# ACTIVATION MECHANISMS FOR VENOM PHOSPHOLIPASE $A_2$ ENZYMES.

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**Declaration:**

I declare that this thesis represents, except where note is made to the contrary, work carried out by myself. The text was composed by myself.

Salah Chettibi. 1990

To My Parents

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**List of abbreviations:**

| | |
|---|---|
| p-BPB | : para-Bromophenacyl bromide. |
| CMC | : Critical micelle concentration. |
| $DiC_8PC$ | : Dioctanoyl phosphatidyl choline. |
| DMF | : Dimethyl formamide |
| DTT | : Dithiothreiotol. |
| EDTA | : Ethylenediaminetetracetic acid. |
| GPC | : Glycerophosphoryl choline. |
| $^3H$ | : Tritium. |
| IRS | : Interface recognition site. |
| MLD | : Manoalide |
| MOPS | : Morpholinopropane sulphonic acid |
| PAS | : Periodic acid Schiff. |
| PC | : Phosphatidyl choline. |
| PE | : Phosphatidyl ethanolamine |
| $PLA_2$ | : Phospholipase $A_2$ |
| PS | :Phosphatidyl serine. |
| SDS | : Sodium dodecyl sulphate |
| TEMED | : N,N,N,N,-Tetramethylethylenediamine. |
| TLC | : Thin Layer Chromatography. |
| TNM | : Tetranitromethane. |

## Summary

Acyl imidazolide activation of venom phospholipase $A_2$ enzymes was studied by a variety of kinetic and chemical means in order to determine the nature of the reaction between the protein and the activator and to identify the amino-acid residues involved. The conductimetric methods of Lawrence, (1971) were extensively modified and improved.

The proposal of Drainas, (1978) that activation by acylation was complementary to activation by calcium was studied in detail. Nitrogen-based chelators were used to show that the metal ion dependence was dominated by the inhibitory effects of transition metal ion impurities, with copper and zinc being the most powerful inhibitors. A kinetic investigation showed that these metal ions were not simple competitive inhibitors for calcium. The kinetic results showed that long-chain fatty acylation had no effect on the metal-ion dependence. The importance of using transition metal ion chelators (eg o-phenanthroline) in such kinetic studies was demonstrated and this study revealed that the affinity constant for calcium was ca 5μM (ie 50 fold less that previously stated).

A new polyacrylamide gel method was developed to study small basic peptides; this had a lower detection limit at octapeptides, but could routinely detect decapeptides using the Coomassie blue detection method. This was used to monitor purification of bee venom components and to characterise peptide fragments of bee venom phospholipase $A_2$.

Short chain acyl imidazolides were found to block the activation site without giving full activation. This was consistent with models in which a long hydrophobic chain was required either to act as a substrate penetration probe or else to change the conformation of the enzyme. The conformation change model was supported by further studies of the resistance of the activated enzyme to a variety of inhibitory reagents.

Use of radiolabelled acyl imidazolide activators showed that the acyl residue did not remain bound to the activated enzyme under acidic denaturing conditions. Cyanogen bromide fragments and fragments obtained by peptidase treatment retained insignificant amounts of the labelled acyl residue. Group modifying agents were tested as blockers or modifiers of the activation reaction. The results eliminated any direct involvement of the N-terminal residue or of lysine side chains. Modification of tyrosine and arginine residues changed the nature of activation but did not affect reaction with the activators. Demonstration that the enzyme could be activated by p-bromophenacyl bromide in the presence, but not in the absence of long-chain fatty acid pointed to the direct involvement of an active histidine residue. Comparison of the sequences of activatable and non-activatable enzymes indicated that a histidine residue at **20-26** might be important.

# Chapter One
# Introduction

## 1.1 Phospholipase $A_2$ enzymes.

Phospholipase $A_2$ enzymes catalyse the hydrolysis of the fatty acid ester bond at the 2 position of 1, 2-diacyl sn-phosphoglycerides (van Deenen & deHaas, 1963). The enzymic activity of phospholipase $A_2$ is rather special since it can hydrolyse water-insoluble molecules (Brockerhoff & Jensen, 1974). Although the enzyme does catalyse *in vitro* hydrolysis of phospholipids in monomolecular solution (Roholt & Schlamowitz, 1961; de Haas et al., 1971 and Wells, 1972), the same substrate present in an organized lipid interface is degraded at a much higher rate. For catalytic activity, the enzyme must have one molecule of calcium bound per enzyme molecule. These enzymes can be divided into two broad classes and quite a number of these enzymes have been described in details by many investigators.

The first class are intracellular enzymes found in almost every tissue examined so far (van den Bosch, 1980 and Waite, 1987). These enzymes are believed to regulate many important process. Some of them are known to be associated with the arachidonate cascade (Flower & Blackwell, 1979; Blackwell et al., 1980; Lewis & Austen, 1981, and Gupta et al., 1984). This cascade leads to the formation of bioactive metabolites, generally, known as the eicosanoids ($C_{20}$ derivatives), a term used for all the oxidative bioactive products of arachidonate (prostaglandins, leukotrienes, thromboxanes and platelet activating factor). Others are involved in the deacylation-reacylation cycle of membrane phospho-lipids,

protection of membranes from lipid peroxidation damage (van Kuijk et al., 1987), modulation of cell adhesiveness (Curtis et al., 1975 and Freitas & de Sousa, 1976) and in the degradation of bacterial phospholipids in relation to host defense against infection (Elsbach et al., 1979,1985; Elsbach & Weiss, 1983 and Forst et al., 1986a,b).

The second class are the extracellular enzymes found in most animal venom such as those of arthropods (Shipolini et al., 1971), snakes and scorpions (Tu, 1977; Habermann & Breithaupt, 1978 and Glenn & Straight, 1982), some of these enzymes are involved in the venom toxicity (Marlas & Bon, 1982), but the general role of phospholipase $A_2$ in most venoms is not fully understood. They are also a major component of mammalian pancreatic secretion (de Haas et al., 1963) and are responsible for the hydrolysis of dietary phospholipids.

### 1.2 Activation and regulation of $PLA_2$ enzymes.

Because unregulated phospholipase $A_2$ enzyme activity could be very damaging to the host cell membranes, the regulation of this activity is very important in many pathological and pharmacological events. There is a great interest in studying possible regulatory mechanisms for these proteins. Irvine, (1982) and Waite, (1987), reviewed some of these regulation mechanisms concerned these enzymes but many details remain obscure.

Recently, a family of proteins named lipocortins (lipomodulin, macrocortin and renocortin) which block inflammation have been found to inhibit phospholipase $A_2$ enzymes activity by interacting directly with the phospholipid substrate to form lipocortin-phospholipid derivatives, thus altering the substrate rather than competing for or binding to sites on the enzyme, (Cloix et al.,1983; Flower et al., 1984 and Hirata, 1984). These proteins were reported to be regulated by steroid hormones which also increase the sphingolipid content of cellular membrane (Nelson et al., 1982; Nelson & Murray, 1982; Bonney et al., 1987 and Sorenson et al., 1988).

Three other class of agents have been reported in the litreature to inhibit phospholipase $A_2$, p-bromophenacyl bromide (BPB) and its analogues, mepacrine (quinacrine, $N^4$-(6-chloro-2-methoxy-9-acridinyl)-$N^1$,$N^1$-diethyl-1,4-pentanediamine) and the marine natural product manoalide (MLD) a sesterterpenoid isolated from the sponge *Luffariella variabilis*. p-BPB covalently modifies histidine and methionine residues of proteins and has the potential to modify other nucleophilic residues, (Roberts et al., 1977), whereas, mepacrine, has been reported to interact directly with membrane phospholipids and form mepacrine-phospholipid derivatives. MLD, on the other hand, has been reported to be a potent irreversible inhibitor of bee venom phospholipase $A_2$ *in vitro* (de Freitas et al., (1984) and cobra venom (*Naja naja naja*) phospholipase $A_2$ (Lombardo and Dennis, 1985) the inactivation was pH dependent, time dependent and concentration

dependent. This inactivation of phospholipase $A_2$ may involve an ordered reaction with the peptide in phospholipases $A_2$ containing 1 to 4-lysine arrangement, (Glaser & Jacobs, 1986-87).

De Haas et al., (1968) showed that pancreatic phospholipase $A_2$ was synthesized as a pro-enzyme. Upon secretion into the gastro intestinal tract this pro-enzyme was activated by trypsin, which cleaves off a seven amino acid peptide at the N-terminal end and in contrast the regulation of the activity of venom phospholipase $A_2$ enzymes, which were found in the active form in the host venom sac is still incompletely understood, but in several cases these enzymes were show to be inhibited by heavy metals such as zinc and copper ions which were found at relatively high concentrations in the venom, (Tu, 1977), or else by the structural arrangement of cell-membrane phospholipids, such as the high sphingolipid content of the venom sac (van den Bosch, 1982 and Dawson et al., 1985).

Broekman et al., (1980) and Billah & Lapetina (1982), reported that the activity of phospholipase $A_2$ was induced by physiological stimuli such as thrombin, as well as calcium ionophores (Rittenhouse-Simmons & Deykin, 1981). Dawson et al., (1983, 1984) and Kramer et al., (1987), have shown that diacylglycerols have a unique stimulatory effect on a number of phospholipase $A_2$ enzymes. Also, Jelsema (1987) and Jelsema & Axelrod (1987), showed that phospholipase $A_2$ activity in rod outer segments of bovine retina was increased several fold by both light and guanosine 5'-[$\gamma$-thio] triphosphate (GTP[$\gamma$-S], which induced dissociation of the transducin

subunits of (G-proteins); this stimulation was mediated by the action of the $\beta\gamma$ subunits. Very recently Goldman et al., (1988) reported a stoichiometric activation of phospholipase $A_2$ from snake venom (*C. adamanteus*) and phospholipase $A_2$ from mammalian tissue with activators termed lipokinins, which are found to be produced by testosterone.

### 1.3 Bee venom phospholipase $A_2$.

Bee venom phospholipase $A_2$ is an example of the second class of extracellular phospholipase $A_2$ enzymes which are widely distributed in nature and are easily isolated and purified in a high quantity from different venom sources and pancreatic glands and are relatively stable. Because of these properties, there is a considerable interest in elucidating the detailed mechanism of their action as a pattern for the lipolytic enzymes and membrane-bound enzymes.

#### a- Purification and properties.

Most of the venom of the Hymenoptera contain phospholipase $A_2$ but the venom from the common European honey bee (*Apis mellifera*) was found to be the richest source for this enzyme, (Banks & Shipolini, 1986).

The chemical properties, and biological action of this enzyme have been extensively studied by many investigators. Shipolini et al., (1971) described a five step procedure for preparing the highly

purified enzyme. They showed that this enzyme is very stable and highly basic with pI=10.5. The amino acid sequence analysis of bee venom phospholipase $A_2$ was carried out by Shipolini et al., (1974a) using reduction and carboxymethylation followed by proteolytic cleavage. The sequence of the N-terminal was obtained from a direct application of the Edman degradation technique and the C-terminal by the digestion with carboxy- peptidase A and B. Eighteen peptides were recovered from the reduced carboxymethylated enzyme after chymotrypsin digestion and fifteen peptides were recovered after digestion of the 2-aminoethylated enzyme with *A.mellea* protease.

The amino acid sequences were determined by the dansyl Edman method. From the sequence studies it appear that this enzyme contains 128 amino acids and carbohydrate residues which are responsible for the increase of the molecular weight from 14555 to 15800 Da. These carbohydrate moieties are fucose, galactose manose and glycosamine in the molecular ratio 1:1:8:4 and their point of attachment is on the amino acid number 13 (asparagine).

All phospholipase $A_2$ enzymes studied so far are stabilized by 4, 6 or 7 disulphide bridges. Shipolini et al., (1974b) demonstrated that bee venom phospholipase $A_2$ consist a single chain of 128 amino acid group residues with eight half cystines. Shipolini et al., (1971) reported that the treatment of the enzyme, with p-chloromercuri-benzoate both in the presence and absence of 8M urea resulted in no detectable spectrophotometric change. These results indicate either

that the enzyme does not contain free sulphahydryl groups or that any such groups are deeply buried in the interior of the molecule. These workers proposed that the four disulfide bridges were present with the linkage being between the residues 37:107; 59:99; 9:30 and 31:89, this proposal was confirmed by using tryptic digestion.

Maraganore et al., (1986) used the primary sequence data of many phospholipase $A_2$ enzymes to devise a hypothetical ancestor sequence. When this was aligned with bee venom phospholipase $A_2$ enzyme it was concluded that the disulphide bridges should link residues 9:30; 31:107; 37:99; and 59:89 rather than these proposed by Shipolini et al., (1974)b.

Although bee venom phospholipase $A_2$ enzyme is from the phospholipase $A_2$ tree, it appears to be in a remote branch of it. However, it was reported to be activated in the presence of calcium ions and to be inactivated by EDTA and p-bromophenacyl bromide in the same manner as other phospholipase $A_2$ enzymes (Shipolini et al., 1971 and Abe et al., 1977 ). It is also stimulated by a wide variety of direct lytic peptides, such as melittin from bee venom (Dempsey & Watts, 1987), mastoparans from wasp venom (Argiolas & Pisano, 1983) and albumin (Gul & Smith, 1974). The two first agents appear to work by complexing with phospholipid substrates and presenting them to the enzyme in a more susceptible physico-chemical form. Albumin could act in two distinct ways:

1- It can complex phospholipids in free solution and enhance their

susceptibility to the enzyme (A. J. Lawrence, unpublished data), but it does not normally extract phospholipid from membranes so that this would not happen with membrane substrates.

2- It can binds the reaction products, free fatty acids and lysophosphatides, and remove them from the membrane (Deuticke et al., 1981). These products have very significant feedback effects on enzyme activity which may well be the dominant control mechanisms for the action of the enzyme on biological membranes.

### 1.4 Phospholipase $A_2$ assays.

Numerous methods have been described for assaying the action of phospholipase $A_2$ on different forms of phospholipid substrate, these assays were showed to vary from each other in their sensitivity in detecting the enzyme activity.

Marinetti (1965) described a simple method for detecting the product released from the action of snake venom phospholipases on egg yolk lipoproteins using silica acid impregnated paper, but this assay was not suitable for the kinetic studies of these enzymes. Wells (1972) and de Haas et al., (1971), used a titration assay to study the kinetic mechanism of phospholipase $A_2$ from the snake venom of *C. adamanteus* and pancreatic gland. This assay, which was carried out in a $CO_2$ free atmosphere at 37°C maintaining the pH at 8.0 by the addition of NaOH, claimed to detect the activity of as

little as 1 μg protein.

Aarsman et al., (1976) developed a spectrophotometric assay for phospholipase $A_2$ using acyl-thioester analogs of substrates (2-hexadecanoylthiol-1-ethyl-phosphorylcholine) in the presence of Elman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid)] (DTNB) and 10mM calcium. This assay was ca. 1000-fold more sensitive than the titrametric assay and it was also much more convenient to use. Volwerk et al., (1979) improved this method by using diacyl thiol analogues of lecithin. The disadvantage of this type of assay is that most phospholipase $A_2$ enzymes are inhibited by thiols which limits the usefulness of the method.

Hendrickson & Raude (1981), developed another assay for measuring the activity of snake and pancreatic phospholipase $A_2$ by synthesizing a fluorogenic substrate 1,2-bis[4-(1-pyreno) butanoyl]-sn-glycero-3-phosphorylcholine. This assay, like any assay of phospholipase $A_2$, was claimed to be sensitive to the physical state of the substrate and could detect the activity of as little as 8 ng of pure pancreatic enzyme.

Thuren et al., (1988), reported a new assay for pancreatic phospholipase $A_2$ using a phospholipid analogue, 1-palmitoyl-2-6(pyren-1-yl) hexanoyl-*sn*-glycero-3-phospho-*N*-(trinitrophenyl) amino-ethanol (PPHTE) in which pyrene fluorescence was intra-molecularly quenched by the trinitrophenyl group. Upon the hydrolysis of this molecule by phospholipase $A_2$, an increase in the

pyrene monomer fluorescence emission intensity occurred as a result of the transfer of the pyrene fatty acid to the aqueous phase. The detection limit of this assay was 10 ng/ml of porcine pancreatic phospholipase $A_2$.

Other colorimetric assays for phospholipase $A_2$ activity were described by (Wells, 1972; Canziani et al., 1982; Bon & Saliou, 1983 and Lob de Araujo & Radvanyi, 1987) using pH indicators such as phenol red, which was found to be the most sensitive dye. These assays were based on the fact that the enzymatic reaction acidifies the medium by liberating fatty acid from phospholipids and thus changes the absorption spectrum of the dye. Their use was limited because the pH indicators inhibited some phospholipases.

Intracellular phospholipase $A_2$ enzymes play an important role in liberating arachidonic acid, which is the limiting precursor in the biosynthesis of inflammatory mediators, such as prostaglandins, thromboxanes and leukotrienes ( Blackwell et al., 1980 and Gupta et al., 1984). These enzymes are difficult to purify and the specific activity after purification is usually found to be very low (1-10 nmol/mg/min).

Only assays employing radioactive substrates have adequate sensitivity for detecting intracellular enzymes, and are normally used for this purpose, (Grossman et al., 1974; Shakir, 1981; Dey, 1982 and Katsumata et al., 1986). Typically the enzyme was incubated with a phosphatidyl choline substrate radiolabelled in the acyl side chains

and after the end of the reaction the radioactive products, $^{3}H$-fatty acid or $^{3}H$-lysophospholipids were separated from the radioactive substrate by TLC, ( Upreti & Jain, 1978). An alternative method in which the products were separated on a silica column, was developed by (Feyen et al., 1983). Grossman et al., (1974), used hexane extraction as a very simple method to separate substrate and products and employed a double isotope technique to correct for contamination of the substrate by the products. Katsumata et al., (1986), improved this method by using hexane containing acetic acid in the presence of anhydrous sodium sulfate. These assays are sensitive but time consuming and are subject to the disadvantages inherent in a fixed-time assay.

Lawrence (1971) showed that esterase enzymes could be assayed by measuring changes in solution conductance, and developed a very sensitive assay system that could be applied to a very wide range of enzymes. The method has many attractive features. It is simple, rapid, direct and normally produces good results using the natural substrates of the enzyme. This was applied to the studies of bee venom activity and activation (Lawrence, 1972; Lawrence & Moores, 1975 and Lawence et al., 1974 ), also was used in studying enzyme kinetics and regulation, (Drainas & Lawrence, 1978 and Drainas & Drainas, 1985).

The assay is carried out in small open conductivity cells of 1-2 ml capacity, which have small flat, bright platinum electrodes in contact

with the solution and are continuously stirred by a small magnetic pellets. The cells (usually 6) are in a water bath controlled with a very well regulated thermostat and conductance changes are recorded continuously. Conductimetric assays had been described by other workers but the major new feature was the use of a cell which could follow changes in conductance very rapidly; without this, initial rates are obscured by changes due to thermal equilibration and by inadequate mixing. The multi-cell system also allowed comparisons to be made with a variety of blank controls, enabling the conductance changes due to the reaction of interest to be clearly distinguished from those produced by other factors.

Solution conductance changes are determined by a variety of factors Lawrence(1971), but for esterases and phospholipase $A_2$ enzymes they are dominated by the production of a fatty acid anion and a proton, which is then absorbed by the buffer as shown in this reaction:

$$\text{Phospholipids} \xrightarrow{PLA_2} RCOO^- + \text{Lysophospholipids} + H^+ + PLA_2$$

The conductance change is dependent on the buffer used if the buffer is cationic such as triethanolamine the result will be positive (an increase in conductance), eg:

$$(CH_2CH_2OH)_3N + H^+ \longrightarrow (CH_2CH_2OH)_3NH^+$$

But if the buffer is anionic ( bicine ) the result will be negative ( a

decrease in conductance), eg:

$$(CH_2CH_2OH)_2NCH_2COO^- + H^+ \longrightarrow (CH_2CH_2OH)_2HNCH_2COO^-.$$

This multi-channel system is highly advantageous for monitoring phospholipase $A_2$ purification because a large number of fractions can be assayed for enzyme activity in a short period of time. It has relatively high sensitivity and can detect the activity of about 5 ng protein. The apparatus also can be used for measuring leakage of electrolyte from erythrocytes which can occur as a consequence of phospholipase $A_2$ attack , and serves as an interesting alternative assay method for cell lysis. The major advantage over the more sensitive radiochemical methods is that it gives continuous progress curves with data being valid within ca. 7 seconds of mixing. It is therefore an ideal method for studying modulation of the kinetic properties of these enzymes. Finally it is a very general method which can be used to study a wide variety of enzyme-catalysed reactions and many of the chemical reactions which are used for modifying proteins and peptides.

### 1.5 Phospholipids susceptibility to $PLA_2$ enzymes.

Pure phospholipids exist in three different forms in aqueous solution these are the free monomer, micellar, and the lamellar forms. The critical micelle concentration (CMC), below which only the free monomer is present falls rapidly with increasing aggregate chain

length; above the CMC excess phospholipid adopts either the micellar or liposome structure.

The micelle is effectively a closed monolayer, which can be spherical or cylindrical with the head groups pointing to aqueous environment, and with the random packing of the side chains in the interior. The bilayer has two monolayers with the hydrophobic side chain in close contact, and can form closed shells which are models for cell membranes.

Phospholipase $A_2$ enzymes attack all three forms, but in the vast majority of cases it attacks micelles much more rapidly than the other forms. It is clear from the previous studies that the catalysis of phospholipases $A_2$ is characterized by three types of specificity:

1- substrate specificity, which requires a phosphate ester adjacent to a fatty acid ester, (Bonson et al., 1972).

2- surface specificity, which requires aggregated substrate with a suitable chain length for optimal activity, (de Haas et al., 1971; Pieterson et al., 1974).

3- stereospecificity, which requires an *L*-conformation at the $C_2$ of the lecithin, (Bonson et al., 1972).

Bee venom phospholipase $A_2$ enzyme shows maximal activity against pure micellar phosphatidyl choline species, but in contrast to some other phospholipases it does attack the monomer at a significant

rate (Shipolini et al., 1971).

The effect of the surface pressure and hence of substrate packing at the interface have been studied by (Verger et al., 1973) and they showed that bee venom phospholipase $A_2$ was able to penetrate the phospholipid film more rapidly than the pancreatic phospholipase $A_2$, but at relatively high surface pressures the penetration of the phospholipid film by the bee venom phospholipase $A_2$ enzyme became slow enough to give a significant lag.

Wells (1972), showed that the hydrolysis of short-chain substrate was much faster when the compound are present as micelles than when present in monomeric dispersion.

De Haas et al., (1971) studied the action of pancreatic phospholipase $A_2$ on homologous lecithins with acyl chain lengths varying from 6 to 10 carbon atoms. Under similar condition of ionic strength the enzyme hydrolyzed the dioctanoyl phosphatidyl choline with a specific activity of 6 mmoles /min/mg, but did not attack didecanoyl phosphatidyl choline at all. In contrast it was found by the monolayer technique that lecithin with acyl chain length varying from 8 to 12 carbon atoms were hydrolyzed at about the same rate. From this difference in response to chain length it was suggested that the enzyme activity is strongly dependent on the quality of the interfacial structure of the substrate.

Many studies concerning the effect of the interfacial properties on

phospholipase $A_2$ enzyme activity have been carried out with pancreatic enzymes. The physiological substrate for these enzymes is normally emulsified by means of bile salts, in contrast to the venom enzymes which must attack unmodified cell membranes to exert a toxic effect. The pancreatic enzymes are released as low activity zymogen precursors.

Pieterson et al., (1974); van Dam Mieras et al., (1975) and Volwerk et al., (1979) showed that the zymogen and the active enzyme from pancreatic phospholipase $A_2$ bind to the monomeric substrate analogues with comparable affinity and catalyse the hydrolysis with comparable efficiency. When aggregated phospholipid substrates were used, the zymogen did not show any catalytic activity, whereas, the catalytic activity of the active enzyme was greatly increased.

