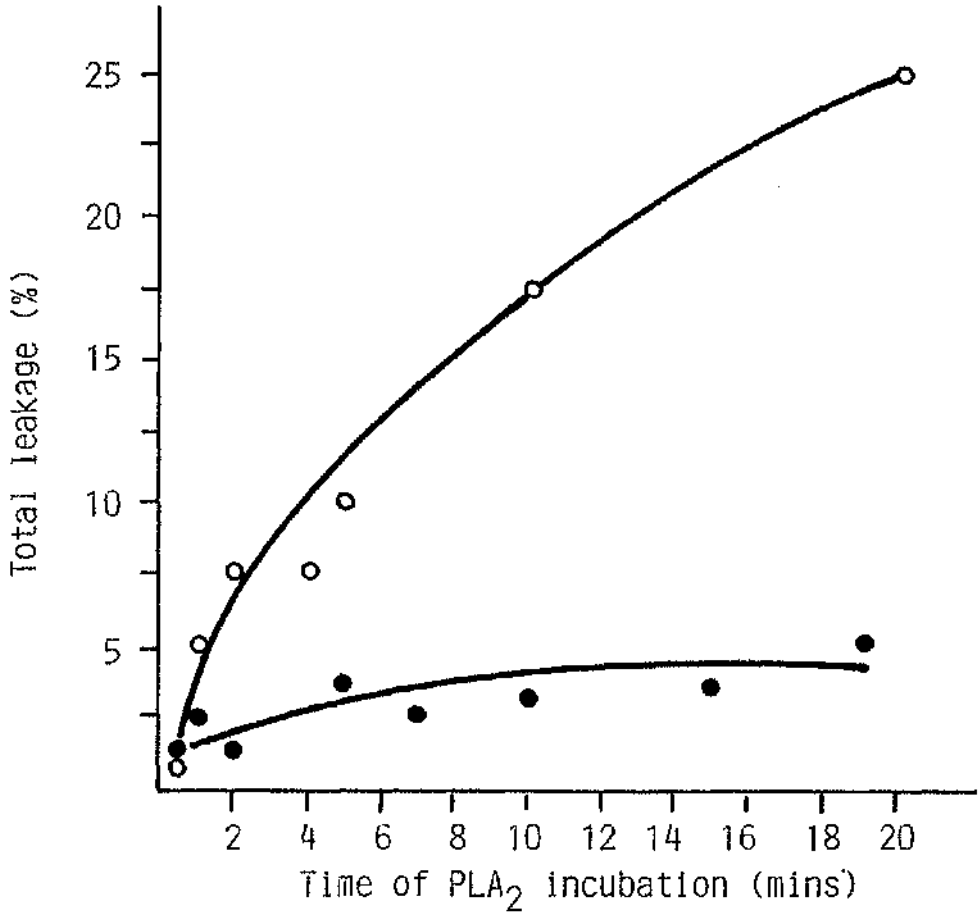
D: Activated PLA_2 (lysolecithin added before addition of albumin)

E: Activated PLA_2 added after treatment F

F: Native PLA_2

G: Native PLA_2 'preincubated with lysolecithin)

Fig.8 The effect of lysolecithin on the "accumulation of reaction products" in the membrane, determined by the leakage response on addition of albumin.



Erythrocytes were incubated with native PLA_2 (5µg), in the presence (●—●) and absence (o—o) of 1µg of lysolecithin. The total amount of leakage on addition of albumin was determined as a function of incubation time.

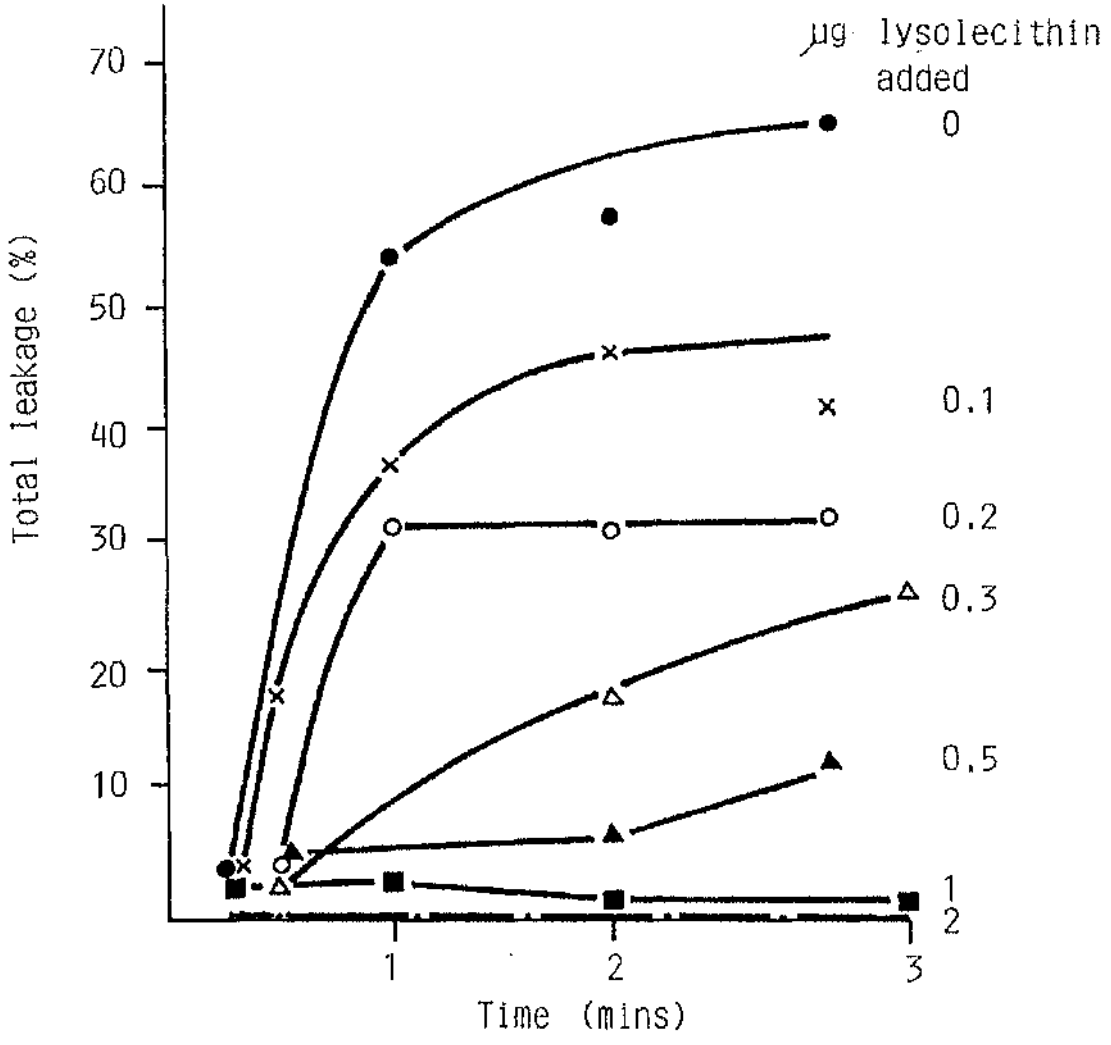
different amounts of lysophosphatidyl choline are shown. The total increase in leakage after the addition of albumin was plotted, showing that there is a dose dependent inhibition, with 1µg producing maximum inhibition (fig.9).

The affinity of native and activated PLA_2 for membranes.

Some of the results suggest that activation increases the affinity of the enzyme for cells. Experiments were designed to find out what proportion of normal and activated PLA_2 binds to cells, determined from the amount remaining in free solution. For experiments with normal PLA_2, 25µl (1mg/ml) was added to cells, left for one minute, then one ml of cells was removed and pelleted in a micro centrifuge. Under these circumstances no lysis occured. 100µl of the supernatant was then assayed on dinonanoyl phosphatidyl choline substrate. With activated PLA_2, 10µl of PLA_2 was used instead because 25µl caused some cell lysis. The results expressed as conductance change/minute (and in parenthesis % of activity bound) on the table below shows the mean of three experimental assays and were compared with the amount of activity when the enzyme was added to buffer alone.

	NORMAL PLA_2	ACTIVATED PLA_2
BUFFER ALONE	2%/min (100%)	1.4%/min (100%)
BUFFER + CELLS	2%/min (100%)	1.32%/min (94%)

Fig.9 The effect of lysolecithin concentration on the "accumulation of reaction products" in the membrane



Activated PLA_2 (1µg) was incubated in the presence and absence, of different concentrations of lysolecithin. The total amount of leakage on addition of albumin was determined as a function of incubation time

The results show that the normal enzyme does not appear to be strongly bound to the cells. With the activated enzyme again most of the enzyme appears to be in free solution but this time some of the PLA_2 had remained bound to the cells.

The effect of erythrocyte concentration on (a), the response to PLA_2 and (b), inhibition by lysophosphatidyl choline.

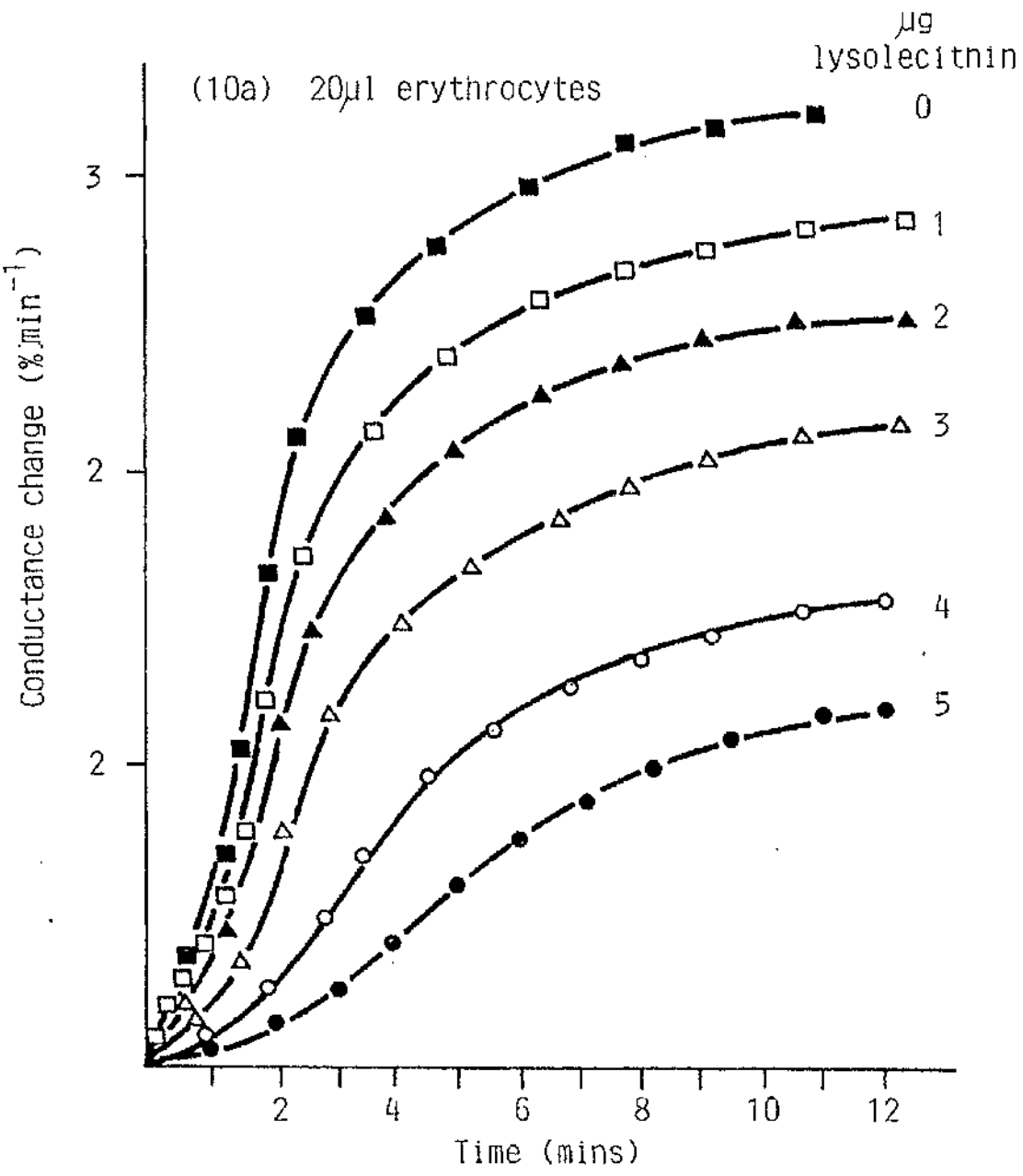
(a)

The effect of cell concentration on the activity of activated PLA_2 was investigated (fig.10). If the enzyme has a very low affinity for the cells then as the cell concentration increases, the conductance change due to PLA_2 attack would be expected to increase in proportion, whilst if the enzyme has a vey high affinity then all the enzyme would be bound at all cell doses and response rates might be independent of cell concentration. Interestingly enough, when the effect of varying cell concentration was measured, the results fell between these extremes.

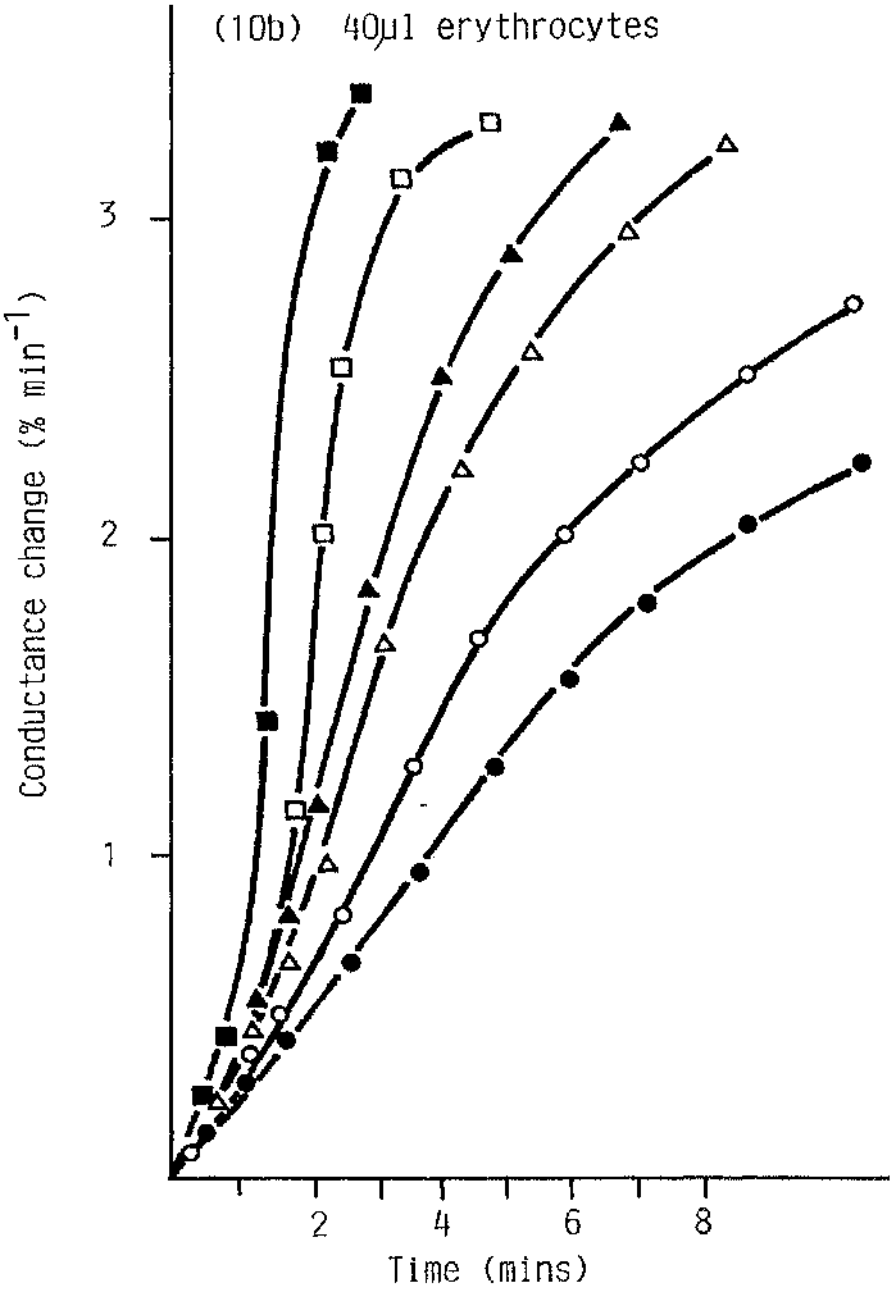
(b)