Van Dam Mieras and co-workers, using tryptic hydrolysis of the active enzyme in the presence of the non-degrading substrate analogues showed that a rather hydrophobic n-terminal sequence of the enzyme **A-L-W-G-F-R** was directly involved in the interaction with the lipid-water interface. This n-terminal was named as the interface recognition site **"IRS"**. No such site has been characterised for venom phospholipase $A_2$ enzymes.

Van Scharrenburg et al., (1981, 1983) showed that the amino acid residues **L-W-R** located in the n-terminal part of the pancreatic enzymes are important for optimal lipid binding properties.

Van Scharrenburg et al., (1984) prepared a series of phospholipase $A_2$ analogues which the N-terminal amino group was varied. These enzymes have greatly reduced affinity towards neutral lipid-water interfaces and correspondly low catalytic activity on micellar substrate. However most of these analogues retained some of their enzymic activity against monomeric substrates.

Meijer et al., (1979)a,b studied a series of modified enzyme species and showed that also **Y**-69; **L**-19; and **M**-20 are involved in the interaction of porcine pancreatic phospholipase $A_2$ with lipid-water interfaces.

Jain & Cordes (1973) and Verger & de Haas (1976) reported that phospholipase $A_2$ enzymes generally do not hydrolyze the natural phospholipids dispersed in water. In this cases the action of the enzymes was facilitated using short-chain substrate, Wells (1974), (1978) or by including a variety of agents, such as the mixed micelles of phospholipids with the nonionic detergent.

Dennis (1973a,b; 1974a,b) used Triton X-100 in his assay mixtures because this gave an isotropic solution of long-chain phospholipids. The activity of bee venom phospholipase $A_2$ was increased towards the hydrolysis of long-chain phospholipids by the presence of Triton X100, (Upreti & Jain, 1978 and Tsai et al., 1985), organic solvents (Coles, 1974), including alkanols (Jain & Cordes, 1973 and Upreti & Jain, 1978).

Other factors has also been reported to be involved in controlling the action of phospholipase $A_2$ enzymes on the hydrolysis of phospholipid substrates such as:

1- The nature of their polar head groups (Thuren et al., 1984). In a result obtained by Thuren et al., (1987), it appeared that pancreatic and intestinal phospholipase $A_2$ enzymes preferred acidic phospholipids as substrates and hydrolyzed 1-palmitoyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidyl-monomethyl ester with a faster rate than phosphatidylserine and phosphatidylglycerole (**PM > PS > PG**). In contrast snake venom enzymes preferred the 1-palmitoyl-2-[6-(pyren-1-yl)] hexanoyl-sn-glycero-3 -phosphatidyl choline as a substrate.

2- The physical state of the phospholipid substrate, in particular the electrical charge of the lipid surface, proved to be an important parameter in governing enzyme activity.

Hill et al., (1983a-b) synthesized a series of negatively charged detergents *S-n*-alkanoylthioglycol sulphated to study the binding properties and the kinetic behavior of pancreatic phospholipase $A_2$ and its zymogen showed that at pH 6.0 and detergent concentrations up to 0.08 x critical micelle concentration (CMC), the activities of both the enzyme and its zymogen are similar and very low, but when the concentration increased from 0.08 to 0.12 x CMC the enzyme activity was greatly increased with no change in the zymogen activity.

Radvanji et al., (1987) using neutral and negatively charged micellar substrates to compare the activity and binding of two neurotoxic phospholipase enzymes (ß-bungarotoxin and crotoxin) with other non-neurotoxic phospholipases. They found that both neurotoxic enzymes were able to bind and hydrolyze only the negatively charged micelles, whereas the non-neurotoxic enzymes bound to and were active towards both neutral and negatively charged micellar substrates.

### 1.6 The binding studies of the metal ions to phospholipase $A_2$ enzymes.

Extracellular phospholipase $A_2$ enzymes show a high degree of sequence homology and are very similar in their three dimensional structures (Renetseder et al., 1985 ). Crystallographic studies of phospholipase $A_2$ enzymes reported a single ion binding site (Dijkstra et al., 1981 and Brunie et al., 1985).

These enzymes are known to have an absolute specific requirement for calcium ions for their activity and no other metal can be substituted for the calcium with retention of significant enzymatic activity (Peiterson et al., 1974) and to be inhibited by the presence of heavy metal ions such as zinc and copper.

Long & Penny (1957) were the first to show that phospholipase $A_2$ was activated by calcium and inhibited by zinc. Roholt & Schlamowitz (1961), demonstrated that calcium acted by binding to

the enzyme. Studies using the barium ion as an inhibitor indicated that a metal ion-substrate complex was not involved.

Tu et al., (1977), using phospholipase $A_2$ from the venom of *Laticauda semifasciata;* observed an increase in the activity of the enzyme in the presence of calcium ions and to lesser extent with $Mg^{2+}$, but this activity was completely inhibited by zinc and cadmium even in the presence of a high calcium concentration.

Early studies by Shipolini et al. (1971) on ion dependence of bee venom phospholipase $A_2$ produced rather obscure results, where the activity of this enzyme was increased by the addition of calcium ions and abolished completely by excess EDTA. Surprisingly activation by calcium was potentiated by sub-stiochiometric levels of (0.1:1 EDTA/$Ca^{2+}$). These results suggested either that an inhibitory metal was present or else that the calcium/EDTA complex had a direct activating effect on the enzyme. They considered the second mechanism to be more probable because the enzyme did not contain sulphydryl groups normally present in enzyme sensitive to non-specific inhibition by heavy metal ions.

On the basis of kinetic studies on phospholipase $A_2$ Wells (1972) using *Crotalus adamanteus* phospholipase $A_2$ and short-chain substrate (dibutyryl lecithin ) demonstrated that the addition order of the reactants and the product release for this enzyme can be represented as follows:

E+ Ca <==> E-Ca

E-Ca+ lecithin <==> E-Ca-lecithin

E-Ca-lecithin <==> E-Ca-lysolecithin-fatty acid

E-Ca-lysolecithin-fatty acid <=> E-Ca-lysolecithin + fatty acid

E-Ca-lysolecithin <==> E-Ca + lysolecithin

E-Ca <==> E + Ca

**a -The use of spectrophotometer in calcium binding studies to phospholipase $A_2$.**

Wells (1973a), showed that the binding of certain cations to *Crotalus adamanteus* phospholipase $A_2$ affected the UV absorption of the enzyme in the region 286-292 nm and used this to determine the affinity constants for metal binding (affinities fall in the order $Ca^{2+} > Br^{2+} > Sr^{2+}$ ). Zinc and cadmium which were inhibitors for the enzyme also perturbed the UV absorption.

However, the character of the perturbation differed from that seen with alkaline earth cations and pH dependent spectral changes which occurred in the absence of zinc and cadmium were shifted to the acid region in the presence of calcium. The results of kinetic investigation (Wells, 1972) and spectrophotometry investigations (Wells, 1973b) gave association constants for calcium and zinc binding to *C. adamanteus* phospholipase $A_2$ of 4 to 8 x $10^{-5}$ M, and 1 to 3 x $10^{-6}$ M, respectively.

Pieterson et al., (1974) found that both porcine pancreatic phospholipase $A_2$ and its zymogen bind calcium ions in 1:1 molar

ratio. They proposed that binding caused a conformational change of the enzyme which altered the absorption spectrum; characterized by a very small change in the 280 and 290 nm region and a change at 242 nm.

The dissociation constant of the enzyme and its zymogen for the metal complex ranged from $10^{-1}$ M at pH 4.0 to 2 x $10^{-4}$ M at pH 10.0, suggesting that the metal ion binding site contains one or more carboxylate groups with an additional contribution of a residue with a $pK_a$ of 6 to 7 most probably histidine. This enzyme was partially protected against inactivation by p-bromophenacyl bromide in the presence of calcium ions.

Similar results were obtained by Halbert et al., (1976), using native and p-bromophenacyl bromide treated notexin from Australian tiger snake *Notechis scutatus scutatus*. Native and modified notexin bind calcium in a 1:1 molar ratio at calcium concentration 2 x $10^{-5}$ to 2 x $10^{-1}$ M. At pH 7.4, the dissociation constant of the native and the modified notexin were 1.4 x $10^{-4}$ and 2.5 x $10^{-2}$ M respectively.

Abe et al., (1977) used equilibrium dialysis studies and fluorescence spectroscopy to demonstrate interaction between calcium ion and the neurotoxin (ß-bungarotoxin). The result were consistent with a single binding site for the calcium with a binding constant of 0.15 mM. The neurotoxin ( ß-bungarotoxin) was also protected against inactivation by p-bromophenacyl bromide in the

presence of calcium ions and lysolecithin.

Fohlman et al., (1979), showed that the addition of calcium to the taipoxin produced ultraviolet difference spectra that were almost identical to those observed for porcine pancreatic phospholipase $A_2$. This dimeric protein bound two calcium ions, one in the alpha-subunit and one in the gamma-subunit. The $k_iCa^{2+}$ values were lower than those of porcine pancreatic phospholipase $A_2$.

Viljoen et al., (1975) using *Bitis gabonica* phospholipase $A_2$ showed that this enzyme binds two molecules of calcium per dimer, causing a change of tryptophan absorption, suggesting that both charge and solvent effects were involved. This enzyme could bind calcium after inhibition with p-bromophenacyl bromide which abolished the calcium induced shift at 242 nm but not at 279 nm. The pH dependence showed that the amino acid residues involved in interaction with calcium had pK values of (5.66, 6.75 and 9.15) these were proposed to be **D**-46, **H**-45 and **T**-25 respectively (Viljoen & Botes, 1979).

Asparagine occupies position 49 in most phospholipase $A_2$ enzymes sequenced so far (Botes & Viljoen, 1974; Joubert, 1975 ; Roberts et al., 1977; Fleer et al., 1978 and Kondo et al., 1978) is reported to be involved in the calcium binding loop. This has been confirmed by (Fleer et al., 1981) in a study of the modification of carboxylate groups in bovine pancreatic phospholipase $A_2$ using $^{14}C$ labelled semicarbamazide. Addition of one mole per mole of protein abolished

both calcium binding and enzyme activity. The binding of labelled semicarbamazide to the protein was controlled by a group with $pK_a$ of 5.2, identified as **Asp**-49. The same group was reported to be essential for the calcium binding by (van Eijk et al., 1984).

Donne-Opden Kelder et al., (1983), reported an additional low affinity binding site for the calcium for porcine pancreatic phospholipase $A_2$ at alkaline pH. Occupation of this site greatly increased the affinity of the enzyme for lipid-water interfaces at high pH. This site contains an essential group which has a $pK_a$ of 6.0 in the absence of calcium, and was identified as carboxylate group, the calcium determined pH shift is strong evidence that a carboxylate identified as **Glu71** is directly involved in calcium binding. Kinetic experiments on various phospholipases showed that only the enzyme species which contain **Glu71** have a second calcium ion binding site (the low affinity site).

The binding of calcium to bee venom phospholipase $A_2$ was studied by (Tsai et al., 1985) using ultraviolet difference spectroscopy, which showed a large peak at 249 nm and two small negative peaks at 287.6 and 302 nm. This suggests that the last two peaks are due to perturbations of aromatic side chain, probably tyrosine. The dissociation constant of this enzyme for calcium was $K_{Ca^{2+}} = 2.5mM$, this is in agreement with the study by (Shipolini et al., 1971). They also demonstrated competitive inhibition of the enzyme by cadmium, and proposed that the cadmium binds to the same site as calcium,

nevertheless the ultraviolet difference spectrum suggests that enzyme-cadmium complex may have a different conformation from enzyme-calcium complex, which probably explain why calcium activates whilst cadmium inhibits the enzyme.

A comparative study of the amino acid sequence of bee venom phospholipase $A_2$ with the proposed ancestral enzyme sequence (Maraganore et al., 1986) strongly suggested that the binding region of the calcium is on the carbonyl oxygens of **Trp**-8, **Gly**-10, **Gly**-12 and the free carboxyl of **Asp**-49, (Waite, 1987).

### 1.7 Erythrocytes membrane and their application in $PLA_2$ enzymes studies.

The erythrocyte lysis assay in the presence of albumin was first used by (Gull & Smith, 1974) to study phospholipase $A_2$ activity.

Drainas and Lawrence, (1978) used a variant of this assay in which electrolyte leakage was measured by conductimetric assay when cells were suspended in a low-conductance isotonic medium. They showed that in the presence of albumin, the activity of bee venom phospholipase $A_2$ was very greatly increased by covalent addition of long-chain fatty acyl groups to the enzyme. This assay has the advantage that albumin sequester any free fatty acids so that the study of the effects of acylation is unambiguous, although, the mechanism of phospholipase $A_2$ induced leakage is complex and not yet understood.

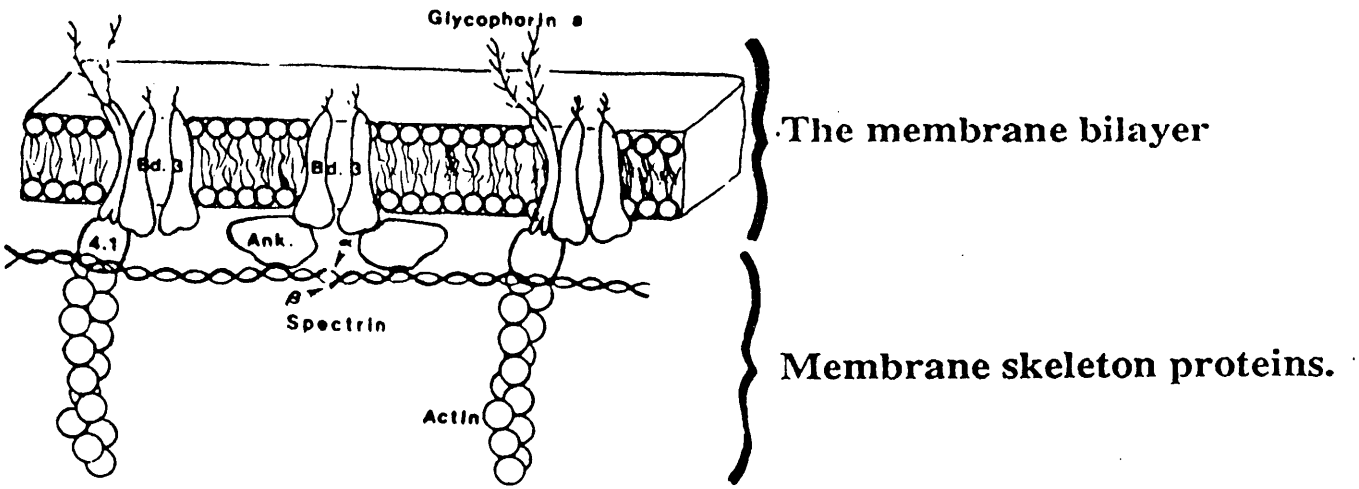
The structure of mammalian red blood cell membrane has been the subject of intense study and the **fig. I-1** shows the generally accepted model in which the lipid bilayer membrane is linked through some of its integral proteins to the cytoskeleton beneath.

There have been many studies comparing the properties of ghosts (membrane that have been broken and resealed) with intact cells and these reveal that the native structure of the membrane cannot always be successfully restored after fracture. In particular the intact cells have very high susceptibility to phospholipase $A_2$ and other lytic agents. This suggests that the activation of the enzyme may be determined by much more than the nature of the exposed phospholipid surface. Lawrence has suggested that the detailed topography of the surface is important in modulating the enzymic activity and has invoked this to explain the powerful inhibitory action of lysophosphatides in this system.

It is clearly important to study the effects of both products of phospholipase $A_2$ (fatty acids and lysophospholipids) and their extraction by albumin from the cells on the membrane protein components and also the mechanism of action of phospholipase $A_2$ on the phospholipids of the mammalian red blood cell membrane. In order to understand these two important biological effects it is of importance to know the general structure and the role of membrane components in the red blood cells.

The mammalian red blood cell ghost contains 49 % protein, 43%

Fig. I.1 Model for association of erythrocyte skeleton with membrane (redrawn from Carraway and Carraway (1989)) .



lipid and 7% carbohydrate (Rosenberg & Guidotti, 1968). This membrane has provided a simple, experimentally accessible system for studying the membrane-cytoskeletal interaction at a biochemical and a physical level. In a study on the structural organization of this membrane, it was described having two major structural units, the peripheral proteins (membrane skeleton) which forms about 50% of the membrane proteins (Bennett, 1985) and the membrane bilayer which contains the lipid bilayer and the integral membrane proteins (Branton et al., 1981 and Lux, 1979).

**a- Membrane skeleton proteins.**

The erythrocyte skeleton was first characterized by (Yu et al., 1973) by extracting erythrocyte ghosts with a nonionic detergent that removed the membrane bilayer. Electron microscopy showed that the cytoskeleton was located within about 10nm of the lipid bilayer (Tsukita et al.,1981).

The membrane skeleton is responsible for many of the structural properties of the erythrocyte membrane, which behaves like a semisolid with elastic properties, which is not observed with simple lipid vesicles (Kwok & Evans, 1981). It is also responsible for maintaining red cell shape, and restricting the lateral mobility of integral membrane proteins. Surprisingly, it is involved in maintaining the transbilayer membrane asymmetry of the phospholipids (Middelkoop et al., 1988).

**The components of the membrane skeleton.**

**1- Spectrin.**

Spectrin is the major protein of the membrane skeleton, isolated from erythrocyte ghost membranes by low salt extraction. The purified protein is a flexible rod-shaped molecule about 100 nm in length composed of two parallel polypeptide chains of Mr = 240 KDa ($\alpha$-chain) and 225 KDa ($\beta$-chain). In solution,spectrin dimers ($\alpha$, $\beta$) can self associate to form tetramers ($\alpha$–2, $\beta$–2) which is apparently the form of the molecule on the membrane as shown by chemical cross-linking experiments (Ji et al., 1980).

Cohen et al., (1980) reported that the binding sites for actin and band 4.1 localise to the same region at the tails of the spectrin tetramers, and the binding sites for ankyrin are localized symmetrically at the mid region of tetramers about 40 nm from each other. Haest (1982) using monomolecular lipid films, constituted at the air/water interface, and liposomes, reported the interaction of purified spectrin with phosphatidylserine.Similar results were obtained in red blood cells, (Haest et al., 1978). Another important role for spectrin reported by ( Berglund et al., 1984), was its association with calmodulin in the presence of calcium ions

**2- Actin.**

Purified erythrocyte actin is capable of polymerising to form 7 nm

filaments, it also activates myosin ATPase, and has all of the properties of other cellular actins. Short actin filaments have been visualized in erythrocyte membrane skeletons by electron microscopy (Shen et al., 1984).

Fowler & Bennett (1984), suggested that these short actin filaments may be stabilized on red cell membrane by a membrane-bound form of tropomyosin, which may also regulate association of actin with proteins such as spectrin or band 4.1 and possibly by an actin bundling protein which is described as band 4.9 (Siegel & Branton, 1985).

**3-Band 4.1.**

Protein 4.1 is an essential component of the erythrocyte membrane skeleton. It has been purified from high salt extracts of inverted vesicles (Tayler et al., 1979). It appears as a single polypeptide of 78 KDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS), (Fairbanks et al., 1971), but in discontinuous buffer gel systems it migrates as a closely spaced doublet designated 4.1a and b. These two members of the protein 4.1 are capable of binding to the end of spectrin tetramers, (Goodman et al., 1982) and mediates the spectrin-actin interaction through the formation of a spectrin-protein 4.1-actin complex.

Protein 4.1 also appears to form high affinity association with the cytoplasmic segments of glycophorin A (Anderson & Lovrien, 1984);

glycophorin C ( Mueller & Morrison, 1981), band 3 (Pasternack et al., 1985) and phosphatidylserine domains (Sato & Ohnishi, 1983; Rybicki & Schwartz, 1985 and Cohen et al., 1985).

Very recently Shiffer et al., (1988), reported that the interaction of protein 4.1 with phosphatidyl serine vesicles suggests that negatively charged phospholipid domains around the high-affinity protein-binding sites of protein 4.1 may contribute to the anchoring of the protein to the erythrocyte membrane.

**4-Ankyrin.**

A 210 KDa protein present in erythrocytes and other cell types (Hargreaves et al., 1980) is the best characterized example of this class in erythrocytes, it binds to band 3 with one of its two domains and to spectrin with the other (Weaver et al., 1984). Ankyrin has been purified from high salt extracts of inverted vesicles (Tayler et al., 1979). It is largely responsible for maintaining the close association of the membrane skeleton proteins with the lipid bilayer.

**b-The membrane bilayer**

**b.1-The integral proteins.**

Erythrocyte membranes contains two major integral proteins, band 3 and glycophorin (A) (B) (C), that span the phospholipid bilayer with distinct domains expressed on outer and cytoplasmic surfaces ( Bennett, 1985).

**1.a-Band** 3.

This important integral protein, exists in dimeric and tetrameric forms of 95 KDa per monomer (Haest, 1982). The inner surface of the membrane contains a large water-soluble domain of MW = 43 KDa (Bennett & Stenbuck, 1980). This cytoplasmic domain is attached to ankyrin which in turn is linked to spectrin and the cytoskeleton, (Bennett & Stenbuck, 1980 ) and is also reported to be associated with band 4.1, this association may modulate the attachment of the membrane skeleton to the membrane, (Pasternack et al., 1985). Band 3 serves as a major anion channel of the erythrocyte (Cabantchik et al., 1978). It is also a binding site for haemoglobin, protein 4.2, and several glycolytic enzymes, (Walder et al., 1984).

**1.b- Glycophorin A.**

This protein is the major sialic acid-containing glycoprotein present in dimeric form of 31 KDa per monomer (Marshesi et al., 1976). Its cytoplasmic domain is small (MW=3.000),and is attached directly to band 4.1, (Anderson & Loverien, 1984). The function of this protein is still obscure.

**b.2- The lipid bilayer.**

Approximately equal molar amounts of phospholipids and unesterified cholesterol make up about 50% of the mass of the erythrocyte membrane, and 60% of the total amount of the lipid is reported to be phospholipid, (Van Deenen & de Gier, 1974). Using

phospholipase, for establishing the arrangement of phospholipids in erythrocytes membranes, it has been concluded that these phospholipids are asymmetrically distributed over both halves of the lipid bilayer, this asymmetry is manifested by the enrichment of the outer leaflet in choline-containing phosphatidylcholine and sphingomylin, and the inner leaflet in the amino phospholipids phosphatidylethanolamine and phosphatidylserine (Zwaal et al., 1975; Op den Kamp, 1979 and Van Deenen, 1981).

### 1.8 Surface active agents.

Verger et al., (1973) emphasised the role of the properties of the interface in affecting phospholipase $A_2$ activity and termed this 'the quality of the interface'. It is clear that many lipophilic reagents enter phospholipid structures and modify their properties, especially the interfacial properties. Activation or inhibition by detergents may be explained in this way and Lawrence and coworkers, have stressed the role of lysophospholipids and free fatty acids as modulators of phospholipase $A_2$ activity especially in membrane systems. At sub-lytic concentration, free fatty acids activate the enzyme when attacking the erythrocyte membrane and lysophosphatides act as powerful inhibitors of this stimulation. Pure phospholipid substrate systems do not provide a model for this inhibitory action of lysophosphatides and it was argued that this is due to properties of the whole membrane. Both products can also be metabolised into bioactive substances such as platelet activating factor, (Albert &

Snyder, 1983).