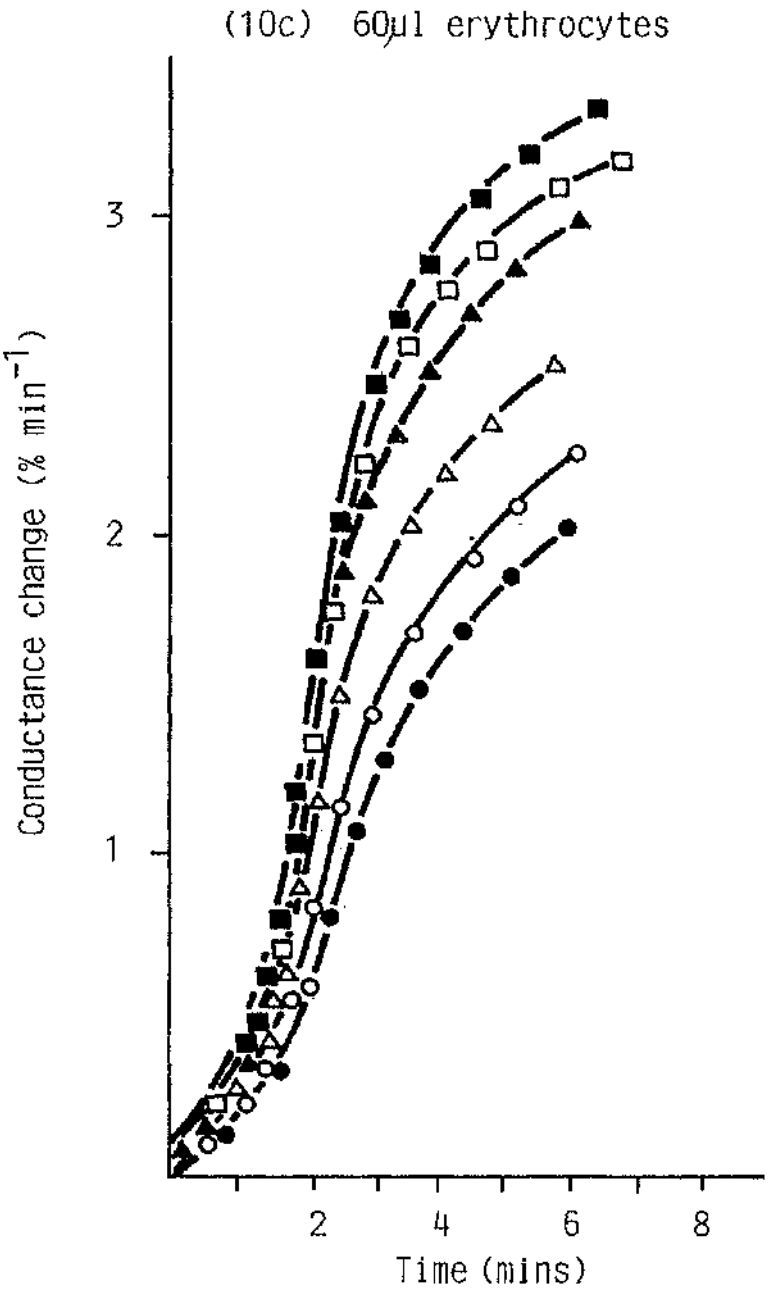
The effect of increasing the number of cells on lysophosphatidyl choline inhibition was investigated (fig.10). In these experiments 20 40 or 60µl of erythrocytes were incubated with albumin and then the appropriate quantity of lysophosphatidyl choline was added. Inhibition became progressively smaller as the quantity of cells was increased. These results could be explained

Fig.10 The effect of erythrocyte concentration on the response to activated PLA_2, and on inhibition by lysolecithin



Erythrocytes (20ul, fig.10a; 40ul, fig.10b and 60μl, fig.10c) were incubated in the presence and absence of different lysolecithin concentrations. The leakage response on addition of activated PLA_2 was then determined


(10b) 40µl erythrocytes
Conductance change (% min^{-1})
3
2
1
2
4
6
8
Time (mins)


(10c) 60µl erythrocytes
Conductance change (% min^{-1})
3
2
1
2
4
6
8
Time (mins)

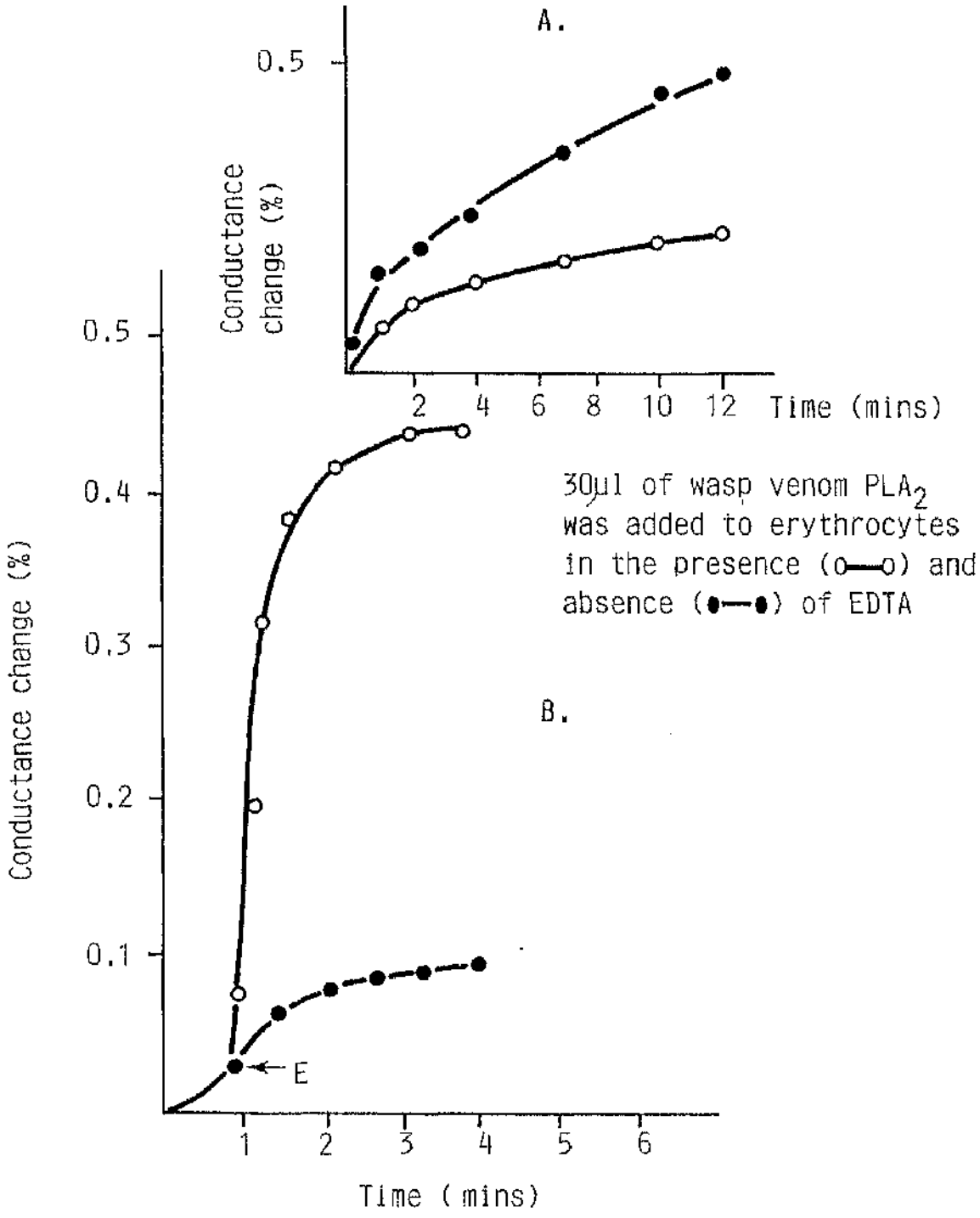
by the albumin and cells being in competition for the lysophosphatidyl choline.

The activation of wasp venom PLA_2

It was of interest to find out if the activation of bee venom PLA_2 was shared by other phospholipases A_2. Preliminary experiments on assorted bumble bees confirmed many of the observations with honey bees. Another suitable insect source of PLA_2 is wasp venom (section 2:12). The venon sac contents were assayed on dinonanoyl phosphatidyl choline substrate. On average each sac appeared to contain about 1/4 of the activity of bee venom enzyme. Fig.(11a) shows the effect of 30 µl of wasp venom on the leakage of erythrocytes in the presence and absence of EDTA. The cells treated with wasp venom showed an increase in leakage compared with those which had been preincubated with EDTA. Most of the increase in conductance in the presence of EDTA was shown to be due to salts in the venom. Under the conditions of this assay no direct lytic activity could be detected. Fig.(11b) shows the increase in conductance produced by the addition of albumin to cells which had been incubated with 30 µl of wasp venom for 15' either in the presence or absence of EDTA. Like the bee venom enzyme, reaction products can accumulate in the membrane when the enzyme is not inhibited by EDTA and these can be detected by removal with albumin.

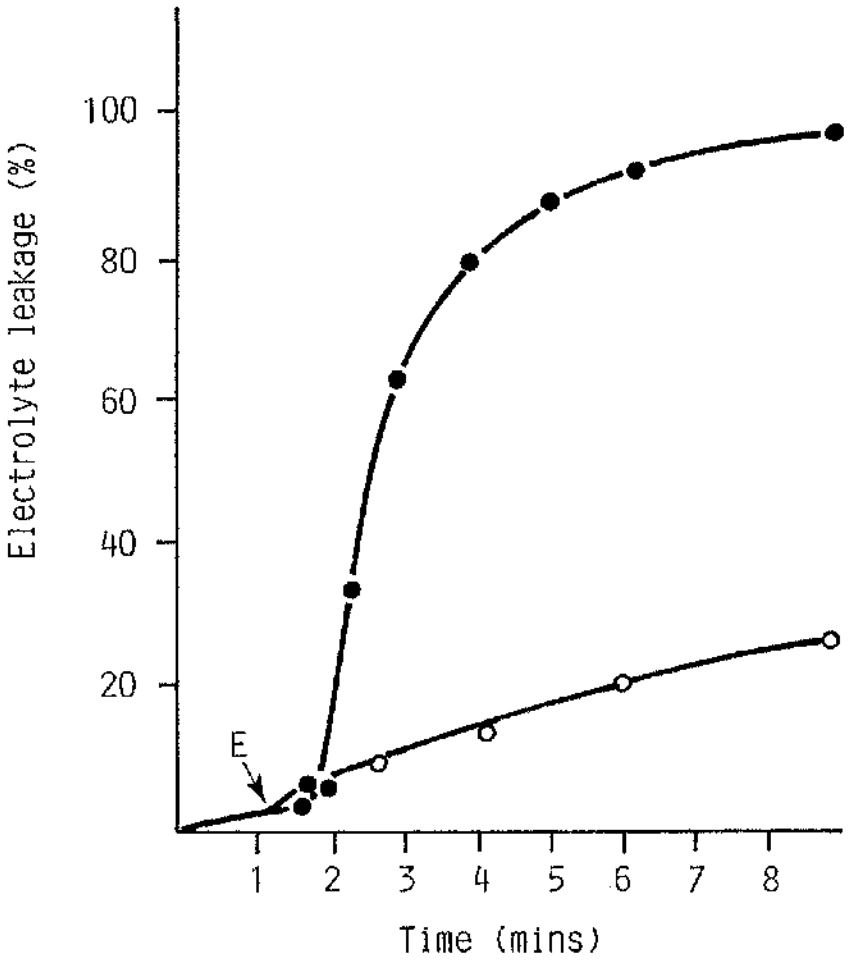
The activation of wasp venom PLA_2 by oleoyl imidazolide was first tested in the erythrocyte assay. Fig.(12) shows the leakage response from erythrocytes in the presence of albumin when normal

Fig.11 The response of erythrocytes to wasp venom PLA_2



The response to albumin, on cells incubated for 15' with 30µl of wasp venom PLA_2 in the presence (•—•) and absence (o—o) of EDTA was determined. The arrow signifies when the enzyme was added

Fig.12 The activation of wasp venom PLA_2 by oleoyl imidazolide



The leakage response of erythrocytes in the presence of albumin was determined when 20µl of native wasp PLA_2 (o—o) or PLA_2 treated with oleoyl imidazolide (●—●) was added to the assay system. The arrow signifies when the enzyme was added

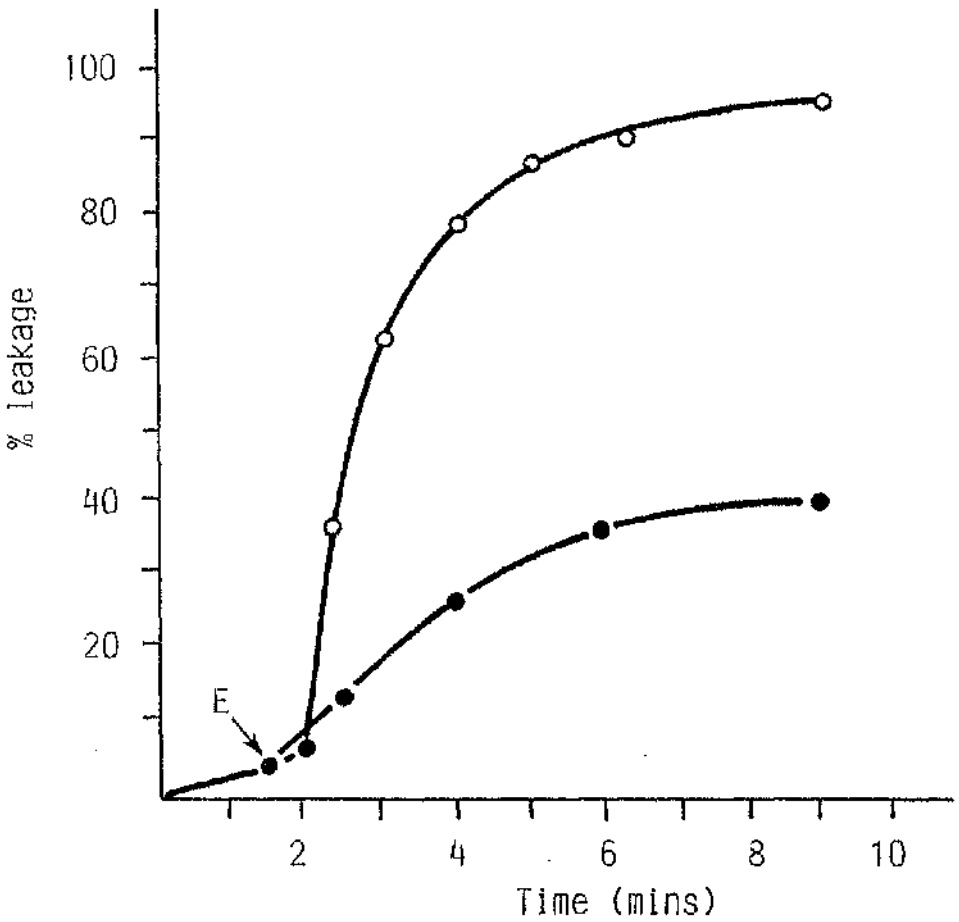
or wasp venom PLA_2 treated with oleoyl imidazolide were added to the assay system. The wasp enzyme showed the typical activation response that the bee venom enzyme produces, a slow phase followed by a rapid increase in activity thus it seems that the covalent modification of PLA_2 is shown by both the bee and wasp venom enzymes.

The action of lysophosphatidyl choline on the activated wasp venom PLA_2 is shown on fig.(13). Like the bee venom enzyme, the addition of lysophosphatidyl choline to the erythroctye/albumin mixture inhibited the action of this enzyme.

The effect of lysophosphatidyl choline on the activity of bee and wasp venom PLA_2 using oleic acid treated erythrocytes.

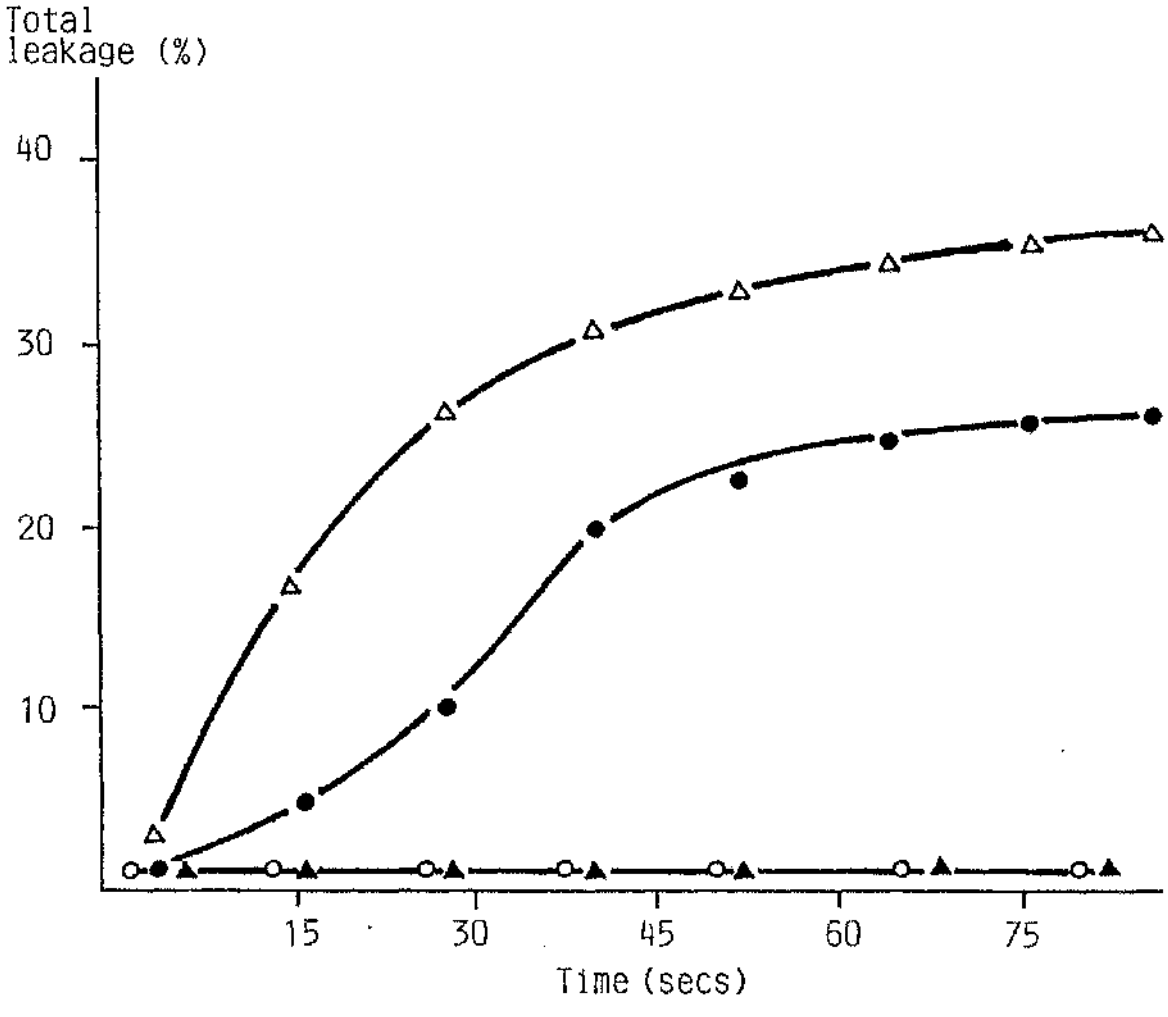
The incubation of erythrocytes with sublytic levels of oleic acid (5μg) resulted in a large increase in the cell leakage induced by both bee and wasp venom PLA_2 (fig.14). The results show the leakage response after subtraction of the leakage due to enzyme alone. If the cells were preincubated with 2 μg of lysophosphatidyl choline before the addition of oleic acid the stimulatory effect by oleic acid was totally abolished both for the bee and the wasp venom enzymes. This again suggests that lysophosphatidyl choline can prevent the accumulation of oleic acid in the membrane.

Fig.13 The effect of lysolecithin on the leakage response to activated wasp venom PLA_2



Activated wasp venom PLA_2 (20µl) was added to the erythrocyte assay in the presence (●—●) and absence (o—o) of lysolecithin (1µg). The arrow signifies when the enzyme was added

Fig.14 The effect of lysolecithin on bee and wasp venom PLA_2 induced leakage of fatty acid treated erythrocytes



Erythrocytes were treated with sublytic levels of oleic acid and the response to bee and wasp venom PLA_2 determined. The effect of preincubating the cells with lysolecithin before addition of oleic acid was also measured.

- Δ : 5μg oleic acid; 2μg bee venom PLA_2
- ▲ : 2μg lysolecithin; 5μg oleic acid, 2μg bee venom PLA_2
- ● : 5μg oleic acid, 30μl wasp venom PLA_2 (2.5μg)
- o : 2μg lysolecithin; 5μg oleic acid; 30ul wasp venom PLA_2

The activation of wasp venom PLA_2 on synthetic substrates.

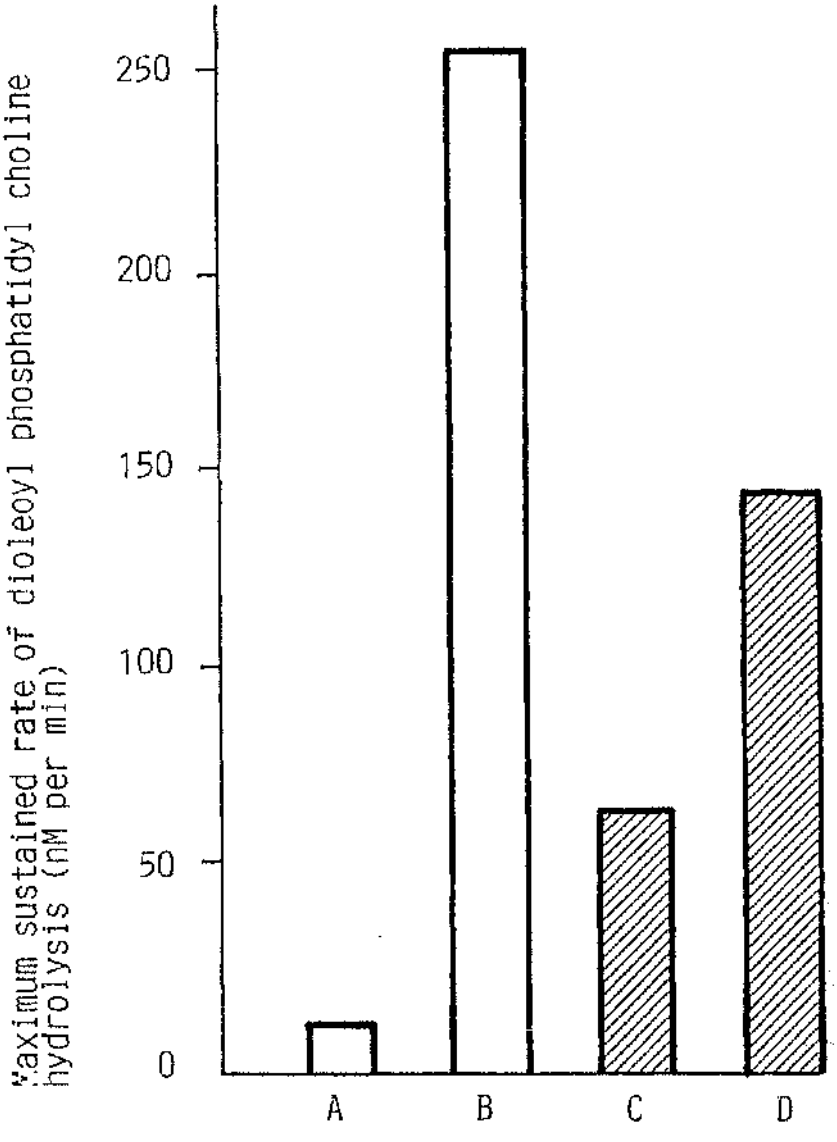
The hydrolysis curve of dioleoyl phosphatidyl choline by bee venom PLA_2 is biphasic, with a slow start followed by an increase in activity due to product activation by fatty acids and to a lesser extent by lysophosphatidyl choline. Activation abolished this biphasic curve and increased the reaction rate. As with the bee enzyme, when activated wasp venom PLA_2 was added to this substrate the hydrolysis rate was greatly increased (fig.15a).

On egg phosphatidyl choline substrate, the native wasp PLA_2 produced the characteristic biphasic reaction which occurs with the bee venom enzyme. Again, activation resulted in an increase in catalytic attack and abolition of the biphasic curve (fig.15b).

The effect of oleic acid on activated PLA_2 .

In direct chemical assays, the covalently activated PLA_2 was not susceptible to further activation by free fatty acids, but in the red cell leakage assay it is possible that fatty acids might act by more than one mechanism therefore the effect of sublytic levels of oleic acid on activated phospholipase A_2 was investigated. Fig.(16) shows the responses to activated PLA_2 in the erythrocyte assay in the absence of albumin. The activated enzyme produced a small increase in conductance which subsequently leveled off. In the presence of oleic acid however, the activated enzyme showed a large increase in activity. The results therefore show that fatty acids can enhance enzyme attack either by weakening the membrane to enhance the leakage response

Fig.15a The effect of native and activated bee and wasp venom PLA_2 on the hydrolysis of dioleoyl lecithin



The maximum rate of dioleoyl phosphatidyl choline hydrolysis was measured for

A: native bee venom PLA_2 (5µg)
B: activated bee venom PLA_2 (5µg)
C: wasp venom PLA_2 35µl (3µg)
D: activated wasp venom PLA_2 35µl (3µg)

Fig.15b The hydrolysis of egg lecithin by native and activated wasp venom phospholipase A_2

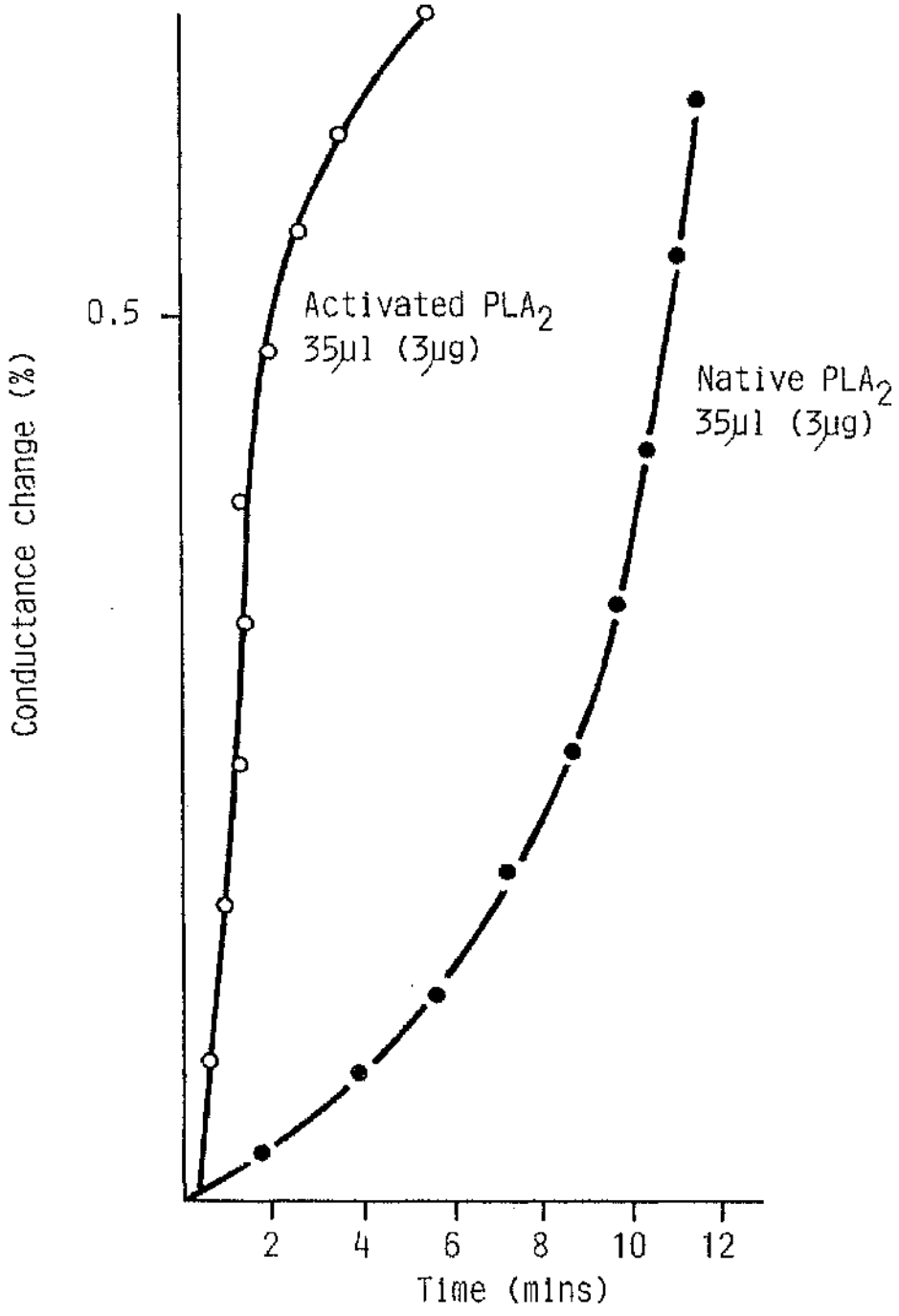
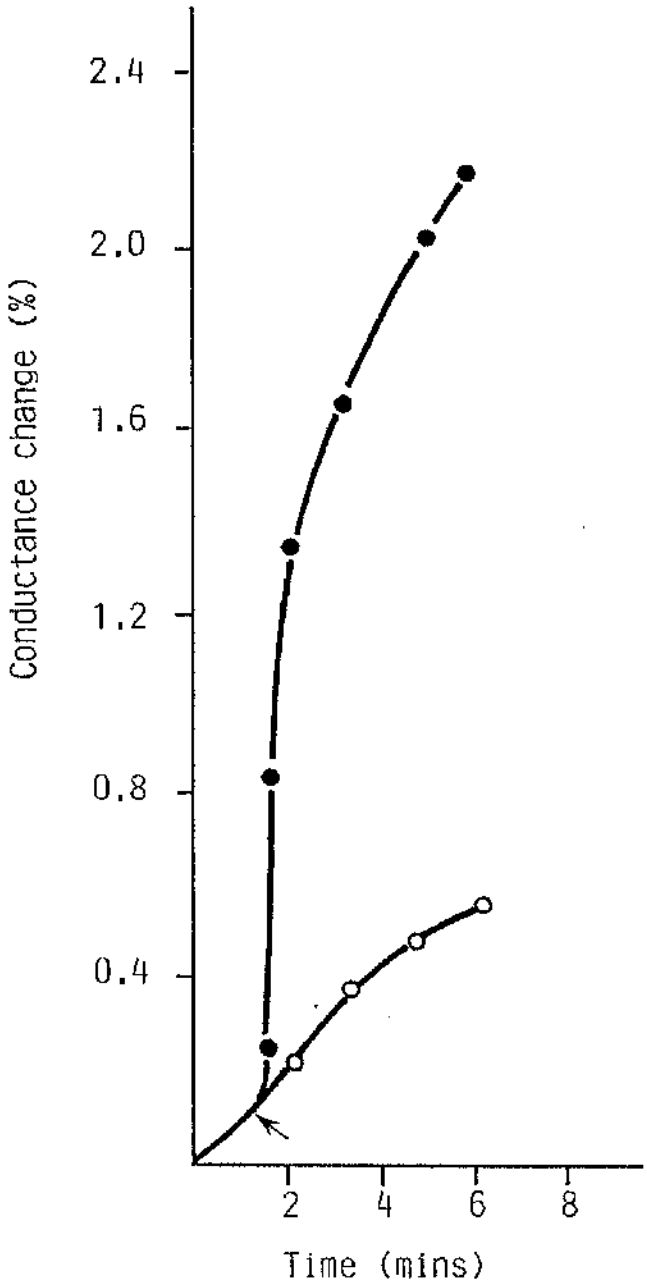


Fig.16 Stimulation of activated PLA_2 by oleic acid in the erythrocyte leakage assay.