**a-Lysophospholipids**

Previous data have demonstrated that these polar phospholipids (lysophospholipids) can directly inhibit the action of phospholipase $A_2$ on red blood cell membranes (Lawrence, 1975), but they also could reverse the inhibitory effect of the phospholipase $A_2$ inhibitors p-bromophenacyl bromide or mepacrine. Metz, (1988) has reported that they are involved in stimulating the mobilisation of cellular calcium. In addition, lysophosphatidylcholine is thought to exert a direct cytoxic effect on cells, (Nalbone & Hostetler, 1985) and may contribute to ischemic injury in the heart, (Sedlis et al., 1983)

**b- Free fatty acids.**

Fatty acids are reported to serve as a modulators of membrane functions and are found to stimulate guanylate cyclase (Wallach & Pasten, 1976), cyclic nucleotide phosphodiesterase (Hidaka et al., 1978), brain phosphatidylinositol phosphodiesterase (Irvine et al., 1979), increasing the activity of $Ca^{2+}$-ATPase (Schmalzing & Kutschera, 1982) and either to stimulate or to inhibit adenylate cyclase. These acids are also involved in modifying the surface charge of the lipid in such a way that interaction with the phospholipase $A_2$ enzymes should be facilitated. Activation of these enzymes by fatty acids has traditionally been explained either by invoking the charge interaction mechanism or else by other

interfacial phenomena.

The work published by (Lawrence & Moores 1975) suggested a different interpretation, that the enzymes may possess an allosteric activation site for fatty acids, which could be distinct from a hydrophobic attachment site. The key evidence was that bee venom phospholipase $A_2$ enzyme could be stabilized in a low activity state by treatment with glutaraldehyde or in a high activity state if the glutaraldehyde treatment was done in the presence of a long-chain fatty acid.

It was also shown that treatment with long-chain fatty acylating agents could stabilize a high activity form of the enzyme (Drainas and Lawrence., 1981). These results suggested that the free fatty acids or fatty acyl group bind to a specific site in the protein to produce a conformation change. This is in contrast to the mechanisms where the bound fatty acid or acyl residues would contribute to the hydrophobic interaction between membrane and the enzyme.

Very recently Van der Wiele et al., (1988)a,b reported that the acylation of pancreatic phospholipase $A_2$ by long-chain fatty acid (capric, lauric, palmitic and oleic acid ) increased its penetrating power and that palmitoyl-phospholipase $A_2$ completely hydrolysed all lecithin in the outer monolayer of the human erythrocyte at a rate comparable with *Naja naja* phospholipase $A_2$, which they stated to be the most lytic known phospholipase $A_2$

enzyme, although there is good evidence that it is not among the most active of these enzymes in leakage assays.

### 1.9 The covalent modification of proteins with lipids

Acylation of several membrane proteins by acyl residues is a relatively recent discovery (Schmidt et al., 1979) and many recent reviews were reported in the field of acylation of proteins with long-chain fatty acyl residues in prokaryotic and eukaryotic cells, (Sefton & Buss, 1987, and Towler et al., 1988)

#### a.The biology of the acyl linkage to the proteins.

The biological function of acylated protein with lipids still not clear, but in most cases lipids are found to be required for the binding of proteins to the membrane (Rose et al., 1984; Buss et al., 1986 and Ikehara & Takami, 1988) as well as for protein solubility (Olsen et al., 1985). Also very recently Aderem et al., (1988), showed that the exposure of the activated macrophages to interferon$_\gamma$ lead to the myristoylation of a 48 K.Da protein suggesting that acylation may be an early intermediate in the activation of macrophages.

Fatty acids, are one of the two products resulting from the action of phospholipase $A_2$ on phospholipid membranes, can activate some of these enzymes under certain assay conditions, producing a possible positive feedback effect.

Moores & Lawrence (1972), using the conductimetric assay showed, that in dilute aqueous solutions of organic solvents (n-propanol) bee venom phospholipase $A_2$ showed product activation which became more pronounced as the solvent concentration was decreased. This activation phenomenon was extended with more studies by Lawrence & Moores (1975) using fatty acids, aliphatic anhydrides and gluteraldehyde. They showed that the enzyme was activated by the addition of long-chain acyl groups ($R>C_6$) or by free fatty acids ($R>C_{12}$). Treatment of the enzyme with dilute glutaraldehyde made it insensitive to either from of activation, but similar treatment in the presence of a fatty acid which could not activated the enzyme directly, produced a stable activated form. This was considered to show that the enzyme could be stabilised in a low activity and in a high activity conformation and it suggested that the fatty acid or acyl group acted as true allosteric activators which operated by producing a conformation change in the enzyme. Drainas, (1978) using long-chain substrate showed that the addition of a long-chain fatty acylimidazole to bee venom phospholipase $A_2$ caused a very rapid irreversible activation with about 60 fold increase in its activity and the most effective activating agents were showed to be the imidazolide derivatives of oleic and linoleic acids (Drainas & Lawrence, 1978, 1981).

Similar activation was also obtained for other phospholipases $A_2$ from different sources such as wasp venom (Lyall, 1984) and some snakes venom (Drainas, 1978). The acyl derivatives were found to

activate bee venom phospholipase $A_2$ when used at a 1:1 stoichiometric ratio and the fully activated enzyme was shown to be stable against inactivation by p-bromophenacyl bromide (Lyall, 1984).

Analytical studies of activated bee venom phospholipase $A_2$ posed the question; does the activator produce its effect by interacting directly with the enzyme or by modifying the substrate water interface?.

The results obtained by Drainas (1978) using radiolabelled acylating agents and by Lyall (1984) using the erythrocyte leakage assay in the presence of albumin which bind the free fatty acids and lysophosphatides, eliminated the last possibilities and supported the first one. Camero Diaz et al., (1985) showed that acylation of the protein greatly increased its stability towards proteases and thiol agents suggesting that it caused a major conformation change. This provided strong evidence for the presence of an allosteric activation site.

**b. Chemistry of acyl linkage to proteins.**

The attachment of fatty acids to proteins has been divided into three categories (**fig. I.2**):

a)- Co-translational myristylation of proteins, where the myristoyl residue is linked through an amide bound to either N-terminal glycine

residues (Aitken et al., 1982; Henderson et al., 1983; Buss et al., 1984; Ozols et al., 1984; Schultz et al., 1985 and Paul et al., 1987) or to non N-terminal amino groups (Kellie & Wrigglesworth, 1987).

b)- Post-translational modification, where long-chain fatty acyl residues other than the myristoyl residue are linked through a thioester or an ester bond to cysteine, serine or threonine residues (Kaufman et al., 1984; Olson & Spizz, 1986 and Jing & Trowbridge, 1987).

c)- The addition of the complex glyco-phospholipids to proteins shortly after the completion of polypeptide bio-synthesis (Cross, 1987; Low, 1987 and Ferguson, 1988).

Very recently, Towler & Glaser (1986a,b) and McLlhnney et al., (1987), showed that amide-linked palmitoyl and stearoyl residues were present in some proteins. These results suggest that amide linkages were not necessairely restricted to myristoylation of proteins.

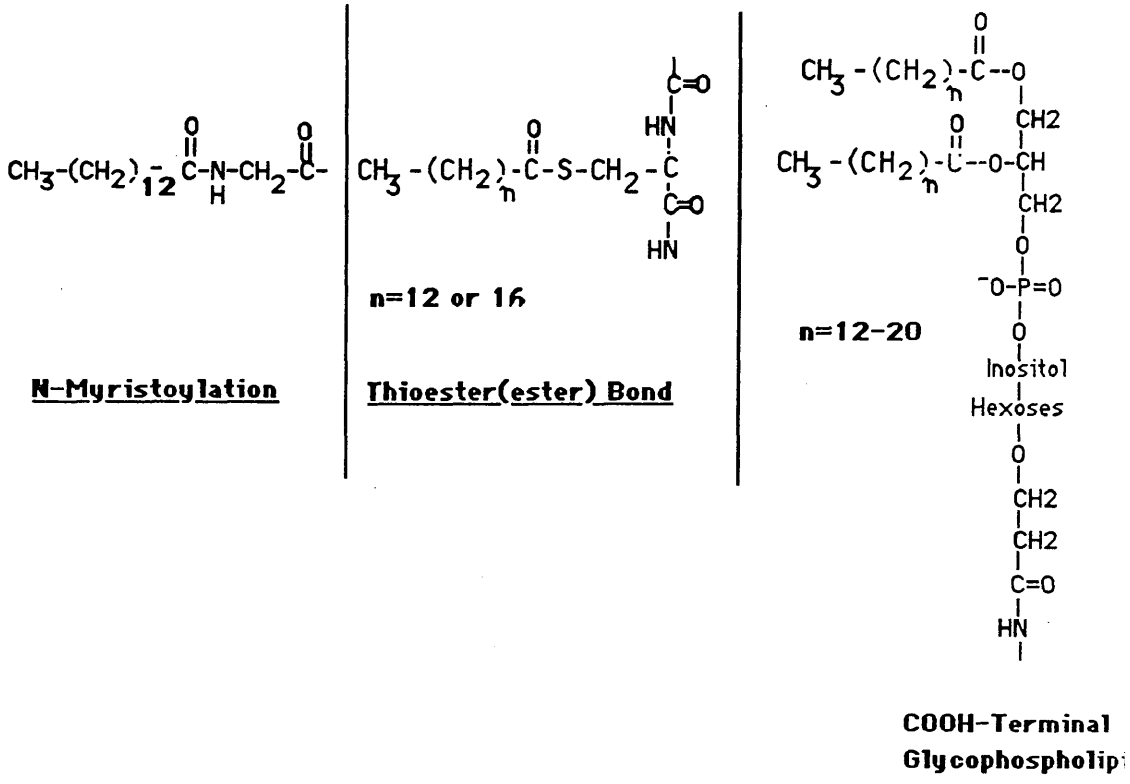



**Figure I.2**: Fatty acid linkage to eukaryotic proteins, (Towler et al.,1988).

## 1.10 The aim of this work.

As many of the proteins recognized as peripheral membrane components are known to possess a covalently bound long-chain fatty acyl residue, this covalent attachment of lipid to eukaryotic cellular proteins is not simply a biochemical curiosity. In several cases lipid is, clearly required for the binding of proteins to particular cellular membranes, or permitting them to interact with other polypeptides that reside in either the cytoplasm or membranes.

Buss et al., (1986) reported that myristoylation of $p60^{v\text{-}src}$ is required for its association with cellular membranes and morphological transformation of cells. Also studies with retroviral gag precursor mutants by Schultz et al., (1985) provided additional evidence that acylation can play a role in targeting proteins to cellular membranes. Another very important role of the acylation of proteins was reported by (Drainas et al., 1978), using long-chain phospholipid substrate in 20 % n-propanol and they showed that both long-chain free fatty acids and their acyl derivatives were able to increase the activity of bee venom phospholipase $A_2$ by 65 fold. Using the mammalian red blood cells assay in the presence of albumin which binds the two products resulted from the action of phospholipase $A_2$ on phospholipid membrane, the activation by the free fatty acids was abolished completely, but the addition of an equal molar of fatty acyl residue produced high degree of an irreversible activation (50-100 fold) this enzyme.

These results suggested that the enzyme has a highly specific allosteric binding site which can be occupied reversibly by the free fatty acids and irreversibly by the acyl derivatives of these acids. Further work by Camero-diaz et al., (1985), on the activation of this enzyme, showed that the occupation of this acylation site by the fatty acyl residue provided the protein with a high resistance against thiols and proteases and suggested that the occupation of this acylation site causes a change in the protein conformation.

From these results it appears that the reaction products resulted from the action of these enzymes on the phospholipid membranes may play an important role in regulating in vivo the activity of these enzyme since the lysophospholipids were reported to be an inhibitor and the fatty acid are shown to be an activator to these enzymes, as well as the calcium which was also reported by many workers in the field to play a direct role in regulating these enzymes. The aims of this work are:

a) To determine the mechanism of addition and mode of attachment of the acyl residue to the enzyme.

b) To study the properties of metal ion binding to bee venom phospholipase $A_2$ in order to determine the relationship between activation by metal ions and by acyl residues.

c) To distinguish between the two possible mechanisms for activation by acylation, *vis* that the acyl chain acts as a hydrophobic

anchor and facilitates binding to the lipid phase or that it is deeply buried in the protein core and changes the catalytic properties of the enzyme by an allosteric mechanism.

# Chapter Two
# Materials and Methods

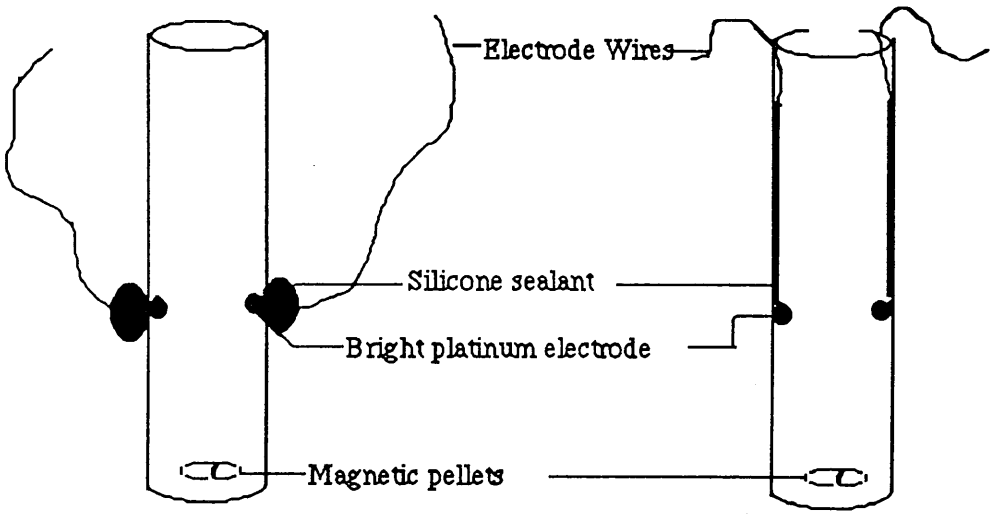
## 2.1 The conductivity apparatus.

This was developed by Lawrence et al., (1971) for kinetic measurements, and to investigate the efflux of electrolyte from erythrocytes. The original apparatus was a six-cell system with a multichannel point-printing recorder. The cells were constructed from glass tubes with platinum electrodes where the leads were sheathed in glass and stuck to the inside walls of the tubes. In an improved system, the electrodes were firmly embedded in the tube wall with the lead passing through the wall. Because platinum does not form a good seal in boro silicate glass, a leak-proof seal was established by means of silicone adhesive, **(fig. II.1)**. The cells were temperature controlled in a precision thermostat with on/off regulation controlled by a thermistor sensor. The measuring system was a low voltage AC bridge, (3000Hz ± 0.5V with high amplitude-stability) and the cell formed one arm of the bridge. Sensitivity was limited by stability which was typically ± 0.001% and did not appear to be limited by the temperature regulation, but by electrical noise present in the cells. The solutions in the cells were stirred continuously with small magnetic pellets.

### a. Stirring.

Each cell was stirred by a magnetic pellet (0.5 cm long) and the major technical problem with the apparatus was to maintain efficient stirring for a large number of cells. This required very careful design

Fig. II.1 The conductivity cell.




The developed conductivity cell

The original conductivity cell

of the rotating magnet beneath the cells, which was done by a trial and error method. In this work the aim was to expand the system to hold eight cells; this required an increased radius for the stirrer with a strong field gradient in the vicinity of the cells. By placing a single cell above the magnets and rotating them slowly it was possible to find the radial position where the magnets remained horizontal, and then to adjust the positions of the activator magnets to give the strongest stirring field.

**b.The circuit.**

This was an AC bridge operating on 3000 Hz at ± 0.5 volts peak to peak. One arm contained the cell and the gain of this arm was a linear function of cell conductance. The other arm contained the balance control and the gain was a linear function of balance resistance. Using this arrangement the cell conductance could be read directly from the resistance reading. The AC outputs for the bridge arms were arranged to be 180°C out of phase and were fed through capacitors into a rectifier network which subtracted the reference from the cell reading. The output from this network was fed into a unity gain amplifier/smoothing circuit. Up to 8 of these outputs were connected to an analogue switch and then fed to a 12 bit (+ polarity) analogue/digital converter which had a sampling time of ca. 30 msec. Each output was connected for a period of second, measured 12 times and the results summed and averaged. This greatly reduced the variation present when only single measurements were made.

The analogue switch was computer-controlled so that cells could be read in any combination. Output data was stored in the computer and could be recalled and printed as values or as differences and also plotted in the same way. The procedure for running experiments was to fill the cells and to balance roughly the cell readings using the balance resistors. When the readings were stable (after ca. 2 min of incubation) an automatic zero subtraction was performed and the data stored in this processed form.

The conductivity cell readings were very sensitive to bubbles forming on the electrodes. This caused readings to drift and vary erratically, but the problem was be reduced or eliminated by degassing the warmed reaction solution at a vacuum pump on the day of use.

**c. Calibration.**

Because of the variation of the distance L between the two electrodes and the difference of the area A of the electrode, the specification of the cells tended to vary by about ± 10 %. This was originally corrected by an electrical adjustment of circuit gain of the arm of the bridge which contained the cell. In the present system the correction was made by computation. Two methods were used to find the correction. In the first method, 2 ml of 10 mM triethanolamine buffer was added to each cell and when the conductance readings became steady, a series of 2 µl aliquots of isotonic saline was added to each cell, with a delay between each addition, and the conductance

change measured each time. These readings were plotted to show linearity, **(fig.II.2)** and the gradients used to determine the correction factors for each cell. These were simply multiplying factors which made all of the readings identical. One of the cells was used as an arbitrary standard.

The alternative method was to lower the sensitivity of the bridge by ca. 20-fold and to read the conductance of a standard buffer present in all cells with the balance arm of the bridge adjusted to give zero output. These conductance values should be directly proportional to the cell constants, but they do not contain a correction for the gain of the output circuit (although this should be the same for all of the individual bridge units). At present, the first method is preferred, although it is more time-consuming than the second.

### 2.2 Buffers

In conductimetric assays, cationic buffers normally enhance conductance rises produced by ester hydrolysis, the exception being when the substrate itself carries a net positive charge. Cationic buffers were required for most chemical and kinetic studies and triethanolamine was the preferred base because of its low reactivity and its low tendency to form ion pairs.

For other studies, where pH change did not form the basis of the measurement, the univalent zwitter-ionic buffers, MOPS or bicine,

were preferred. MOPS was used for the work with erythrocytes. Bicine was used for biochemical studies.

All buffers were used used at 10 mM concentration of the univalent ionised species and the concentration was specified by the amount of univalent acid (HCl) or base (NaOH) present.

**a. 10 mM basic (cationic buffers)**

These were all prepared according to the procedure given here for triethanolamine buffer:-

200mM stock solution of triethanolamine buffer was prepared by mixing 18 ml of 11 M hydrochloric acid (HCl) with 800 ml distilled water and the pH was brought to 8.0 with Triethanolamine (Sigma, Chemical, Company Ltd). The final volume was made up to one litre with distilled water. The stock solution was diluted to 10 mM for use.

**b. 10mM anionic buffers.**

These prepared as above, but substituting 200 ml of 1 M NaOH for HCl, and adjusting the pH by addition of the solid buffer acid.

**c. Erythrocyte assay buffer.**

These assays required a low conductance, isotonic buffer which was prepared by dissolving 98 g of sucrose in 800 ml of distilled water plus 10 ml of 1 M NaOH. The pH was adjusted to 7.4 with MOPS

(Sigma Chemical Company Ltd), and the final volume was made up to one litre with distilled water. Although this buffer could be made by dilution of concentrated stock buffer into the appropriate sucrose solution, the pH differed significantly from that obtained when the sucrose was omitted.

**d. Buffered Saline.**

This solution, used in the preparation of mammalian red blood cells, was standard isotonic saline buffered with 10 mM MOPS, pH 8.0. It was prepared by dissolving 9 g of sodium chloride (NaCl) in 900 ml distilled water in the presence of 10 ml of 1 M NaOH, adjusting the pH to 7.4 with MOPS, and bringing the volume to 1 litre with distilled water.

**2.3 Preparation of substrate.**

**a. Thin layer chromatography (TLC)**

Phospholipids were routinely analysed by thin layer chromatography (TLC), as follows. Solutions were spotted onto silica gel coated, plastic TCL sheets (Mark AG Darmstaf, West Germany) and allowed to dry. The chromatogram was developed by a solution of chloroform/methanol/ acetic acid/water, 25/15/4/2 respectively. The chromatograph was dried and phospholipids were detected by dipping the sheet into a beaker containing the molybdenum blue reagent and then rinsing the sheet in cold water.

Monoacyl and diacyl phospholipids gave stable blue spots, but glycerophosphoryl choline gave a blue/green spot which faded very rapidly. On occasions, the chromatogram was developed with ninhydrin to distinguish phosphatidyl ethanolamine from phosphatidyl choline. In this case it was essential to stain with ninhydrin before applying the phospholipid stain.

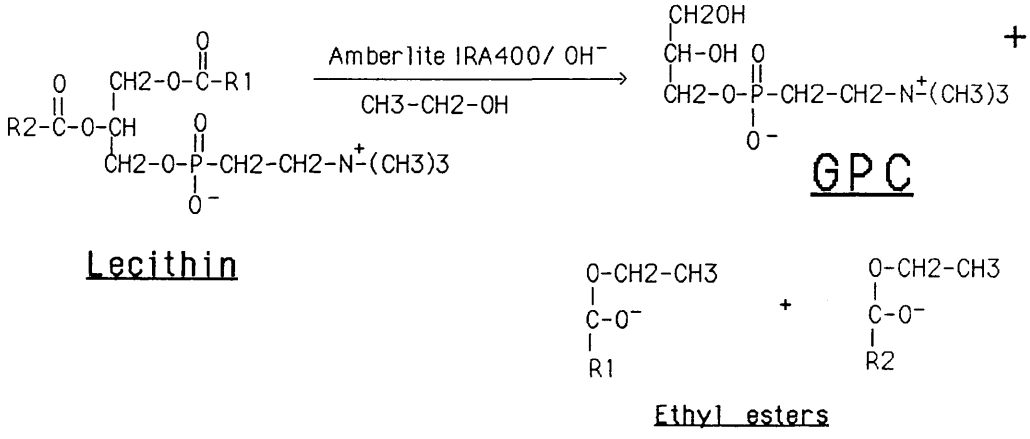
**b. Preparation of lecithins.**

Lecithins (1,2 acyl-3-snglycerophosphatidyl choline) were prepared by the method of Brockerholf & Yurkowski, (1965). Two dozen egg yolks were separated, homogenized in 500 ml acetone, and filtered to remove the acetone along with the yellow pigment and oils. This was repeated three times to give a pale voluminous precipitate. It was found to be important to use a high volume of acetone at each stage rather than more washes with a smaller volume. The white precipitate was extracted with ethanol (1 litre) by stirring for 30 minutes and filtering. The precipitate was discarded and the solution evaporated to dryness. The product was checked by TLC. This material contained two major phospholipid bands corresponding to phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE).

**c. Preparation of GPC (Glycerophosphorylcholine)**

GPC was prepared by a new developed method( Lawrence et al unpublished results). 48 g of lecithin (PC+PE) prepared as described above was dissolved in 500 ml ethanol and then mixed with a strong-

base anion exchange Amberlite resin IRA 400 (BDH. Chemicals Poole, Dorset) prepared in its hydroxide form by mixing 100 ml of 1M NaOH solution with 50 g of Amberlite resin IRA 400, stirring for 20 min and washing the resin several times with distilled water followed by four washes with ethanol. The mixture of lecithin-ethanol-resin was stirred at 55°C. The progress of the reaction was judged by spotting the solution on the TLC sheets and staining with the phospholipid detecting reagent (molybdenum blue). Reaction progress was assessed from the colour and stability of the spot (lecithin and lysolecithin give a stable blue spot, but GPC gives a blue-green spot which fades completely within 5 to 10 minutes). Using this test the reaction was found to reach completion in two to three hours.




The resin was removed by filtration and the solvent evaporated off leaving an oily residue which we believed was GPC contaminated with glycerol. Tests showed that the only solvent which could dissolve glycerol, but not GPC was dimethyl-formamide (DMF). The

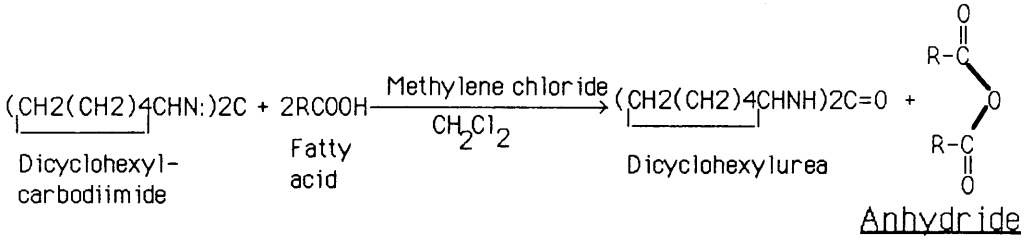
residue was therefore washed with dimethyl formamide (DMF), and the supernatant decanted, leaving the residue glycerophosphatidyl choline (GPC) which was washed three times with ethyl acetate to remove DMF. The residue was then dissolved in ethanol and deionised by mixing the solution with Dowex MR-3 (Sigma Chemical Co. Ltd), stirring continuously and checking of the conductance every 5 minutes. When the conductance reading of the mixture was equal to the conductance of ethanol, the resin was removed by filtration, the solvent evaporated completely, and the GPC washed again with chloroform. The chloroform was poured off and the solid was vacuum-dried and stored in the fridge.

**d. Preparation short-chain phosphatidylcholine derivatives.**

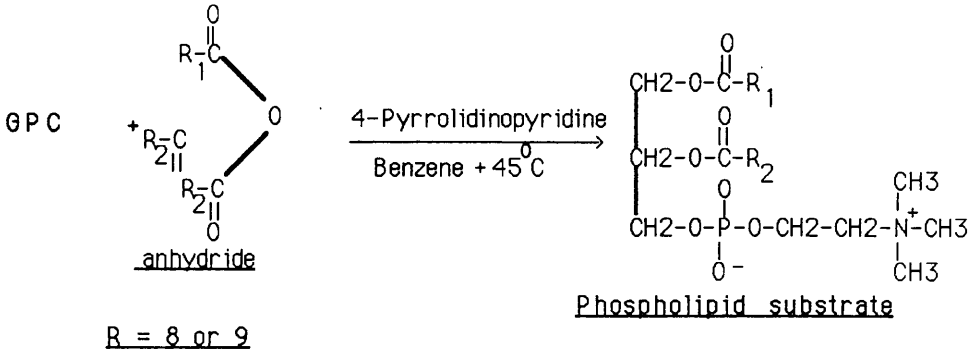
GPC was used for the preparation of diheptanoyl, dioctanoyl, dinonanoyl and didecanoyl phosphatidyl cholines for use as phospholipase $A_2$ substrates. Most of the work was with the dioctanoyl derivative, called here $DiC_8PC$.

This was prepared by a modification of the method of (Patel et al., 1979). These workers used the cadmium chloride adduct of GPC which is soluble in dimethyl sulphoxide, but in the present work the free base was used to minimise the possibility of metal-ion contamination. A solution of octanoic anhydride was prepared by mixing two moles of octanoic acid with 1 mole of 1,3- dicyclohexyl-carbodiimide in dry methylene chloride. After the reaction was over

the solution was filtered and evaporated. The reaction proceeds as:




2 ml of the anhydride was dissolved in 10 ml dry benzene containing 0.145 g (1mmol) of 4-pyrrolidinopyridine and 0.4 g of GPC. This was stirred overnight at 45 °C to 50°C, the progress of the reaction was checked by thin layer chromatography as described above chloroform:methanol:acetic acid:water (25:16:4: 2). After the reaction was over the benzene was evaporated, the product was dissolved in chloroform, and applied to an alumina column (activated, neutral. Aldrich Chemical Co. Ltd) which was washed with chloroform and the product eluted with 1 to 8 v/v methanol/chloroform. The eluate was collected in universal bottles and analysed by thin-layer chromatography. The samples containing diacyl phospholipid were pooled and evaporated to dryness. The solid, dioctanoyl phosphatidyl choline ($DiC_8PC$), was weighed, dissolved in methanol and diionized with 'Amberlite' monobed resin MB-3 (BDH Chemicals Ltd, Poole England).




An alternative method in which the acid anhydride was replaced by the chloride, gave a much faster reaction, but the product was rather less pure as judged by TLC.

This procedure was modified slightly for the preparation of diheptanoyl phosphatidyl choline. This derivative was of interest because its critical micelle concentration is such that it would be monomeric at the concentrations used in this work, whilst the dioctanoyl derivative has a critical micelle concentration which lies within the useful concentration range. Unlike the higher acid anhydrides, diheptanoyl anhydride is cheaper to buy than to synthesise. The product, diheptanoyl phosphatidyl choline, is the highest derivative will partitions into water from chloroform. This could be used to remove unreacted anhydride from the reaction mixture. The phospholipid could then be recovered from water by extraction with butanlol.

## 2.4 Erythrocyte preparation.

20 ml of blood from New Zealand white rabbits (from the animal house, Physiology Dept. University of Glasgow) was collected over heparin, (Sigma, London; 100 units/ml, 250 units for 10 ml of blood). The blood from other animal species was obtained from Glasgow Veterinary School. The blood was centrifuged at 3000 rpm, the serum and white blood cells (which were found to inhibit $PLA_2$ action) were removed by aspiration with a vacuum pump, the red cells were resuspended in isotonic saline at 2500 rpm. The procedure was repeated three times and the erythrocytes were made up to 30 % with saline and stored in the fridge and used within a week.

## 2.5 Preparation of carboxymethyl cellulose column (CM52).

Carboxymethyl cellulose (CM52) was used for the purification of bee venom phospholipase $A_2$. This was prepared by washing 10 g of CM52 with dilute hydrochloric acid for 30 min., filtering and washing with sodium hydroxide for another 30 min. The material was then washed at the filter with distilled water and then with 100 mM bicine buffer. The resin was then poured in a medium size column (2.5 x 10 cm), and washed three times with a strong solution of bicine buffer (100 mM), and five times with a weak solution ( 5 mM ) of the same buffer.

## 2.6 Conductimetric assays:

Cleaned cells were normally rinsed with buffer, emptied and refilled with 2 ml of fresh buffer and left for 2-3 minutes to achieve thermal equilibration. The readings for each cell were then balanced to ±100 by the balance control. For most reactions the computer was then activated to subtract the residual reading from all subsequent readings before the enzyme was added. For some very slow reactions where the enzyme was in large volume, the enzyme was added before the autozeroing process was carried out. Only the data obtained after autozeroing was recorded.

### a. Calibration of the phospholipase $A_2$ assay.

Calibration of the cells for the phospholipase $A_2$ assays was carried out by repeated addition of 0.5 µl of 200 mM octanoic acid to 2 ml of triethanolamine/$Cl^-$ buffer to mimic the ionic change caused by phospholipid hydrolysis ;

$$(CH_2CH_2OH)_3N + R\text{-}COOH \longrightarrow R\text{-}COO^- + (CH_2CH_2OH)_3NH^+.$$

The conductance change was corrected for dilution by carrier solvent and plotted against the fatty acid concentration. **Fig II.2** demonstrate the linearity of the response.

### b. Phospholipase $A_2$ assays.

The kinetic studies of bee venom phospholipase $A_2$ were carried out

using two different conductimetric assay methods:

The first assay measured the catalytic activity of bee venom phospholipase $A_2$ directly by following the hydrolysis of various phospholipid substrates. The second assay, used largely in studies of the activation mechanism of bee venom phospholipase $A_2$, followed the progress of leakage of electrolyte for intact red blood cells.

**b.1- Phospholipid hydrolysis.**

All reactions used 2 ml of 10 mM triethanolamine buffer solution pH 8.0. To this was added phospholipid substrate prepared as a solution in methanol. Where the product fatty acid was shorter $C_{12}$, or shorter, no organic solvent was required for the assay. For long-chain compounds, such as egg lecithin, 20 % v/v on n-propanol was also included. Calcium was either omitted or included at concentrations up to 1 mM, depending on the level of sensitivity required. The standard activity assay used a pure aqueous buffer with 20 µl of dioctanoyl phosphatidyl choline added in methanol to give a final concentration of 0.6 mM.

**Fig.II.3a** and **fig.II.3b** shows the hydrolysis curves plotted both from the conductance values and from the rates of change resulting from the action of native bee venom phospholipase $A_2$ on dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) in the absence and presence of calcium ions. The reaction was as follows;

$$\text{Phospholipids} \xrightarrow{PLA_2} RCOO^- + \text{Lysophospholipids} + H^+ + PLA_2$$

The egg lecithin substrate was less susceptible to bee venom phospholipase and the assay showed strong product activation by fatty acid and also gave a large response to activation of the enzyme by acylating agents.

**b-2. Erythrocyte leakage assay**.

This conductimetric assay was carried out with 2 ml of isotonic sucrose buffer pH 7.4 with 20 µl of 33 % red blood cells and 0.1 mM bovine albumin (Sigma Chemical Co. Ltd). After the readings stabilized the computer was activated for storing the data and 2 µg of the activated enzyme was injected into the reaction mixture. **Fig.II.4a**, shows a typical response curve and **fig.II.4b**, is a plot of difference values. It is worth noting that because the maximum rate is well separated from the initial rate, this assay is ideal for quantitative measurements.

**2.7 Gel electrophoresis**

**a. Propionic acid/urea/ polyacrylamide gels (Acidic gels)** .

The gel stock solution was made by adding 22.5 g acrylamide monomer ($CH_2.CH.CO.NH_2$) (Koch-Light Ltd; Haverhill Suffolk England), 1 g of N,N'-methylenebis-acrylamide (Koch-Light Ltd;

Fig.(II.4)a The leakage response of rabbit erythrocyte to native and activated bee venom phospholipase A2 in the presence of albumin.

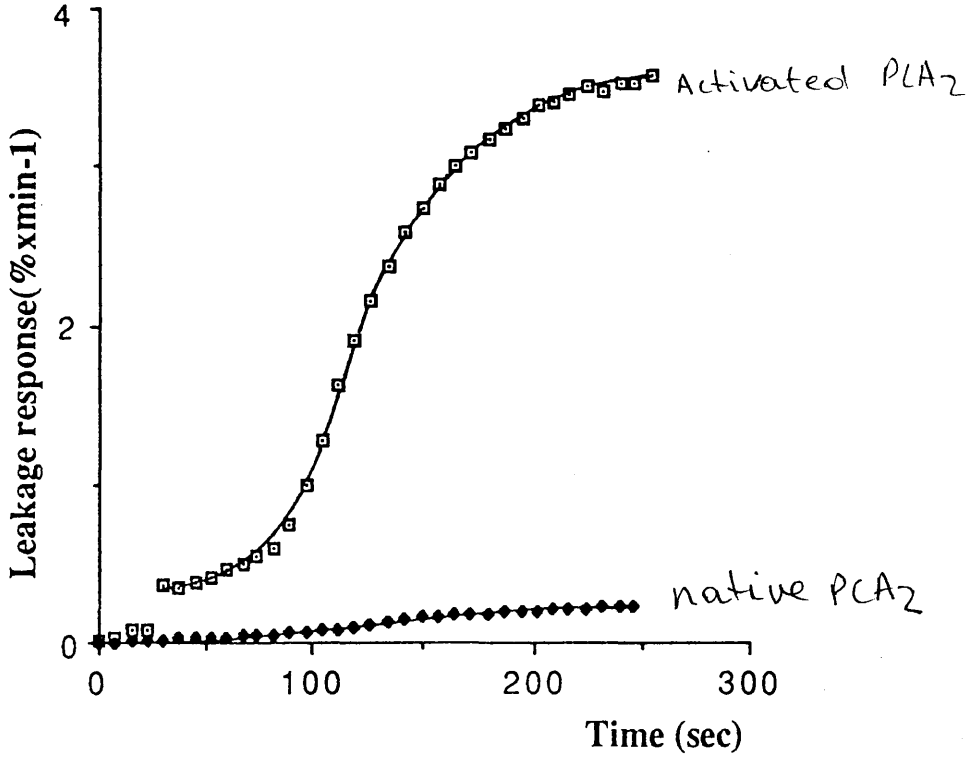
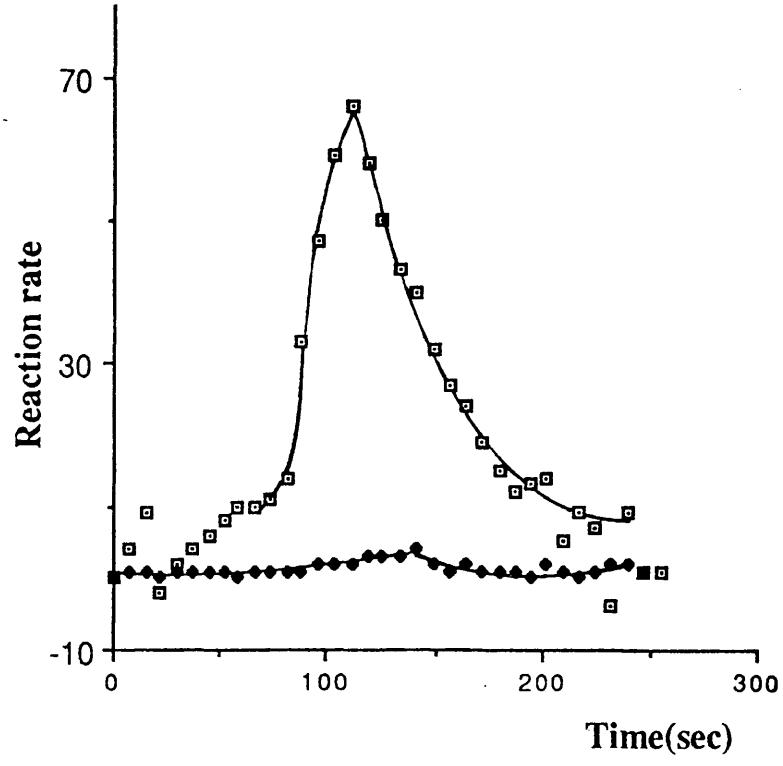



Fig.(II.4)b The reaction rate profile of the rabbit erythrocyte leakage response of the data in Fig.(II.4)a



Haverhill Soffolk England), and 36 g urea (BDH Limited Poole England) to 50 ml of distilled water. The mixture was stirred at 45°C and when all components had dissolved, the solution was cooled and the final volume made up to 100 ml with distilled water. To 60 ml of this stock solution was added 1.2 ml of propionic acid (Riedel-de Haen Ag Seelze-Hannover; West Germany), 0.4 ml of 10 % w/v ammonium persulphate solution (Sigma Chemical Co. Ltd) and 0.05 ml of TEMED (Sigma Chemical Co. Ltd). The solution was mixed gently by pouring 3 to 4 times between two beakers and poured into the gel plates with the comb positioned. The gels were polymerized at 37°C in an incubator in order to obtain uniform setting.

**Gels running conditions**

Samples were prepared by mixing the appropriate amount of protein with the same amount of water containing 50 % glycerol and neutral red marker dye. Gels were run with 2 % acetic acid in both anode and cathode compartments at 20 mA (> 400 V) until the red dye approached the bottom of the gel (2-3 hrs). The gel tended to form adhesions to the spacers and tended to tear on removal. This was prevented by running sharp blade between the gel and the spacers. Apart from this the gels were very robust and easy to handle.

**Gel staining and destaining.**

Gels were stained for 15 min with 0.1% Coomassie brilliant blue G (Sigma Chemical Co. Ltd) prepared with methanol/water/acetic acid,

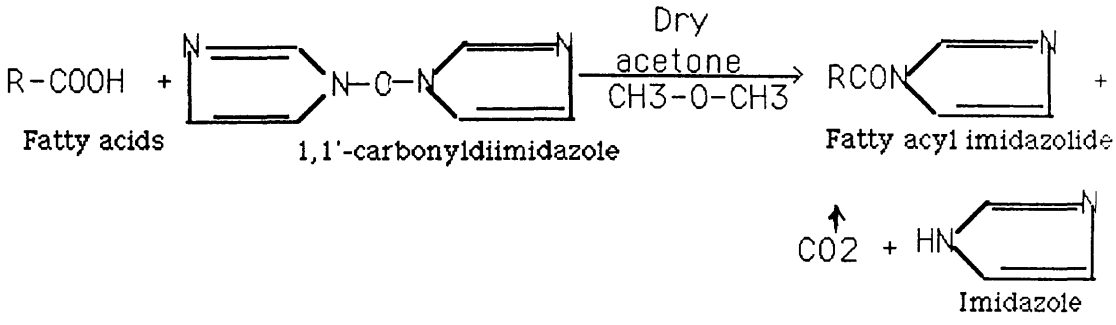
(50 / 50 / 7). The gel was left overnight in a mixture of this stain diluted 10-fold with destaining solution (methanol /acetic acid / water, 50 / 70 / 880). Finally the gel was left in destain solution.

**b. Ammonia / urea / polyacrylamide gels (Basic gels).**

These were prepared by the same procedure described above, but using ammonia instead of propionic acid, as gel electrolyte (Lawrence, A.J, unpublished results). Sample are prepared by mixing 1:1 v/v protein with bromophenol blue marker dye, the gels are run with 2 % ammonia solution in both anode and cathode compartments.

**2.8 Preparation of fatty acylating imidazolide**

Stock solutions of 2 % fatty-acyl imidazolides of different fatty acids were prepared by mixing 100 mM free fatty acid (Sigma Co. Ltd) with 200 mM 1,1'-Carbonyldiimidazole (Aldrich Chemical Co.) in 1 ml dry acetone and used without further purification.




Pure acylimidazolides were prepared by mixing one molar

equivalent of free fatty acid dissolved in acetone (typically ca.1 g of fatty acid in 20 ml of acetone) with two molar equivalents of 1,1'-carbonyldiimidazolide.

After 10 min the acetone was evaporated and the solid extracted with dry petroleum ether. The precipitate (imidazole) was removed from the solvent. The solvent was evaporated to dryness, a second petroleum ether extraction was carried out to remove any trace of imidazole from the fatty acylimidazolide, then the solvent was evaporated and the solid of pure fatty acylimidazole (activator) was weighed and stored at -5°C (Lawrence,A.J, unpublished results).

**a-Preparation of $^{3}H$ Fatty acylimidazolide**

$^{3}H$ acyl imidazolides were prepared by mixing 0.28 mg $^{3}H$ oleic acid with 2 mg cold oleic acid in toluene. The solvent was freeze dried, solid was dissolved again in 1 ml dry acetone, then 3.2 mg of 1,1'-carbonyldiimidazole was mixed with the labelled fatty acid to form $^{3}H$ fatty acyl imidazolide. The acetone was removed and the solid extracted with 5 ml of dry petroleum ether to remove any excess imidazole. The solvent was then removed by freeze-drying and the solid, ($^{3}H$ fatty acyl imidazolide, specific activity of 5 μCi/μmol was dissolved again in 1 ml dry petroleum ether and 100 μl portions put in small glass bottles, dried and stored in a small desiccator under vacuum with sodium hydroxide pellets as desiccant. The final specific activity in each bottle was 0.5 μCi / μmol.

**b- Activation of $PLA_2$ by acyl imidazolides.**

This was carried out as described by Camero-Diaz et al., (1985). 10 µl of 200 mM triethanolamine buffer was added to 90 µl of 1 mg/ml phospholipase $A_2$ and then treated with 2 µl of 0.2 % fatty acid imidazole to give equimolar amounts of activator and enzyme. The reaction reached completion after one to two hours incubation at 37°C.

## 2.9 Preparation of protein-modifying agents.

### a.Amino-group reagents.

Several reagents were used to modify reactive groups on the protein to correlate such modifications with changes in activity or in activation. These included succinic and dimethyl maleic Anhydrides (Sigma Chemical Co. Ltd) acetic anhydride (Aldrich Chemical Co. Ltd), used in the modification of the N-terminal amino group residue as well as the epsilon amino-groups of the lysine residues. One molar stock solutions of these anhydrides were prepared by dissolving the appropriate amount of each compound in 10 ml acetone. Methyl acetimidate hydrochloride was prepared as a one molar stock solution in methanol.

### Measurement of reaction rates.

The reactivity of these reagents could be measured very easily by conductimetric methods and rate constants of half-reaction times

were determined by the following method. 2 ml of triethanolamine buffer was incubated in the conductivity cells either in the presence or absence of an amino-compound. When the conductance change became steady, 1-2 µl of the amino-group reagent was added. By this means the rate of aminolysis of the reagent could be compared with its rate of hydrolysis.

**b. Phenylglyoxal.**

A reagent specific for arginine. This was prepared by dissolving 1.34 g of phenylglyoxal (Sigma Chemical. Co Ltd) in 10 ml acetone.

**c. Tetranitromethane (TNM)**

This was used in the nitration of the amino acid tyrosine residues and it was prepared by dissolving 5 mg of TNM (Sigma. Chemical Co. Ltd) in 5 ml of distilled water ($H_2O$)

## 2.10 Reduction and S-carboxymethylation of native and activated $PLA_2$.

Native and activated bee venom phospholipase $A_2$ were reduced and *S*-carboxymethylated as described by (Crestfield et al., 1963). 3 mg of protein was added to a solution contain 0.361 g urea, 30 µl of 50 mg/ml EDTA, 20 µl 2-mercaptoethanol and 300 µl of tris buffer pH 8.6 which was made up by mixing 5.23 g of tris with 9 ml HCl and the final volume was made up to 30ml with distilled water. The

solution was made up to 0.750 ml with distilled water then a solution of 8 M urea and 0.2 % EDTA was added to make the final volume 1.2 ml. After four hours of incubation at room temperature the contents were transferred to a 2.5 ml glass universal bottle under a nitrogen barrier and a freshly prepared solution of 0.268 g of iodoacetic acid in 1 ml NaOH was added to the reaction mixture. The iodoacetic acid added was slightly less on a molar basis than the amount of mercaptoethanol. After 15 min incubation the reduced protein was precipitated by ethanol and stored at -5°C.

### 2.11 Cyanogen bromide cleavage of $PLA_2$.

Native and activated bee venom phospholipase $A_2$ were reduced and carboxymethylated and then freeze-dried. A 50 fold molar excess of cyanogen bromide (10 mg in 1 ml of 70% (v/v) formic acid) over methionine residues (3 per molecule) was added and the reaction allowed to continue in the dark at room temperature for 24 hours. The reaction mixture was diluted 10 fold with water, lyophillised and then redissolved and lyophillised twice more.

### 2.12 Periodic acid Schiff (PAS) method for detecting glycopeptides.

Phospholipase $A_2$ can be stained on gels by the techniques used to detect sugars on glycoproteins. In this work the PAS technique was used to detect the enzyme and to characterise its cleavage fragments.

The enzyme ($PLA_2$) and its fragments were run on 22.5 % propionic acid urea gel prepared as described elsewhere, the gel which contained the samples was washed with 12.5 % trichloroacetic acid for 30 min, rinsed with distilled water for 3 min or longer, then immersed in 1 % periodic acid and 3 % acetic acid for 50 min. The gel was washed overnight with 200 ml distilled water then put in the dark with 50 ml Schiff's reagent , after 15 min. the Schiff's reagent was removed and the gel was washed with 0.5 % fresh sodium metabisulphite for 30 min. Finally it was destained in distilled water and put in 3 - 5 % acetic acid.

# Chapter Three
# Results

# Part One

### 3.1.1 The development of propionic acid/urea/ polyacrylamide gel.

Polyacrylamide gel electrophoresis has been an extremely useful analytical tool for the separation and quantification of protein species from complex biological sources. In the most commonly used variation of this method proteins are separated according to their molecular weight by electrophoresis on polyacrylamide gel in the presence of Sodium dodecyl sulfate (SDS-PAGE) which binds to proteins at a ratio of about one SDS for every two amino acid residues to give the protein a large net negative charge, roughly proportional to its mass. This method is widely used for the separation of high molecular weight (> 10 KD) species, but gives poor results for peptide mixtures. These need very high concentration gels to achieve resolution, but there is a significant complication, that small peptides are not well retained on the gel during the destain procedure. One method that is frequently used for low MW species is to replace SDS by urea. This presumably acts as a denaturant to improve resolution (by unknown means), but it is also said to pack the pores in the gel and so decrease their effective size (Panyim and Chalkley, 1969).