Conductance changes on addition of activated bee venom PLA_2 (4μg) to erythrocytes in the presence (●—●), and absence (o—o) of oleic acid (2μg) when determined. The arrow signifies where the enzyme was added.

or alternatively by modifying the structure so that it is more susceptible to enzyme attack.

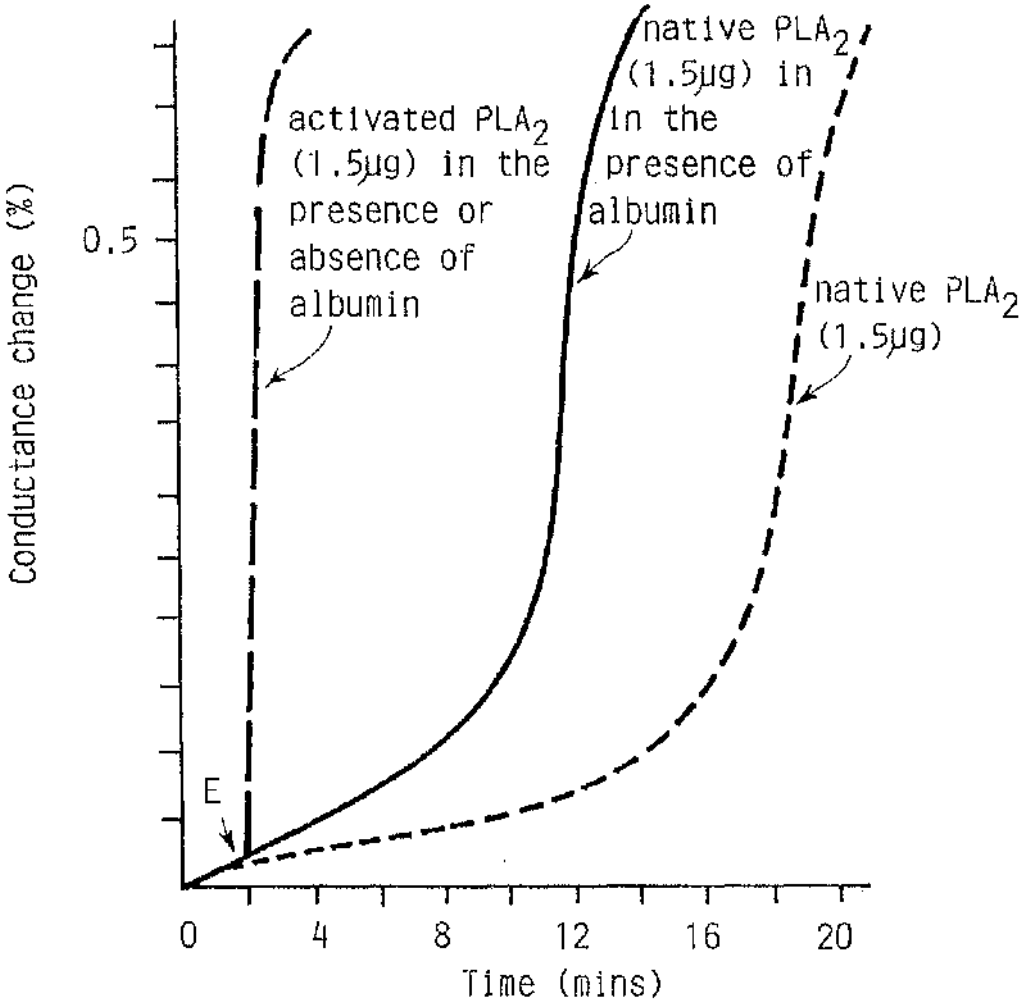
The effect of albumin on the action of PLA_2.

Synthetic substrates should provide simple model systems to elucidate some of the roles of albumin on PLA_2 activity. The simplest substrate is the monomeric dihexanoyl phosphatidyl choline and here albumin is without any effect. With the simple micelle forming compounds such as dinonanoyl phosphatidyl choline there was slight inhibition but albumin stimulated the hydrolysis of 2-octanoyl and 2-nonanoyl phosphatidyl choline (Camero.R.,M.Sc.thesis,1982) by PLA_2. These studies have been extended further and the action of albumin on the hydrolysis of egg phosphatidyl choline by native and activated PLA_2 was investigated. Albumin stimulated the hydrolysis of egg phosphatidyl choline by native PLA_2 but had no effect on the activated enzyme (fig.17). The interaction of albumin with wasp venom PLA_2 was also investigated and fig.(18) shows the stimulatory action of albumin on the hydrolysis of 2-nonanoyl phosphatidyl choline by wasp venom PLA_2 (20µl of 0.5mg/ml).

The action of thiols on native and activated phospholipase A_2.

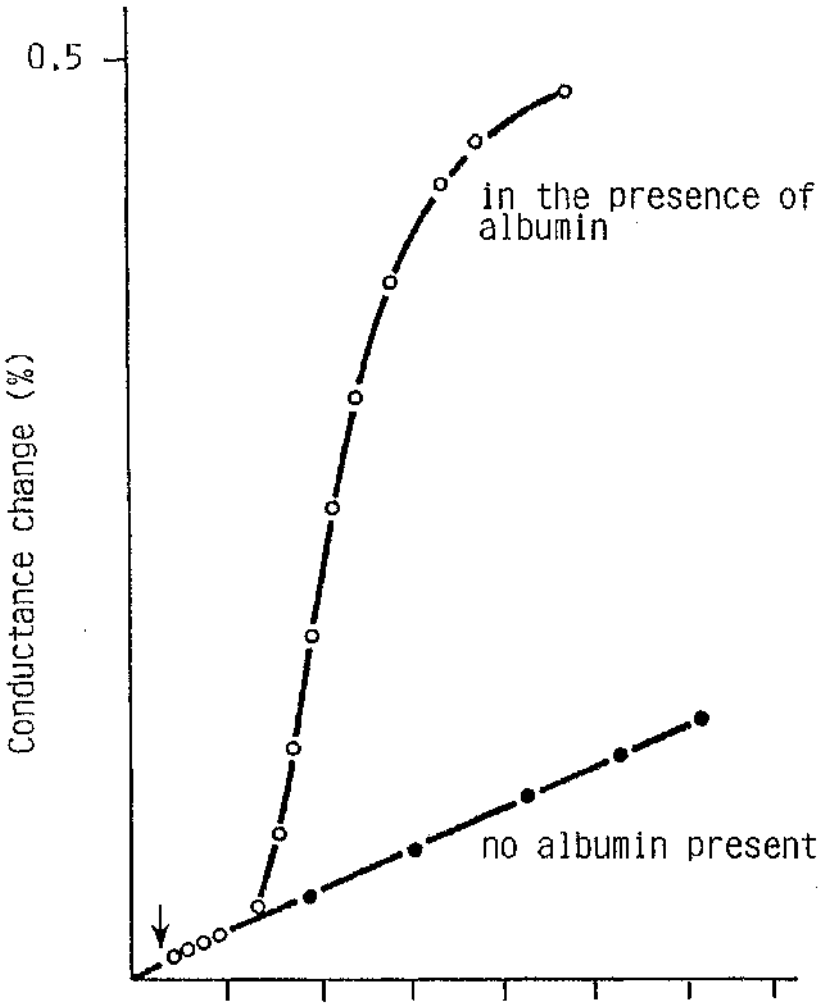
PLA_2 is stabilised by disulphide linkages and these are susceptible to attack by SH reagents. Preliminary tests had shown that activated enzyme was less susceptible to attack and the

Fig.17 The effect of albumin on the hydrolysis of egg lecithin by native and activated bee venom PLA_2



The arrow E signifies where the enzyme was added

Fig.18 The effect of albumin on the hydrolysis of 2-nonanoyl phosphatidyl choline by wasp venom PLA_2



Arrow signifies where the enzyme was added

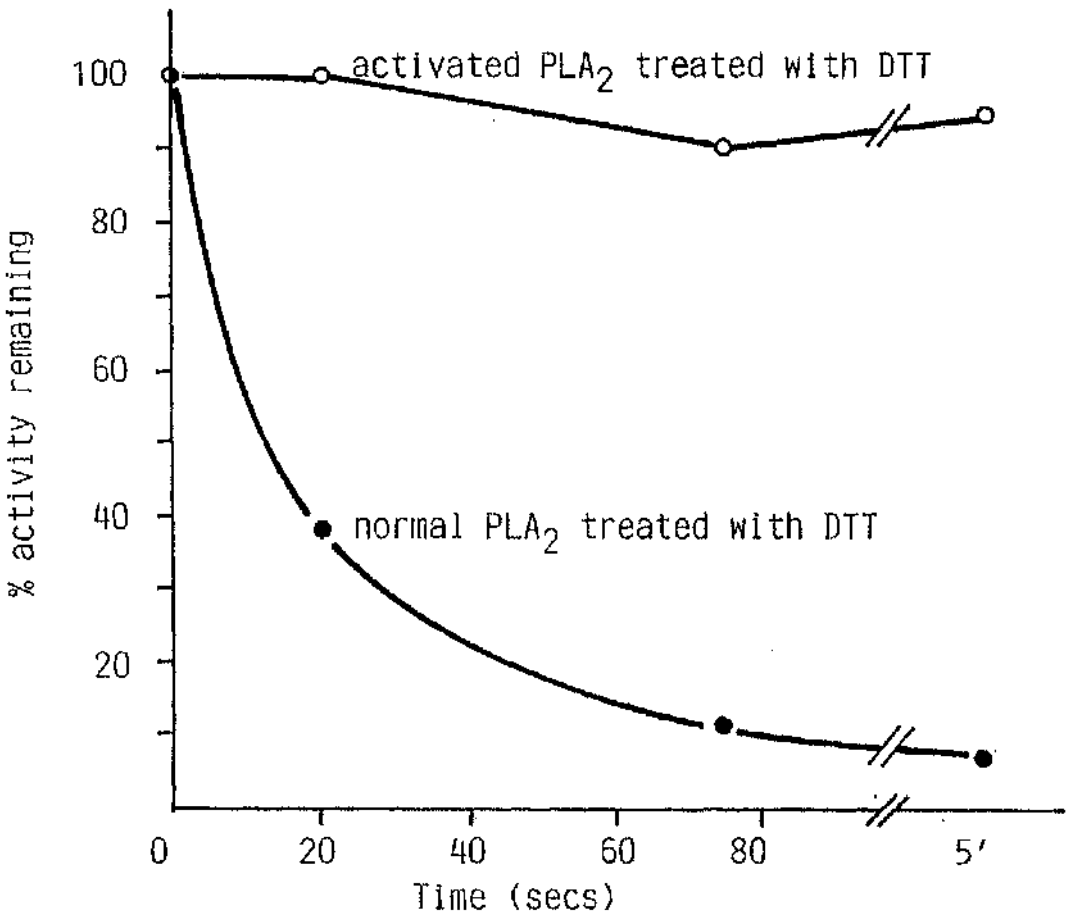
action of dithiothreitol on the activity of the normal and activated form of PLA_2 was investigated in detail. Fig.(19) shows the proportion of activity remaining when either normal or activated bee venom treated with DTT (chapter 2,section 13) were assayed on dinonanoyl phosphatidyl choline substrate. The native enzyme was extremely sensitive to DTT and within 5 minutes less than 10% of the activity remained, while in this time the activated enzyme retained more than 90% of its activity and even after an overnight incubation was still just as active. These results support the idea that activation causes a conformational change in the enzyme which in some way protects the enzyme from the effects of DTT.

Wasp venom PLA_2 showed the same differential stability and the actiavated enzyme could also be protected against the effects of DTT. Fig.(20) shows the result of treating the normal and activated wasp PLA_2 with DTT in the same way as the bee venom enzyme. Although the activated enzyme was protected against the effects of DTT, the activity unlike that of the bee venom enzyme was ultimately reduced. However, although the native enzyme was more susceptible to DTT than the activated enzyme, it was not as sensitive as the bee enzyme.

The effect of p-bromophenacyl bromide on the activity of bee venom phospholipase A_2.

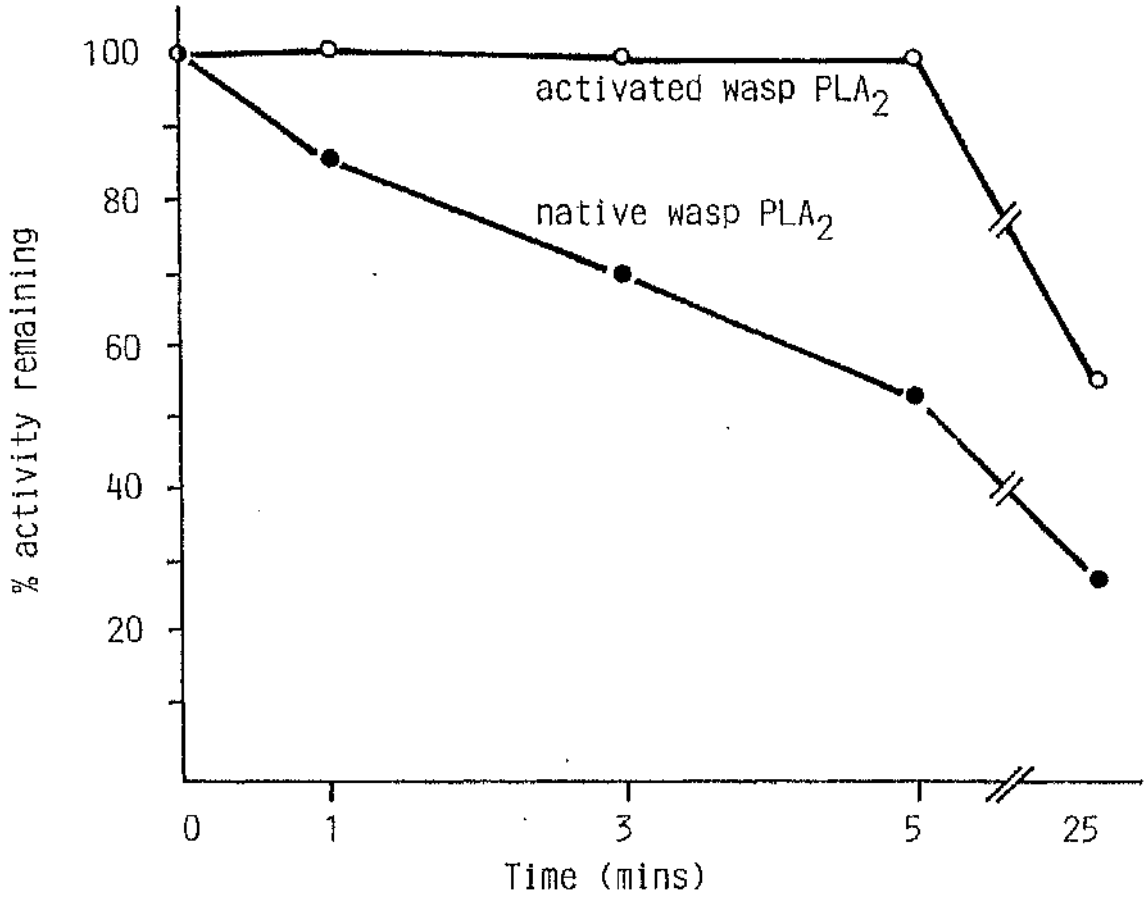
One of the aims of chemical modification studies is to attempt to identify active site residues in order to gain more insight into

Fig.19 Inactivation of native PLA_2 with D.T.T.