In the absence of SDS proteins are separated on the gel according to their net charge and size. Mobility is much lower, but can be increased by working either in very acidic, or very basic, conditions where the peptide is far removed from its isoelectric point. Most

frequently acidic conditions have been employed to suppress carboxylate ionization and hence to increase the net positive charge of the proteins. Panyim & Chalkley, (1969) developed an acid/urea polyacrylamide gel system to study the properties of histones, which are small, highly basic proteins.

This method has been widely used for peptide mapping of basic proteins such as histone variants, (Mardian & Isenberg, 1978) and it was found to be a very suitable method for giving a high resolution with low molecular weight protein species. King et al., (1976) used both SDS-PAGE and acid/urea PAGE to study bee venom components and their results indicated that both methods were equally effective, although their results gave a clear indication that the latter method showed more bands for the low molecular weight species. Based on their work, the acetic/acid PAGE method was used in this laboratory for routine studies of bee venom phospholipase $A_2$.

The original method using 5 % acetic acid in both gel and running electrolyte produced variable results in our hands and it was very noticeable that the gels did not set uniformly. However, raising the pH of the gel mixtures by reducing the acid concentration five fold gave a considerable improvement in resolution and reproducibility, with much more uniform gel setting. At this stage the effect of the TEMED concentration used for setting the gels was investigated because it had a very large effect on the actual setting pH **(fig.1b)**. Remarkably, in view of the above findings, the best separation of

peptides was found at low TEMED concentration (0.083%). Briefly these gels all have a migration front, which must be due to a discontinuity in the electrolyte composition and this is easily seen by visual inspection of an unstained gel. At high TEMED concentration the marker dye (neutral red) migrated on this front as a very sharp line. After destain a large, unresolved peptide band was also found in this position. With low TEMED concentration (0.166 %), the marker dye ran behind the front and migrated as a diffuse band. At first this was considered to be the sign of a poor gel, but it was observed that in these cases a broad number of clear bands were seen migrating ahead of the major band (melittin) found in whole bee venom, **(fig 1a)**. This was found to be highly reproducible, but nevertheless great variability in gel quality was observed and within a given gel some areas were seen to destain much more clearly than others. The improvement given by low TEMED concentrations (0.083%) was hard to explain, particularly in view of the fact that high setting pH was otherwise the most important factor in improving gel setting.

This lead to the idea of investigating the use of other aliphatic acids with higher pKa values than acetic acid to increase the setting pH without changing the overall nature of the electrolyte system.

The pH of 2 % solutions of acetic, propionic, butyric, isobutyric and pivalic acids were found to be 3.59, 3.90, 3.96, 4.02, 4.25 respectively and after the addition of the setting reagents, ammonium persulfate and TEMED the pH rises to 4.17, 4.46, 4.65, 4.70, 4.81

Fig.(1) The effect of TEMED concentration on the resolution of propionic acid/urea/polyacrylamide gel

Plate A; low TEMED concentration (0.083%).

Channels 1, 2 and 3 contains 88μg, 44μg, 22μg of whole crude bee venom.

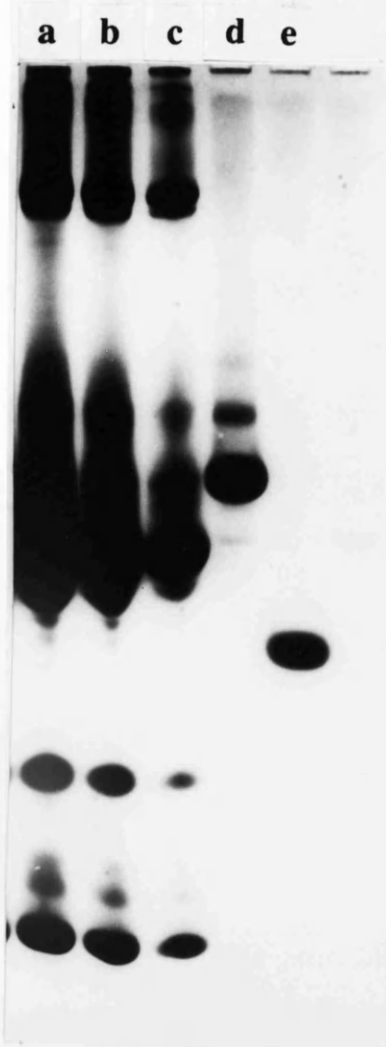
Channels 4 and 5 contained 4μg angiotensinogen and angiotensin I respectively.

Plate B; high TEMED concentration (0.166%).

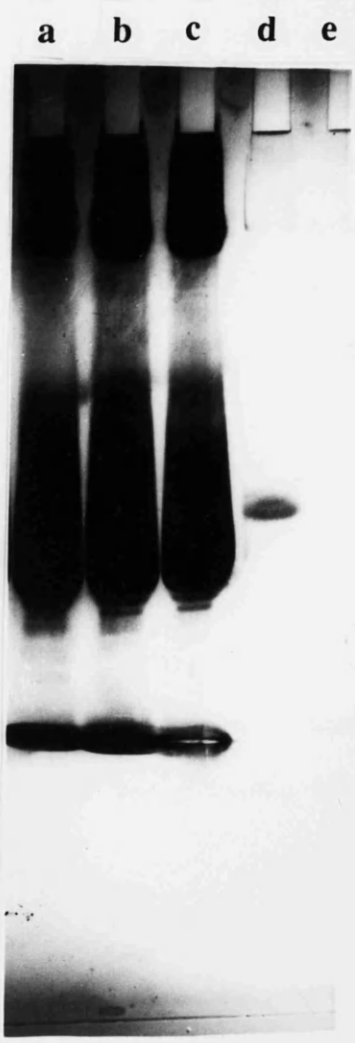
Channels 1, 2 and 3 contains 88μg, 44μg, 22μg of whole crude bee venom.

Channels 4 and 5 contained 4μg angiotensinogen and angiotensin I respectively.

A




B



respectively.

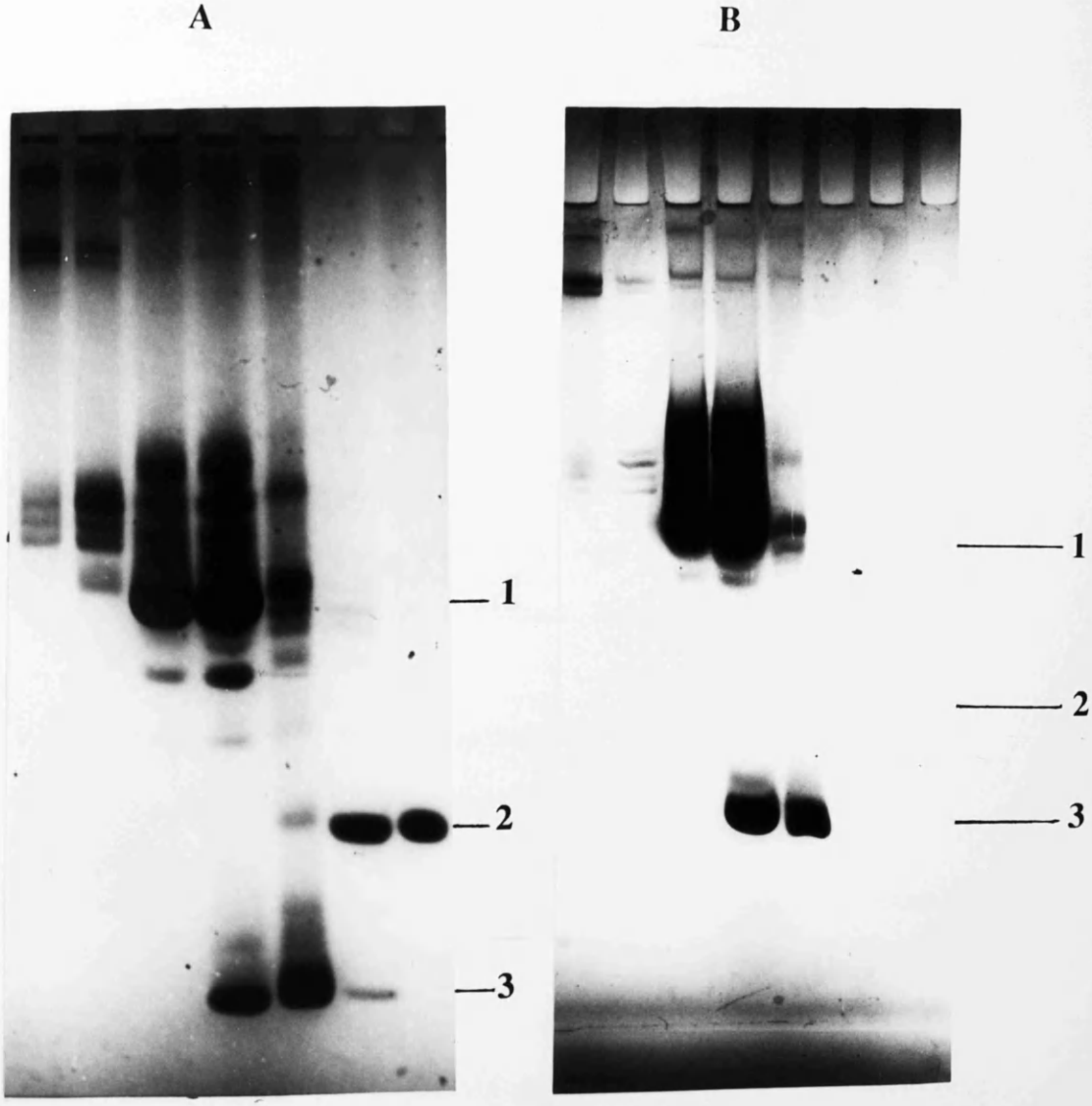
In a comparative study of the effect of these acids on the resolution of the gels, it was found that after 24 hours of the destain period, the gels with higher $pK_a$ (weaker) acids gave a much clearer pattern than those with lower pKa. The most noticeable differences were that destaining was more uniform and the backgrounds much more transparent and also the bands were more uniform in shape for the weaker acid formulations. In contrast the resolution of the bands deteriorated to a noticeable degree. In consequence there was an optimum performance which was found in propionic acid gels. At this stage a very significant feature was noted. Many bands, clearly visible in acetic acid gels at the beginning of destain, faded as the treatment continued and were completely missing by the time that the background was adequately destained. In marked contrast, the corresponding bands did not fade on propionic ( or higher acid) gels. This was most clearly demonstrated with reference to a band which appeared to correspond to the peptide apamin in bee venom, **(fig 2a,b)**.

Investigation of the role of urea showed that it was essential for good resolution and 6 M urea gave considerably better results than 3M urea. The final gel formulation used for the remainder of this work was based on 2 % propionic acid with 6 M urea and a stock solution of 22.5 % acrylamide monomer with 1 % of the cross linking reagent N,N' -methylene-bis-acrylamide. This was varied to give gel

Fig.(2) Comparative study of propionic and acetic acid/urea polyacrylamide gel electrophoresis

100 mg of whole crude bee venom was put through a large column (50x2.5cm) of Bio-Gel P30 in the presence of 20mM ammonium acetate and 2ml fractions were collected. 100µl sample was taken from each tube, mixed with 100µl of 50% glycerol containing neutral red dye marker and applied to 22.5 % of propionic acid (A) and acetic acid (B) urea polyacrylamide gel electrophoresis.Gel staining were carried out with 1% coomassie blue.

The bands 1, 2 and 3 are corresponded to melittin, apamin and mast cell degranulating peptide.


A
B
1
2
3
1
2
3

concentrations in the range 15-22.5 %. For lower gel concentrations a different ratio of bisacrylaminde to monomer was necessary. Setting was with 0.066 % ammonium persulfate (APS) and 0.083 % TEMED, and the electrolyte in both tanks was 2 % acetic acid. Propionic acid produced identical results, but run at rather lower current.

### 3.1.2 Basic gels.

At this stage it was appreciated that a complementary system could be produced by substituting a weak base for the acidic electrolyte in the gel. Ammonia, ethanolamine, Tris and imidazole were investigated and the first two compounds were found to give useful results, whilst Tris and imidazole gave very rapidly setting gels with extremely poor resolution (see part three, figure 26a,b).

### 3.1.3 Protein migration on propionic acid/urea/ polyacrylamide gels

Migration in these acidic gels depends on both charge and size and should therefore be determined by the ratio of net positive charge to the number of amino acids present in the molecule. At the pH of these gels, carboxylate ionisation should be largely, but not completely, suppressed and the dominant effects should be due to the positive charges contributed by the amino acids **H, K, R** plus the **N**-terminal residue.

Results presented in **fig. 3**, were obtained from the electrophoresis of some synthetic peptides, of the related family renin substrate ( 14 amino acids, 4 positive charges ), angiotensin I ( 10 amino acids, 4 positive charges ), Angiotensin II ( 8 amino acids, 3 positive charges ). Angiotensin II faded after a 36 hours of gel destaining, but the other compounds did not. Furthermore a mixture of 5 polypeptides of myoglobin fragments with a molecular weight range 2510-16950, and a low molecular weight poly-lysine marker were easily detected on this gel.

Although the poly-lysine marker was specified as molecular weight 1000-4000, which corresponded to a degree of polymerization from ca. 8-32. >55 bands could be discerned in this and similar gels. Unfortunately the distribution appears to be a near exponential decline from the highest mobility species. Attempts to enrich the sample in higher olegomers, by dialysis or by gel filtration on Bio-Gel P6, to obtain useful markers for the gel were unfortunately unsuccessful.

### 3.1.4 Identification of bee venom components by propionic acid/urea/polyacrylamide gel

Electrophoresis of whole bee venom on 22.5 % propionic acid / urea / polyacrylamide gel gave a band pattern with six major regions migrating ahead of phospholipase $A_2$, (see fig.1a). Many minor bands in the whole crude venom were present, but the unavoidable

Fig.(3) The resolution and migration of some small peptides on propionic acid/ urea polyacrylamide gel electrophoresis.

20μg of different molecular weight markers were mixed with 20μl of 50% glycerol containing neutral red dye marker and applied to 22.5% propionic acid/urea polyacrylamide gel electrophoresis.

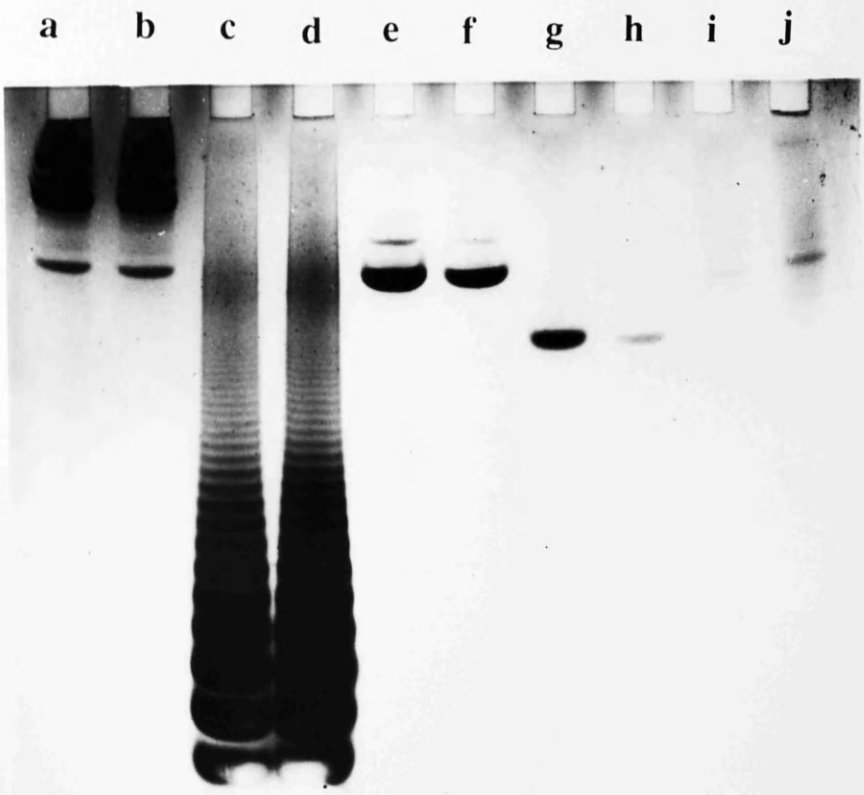
Channels a and b, 20 and 10μl of myoglobin fragments(molecular weight markers 2510-16.950).

Channels c and d, 20 and 10μl of poly-lysine marker (MW 1000-4000).

Channels e and f, 20 and 10μl of rennin substrate.

Channels g and h, 20 and 10μl of Angiotensin I.

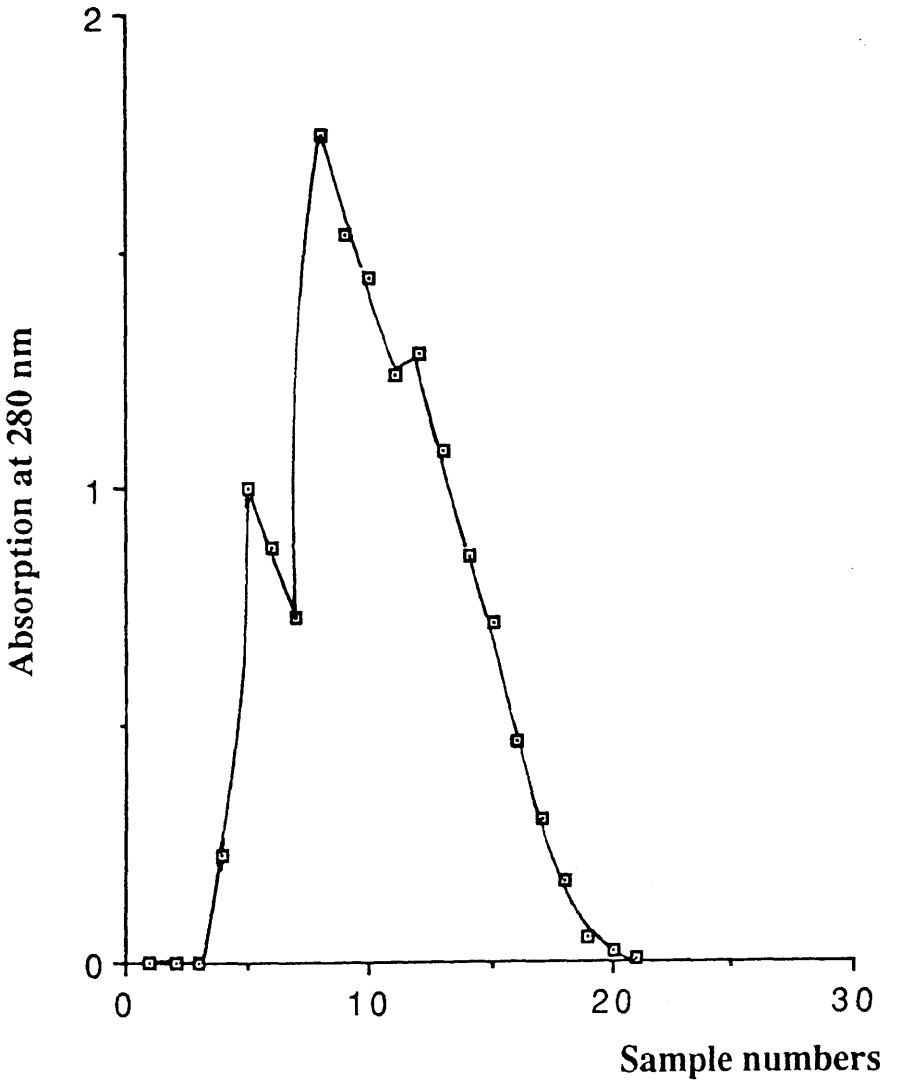
Channels i and j, 20 and 10μl of Angiotensin II respectively.


a
b
c
d
e
f
g
h
i
j

overloading with the major component (presumed to be melittin because of its cell-lytic properties) obscured much of the mid-mobility region. In order to obtain better resolution on the gel, the crude venom was subject to a preliminary MW fractionation by passing it through a column (250 x 15 mm) of Bio-Gel P30 equilibrated in 200 mM ammonium acetate (pH 6.5). 1ml fractions were collected and lyophilised to remove ammonium acetate before analysing by gel electrophoresis. The peaks of the venom components resulted from the separation were determined using a spectrophotometer, **(fig. 4)**.

The result obtained from this separation showed a very clear picture of 14 bands migrating ahead of phospholipase $A_2$, **(fig. 5)**. Comparison of the resolution obtained when the samples were not desalted by lyophilisation showed this procedure to be essential. High concentrations of salt present in the samples gave smearing and bunching of bands on the gel. Desalting was therefore essential for good analysis and because the existing methods were rather slow, the possibility of desalting by precipitation with organic solvents was investigated. A pilot experiment in which 0.5 ml of crude venom solution was subject to ethanol fractionation using a microfuge showed that when the precipitates obtained over a wide range of ethanol concentrations were taken up in the sucrose/glycerol marker dye solution and applied to the gel directly, a high resolution band pattern was obtained which showed good fractionation of venom components.

Fig.(4) The seperation of bee venom components on BIO-GEL P30 column.




500µl of 100 mg/ml of whole crude bee venom was applied to a large column (50x2.5cm) of Bio-Gel P30 in the presence of 20mM ammonium acetate and 2ml fractions were collected. UV absorption at 280nm were obtained by mixing 20µl from each tube with 1ml of Bradford reagent.

Fig.(5) 22.5% propionic acid/urea/polyacrylamide gel electrophoresis of whole bee venom components derived from a Bio-Gel P30 column.

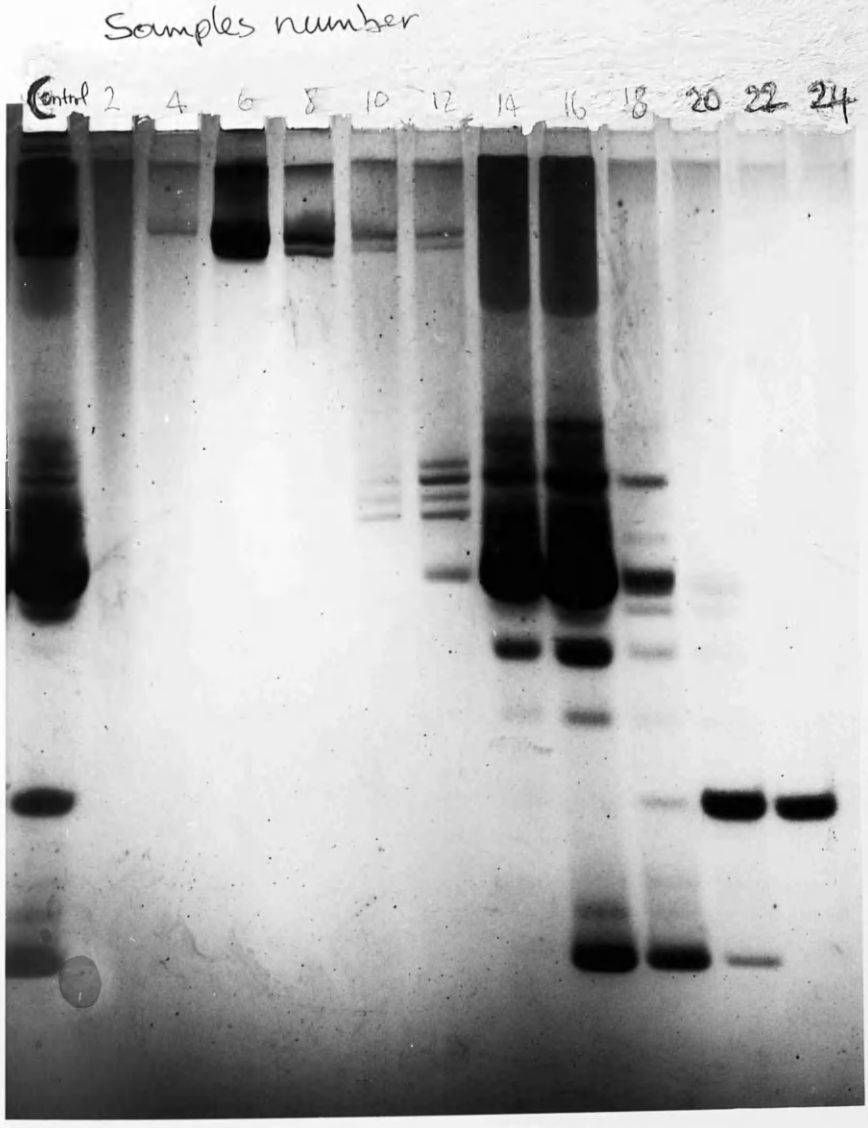
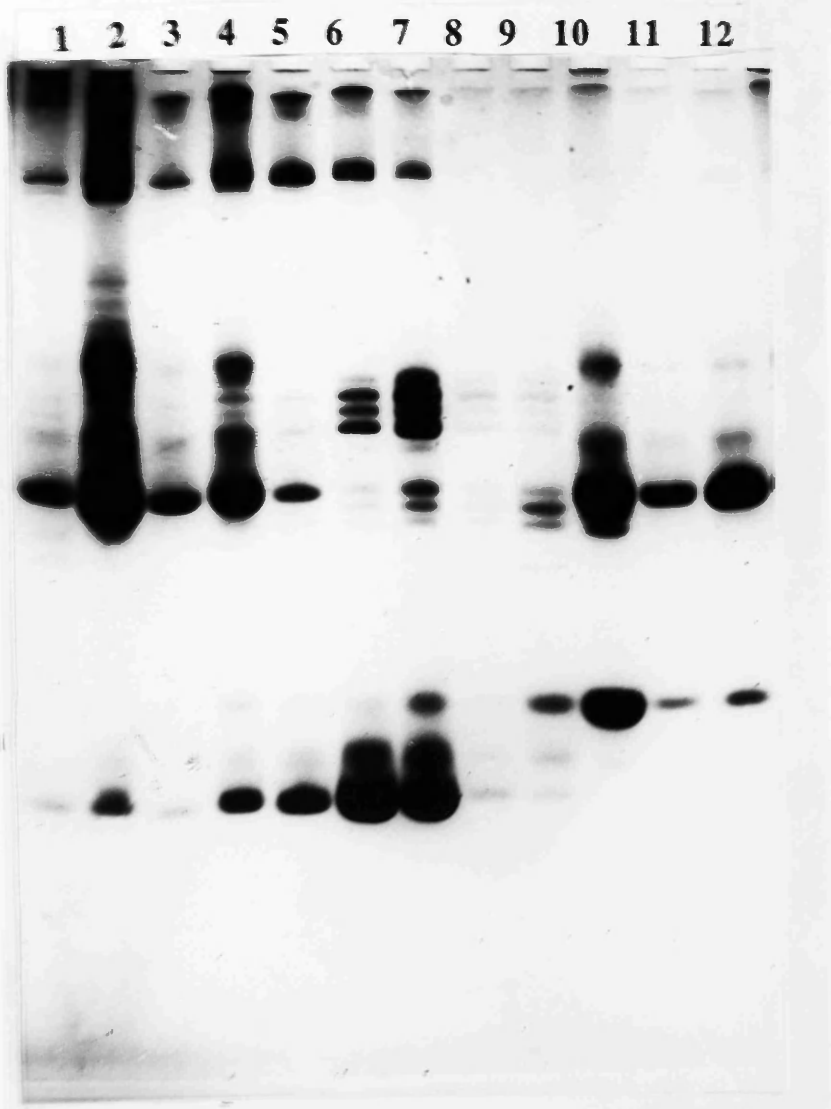

Samples number
Control
2
4
6
8
10
12
14
16
18
20
22
24

**Figure. 6**, shows the results obtained by ethanol precipitation over the range 0.5 / 1 (v/v) to 6 / 1 (v/v) of solvent / venom with a volume interval of 0.5 each time. This fractionation was very quick and reproducible. Comparison of the results with those obtained by gel filtration showed very similar pattern and the separation obtained for the apamin, peptide 401 and secapin suggested that the solubility of bee venom components in organic solvents may depend quite strongly on their size. Although the fate of the salt was not determined, it seemed probable that ammonium salts of weak organic acids would be highly soluble in aqueous ethanol.

Because this method was highly advantageous for the rapid desalting and for the purification of bee venom phospholipase $A_2$ an attempt was made to investigate the effect of calcium on the fractionation. When employed for precipitation of other metal ion dependent proteins, it had been observed that the presence of the ion both increased the stability of the protein during the procedure and also decreased the solvent concentration needed to precipitate the protein. Preliminary results showed that this was not the case here. When calcium was present in excess, the redissolved precipitate solutions were extremely turbid, but if EDTA was present in excess, they were clear. In addition the recovery of activity was significantly greater when EDTA was present throughout the fractionation. Throughout this work the combination of ethanol precipitation and propionic acid/urea polyacrylamide gel electrophoresis was used as an additional method for the purification

Fig. (6) 22.5% propionic acid/urea/polyacrylamide gel electrophoresis of whole bee venom components derived from ethanol precipitation.

100µl of 20mg/ml crude bee venom containing 1mM PMSF with 0.2mM EDTA was chilled on ice and treated with cold ethanol with a concentration range of 2:1, 1.5:1, 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5,1:4, 1:4.5, 1:5, 1:5.5 and 1:6 (v/v) of venom/solvent respectively. The precipitate was removed by centrifugation and dissolved in 100 µl of 50% glycerol containing neutral red marker ( channels 1,4,5,6,7,8,9,10,11 and 12), but the samples applied to channels 2 and 3 were dissolved in 2ml solution. In each case 5µl was applied to the gel.


1 2 3 4 5 6 7 8 9 10 11 12

of bee venom phospholipase $A_2$ for use in chemical and kinetic studies.

# Part Two

### 3.2.1 Metal ion dependence of $PLA_2$ enzymes.

Much of what is now known about the dependence of phospholipase $A_2$ enzyme activity on the calcium ions has come from the studies of Wells (1972) using the enzyme from *Crotalus adamanteus*. He determined the effect of addition order of calcium ion and substrate and also showed that various metal ions were powerful competitors for calcium. The clear message from this work was that valid kinetic studies of calcium dependence could only carried out with metal-free reagents. Shipolini and coworkers (1971) made an extensive kinetic study of purified bee venom phospholipase $A_2$ and concluded that EDTA might have an activating role in the presence of excess calcium. Drainas, (1978) showed that activation of bee venom phospholipase $A_2$ by oleoyl imidazolide apparently changed the calcium-dependence and the results indicated that calcium and acyl chain activation are complementary. The present studies were undertaken to resolve this point.

One simple approach which satisfied the stringent requirements indicated by Wells and also allowed the concept of activation by chelators to be investigated was to use divalent ion chelators which have a little affinity for calcium. A wide range of such compounds were available, the most well known being o-phenanthroline. Studies of this kind have not been described elsewhere, but the results presented below show that they can circumvent most of the problems encountered in this work and give results which illuminate the

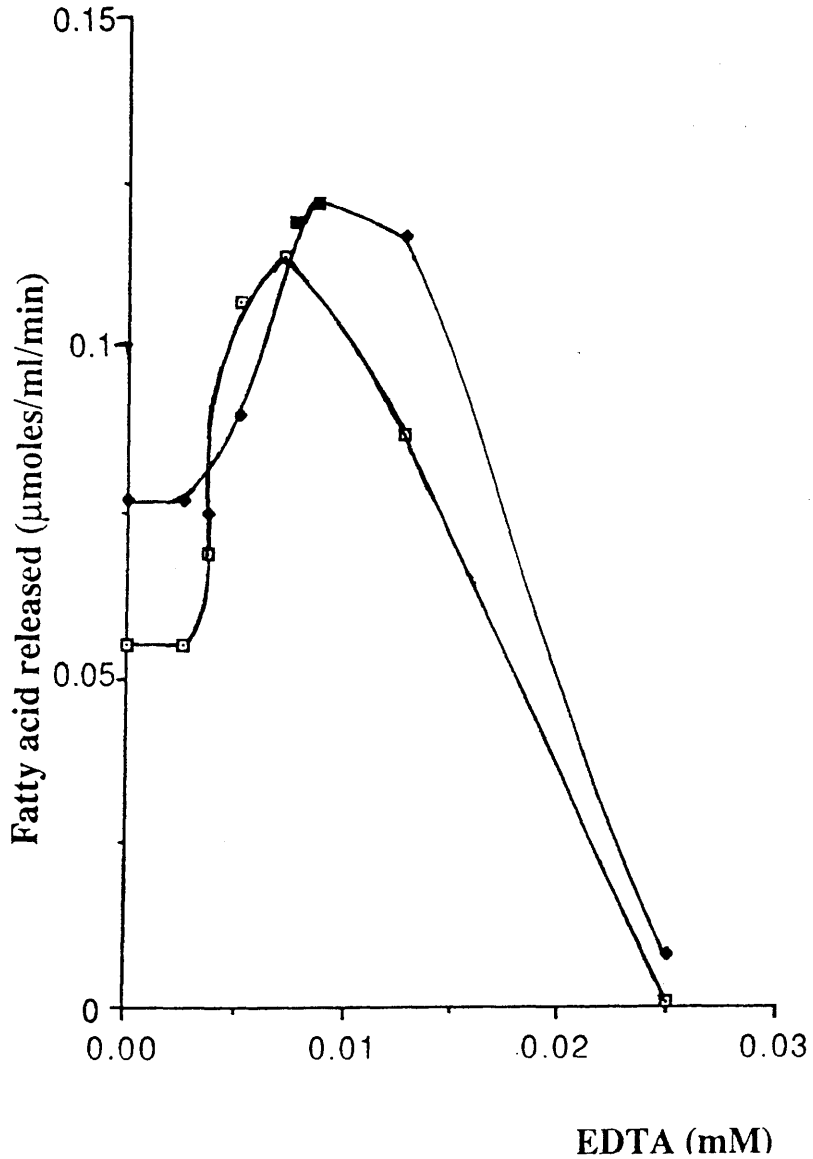
relevant aspects of the behaviour of these enzymes.

### 3.2.2 The effect of EDTA on bee venom $PLA_2$ activity.

A range of EDTA concentrations was used to titrate the calcium present in the assay medium containing 10 mM triethanolamine/$Cl^-$ buffer pH 8.0 and 20 µl of the short-chain substrate dioctanoyl phosphatidyl choline ($DiC_8PC$), in a medium not supplemented with calcium. As expected, excess EDTA was a potent inhibitor for this enzyme, but at lower concentrations there was a pronounced activation, **(fig. 7)**. This contrasted with the plateau level of activity which was observed in an earlier study by Lawrence in this laboratory. Subsequently we found that the difference between the two results was due to the contamination of the assay medium with the heavy metal, the source of which was the automatic syringe used for dispensing the buffer solution into the conductivity cells. (Liberation of heavy metal ion from the syringe was discovered in a conductimetric assay for detecting urease, an enzyme which is extremely sensitive to inhibition by heavy metals).

The activation of bee venom phospholipase $A_2$ by lower EDTA concentrations was also reflected by a very clear change in the shape of the substrate hydrolysis curve. At intermediate EDTA concentrations the curve shape indicated that the enzyme possessed much higher affinity for the substrate than in the absence of EDTA. The simplest interpretation of this result was that another metal was

Fig.(7) The effect of EDTA on native and activated bee venom PLA2




Native and activated bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 1 mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 µl of 30 mg/ml dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) and different EDTA concentrations.

Native phospholipase $A_2$

Activated phospholipase $A_2$

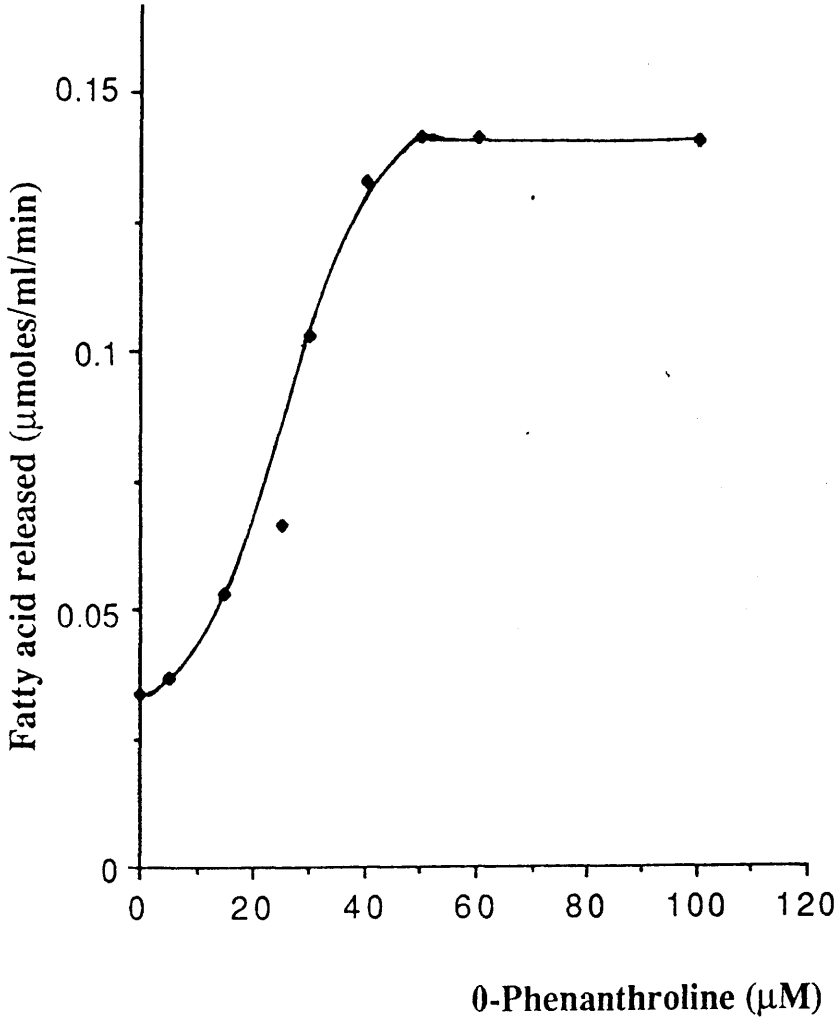
present in the system which had a higher affinity for EDTA than calcium and which acted to decrease the affinity of the enzyme for the substrate.

### 3.2.3 The effect of o-phenanthroline on the activity of bee venom $PLA_2$ enzyme.

O-phenanthroline, a nitrogen ligand which forms a complex with metal ions, has a quite distinct spectrum of affinities to the oxygen ligands typified by EDTA and shows very low affinity indeed for the calcium ion. When it was included in this assay system it caused an increase of ca. 6-fold in the activity of bee venom phospholipase $A_2$ enzyme. This activation was also characterised by the increase in the linearity of the hydrolysis curve. Using a range of o-phenanthroline concentrations it was found that full stimulation of bee venom phospholipase $A_2$ enzyme could be achieved at a concentration of 50 μM ligand, **(fig. 8)**, but an excess of o-phenanthroline did not have any adverse effect on the enzyme activity. The amount of EDTA needed to inhibit bee venom phospholipase $A_2$ activity was significantly reduced in the presence of o-phenanthroline, **(fig. 9)**.

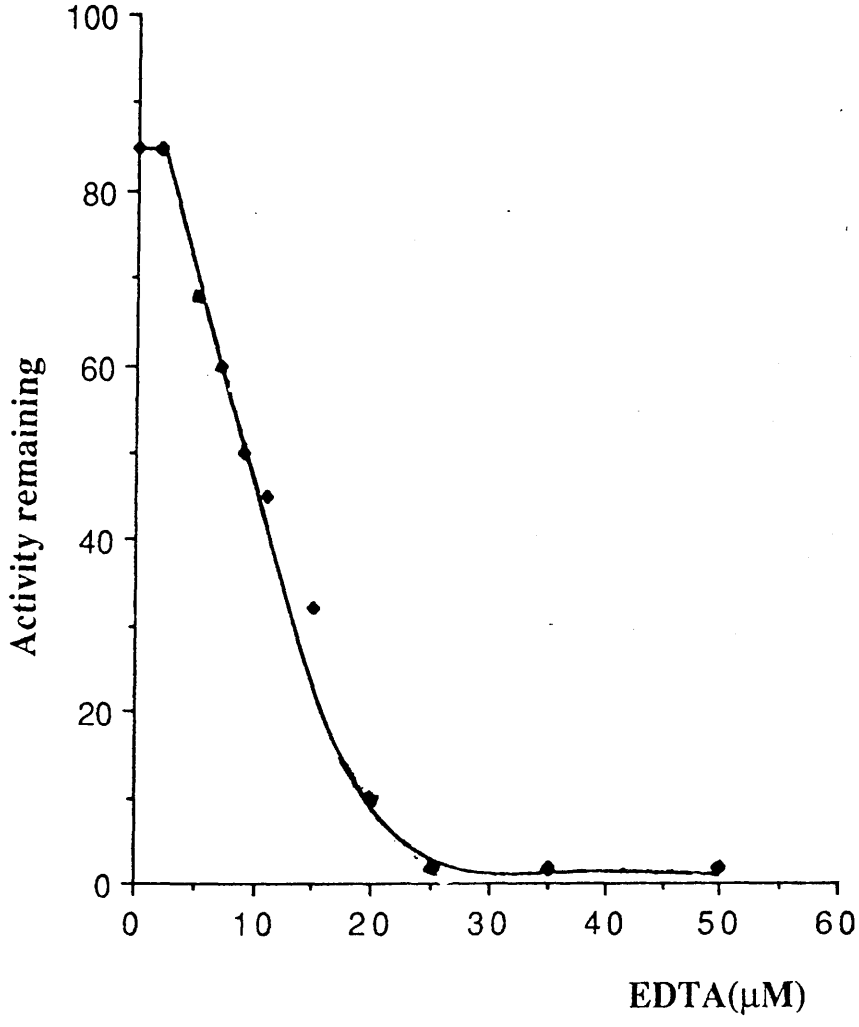
**Figure 10**, compares the shape of the curves obtained for the hydrolysis of the short-chain substrate ($DiC_8PC$) by bee venom phospholipase $A_2$ in the presence or absence of o-phenanthroline and also shows how this is affected by calcium. The results show quite clearly that calcium changes the initial rate, but does not affect the

Fig.(8)The effect of o-phenanthroline on the activity of native bee venom PLA2



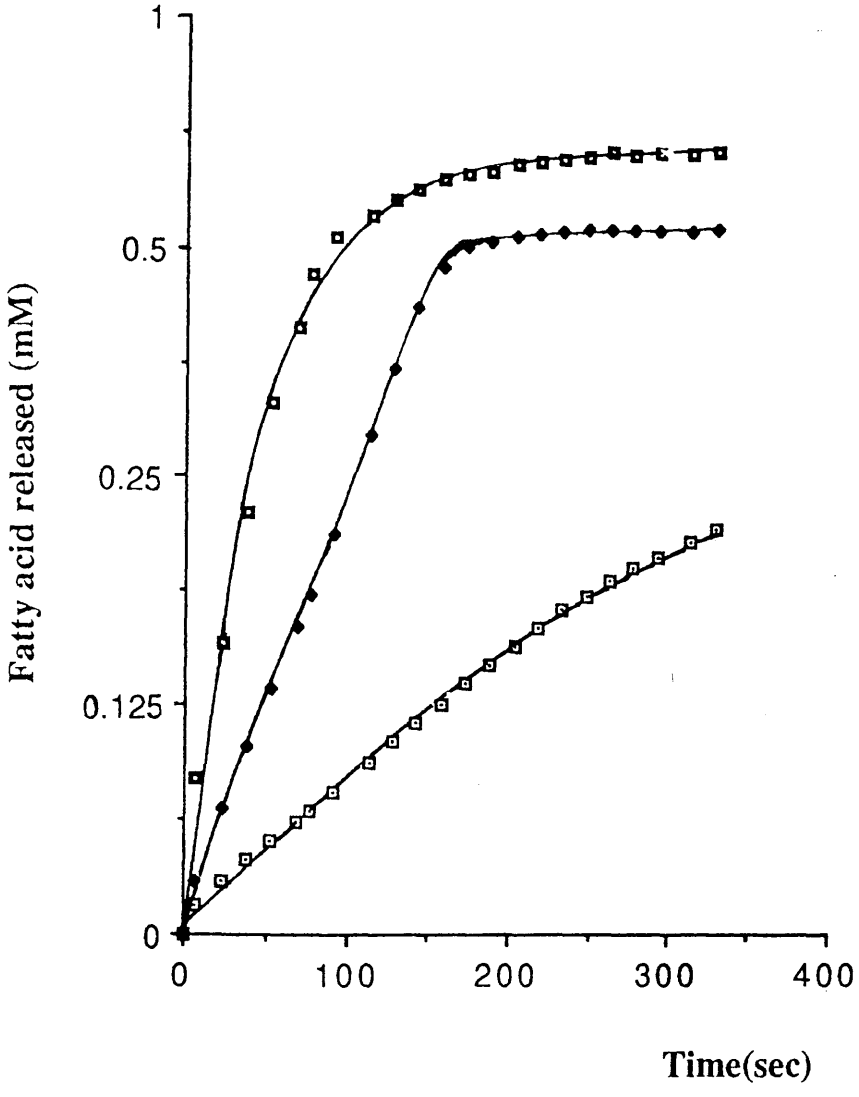

Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5μl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 μl of 30 mg/ml dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) and different o-phenanthroline concentrations.

Fig.(9) The effect of EDTA on the activity of native bee venom PLA2 in the presence of 100μM o-phenanthroline.




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 μl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 μl of 30 mg/ml dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) ,100 μM o-phenanthroline and different EDTA concentrations.

Fig.(10) The effect of calcium and o-phenanthroline on curve shape for the hydrolysis of dioctanoyl phosphatidyl choline by native bee venom PLA2.




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 µl of 30 mg/ml dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) in the absence and presence of 1mM calcium or 100 µM o-phenanthroline.