Activity was measured in the dinonanoyl phosphatidyl choline assay

Fig.20 Inactivation of wasp venom PLA_2 with D.T.T.



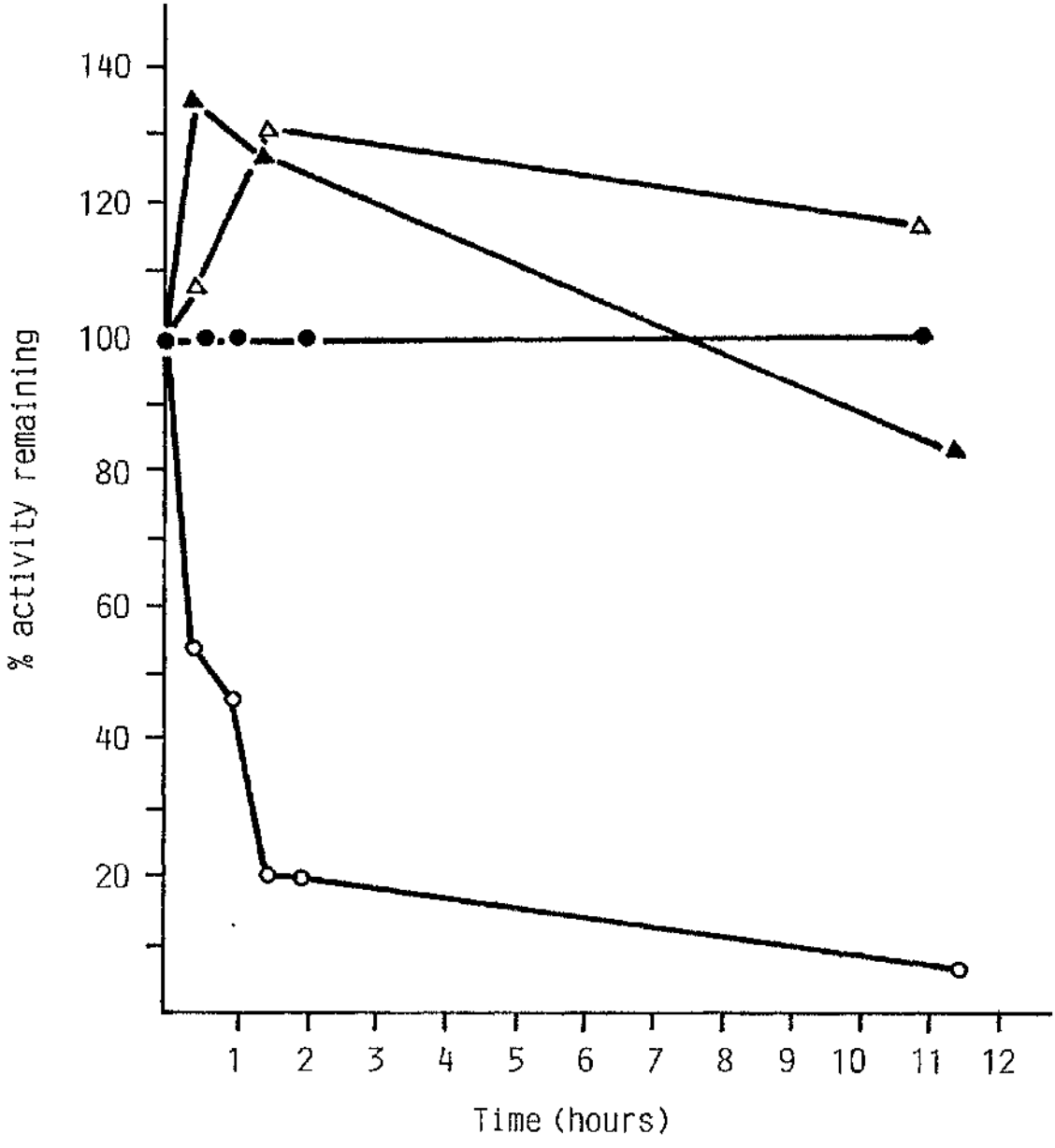
Activity was measured in the dinonanoyl phosphatidyl choline assay

the mechanism of action of PLA_2. P-bromophenacyl-bromide has been shown to inhibit the catalytic activity of porcine pancreatic PLA_2, its zymogen, and other PLA_2 enzymes by alkylating the imidazole side chain of histidine-53. It was of interest to test if the activity of bee venom PLA_2 was also affected by this reagent. PLA_2 was treated with a 5 fold molar excess of p-bromophenacyl bromide and then assayed on dinonanoyl phosphatidyl choline substrate (fig.21). After 90 minutes, only 20% of the activity remained suggesting that this enzyme also has an unusual reactive histidine residue at the reaction centre.

In contrast treatment of activated enzyme with a 10 fold molar excess of the reagent fig.(21) showed that the activated enzyme was strongly protected against this reagent although, eventually some activity was lost compared with an untreated control. A rise in activity in both samples observed in the first hour may be due to the enzyme still being further activated.

The action of P-bromophenacyl bromide on the hydrolysis of egg phosphatidyl choline by PLA_2 was also tested (fig.22). 25µl of 100mg/ml substrate was added to propanol containing buffer and then 2µl of enzyme sample was added. Hydrolysis of this substrate produces a biphasic reaction with a slow initial phase followed by an increase in activity which is due to product activation. Treatment of the enzyme with p-bromophenacyl bromide decreased the rate of all phases of the curve, suggesting a single inhibitory mechanism. Again the activated enzyme was resistant to the reagent as determined in this assay. The dinonanoyl phosphatidyl choline assay was used to minimise the direct effects of activation and the results were consistent with the

Fig.21 The effect of P-bromo-phenacyl bromide on the activity of bee venom PLA_2



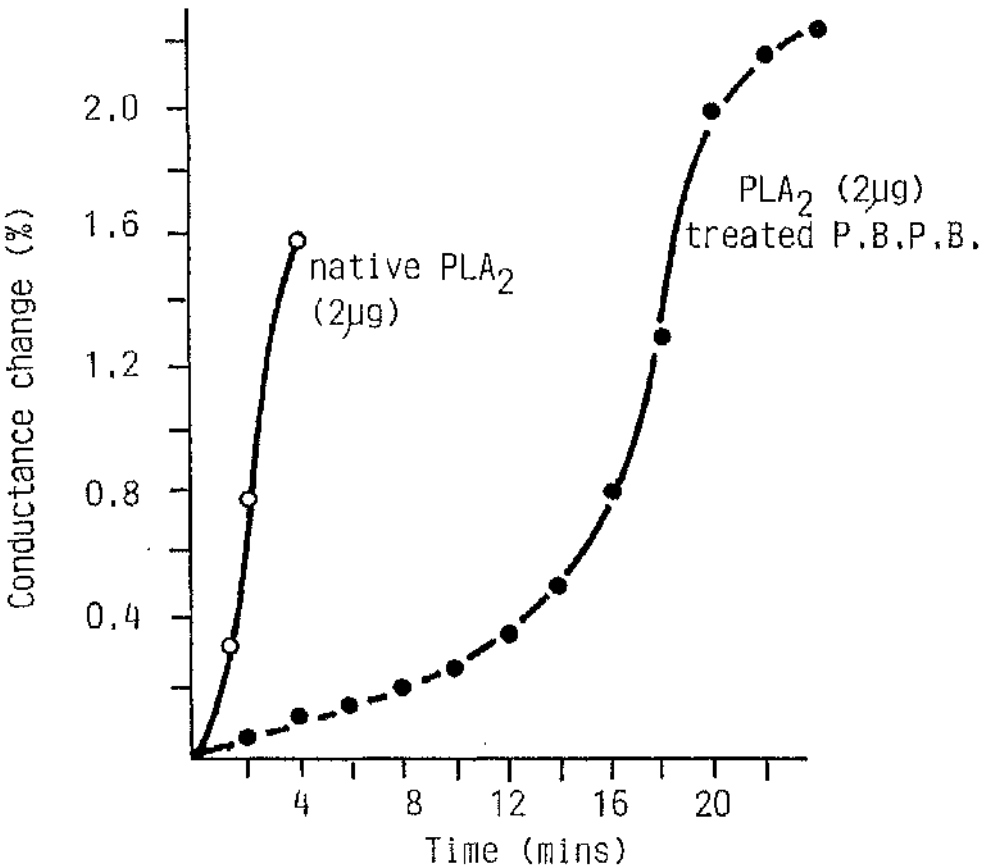
● : native PLA_2

o : native PLA_2 treated with P.B.P.B. (5 fold excess)

△ : activated PLA_2

▲ : activated PLA_2 treated withP.B.P.B (10 fold excess)

Fig.22 The hyrolysis of egg lecithin by native bee venom PLA_2 and PLA_2 treated with P.B.P.B.



possibility that the enzyme may not be fully activated thus some of the enzyme could still be susceptible to modification by p-bromophenacyl bromide and this would only be easily detected in the dinonanoyl phosphatidyl choline assay.

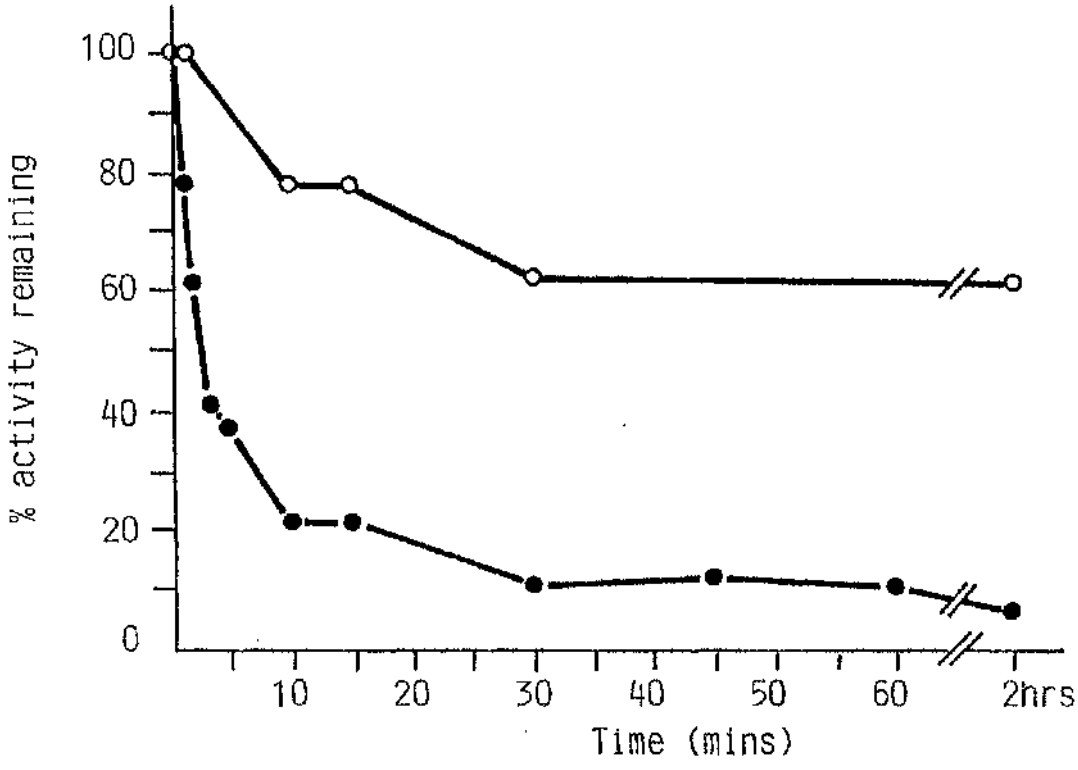
The action of trypsin on normal and activated PLA$_2$

The previous results suggested that the activated enzyme has undergone a conformational change which protects it from the effects of DTT and PBPB. To investigate this further the action of trypsin on the normal and activated bee venom enzyme was tested. Activated PLA$_2$ is protected against the action of trypsin while the native enzyme is quickly inactivated (fig.23). This further confirms that the activated enzyme has undergone a conformational change, protecting it from a range of reagents which inactivate the native enzyme.

The role of the N-terminal peptide in PLA$_2$ activation.

The possibility that the activator was binding to isoleucine, the N-terminal amino acid was investigated using the enzyme leucine aminopeptidase. When activated PLA$_2$ was treated with up to a 500 fold molar excess of leucine aminopeptidase, the degree of activation both in the erythrocyte and egg phosphatidyl choline assays was comparable with untreated controls. In these experiments it was assumed that the leucine aminopeptidase was indeed removing the N-terminal peptide since absolute proof of this would require the detection of free leucine. It was also

Fig.23 The effect of trypsin on the activity of normal and activated bee venom PLA_2



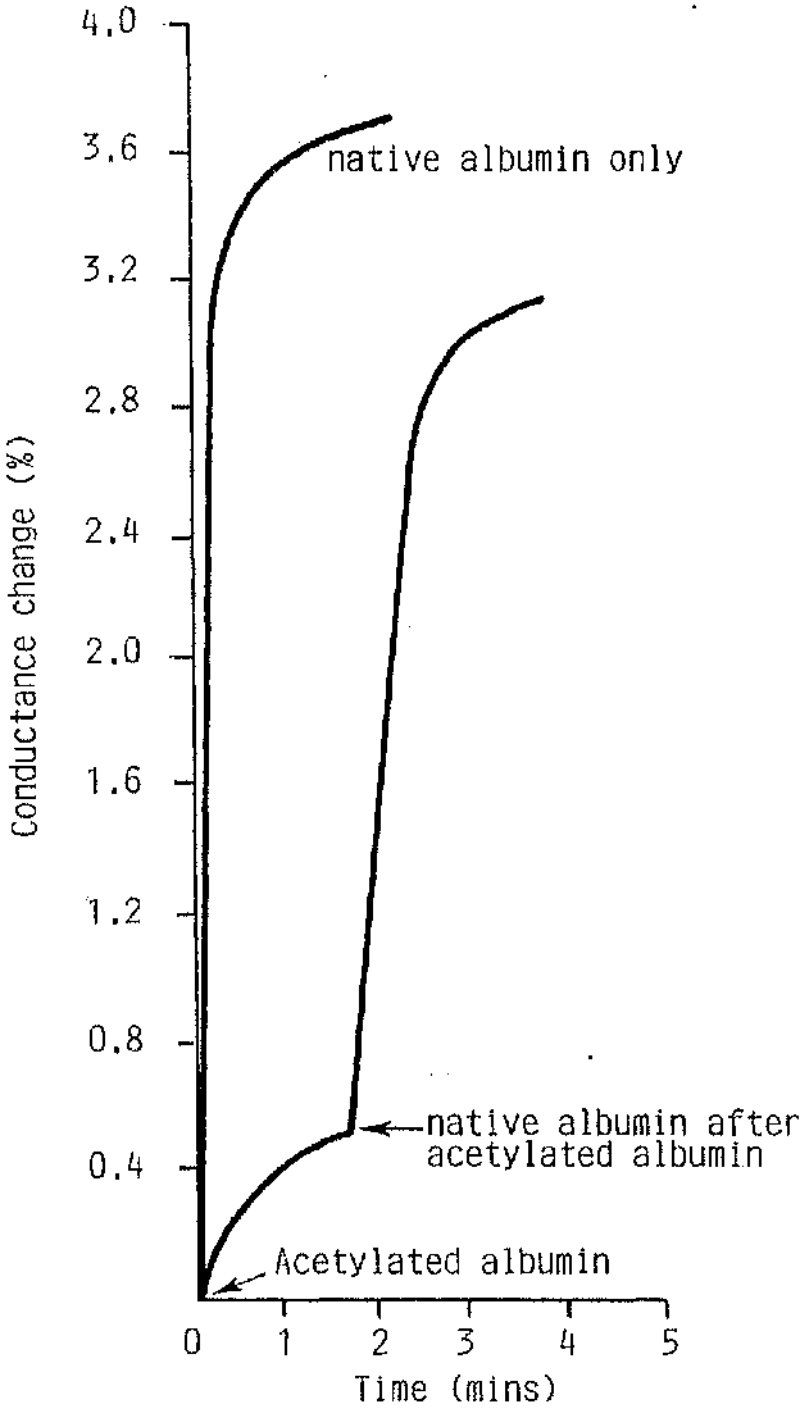
Both normal and activated PLA_2 (50µl of 2mgml^{-1}) were treated with 25µl trypsin (1 mgml^{-1})

possible that the N-terminal peptide was buried within the enzyme and not accessible to the peptidase. This could be detected by cleaving the disulphide bridges, adding the reagent, and then allowing the bridges to reform. Removal of the N-terminal peptide also had no effect on the activity of the native enzyme towards dinonanoyl phosphatidyl choline and in addition the PLA_2 was still activated with oleoyl imidazolide suggesting that the N-terminal peptide is not required to allow activation to occur, for sustained activation or for the activity of the native enzyme on dinonanoyl phosphatidyl choline.