Native enzyme only (control)

Native enzyme +calcium

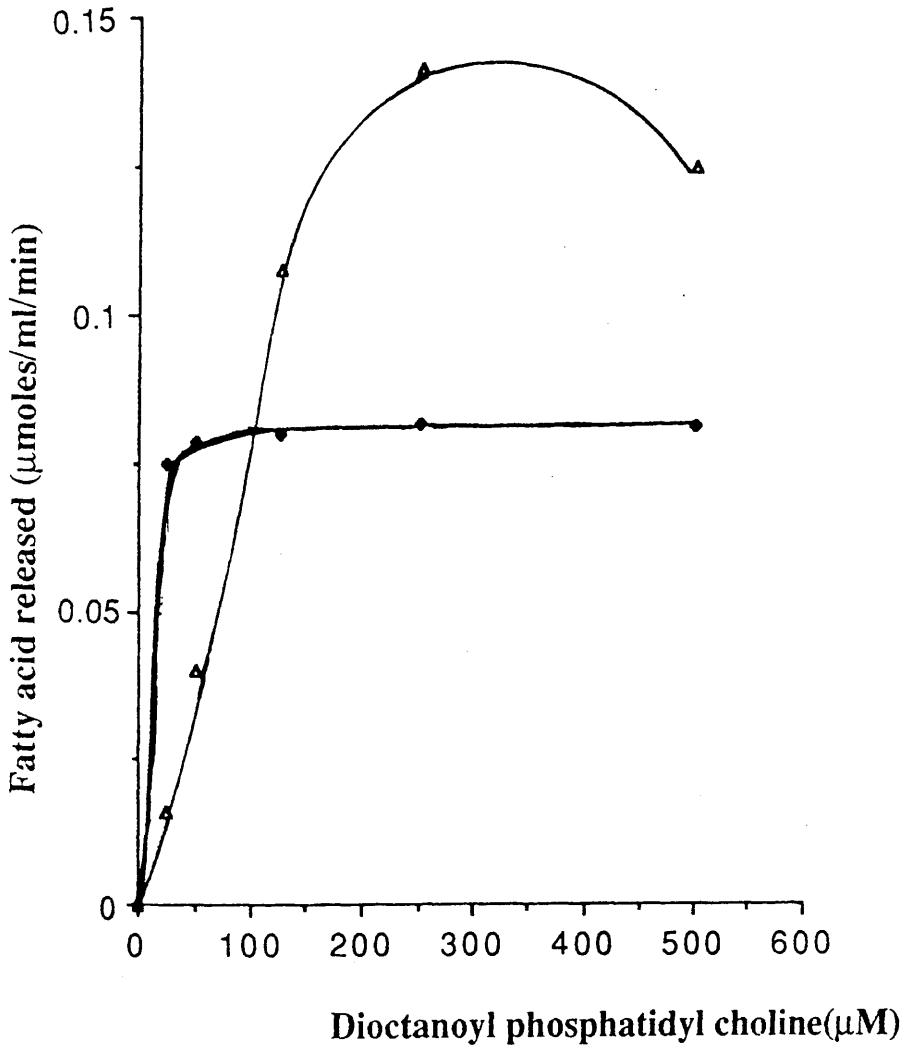
Native enzyme + o-phenanthroline

linearity of the progress curve. In contrast, o-phenanthroline increases both the initial rate and the degree of linearity, suggesting that it makes a large difference to the affinity of the enzyme for the substrate. This probable effect on substrate affinity was investigated further by measuring the initial rates as a function of substrate concentration in the presence of either calcium or o-phenanthroline, **(fig. 11)**. These data suggested that an inhibitory metal ion was present which bound to the enzyme more avidly than calcium and decreased the affinity of the enzyme for the substrate. Excess calcium did not appear to be able to displace this ion very readily. The kinetic interpretation of these results will be discussed later.

Similar activation phenomena were also observed using purified egg phosphatidyl choline as substrate in an assay buffer containing 20 % n-propanol with organic ligands present in the concentration range 50 μM to 100 μM, **(fig. 12)**. It was hoped that the relative affinities of these ligands for different metal ions would help identify the ions involved, but the degree of selectivity of these ligands was quite limited. Penicilamine is relatively selective for copper ions and cyclam has extremely high affinity for nickel, but these ligands will bind any transition metal ion.

Because phospholipid substrates are often synthesised from glycerophosphoryl choline as its cadmium chloride complex, much attention has been paid to the inhibitory action of cadmium, especially as the ionic radii of cadmium and calcium are almost identical. To test whether or not cadmium was the endogenous

Fig.(11) Concentration dependence of the rate of hydrolysis of dioctanoyl phosphatidyl choline by bee venom PLA2 in the presence of calcium and 0-phenanthroline.

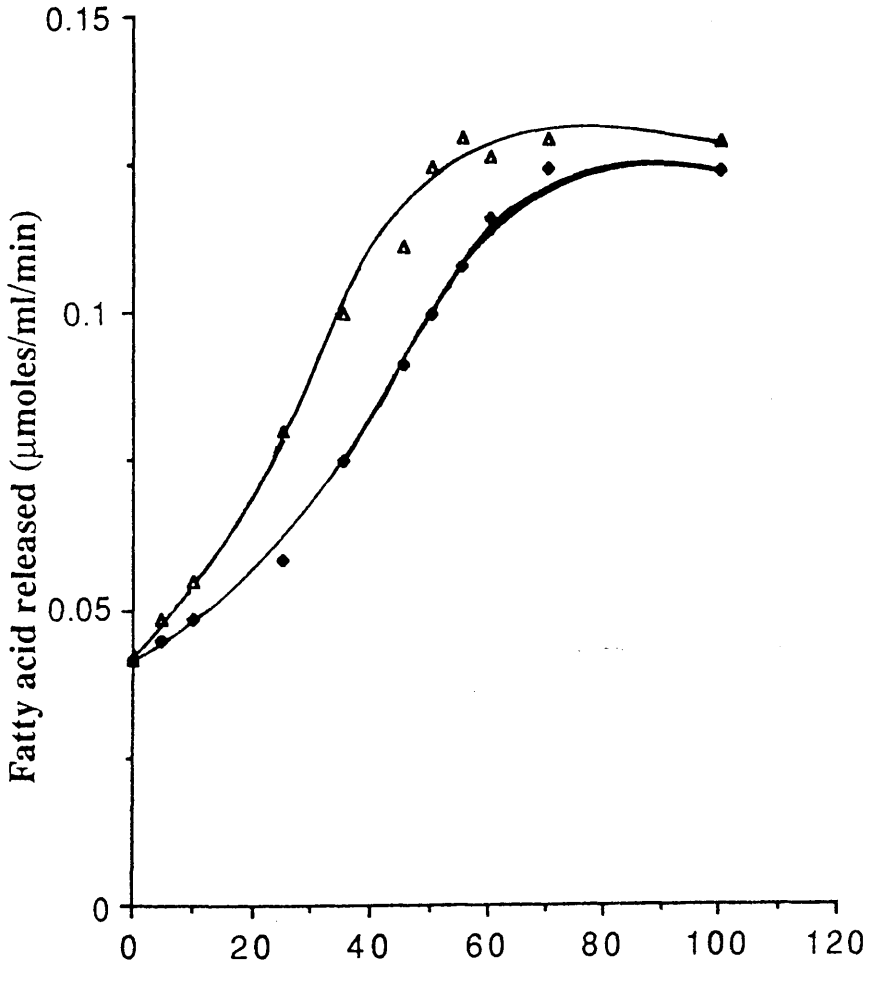



Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 μl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing different dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) concentrations in the presence of 1mM calcium or 100 μM o-phenanthroline.

Δ——Δ Native enzyme +calcium

●——● Native enzyme + o-phenanthroline

Fig.(12) The effect of different concentrations of penicillamine and cyclam on the activity of native bee venom PLA2.




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ in 20 % n-propanol buffer pH 8.0 at 37°C containing 20 µl of egg lecithin substrate in the presence of different cyclam and penicilamine concentrations.

△—△ Native enzyme +cyclam

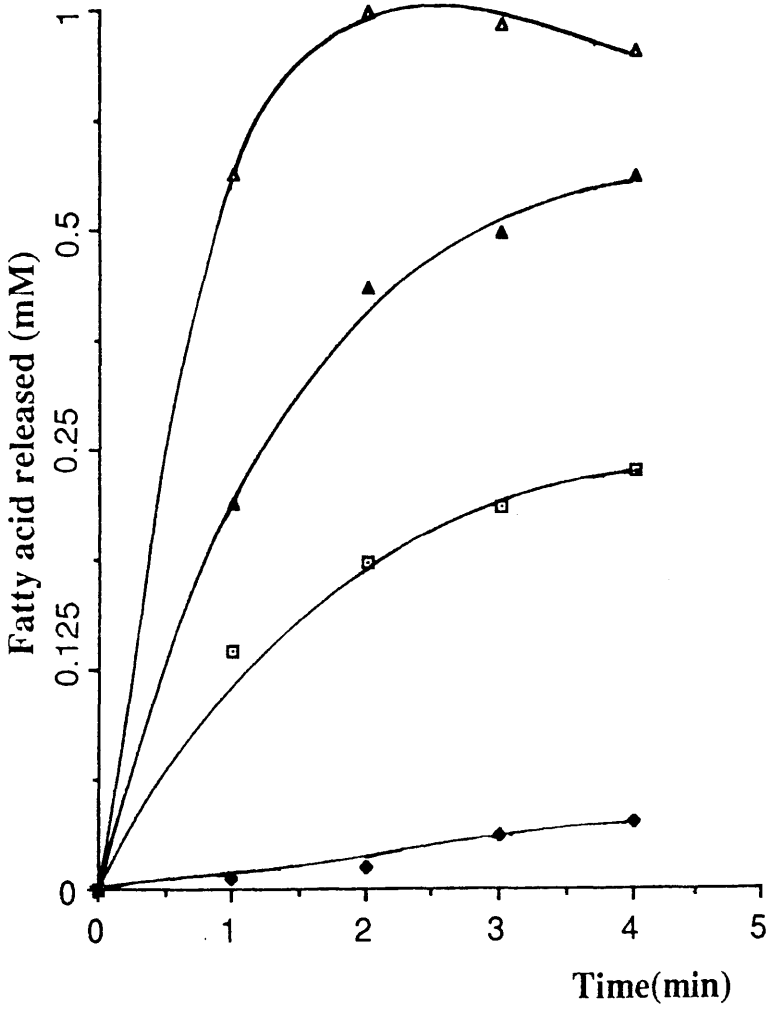
●—● Native enzyme + penicilamine

inhibitor, dioctanoyl phosphatidyl choline ($DiC_8PC$) substrate was prepared from glycerophosphoryl choline free base which had never been converted to the cadmium chloride complex. The response of the enzyme to cadmium-free substrate was equally as sensitive to o-phenanthroline as to substrates purified by the original methods. In order to determine the source of the inhibitory metal ion, the complete assay solution containing the substrate was exhaustively deionised before use. Paradoxically this increased the degree of inhibition, unless o-phenanthroline was also included. This was most readily interpreted by concluding that the major source of exogenous calcium was in the reagent solutions and that the inhibitory ion was largely present in the enzyme sample, **(fig. 13)**. It is clear from these results that the calcium ion dependence determined by other workers might be influenced by the presence of other metal ions. Comparison of the calcium ion dependence in the presence and absence of o-phenanthroline confirmed this and provided an explanation for the different apparent calcium affinities that have been reported for this (and perhaps other) enzymes, **(fig. 14)**.

### 3.2.4 The inhibition of bee venom $PLA_2$ with some heavy metals.

Because of the difficulty of obtaining heavy ion-free reagents, it was hard both to identify the endogenous inhibitory ion and to determine whether or not the enzyme has residual activity in the presence of such a metal but in the absence of free calcium. As

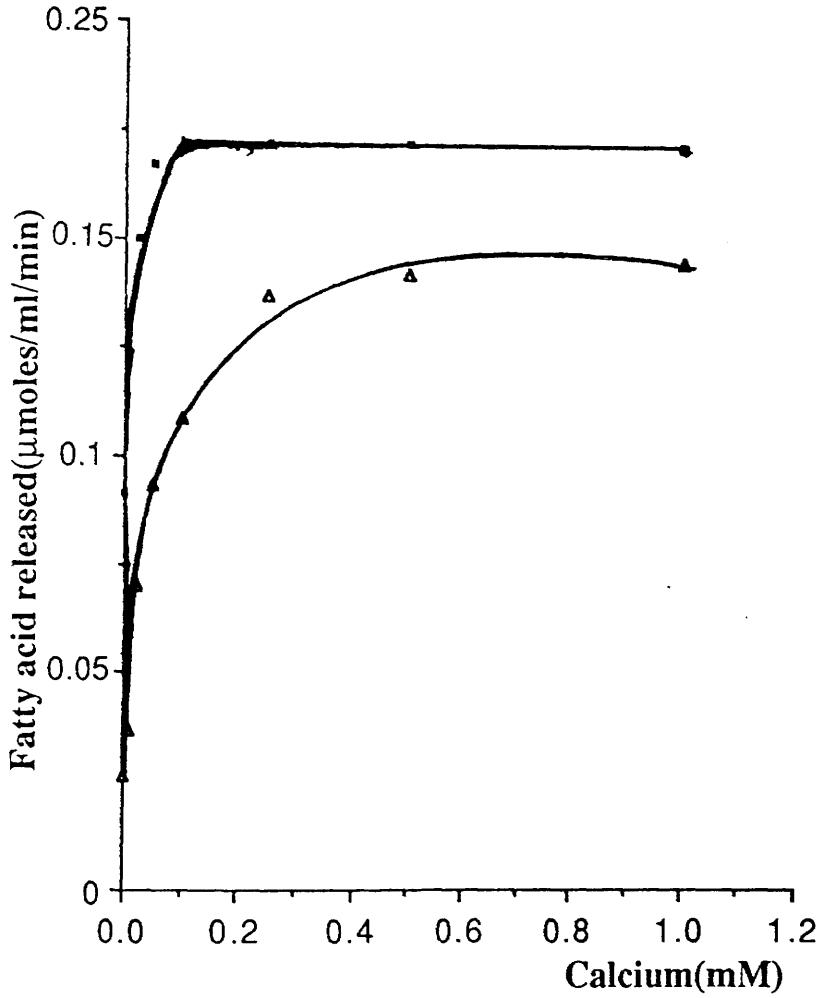
Fig.(13) The hydrolysis of diionized dioctanoyl phosphatidyl choline by bee venom PLA2 in the absence and presence of o-phenanthroline and calcium.




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 µl of 30 mg/ml non-deionised dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) or deionised substrate in the absence and presence of 100µM o-phenanthroline and 1mM calcium.

- Non-deionised substrate + native $PLA_2$
- Diionised substrate + native $PLA_2$
- Diionised substrate + o-phenanthroline + native $PLA_2$
- Diionised substrate + o-phenanthroline + calcium + native $PLA_2$.

Fig.(14) The effect of calcium on the hydrolysis of dioctanoyl phosphatidyl choline by bee venom PLA2 in the absence and presence of O-phenanthroline.




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 μl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 μl of 30 mg/ml of dioctanoyl phosphatidyl choline substrate ($DiC_8PC$), in the absence and presence of 100μM o-phenanthroline and different calcium concentrations.

Δ—Δ Native $PLA_2$ + calcium

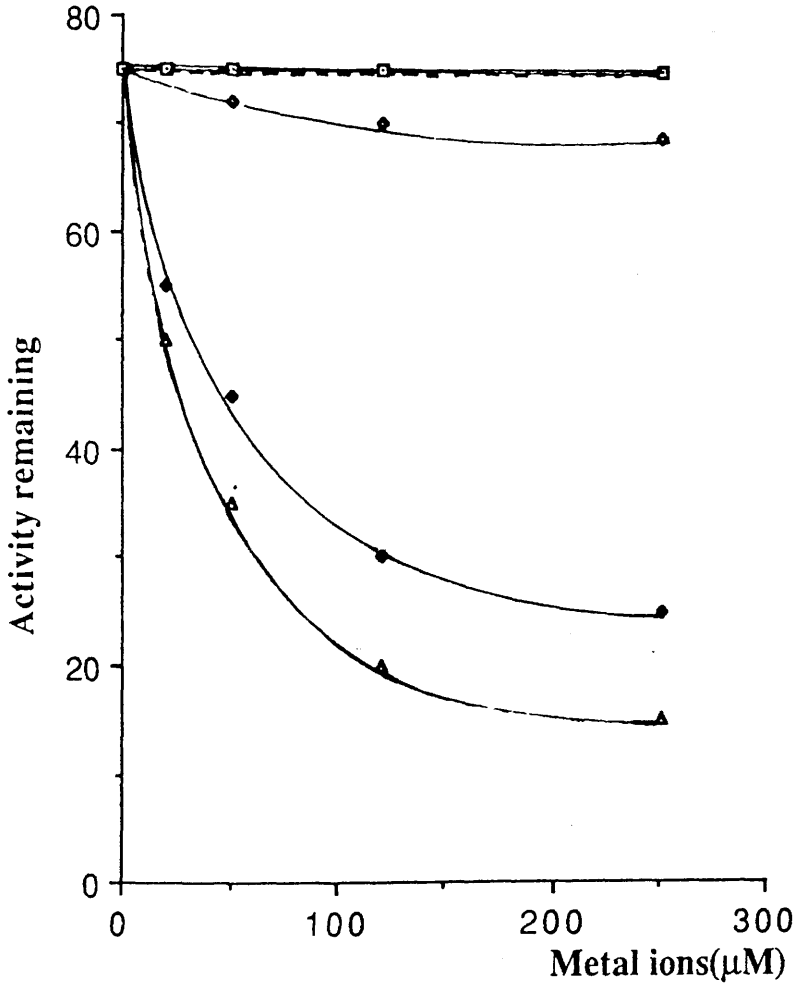
▪—▪ Native $PLA_2$ + o-phenanthroline + calcium.

suitable alternative methods did not exist for controlling the concentration of two ions independently, the effect of a variety of metal ions was studied at a fixed high calcium concentration, thereby swamping and effects of endogenous ions. The data in **fig. 15** showed that copper and zinc were potent inhibitors of the enzyme and that other ions were much less effective. This result shows that even at a very high concentration of the inhibitors (zinc and copper) the enzyme still has the ability to hydrolyse the substrate at a reduced rate. Full investigation of the effect of the metal ion clearly requires a very extensive kinetic investigation. The limited objective of this work was to see whether or not the effect of the metal ion could be attributed to simple competition at a single calcium binding site. The results obtained above show that it is not possible to duplicate the effect of metal ion inhibitors simply by reducing the calcium concentration and support the model that these ions act at different sites in the protein.

### 3.2.5 The effect of o-phenanthroline on some other $PLA_2$ enzymes.

Phospholipase $A_2$ enzymes from different sources such as the non-toxic pI 8.8 isoform from *Naja mossambica mossambica*, phospholipase $A_2$ from *Naja naja* venom (Common Idian cobra), the ß-subunit from the venom of the South American rattlesnake *Crotalus durrissus terrificus*, notoxin II-5 from the crude venom of the Australian tiger snake *Notechis scutatus scutatus* and

Fig.(15) The effect of some heavy metals on the activity of native bee venom PLA2




Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 μl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 μl of 30 mg/ml of dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) in the presence of 1mM calcium and different heavy metal ion concentrations.

- in the presence of Zinc
- in the presence of Copper
- in the presence of Iron
- in the presence of Cadmium
- in the presence of Cobalt

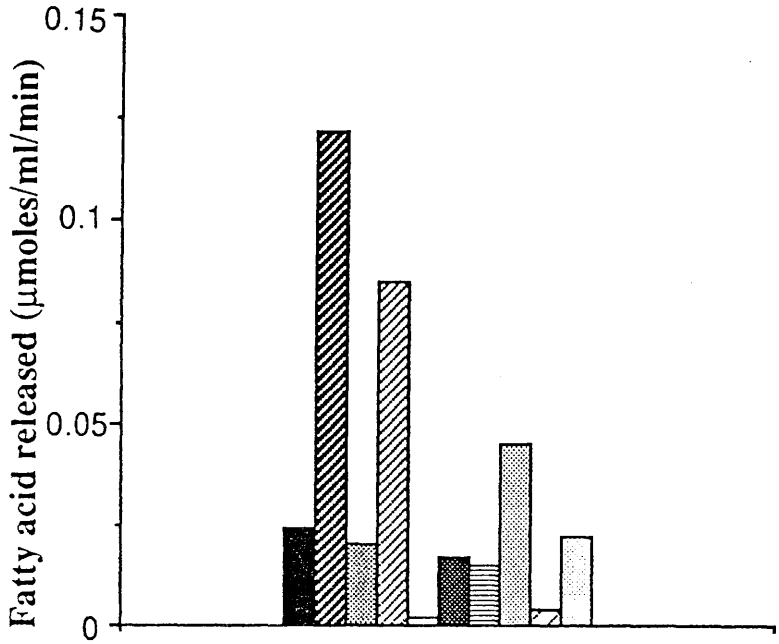
phospholipase $A_2$ from bovine pancreas, were all tested for sensitivity to o-phenanthroline or to calcium, **(fig 16)**.

The results show that all of these enzyme behave in a very similar fashion to the bee venom enzyme, with strong activation by o-phenanthroline or by excess calcium. They therefore present the same problem in understanding the relationship between calcium binding and the binding of the inhibitory ion(s). This work shows very clearly that the inclusion of transition metal ion chelators in assay media is very important for the study of all such enzymes.

### 3.2.6 Calcium requirement for bee venom $PLA_2$.

An alternative way to study the action of calcium in the absence of inhibitory ions is to use a metal ion buffer where the buffer compound has even higher affinity for transition metal ions than for calcium. Investigation showed that nitrilotriacetic acid was an ideal compound for this purpose. **Figure17**, shows the reaction progress curves obtained in this buffer system, indicating that it abolished the inhibitory actions of the contaminating metal ion. In contrast to EDTA/EGTA which buffered calcium at too low a level to support significant phospholipase $A_2$ activity, this compound buffers over an ideal concentration range. The affinity constant of the enzyme for calcium, calculated from the present results, gave a value $K_d(Ca)$ = ca..5 μM, **(fig.18a,b)**. The level of calcium present in distilled water used in this assay system was determined as ca.8-10 μM using a

Fig.(16) the effect of o-phenanthroline on different phospholipases A2 activity.




Enzyme activity from different sources was measured from the hydrolysis of 20 μl of 30 mg/ml dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) by $PLA_2$ in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C and in the absence and presence of 100μM o-phenanthroline.

■ non-toxic $PLA_2$ (CMII)pI 8.8 isoform from *Naja mossambica mossambica*

▨ non-toxic $PLA_2$ (CMII)pI 8.8 isoform from *Naja mossambica mossambica* +100μM o-phenanthroline

▩ $PLA_2$ from *Naja naja* venom

▨ $PLA_2$ from *Naja naja* venom + 100 μM o-phenanthroline

□ ß-subunit from *Crotallus durrissus terrificus*

■ ß-subunit from *Crotallus durrissus terrificus* + 100 μM o-phenanthroline

▤ notoxin (II-5) from *Notechis scutatus scutatus*

▩ notoxin (II-5) from *Notechis scutatus scutatus* + 100 μM o-phenanthroline

▨ $PLA_2$ from bovine pancreas

□ $PLA_2$ from bovine pancreas + 100 μM o-phenanthroline.

FIG17

Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 µl of 30 mg/ml of dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) and 1mM nitrilotriacetic acid in the absence and presence of 100µM o-phenanthroline and different calcium concentrations.

▲—▲ $PLA_2$ activity in the presence of o-phenanthroline

▲-▲ $PLA_2$ activity in the presence of nitrilotriacetic acid only

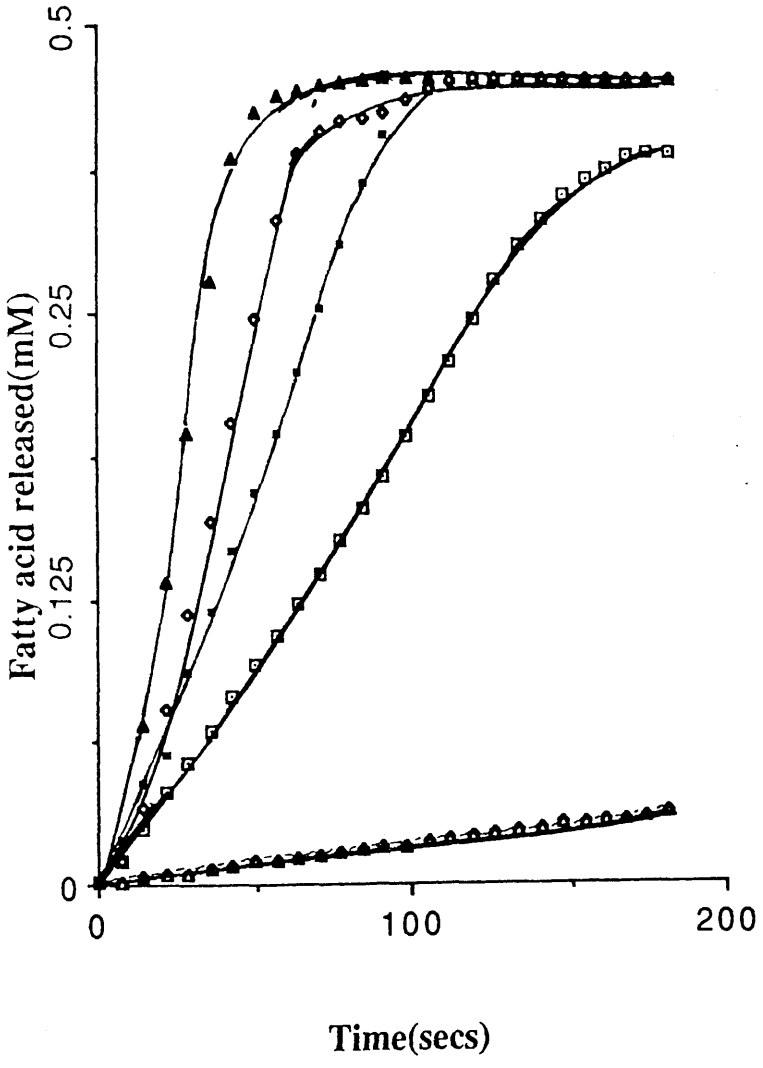
▲-▲ $PLA_2$ activity in the presence of nitrilotriacetic acid + o-phenanthroline

◇—◇ $PLA_2$ activity in the presence of nitrilotriacetic acid + o-phenanthroline +25 µM calcium

•—• $PLA_2$ activity in the presence of nitrilotriacetic acid + o-phenanthroline +50 µM calcium

o—o $PLA_2$ activity in the presence of nitrilotriacetic acid + o-phenanthroline +75 µM calcium.