The modification of albumin by acetylation.

Albumin contains three main fatty acid binding sites which can be acetylated with acetic anhydride (chapter2, section 14). Fig.(24) shows that modified albumin cannot bind free fatty acids. This was tested by incubating erythrocytes in the presence of 3µl of 1mg/ml oleic acid and then testing the ability of normal albumin to extract the fatty acids from the cells. This resulted in a large increase in cell leakage however if acetylated albumin was used this response was abolished though normal albumin could be subsequently added after the acetylated albumin, producing the normal response. Acetylated albumin was also tested for its ability to stop leakage after its onset as a result of lysophosphatidyl choline treatment. Lysis of cells by lysophosphatidyl choline is initially slow and is followed by a rapid increase in leakage of cell contents. A dose response curve of cell leakage, in response to lysophosphatidyl choline showed

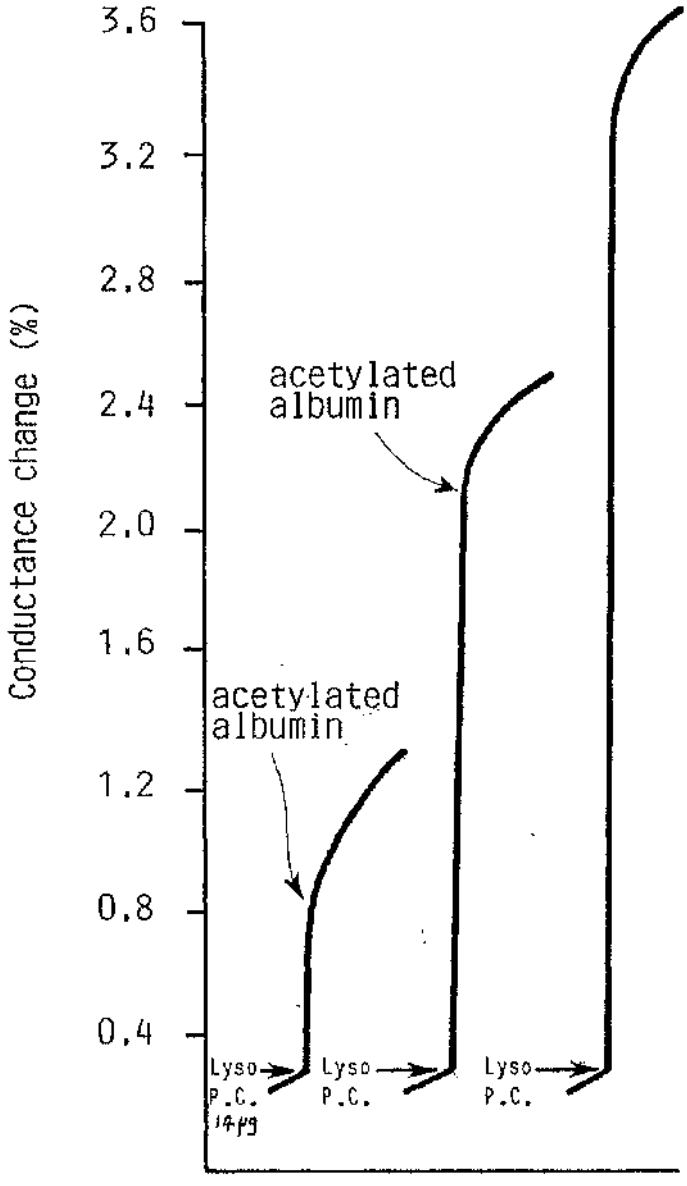
Fig.24 The ability of native and acetylated albumin to extract fatty acids from the erythrocyte membrane



Cells were incubated in the presence of oleic acid (2μg) and then the ability of native or acetylated albumin to extract the fatty acid was tested

a threshold for rapid leakage and a higher level where lysis and leakage responses merged. Earlier experiments had shown that the response to a dose level in between these could be rapidly curtailed by albumin. Fig.(25) shows that acetylated albumin was also able to stop leakage responses and could do this even at a late stage. If acetylated albumin is used in the erythroctye assay, the response to activated PLA_2 is inhibited and this does not agree with the model proposed. If native and acetylated albumin are added together, the response to activated PLA_2 is also inhibited. Thus it appears that acetylated albumin inhibits the enzyme directly and and though it can affect the selective removal of products from membranes it cannot be used to elucidate the role of both fatty acids and lysophospahtides on the activity of PLA_2.

Fig.25 The effect of acetylated albumin on halting leakage induced by lysophosphatidyl choline



Acetylated albumin was added after the lyso phosphatidyl choline (Lyso P.C.) induced leakage of cells

CHAPTER FOUR

DISCUSSION

One of the most important areas in the field of enzyme kinetics is the mechanism of enzyme control and in this respect, the enzyme phospholipase A_2 is subject to control by several mechanisms. The pancreatic enzyme is regulated by the conversion of the zymogen to the active enzyme by the action of trypsin, and in neutrophils and macrophages PLA_2 activity is regulated by a 40K glycoprotein, lipomodulin. Almost all phospholipases have an absolute requirement for Ca^{2+}. Bee venom PLA_2 is simulated by the lytic peptide melittin and it has recently been demonstrated that wasp venom contains mastoparans, tetradecapeptides capable of stimulating PLA_2 activity.

PLA_2 activity is also altered by reactions products. There are many reports of phospholipase being stimulated by fatty acids (e.g.Dawson, 1959) but most of these effects have been attributed to substrate mediated phenomena. The irreversable activation of bee venom PLA_2 by covalent modification of the protein has only been demonstrated in this laboratory and it shows that activation by free fatty acids, must be an allosteric proceess. Our data also shows that wasp venom, Naja-naja & Vipera ammodytes PLA_2 are activated by oleoyl immodazolide. The effects due to activation are large and specific and this makes it one of the most intriguing areas in the control of this enzyme. Strangely, this work has not been repeated, or extended by other workers or even discussed in relation to other work going on in the field. It is extremely simple to activate the enzyme and the activated enzyme itself is a useful tool, being far more effective than the native enzyme, itself the most active of all

phospholipase A_2 enzymes. One possible reason why activation of PLA_2 by fatty acids and acylating agents has been largely ignored is that other workers do not have the equipment to detect activation and that activation is an artefact of the methods used in this laboratory.

The work in this thesis uses a conductimetric assay technique and this is emphasised because to our knowledge there have been no other reports in the field which use this method. It is of importance to show that the results obtained are not due to experimental artefacts and are infact due to specific advantages of the method. Because choline glycerophospholipids are non-conducting, fatty acid contaminants can be easily detected by conductimetry. It is possible that conductimetry detects small amounts of fatty acids in substrates and these might be missed in other assay methods and it is clearly of extreme importance for activation studies that substrates be free of fatty acids. One of the most important aspects of the conductivity cell design, is that stirring is very efficient. Because of the very high stirring efficiency of the cells used here, events which occur within a few seconds can be recorded accurately and initial lag phases cannot be overlooked. Calibration of the apparatus is usually by the addition of fatty acids or by the hydrolysis of known amounts of substrates. In all cases the chemical assays are found to give linear calibrations, so artefacts seem unlikely. Artefacts are possible in this system when long chain substrates are hydrolysed in propanolic media, and the shape of the response curve depends on the concentration of propanol in the buffer. It

also is possible that the enzyme or substrate could be altered in some way in the conductivity cell resulting in a change in activity. This might come about by a reaction at the electrodes although we have no evidence to suggest anything of this nature occurs.

Conductimetry is technically easier to use than for example spectrophotometric or titration methods which are probably the commonest assays for venom PLA_2 .Because conductimetry is so convenient and versatile, it is tempting to use it for all experiments at the expense of other methods and it does have the great advantage that intact cells can be used as substrates. (It is interesting that if lipids are extracted from erythrocytes the lag phase characteristic of other substrates is not observed. This could reflect the asymmetric distribution of lipids in the membrane.

The major assay system for tissue PLA_2 enzymes are radiochemical and although these have the sensitivtiy to detect very small changes, usually only one or two time points are measured and early events may be missed. Radiochemical methods ought to be able to detect activation yet there are no reports of this.

The work with activated PLA_2 stemmed from work by Lawrence & Moores (1975) on the activation of bee venom PLA_2 by fatty acids, aliphatic anhydrides and glutaraldehyde. The hydrolysis curves of egg lecithin due to attack by PLA_2, showed that fatty acids interacted with the enzyme and not the substrate firstly, because the amount of fatty acid required was independent of the

substrate concentration and conclusively because activation could be obtained by covalent addition of an acyl chain to the protein. High activity was obtained using anhydrides and ethoxy formates as acylating agents but these also resulted in a degree of non-specific activation. They subsequently showed that incubation of the enzyme with glutaraldehyde did not inhibit initial hydrolysis but prevented product activation. Treatment of the PLA$_2$ with glutaraldehyde in the presence of oleic acid had a large activating effect suggesting it could stabilise an active conformation of the enzyme.

The results obtained from the work carried out in this thesis, enforces the idea that activation results from a conformational change in the enzyme and suggests changes in the tertiary rather than the quaternary structure of the enzyme as the activated enzyme has all the characterisitics of a rigid stable conformation. Most of the evidence to suggest that the active enzyme is catalytically active as a dimer is now known to be incorrect. Whether the activated state represents the normal state of the enzyme and the low activity state is induced by the substrate, or vice versa remains to be elucidated, and this is discussed in further detail below.

Disulphide bridges of proteins are not formed until they are inserted into the lumen of the endoplasmic recticulum and free from the reducing conditions of the cytosol. They do not determine the secondary structure of the enzyme, but rather they stabilise it since they are probably formed between the closest

cysteine residues determined in the initial folding of the polypeptide chain. An interesting experiment would be to break the disulphide bonds of PLA_2 and see whether activation by oleoyl imidazolide could still take place.

There is no doubt that binding of oleoyl imidazolide is via a covalent linkage because if ^3H labelled fatty acid or oleoyl imidazolide is added to PLA_2, the fatty acid but not the oleoyl imidazolide is removed by albumin and it is possible that the activator may be hidden within the protein.

In the erythrocyte assay system fatty acids stimulate the enzyme, but cannot do so in the presence of albumin because albumin has a higher affinity for fatty acids than cells. Thus fatty acids could never accumulate in the membrane.The case with enzyme treated with oleoyl imidazolide is quite different. There is only a small increase in leakage when activated PLA_2 is added to erythrocytes alone but in the presence of albumin, the enzyme activity was greatly stimulated. The presence of albumin rules out any effects attributable to substrate mediated phenomena and the only possible explanation is that the enzyme has been covalently modified by the activator. The resistance of activated PLA_2 to DTT and trypsin under conditions where the native enzyme is inactivated is again strong evidence that the enzyme has undergone a conformational change which in some way protects it from these agents.

The acylation of PLA_2 is not a unique phenomenom and acylation of certain proteins have been described by several workers. Schlesinger et al, (1980) have presented evidence for

covalent attachment of fatty acids to several glycoproteins that span the membrane of animal enveloped viruses. One to six moles of fatty acid per mole of protein was found for three different glycoproteins. Using Rous sarcoma virus transformed embryo fibroblasts, they showed that fatty acid acylation of virus-specific proteins was a host cell function which could be prevented using cyclohexamide. Of the other reports of fatty acids being covalently bound to proteins the most thoroughly studied is a small protein present in large amounts in the outer membrane of E. Coli (Schlesinger et al, 1980). This protein has one fatty acid in peptide linkage to an NH_2-terminal cysteine residue and two additional fatty acids esterified to a glycerol which is linked to the same cysteine residue by a thioester bond. Acylation of proteins in cells may serve several functions such as histocompatibility antigens or surface hormone receptors. Fatty acid acylation could provide a stabilising anchor for these proteins destined to remain membrane bound, could allow for asymmetric assembly of specific lipids around the protein and it could provide a biochemical signal for transport of a protein to a specific cell membrane.

Covalent modification is important in the regulation of many enzymes. It is interesting to speculate on how covalent modification might alter the activity of an enzyme. About forty enzymes are known to be regulated by phosphorylation (Cohen, P., 1983) and other covalent modifications involve limited proteolysis. In bacteria adenylation and uridylation covalently modify the glutamine synthetase of E. Coli and in the organism

Rhodopseudomonas gelatinosa, it has been shown that citrate lysase is rapidly converted from the inactive SH form to the active S-acetyl form by an enzyme citrate ligase (Cohen, P.,1983) Deacylation is carried out by citrate lysase deacetylase. No enzyme in mammalian cells is kwown to be regulated by reversible acylation, however reversible acylation of histones in the nucleus is well documented and may play a role in gene transcription. Acetylation in this case occurs on lysine residues and not on sulphydryl groups.

Experiments on the pH dependence of activation suggested that activation is controlled by a group with a pk in the range of 6.5. It is possible that this may aid acylation without being the target group. In the absence of peptide maps of the enzyme, it is still possible to speculate on the amino acid involved in activation. There are no free sulphydryl groups in PLA_2 and although S-S bonds can open, it is very unlikely under the conditions used here. Reaction with acid groups may be possible but this derivative would probably be very unstable whilst lysine, tyrosine, serine/threonine, histidine and isoleucine are all possible candidates. Imidazoles have been used by other workers as specific reagents for tyrosine (Simpson et al, 1963). The chemical modification studies discussed below support the idea that a histidine residue could be involved in binding of the activator.

One of the most important questions concerning the study of phospholipase A_2 is in what ways do the structure and properties

of the active site tell us something about the special requirements of substrate organisation seen with these enzymes. Para-bromo-phenacyl-bromide has been used as a specific inhibitor of phospholipases because this reagent irreversibly alkylates active site residues in the porcine pancreatic (Volwerk et al,1974), snake venom PLA_2 and in general inhibits neural-active and calcium dependent phospholipases from a variety of mammalian cells. It has been used to inhibit platelet aggregation (Thakhar et al,1983), agonist induced catecholamine release from brain synaptosomes and adrenal medulla (Bradford et al,1974)and to inhibit hamster sperm acrosome release (Lui & Miezel,1979).

The results of the work of Wells (1973) on Crotalus adamanteus PLA_2 have shown that two tryptophans as well as a single abnormally ionising lysine, appear to be essential for the catalytic activity of this dimeric enzyme, however chemical modification of tyrosine and histidine residues had no effect on enzyme activity. Inactivation of porcine PLA_2 and its zymogen with p-bromo-phenacyl-bromide followed first order kinetics and studies on the pH dependence of inactivation indicated that a group with an apparent pK of 6.1 was involved. When the residual activity was less than 5%, amino acid analysis showed the loss of one histidine per molecule of PLA_2. 90% Of the reagent was bound to histidine-53 (position 46 in the active enzyme) while 10% was associated with histidine-115. The enzyme could be protected against inactivation by Ca^{2+}, Ba^{2+} and non degradable substrate analogs in the monomeric state. From these results it has been concluded that histidine-53 is involved in the active site of PLA_2. P-bromo-phenacyl-bromide is not a histidine reagent and free

histidine is not modified with PBPB thus it seems that the reaction between this reagent and histidine-53 is catalysed by the enzyme.

The experiments with the bee venom enzyme show that this enzyme is also inactivated by p-bromo-phenacyl-bromide. Histidine-53 is conserved in the primary structure of all vertebrate phospholipases A_2 supporting the importance of this amino acid. Bee venom PLA_2 contains six histidine residues, with one of these being at position 48. Obviously further work would have to be carried out to determine which amino acid is being altered nevertheless it is interesting that the bee venom enzyme is also affected by this reagent and this may reflect some similarities in the mechanism of action of vertebrate and non-vertebrate phospholipases A_2. When treated with p-bromo-phenacyl-bromide, the activated enzyme was protected against inactivation. This could mean that either the activated form of the enzyme has undergone a conformational change which in some way prevents inactivation by the reagent or it could be that the site where the reagent binds is still exposed but that the reagent and the activator bind to the same site. As expected the inactivation by p-bromo-phenacyl-bromide could not be reversed by the addition of activator. It would be of interest to see is the enzyme could still bind the activator. A change in conformation without increase in activity could possibly be detected by following the absorbtion spectrum of the enzyme after the addition of activator. Some recent work by Kyger and Franson (1984) has shown that p-bromo-phenacyl-bromide irreversibly inhibits, in a dose

dependent manner, a wide spectrum of enzymes and therefore cannot be considered a selective inhibitor of enzymes in crude cellular experiments nevertheless this reagent has been extremely useful in pin-pointing active site residues in order to gain more insight into the mechanism of action of PLA_2.

Experiments where native and activated PLA_2 were treated with DTT, showed that the native enzyme is extremely sensitive to DTT whereas the activated enzyme is protected. Wasp venom PLA_2 was also sensitive to DTT and again the activated enzyme was protected against its effects. In comparison with phospholipases A_2 of the pancreatic gland and of snake venoms which, have rigid structures ensured by the presence of 6-7 disulphide bridges, phospholipase A_2 of bee venom has four disulphide bonds. Because the pancreatic and snake enzymes are susceptible to thiols it was not unexpected that the bee enzyme would also be highly sensitive to thiols.

Reduction of porcine pancreatic phospholipase A_2 with 2-mercaptoethanol in the presence of 6M guanidinium hydrochloride or 8M urea has been shown to result in the complete loss of all seven disulphide bridges and the concomitant loss of all enzyme activity (Gustaaf et al, 1980). However the fact that activation of both bee and wasp venom enzymes confers protection against DTT is further evidence supporting the idea that the enzyme undergoes a conformational change when activated. Because the native PlA_2 is extremely sensitive to thiol inactivation, this finding now allows the use of the sensitive dinonanoyl lecithin assay to

detect modification of the enzyme.

The experiments involving the action of trypsin on the native and activated enzyme showed that the activated enyme was protected against the action of trypsin under conditions where the native enzyme was rapidly inactivated, further confirming that the activated enzyme has undergone a conformational change. The fact that the activated enzyme maintains its catalytic activity in the presence of trypsin may provide the opportunity to use the enzyme under conditions where proteases would usually destroy the activity.

An explanation for the difference in activity of normal and activated PLA_2 may be explained based on a similar idea to that of Monod (1965). In this theory heamoglobin is assumed to pre-exist as an equilibrium mixture of two conformational states, a tense T-state which binds oxygen with low affinity and a relaxed R-state which binds oxygen with high affinity. In the case of PLA_2 there are two possibilities. In free solution, the native enzyme could exist in an active relaxed state. On reaching a bilayer it could be forced into a rigid inactive configuration and hence very little hydrolysis of the substrate can occur. With micellar substrates the configuration is not altered and hydrolysis of the substrate by the enzyme occurs. Activation would hold the enzyme in its active state, thus on substrates such as dinonanoyl lecithin which are micellar, one would not expect to see much change in activity and in keeping with this it is difficult to detect any differences in native and activated PLA_2 on this substrate. On bilayers however, because the enzyme is held in its

active configuration by the activator it will not be distorted by contact with bilayers and so catalysis can occur. This argues that activation does not increase the activity of the enzyme above its normal level but stabilises the active form of the protein (fig. ii). This model is not consistent with the results obtained from work with Para-bromophenacyl-bromide. If, according to this model, the enzyme is in its active state in free solution, then, as with the activated enzyme, it should not be susceptible to attack by the reagent. However, the results obtained demonstrated that the native enzyme is highly susceptible to modification by this reagent.

The other hypothesis is that the enzyme exists in free solution in a low activity state and when binding micellar substrates it is forced into its high activity configuration Bilayers, however do not cause this change and the enzyme remains in its normal low activity state. If the enzyme is activated, micellar substrates, would be able only to increase the activity by a small amount but the activated enzyme will be able to attack bilayers at a high rate (fig.iii).

Perhaps the most obvious explanation of fatty acid activation is that the acyl chain helps to bind the enzyme to the substrate surface to increase the apparent affinity of the enzyme for the substrate. It is actually quite difficult to imagine the mechanism for such a process because the fatty acid would have to interact with the hydrophobic part of the substrate, and

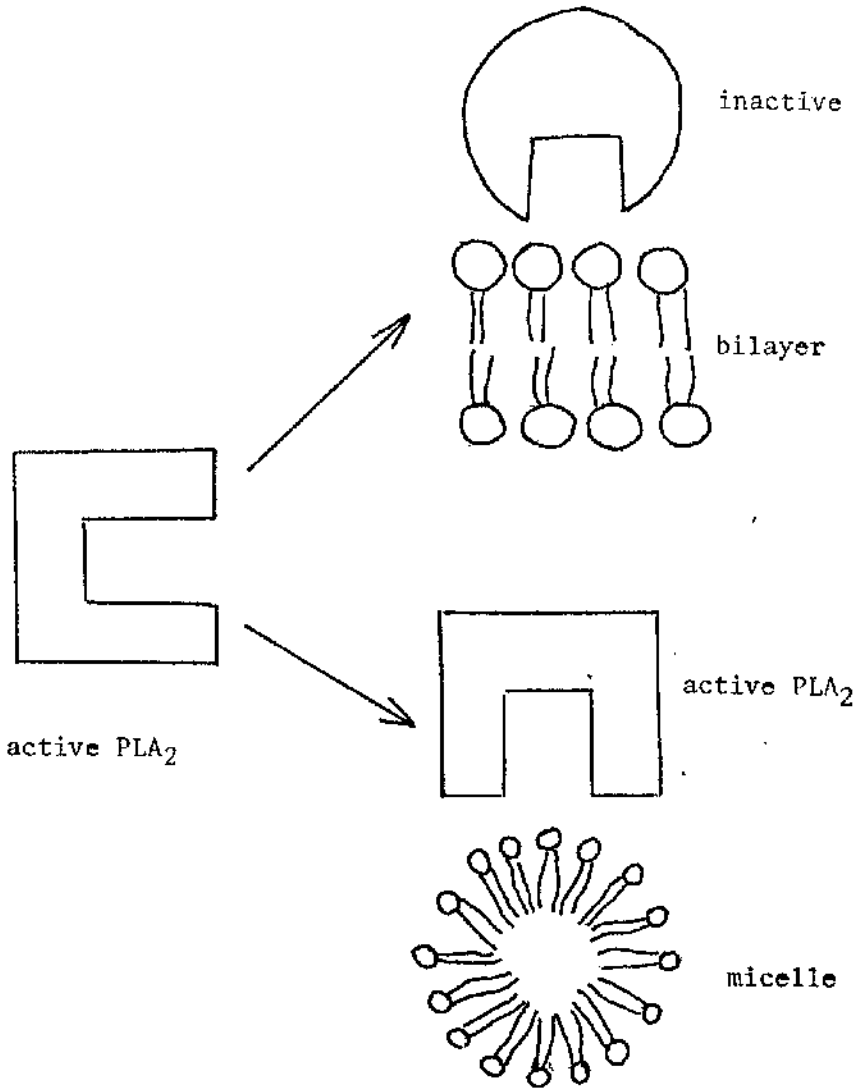


Fig. (ii) Model for the hydrolysis of micellar substrates and bilayers by native PLA_2.

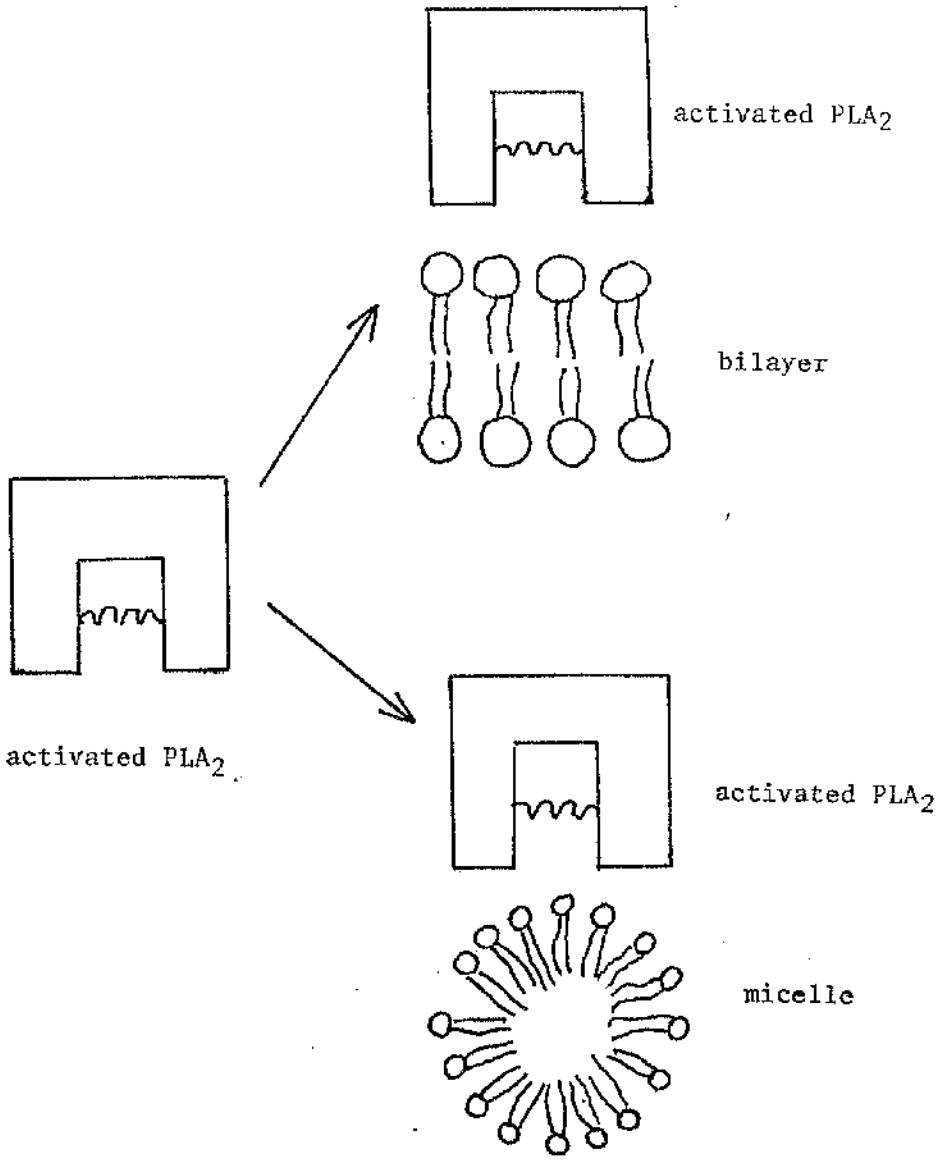


Fig.(ii) Model for hydrolysis of micellar substrates and bilayers by activated PLA_2.

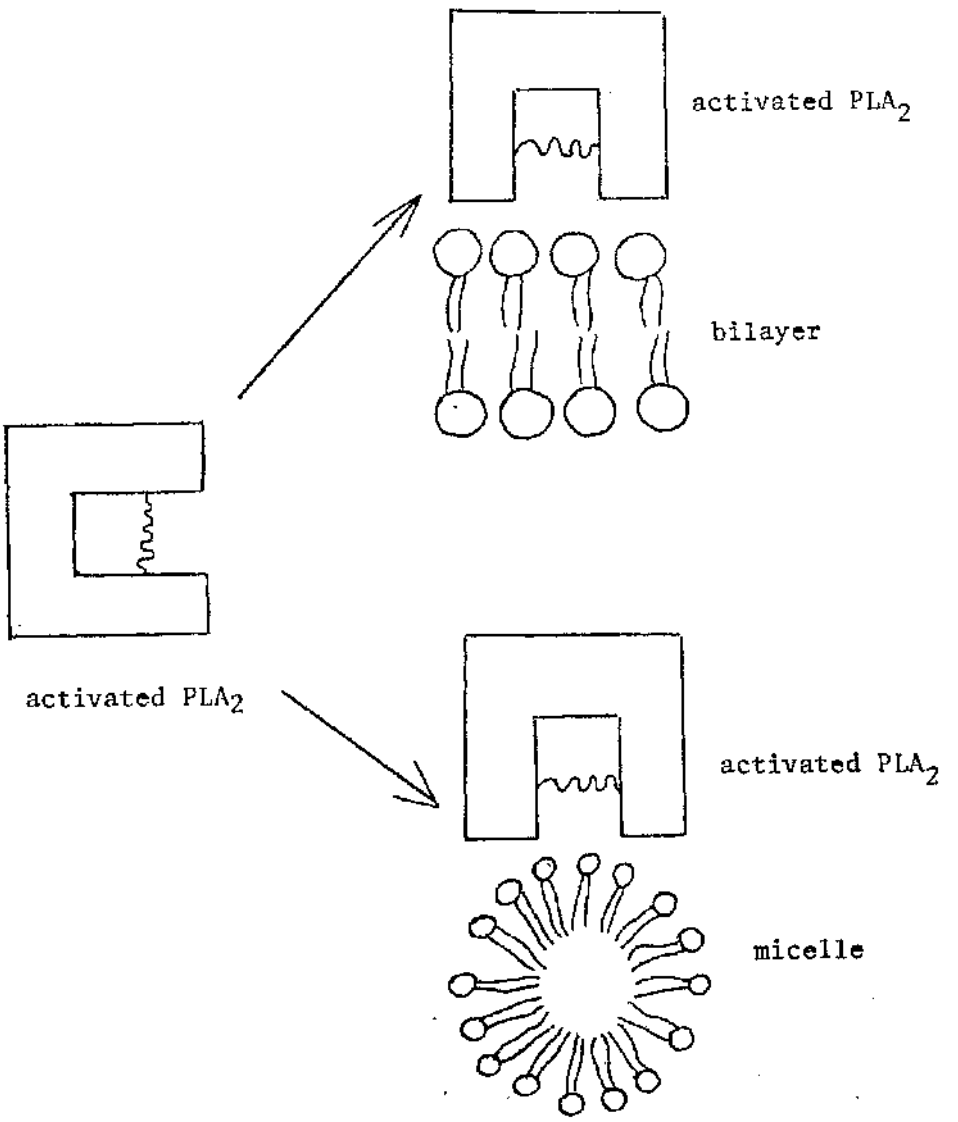


Fig. (III) Alternative model for hydrolysis of micellar substrates and bilayers by activated PLA_2.

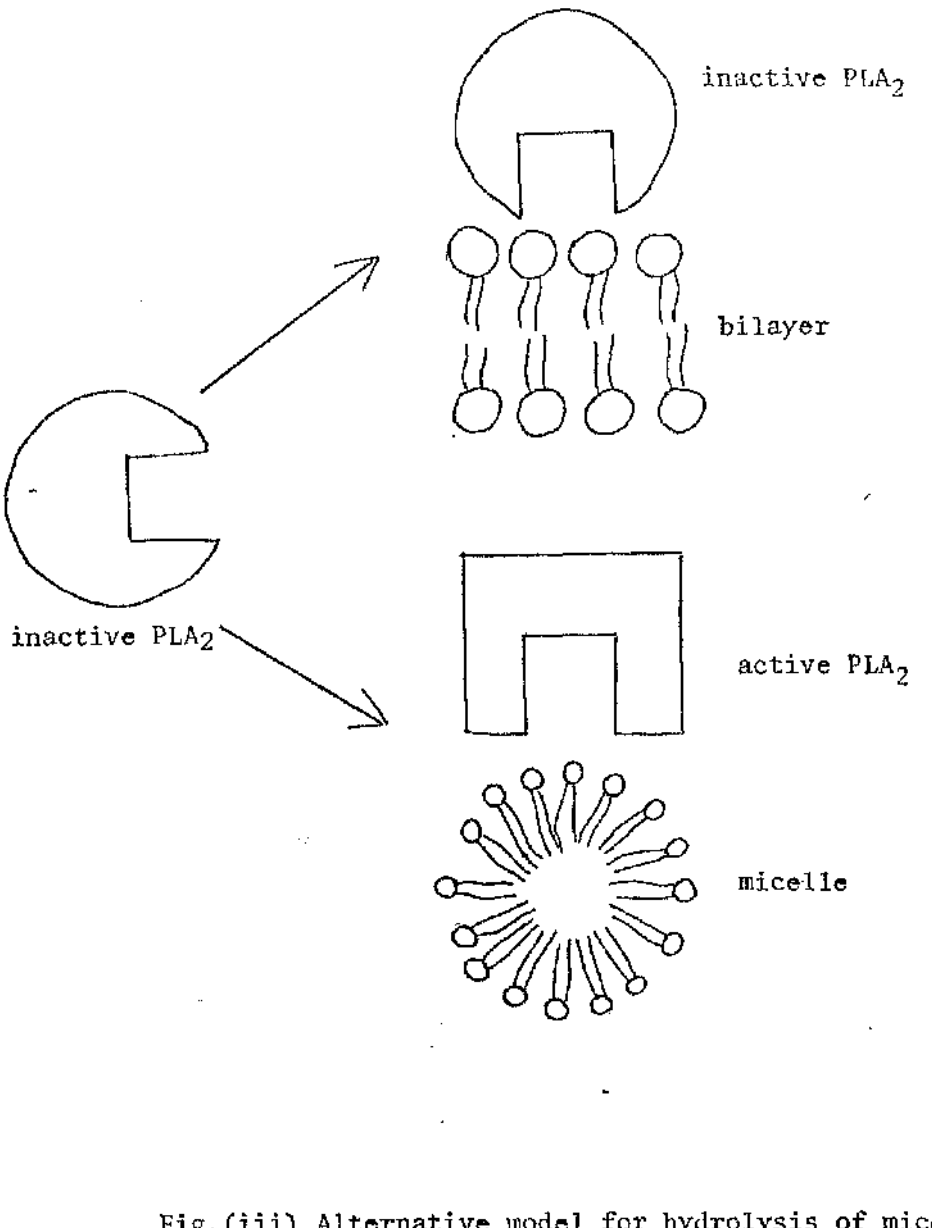
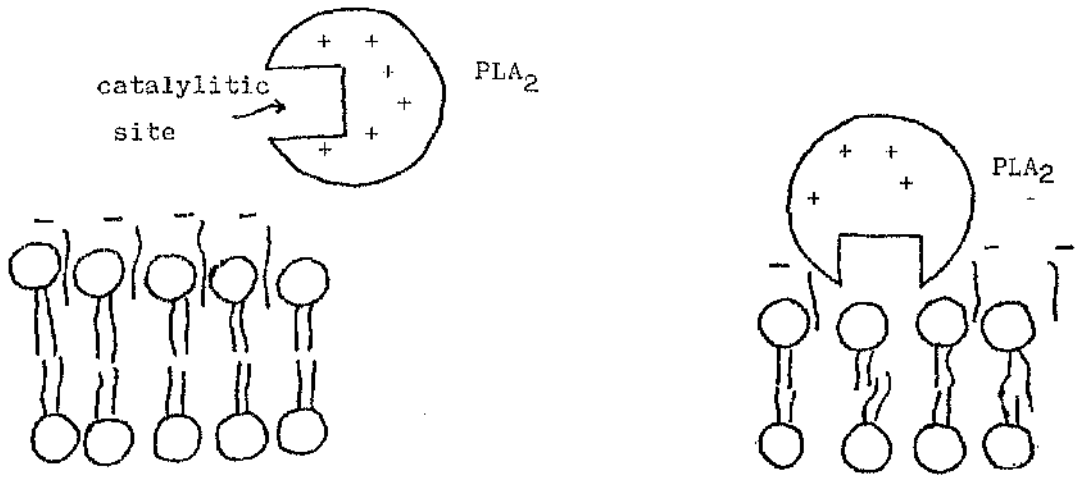


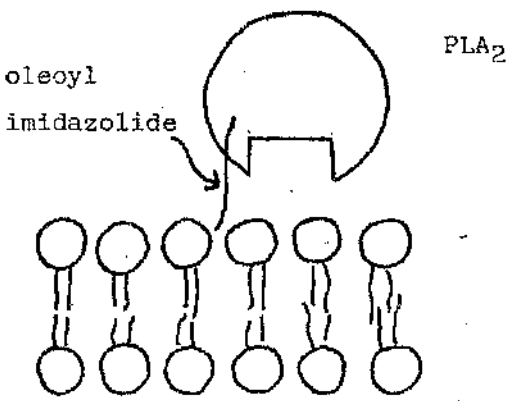
Fig.(iii) Alternative model for hydrolysis of micellar substrates and bilayers by native PLA_2

presumably of the enzyme also. It does not seem probable that a single carboxylate group could produce a substantial interaction with the protein but if the fatty acid was partially involved in a hydrophobic interaction with the protein (fig. iv) it should follow that medium chain length fatty acids would block activation by longer acids and although this might not be straightforward to test, there are no indications that the competitive inhibition of activation occurs. With acyl group activation however, a very simple model is possible where the acyl chain can act as a hydrophobic anchor for the protein. Apart from the problen that this gives no analogous mechanism to explain activation by the free fatty acid, it proposes that the interaction of the acyl chain with the functional protein is minimal and this is not supported by the results of the present study where acylation has been shown to produce far reaching changes in the stability of the protein which suggest very strongly that large changes in conformation have occured.

Fig. (iv) Models for activation of PLA_2 by free fatty acids and oleoyl imidazolide.



(a) Model of PLA_2 activation by free fatty acids.



(b) Model of PLA_2 activation by oleoyl imidazolide.

An important aspect of PLA_2 control is whether enzyme activity is altered in the presence of albumin. The results obtained here show that albumin enhances the response of native and especially activated PLA_2 in the erythrocyte assay. This means that modulation of PLA_2 activity in vertebrate systems is different from non-vertebrate systems since vertebrates do and non-vertebrates do not have albumin. The results obtained with synthetic substrates suggest that albumin can complex phospholipids and make them more susceptible to PLA_2 attack. The situation with red cells is different because albumin does not remain bound to the cells. In the presence of albumin products are continuously removed from the membrane and this can result in both lysis and sublytic leakage of the cells however these effects are tightly coupled to continuous enzyme action and are inhibited by EDTA. This means that the leakage is not due to the continual disruption of the outer lipids because to produce the amount of leakage caused by the removal of fatty acids, there has to be a certain amount of fatty acid in the membrane and because albumin is present all the time it is unlikely that this amount of fatty acid could ever accumulate. Cell lysis produced by PLA_2 attack has been thought to be due to build up of lytic products in the membrane.The experiments with albumin show that this cannot be the reason because it prevents accumulation of these products in the membrane. Also, leakage would depend on the concentration of albumin. At high concentrations there would be very little fatty acid in the membrane and leakage should be reduced by excess albumin but this is not observed.

Both lysolecithin and fatty acids have feed-back effects on

PLA activity and this may be important in the regulation of the enzyme. The experiments described in this thesis show that the activity of PLA$_2$ is strongly inhibited by sublytic levels of lysolecithin and stimulated by fatty acids and that the fatty acid stimulation is overriden by lysolecithin. Because the inhibitory action of lysolecithin is much greater than the stimulatory effect of fatty acids, the main action of albumin is almost certainly to overcome lysophosphatide inhibition. This hypothesis could be critically tested using chemically modified albumin which could only remove lysophosphatides and not fatty acids from the cells. Unfortunately, chemically modified albumin also inhibited the catalytic action of the enzyme and was therefore of no use in these studies.

The results described above suggested the mechanism of action when PLA$_2$ attacks cells in the presence of albumin. When PLA attacks cells, there is a small increase in leakage rate which is not sustained. This is the type of behaviour which might be expected for a product inhibited reaction but only in this case if leakage was affected by the rate of accumulation of products, not product concentration alone. From the results with lysolecithin and fatty acids, it can be concluded that the inhibitory effect of lysolecithin in the cells should dominate over any effects caused by fatty acids.

Inhibition by lysolecithin appears to be unique to membranes and this suggests that there is something special about the membrane strucure which only allows inhibition to be seen with

bilyers. It is well known that PLA_2 prefers micellar structures rather than bilayers. It is possible that there are areas of high curvature on erythrocytes which are preferentially attacked by PLA_2 and that when lysolecithin is added to cells either directly or by PLA_2 attack it could preferentially occupy these sites and inhibit the catalytic action of the enzyme. In the presence of albumin however the inhibitory lysolecithin (and fatty acids) are continually removed from the membrane, however the mechanism of inhibition is not known. If the PLA_2 attacks at preferred sites on the membrane, there would always be a high concentration of lysolecithin at these focal points. To explain why leakage is tightly coupled to the catalytic action of the enzyme, if the enzyme was inhibited by, for example, the addition of EDTA then the products would diffuse away over the cell surface and leakage would terminate. If PLA_2 attacks uniformly over the membrane, lysoproducts would not accumulate in high enough levels to cause lysis and it would be difficult to explain why leakage was linked to the continual action of the enzyme.

The leakage response due to addition of sublytic amounts of fatty acids to erythrocytes followed by their extraction with albumin depends in a dose dependent manner on the concentration of fatty acids added. The results suggest that the leakage response due to fatty acid removal by albumin is a transient processs, since after the fatty acids have been removed, leakage of electrolytes ceases. However, after sensitisation of the cells by this procedure, the erythrocytes are highly susceptible to attack by PLA_1, suggesting that there has been a change in the

membrane.

The extraction of lysophosphatidyl choline from cells is also a non-destructive process and the results obtained show that even when the cells are losing their contents very quickly, when albumin is added the leakage is halted almost immediately.

If lysophosphatidyl choline is added to cells which have been pre-treated with oleic acid, the response due to their extraction with albumin is not altered however if the order of addition of fatty acid and lysophosphatide is changed then very different results are obtained. The addition of fatty acids to erythrocytes results in stimulation of PLA_2 and this is strongly inhibited by lysophosphatidyl choline. If the lysophosphatidyl choline was added after the fatty acids, typical results were obtained, however if lysophosphatidyl choline was added before the addition of fatty acids then the inhibition of the lytic effects of extraction, produced by lysolecithin was much stronger (Elansari O, Lawrence A.J. & Lyall,F, unpublished data). It was also found that if lysophosphatidyl choline was added before fatty acids then the cells were not sensitised, but if they were added in the reverse order, then the cells were more sensitised than controls.

It is possible that the sensitisation of cells by treatment with fatty acids, followed by their subsequent removal with albumin is due to removal of cholesterol by albumin, however it has been demonstrated in our lab (Elansari, O. & Lawrence, A.J., unpublished results) that this is not the case.

Although albumin is the best kwown fatty acid binding protein, fatty acid binding proteins have been isolated from other sources for example an 11,800 (±1000) dalton protein has been isolated from oat seedlings (Rickers et al, 1984) and two 8,700 (±500) dalton proteins have been isolated from bovine liver cytosol (Haunerland et al, 1984). An ideal tool in the studies with albumin would be a fatty acid binding protein which could not bind lysolecithin since the effects due to lysolecithin would then be easier to establish.

Venom enzymes differ in many ways from membrane enzymes and one of the reasons for their study is that they appear to be under many of the same control mechanisms as the membrane phospholipases A_2 thus venom enzymes can be used as a basis of investigation for the tissue enzymes.

Bee venom PLA_2 seems to have two activating factors, fatty acids and the lytic peptide melittin. Although wasp venom contains PLA_2 it does not contain melittin (although the mastoparans may have an equivilent role to melittin) and it was of interest to see how many of the control mechanisms which operate for PLA_2 also stand for the wasp enzyme. Preliminary tests by Drainas (Ph.D thesis, 1978) showed that the phospholipases A_2 from Vipera ammodytes venom and fron Naja naja venom are indeed activated by oleoyl imidazolide although the effects seen were not as large as those seen with the bee venom enzyme. The results suggested that the mechanism of action with acyl-imidazolides might be a general mechanism and that these agents might also be irreversible activators for many other fatty

acid activated enzymes. One of the things investigated, was a comparison of the activation of bee and wasp venom PLA_2. The wasp venom PLA_2 was also activated by oleoyl imidazolide and the activated enzyme was strongly inhibited by the presence of lysolecithin. Bee venom PLA_2 was sensitive to thiol agents and was rapidly inactivated by DTT. Wasp venom PLA_2 shared this sensitivity to DTT. Both the activated wasp and bee PLA_2 were sensitive to trypsin. Activation of both these enzymes conferred on them protection against DTT and trypsin under conditions where the native enzymes were inactivated. It would be interesting to see how the pancreatic and other tissue phospholipases A_2 responded to treatment with oleoyl imidazolide.

Because of the complexity of the erythrocyte assay, synthetic substrates have been used to try and find more about the mechanism of action of albumin. Albumin has no effect on the hydrolysis of dihexanoyl phosphatidyl choline, had a slight stimulatory effect on dinonanoyl phosphatidyl choline and stimulated the hydrolysis of 2-octanoyl and 2-nonanoyl phosphatidyl choline (Camero,R. M.Sc.thesis,1982). The hydrolysis of 2-nonanoyl phosphatidyl choline is biphasic with a slow start followed by followed by a faster phase which resembles the hydrolysis of micellar structures. When sufficient lysolecithin accumulates the substrate is probably distorted so that the micelle is the most stable form. Albumin also stimulated the hydrolysis of this substrate by wasp venom PLA_2 and the hydrolysis of egg lecithin by bee venom PLA_2. It is possible that albumin complexes phospholipids in such a way as to make them

more susceptible to PLA_2 attack. In the presence of albumin it might at first seem suprising that albumin does not bind lysolecithin and prevent the phase change but it is possible that phospholipid molecules saturate the binding site for lysocompounds in the albumin and the lysolecithin left in solution would then promote the phase change.

PLA_2 in this work was purified most successfully by passage through an ion-echange column followed by a further purification step on Con.A Sepharose-4B. The total yield in activity was always higher after purification on these columns, suggesting that an inhibitor was being removed during the purification. Several methods were attempted to identify such an inhibitor but these all failed, for example when fractions collected were pooled, the original activity was not restored. Activity peaks obtained from the columns were often broad rising peaks at one side but falling sharply at the other suggesting the presence of an inhibitor in the later fractions. When acrylamide gels were run of these fractions we could not detect the presence of any component in the early fractions compared with the later ones. It would be interesting to further characterise the non-glycosylated protein and to include inhibitors of protein glycosylation in the diet of bees and, if they survived, to examine the contents of the venom sac for the front-running component. The effect of this on the overall potency of the venom sac could also be investigated.

diet of bees and, if they survived, to examine the contents of the venom sac for the front-running component. The effect of this on the overall potency of the venom sac could also be investigated.

It appears that in the case of the honey bee both a glycosylated and a non-glycosylated form of phospholipase A_2 are secreted into the venom sac.

The partial glycosylation of proteins is not unprecedented. Glycosylation of proteins requires that the protein contains the sequence Asn.-X.-Ser.or Thr. Many proteins contain the required sequence but are not or only insufficiently glycosylated in the course of normal cellular biosynthesis thus although the tripeptide sequence is a necessary requirement, it is not always sufficient to allow glycosylation to occur. The oligosaccaride transfer to asparagine in the pancreatic enzyme, ribonuclease, is an excellent example of this. Only about 20% of the protein molecules are glycosylated on asparagine-34 (Bause, E., 1979). Similarly, α-lactalbumin contains two recognition peptides Asn.-Gln.-Ser. & Asn.-Thr.-Ser. but the majority of these sequences are unglycosylated (Hart, 1979).

CHAPTER FIVE

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