Fig.(17) The effect of calcium and o-phenanthroline on the hydrolysis of dioctanoyl phosphatidyl choline by bee venom PLA2 in the presence of nitrilotriacetic acid.



linear extrapolation to zero enzymic activity as a measure of calcium concentration.

Fig(18)a

Calculation of free calcium ion (M) in 10 mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C with ligand nitrilotriacetic acid (L) was carried out using these equation

$K = M \times L/ML$ $\quad (K = (1 + H/K_a)$

$M + ML = M_t$

$L + ML = L_t$

Fig.(18)b

Native bee venom phospholipase $A_2$ enzyme activity was measured by injecting 0.5 µl of 0.5mg/ml enzyme into 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C containing 20 µl of 30 mg/ml of dioctanoyl phosphatidyl choline substrate ($DiC_8PC$) and 1mM nitrilotriacetic acid in the absence and presence of 100µM o-phenanthroline and different calcium concentrations.

Fig.(18)a. Free(Ca++) concentration calculated by1mM nitrilotriacetic acid after the addition of different calcium concentrations.

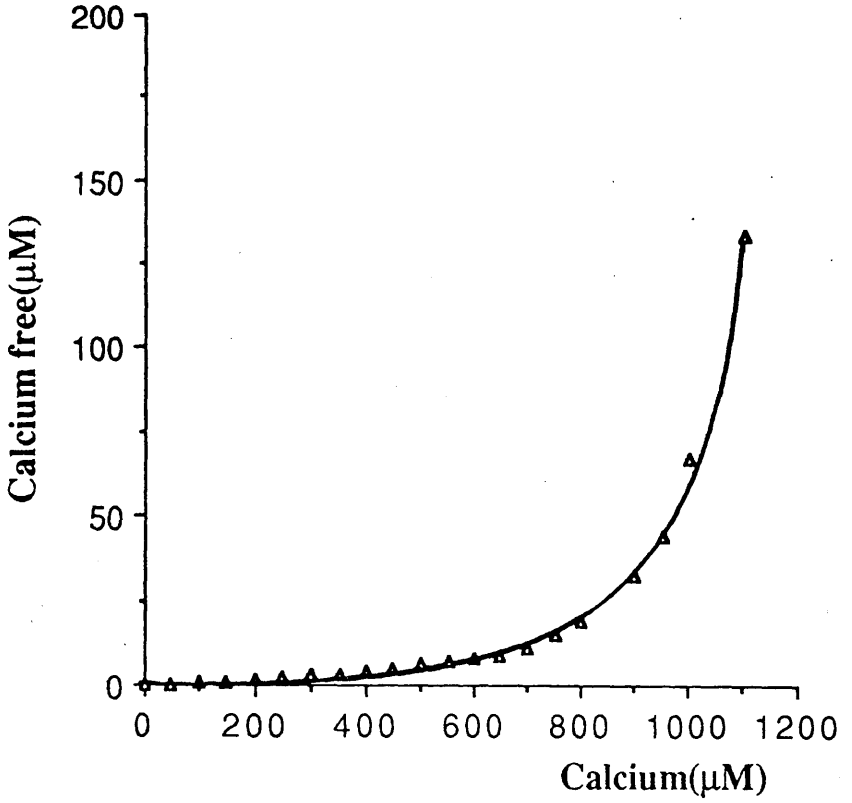
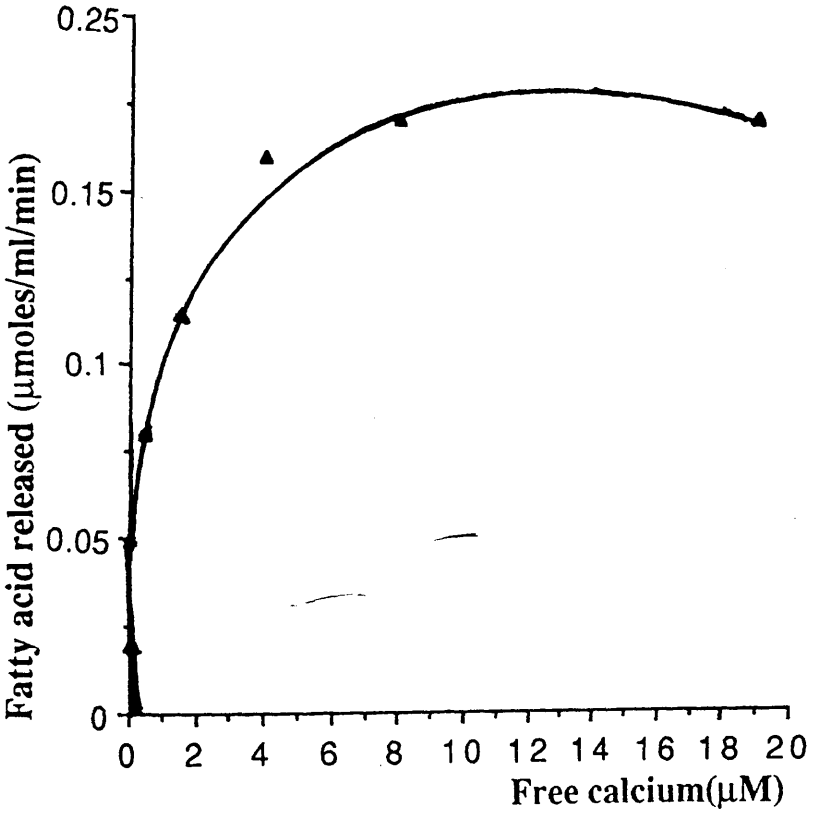



Fig.(18)b. Use of a calcium/nitrilotriacetic acid buffer to measure the the calcium dependence of native bee venom PLA2.



### 3.3.1 The application of erythrocyte leakage assay for phospholipase $A_2$ activation studies.

Bee venom phospholipase $A_2$ can be activated towards hydrolysis of long-chain phosphatidyl choline derivatives by treatment with a variety of reagents which are believed to add a long-chain acyl residue to a specific but as yet uncharacterised site on the protein (Drainas & Lawrence, 1978; Lyall, 1984). Free fatty acids can produce the same activation and there is a reason to believe that the enzyme has a fatty acid activation site which is highly susceptible to acylation by derivatives of long-chain, but not short-chain fatty acids.

The response to activation was most clearly demonstrated using long-chain phospholipid substrates in a 20 % n-propanolic assay medium. Drainas and Lawremce, (1978) showed that very similar results could be obtained using erythrocyte leakage in the presence of albumin as an assay method.

The response of erythrocytes to phospholipase $A_2$ enzymes is poorly understood. Washed cells do not leak to any great extent when treated with venom phospholipase $A_2$ enzymes. If the cells are pretreated with sub-lytic levels of fatty acids then they show a large leakage response to the enzyme and interestingly this is strongly inhibited by lysophospholipids. Preliminary results showed that cells pretreated with fatty acid did not distinguish between native and

acylated phospholipase $A_2$ and it was postulated that this was because the enzyme was already fully activated by the fatty acid.

Synergism of phospholipase $A_2$ action by albumin was observed both in lysis assays by Gul and Smith, (1974) and in leakage assays by Drainas and Lawrence, (1978). Gul and Smith proposed that the effect of albumin was to remove fatty acids from the membrane and to cause membrane rupture as a direct result of depletion. Drainas et al., (1981) proposed that albumin acted by removing the inhibitory lysophosphatides released by enzyme action. They also showed that the removal of fatty acid from the membrane by albumin stimulated leakage directly, although it was not clear that this effect contributed to the synergistic response.

In the present work a preliminary study was undertaken to try to increase the sensitivity of erythrocyte assays for phospholipase $A_2$ activation. The main approach was the study of responsiveness of a range of different erythrocyte types and using albumin or free fatty acids as synergants. In addition a novel system was investigated in which the sensitivity of the cells was modified by adding fatty acids and then removing them with albumin.

Preliminary studies showed that different species of red blood cells responded differently to both albumin and free fatty acids in the leakage assay. Erythrocytes were collected from Rabbit, Mouse, Rat, Dog and Guinea Pig. The cells from Dog and Guinea Pig were similar to those from rabbit in their response pattern, but those from

rat and mouse cells showed significant differences. The most important feature was that the sensitivity of the cells to phospholipase $A_2$ in the presence of albumin followed the series:- Mouse> Rat >>Rabbit, Dog, Guinea-pig. This showed that the sensitivity of the assay could be improved by a judicious choice of cells.

When free fatty acids were used as synergists for phospholipase $A_2$, very little difference was seen between the response to native or to acylated phospholipase $A_2$ enzymes, so this did not form the basis of a useful assay of activation. It was of interest here that the synergistic response was also dependent on cell type and paradoxically the order was reversed in comparison with the synergism obtained by albumin, ie Guinea-pig, Dog, Rabbit >> Rat > Mouse **(Table 1)**.

Elansari, (1986) had made an extensive study of the effect of the addition of free fatty acids to erythrocytes; he extended the work by Drainas et al., (1981), which showed that these cells gave a transient leakage response when exogenous fatty acids were extracted by albumin. He also showed that this was inhibited by lysophosphatide and that the effect depended on the addition order of the reagents. In this study it was now found that cells treated by this method became extremely sensitive to phospholipase $A_2$, thus providing a third type of leakage assay for phospholipase $A_2$ activity. This was now investigated in detail.

Table.1. The effect of oleic acid or albumin on the sensitiztion of erythrocytes from different animals to native or activated bee venom $PLA_2$.

| Erythrocyte types | Erythrocytes + oleic acid | | Erythrocytes + albumin | |
|---|---|---|---|---|
| | native $PLA_2$ | activated$PLA_2$ | native $PLA_2$ | activated $PLA_2$ |
| Rabbit | +++ | +++ | _ _ _ | +++ |
| Dog | ++ | ++ | _ _ | ++ |
| Guinea pig | ++ | ++ | _ _ | + |
| Mouse | _ _ _ | _ _ | ++++ | +++++ |
| Rat | _ _ _ | _ _ _ | +++ | ++++ |

20 µl of 30 % washed erythrocytes in saline were added to 2 ml of 10 mM isotonic sucrose buffer pH 7.4 and at 37°C in the conductivity cells, and either 1 µl of 1 mg/ml oleic acid or 20 µl of 66 mg/ml was added to the mixtures; after the reading of the cells were stabilized, 2 µl of 1 mg/ml native or activated enzyme with 0.2% oleoyl imidazolide activator were injected to the conductivity cell. (-) not sensitive to the enzyme, (+) sensitive to the enzyme.

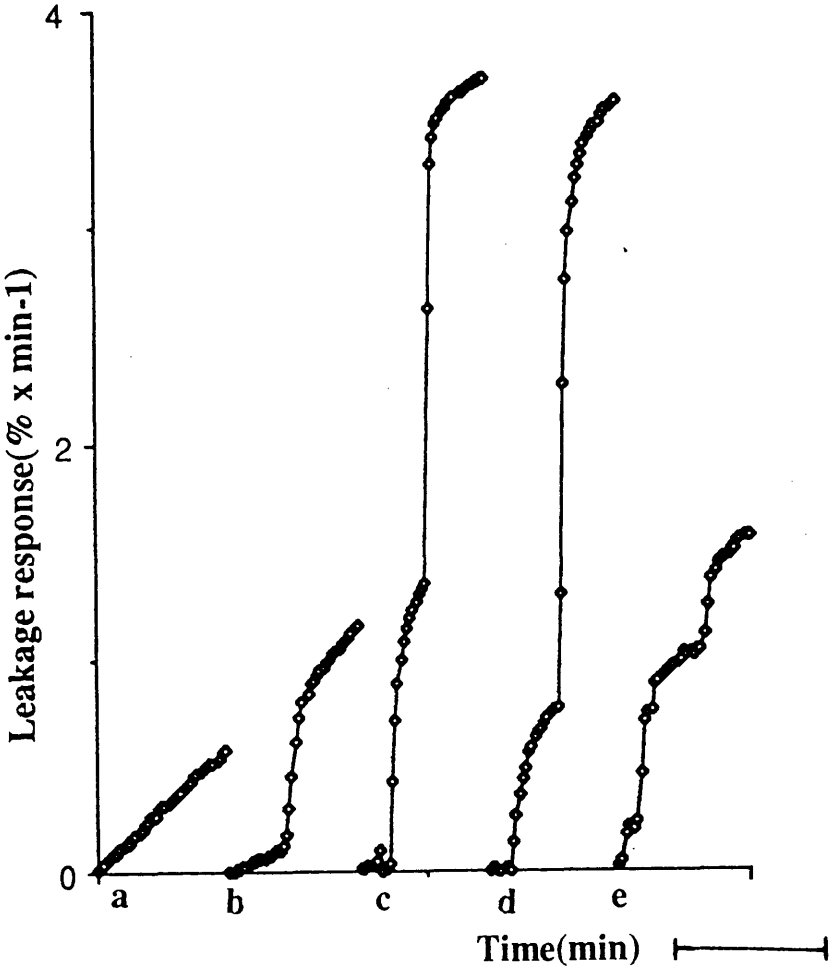
**Figure 19** shows the effect of concomitant extraction of lysophosphatides and fatty acids from rabbit erythrocytes by albumin. The results show two different aspects of the responsiveness of these cells; firstly, they show the leakage produced when albumin was added to fatty acid treated cells and secondly, they show the leakage obtained when these 'extracted' cells were treated with native bee venom phospholipase $A_2$. This effect which was dependent on the nature of fatty acids and enhanced response of red blood cell to the enzyme is here termed 'sensitisation'.

Lysophosphatidyl choline added to the cells before oleic acid was a potent inhibitor of sensitisation, but was without effect when the addition order was reversed. Full inhibition was obtained when lysophosphatide was present in one third the molar concentration of oleic acid. When myristic acid was used in place of oleic acid the results were completely different; with their effects of addition order being reversed, **(fig. 20)**. It should be noted that myristic acid was both more lytic than oleic acid and a more powerful stimulator of phospholipase $A_2$ activity in rabbit erythrocytes.

Further study of time-dependence of addition showed that lysophosphatide was inhibitory if added very soon after oleic acid, but inhibition fell rapidly with increased time lapse between additions, a very long delay between addition of lysophosphatide/ oleic acid and albumin also reduced the magnitude of inhibition.

This investigation was repeated using a range type of erythrocytes

Fig(19) The effect of the addition order of oleic acid and lysophosphatide and their extraction by albumin on the susceptibility of rabbit erythrocyte to bee venom PLA2.




Rabbit erythrocyte leakage response caused by 2 µl of 1mg per ml native bee venom phospholipase $A_2$ in the absence (a) and presence of oleic acid (b), oleic acid + albumin (c), oleic acid + albumin + lysophosphatidyl choline (d) and lysophosphatidyl choline + oleic acid + albumin (e).

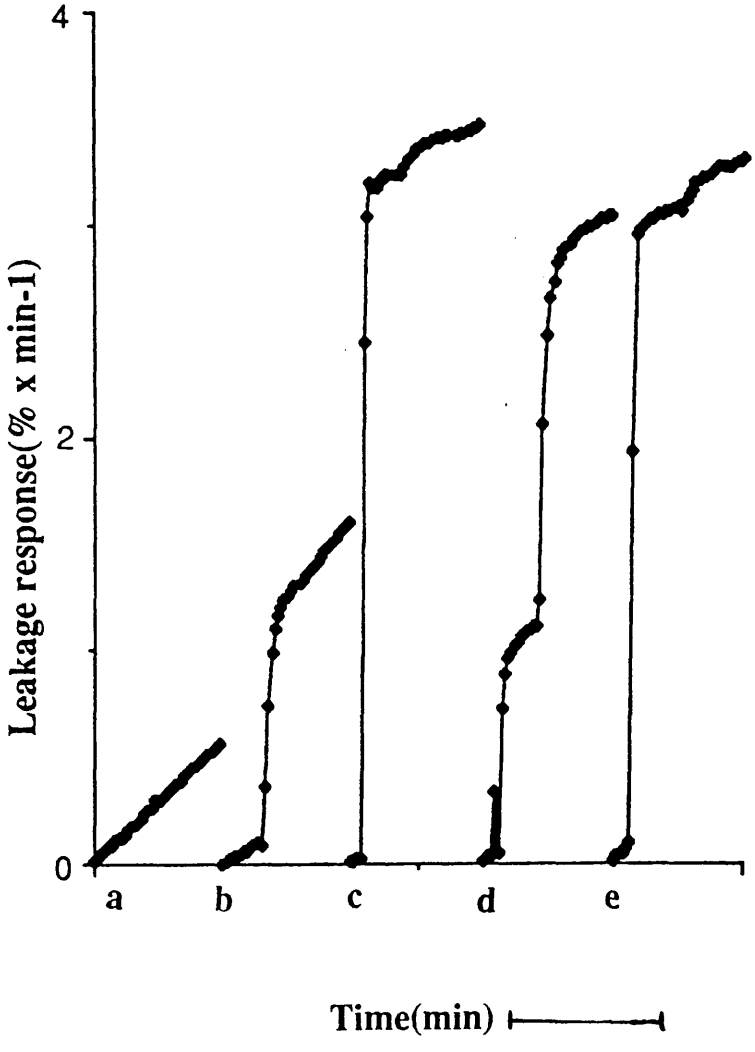
and it was found that the amount of leakage stimilated when fatty acid were extracted by albumin was in the order of Rabbit > Dog > Guinea Pig > Rat > Mouse. However the sensitisation to phospholipase $A_2$ enzymes produced by this treatment was very similar for all cell types.

Preliminary result showed that the response of sensitized cells gave qualitative differences to the native and activated bee venom phospholipase $A_2$ but the effect was not very large. Most of the assays for activation were therefore carried out using rabbit or rat erythrocytes in the presence of albumin, the later is five times more sensitive than the former for all phospholipase $A_2$ enzymes tested and this difference was maintained when phospholipase $A_2$ enzymes were activated by treatment with acyl imidazolide.

### 3.3.2 Bee venom phospholipase $A_2$ activation

Two parameters were of interest in studies of the activation of phospholipase $A_2$ enzymes; the degree of activation achieved, (ie. the activation factor) and the rate at which the enzyme reacted with the activator. There was reason to believe that the activation factor would depend on the type of assay used. For example, in the erythrocyte leakage assay, where the activation factor was calculated as the ratio of the maximum leakage rate produced by the activated enzyme to that produced by the native enzyme, it was affected by the non-linearity of the dose response curve. In contrast, activation rates

Fig.(20) The effect of the addition order of myristic acid and lysophosphatide and their extraction by albumin on the susceptibility of rabbit erythrocyte to bee venom PLA2.




Rabbit erythrocyte leakage response obtained from their incubation with 2 µl of 1mg/ml native bee venom phospholipase $A_2$ in the absence (a) and presence of myristic acid (b), myristic acid + albumin(c), myristic acid + albumin + lysophosphatidyl choline (d) and lysophosphatidyl choline + myristic acid + albumin (e).

should not depend on the nature of the assay used, but should be highly dependent on the conditions under which the enzyme was treated with the activator.

In previous studies, Drainas used 20 % aqueous n-propanol both in the assay medium and in the activation medium. In this work his experiments were repeated and extended using the erythrocyte leakage assay for activation and also confining the activation studies to an entirely aqueous reaction system.

One question whether or not activation was simply determined by site occupancy or whether it was affected by the quality of the acyl chain of the acid moiety?. This had been tested by Drainas using lauroyl imidazolide and oleoyl imidazolide as activators. The former was slow to act and produced much lower activation than the latter. Enzyme partially activated by lauroyl imidazolide reacted rapidly with oleoyl imidazolide to produce maximal activation. The interpretation was that both residues produced the same degree of activation but that lauroyl imidazolide failed to activate fully due to a lower degree of specificity and to hydrolysis of the reagent.

This was now tested further by measuring the activation factors for a wide variety of compounds and comparing the rate of activation found for weak and for strong activators. In particular the effect of strong activators on enzyme that had been given long exposure to a weak activator was studied in greater detail.

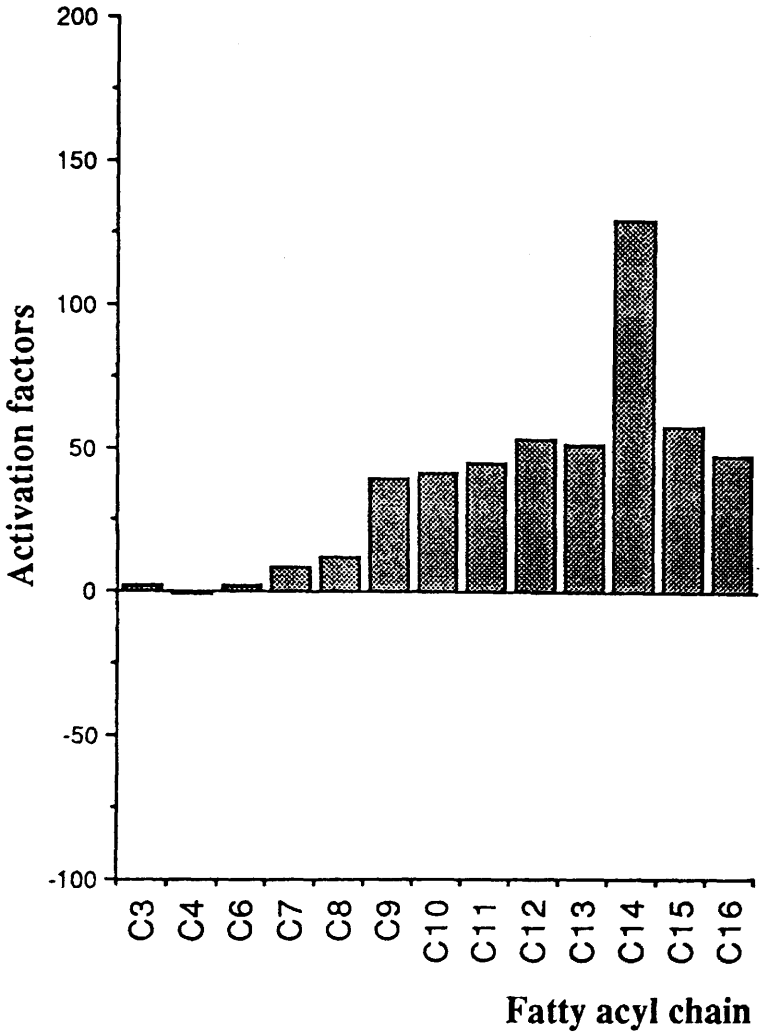
The activation factor for bee venom phospholipase $A_2$ treated with saturated fatty acyl derivatives increased smoothly as the acyl chain length increased. However, the highest activation was obtained with the C14:0 saturated fatty acid (myristic acid ) and then declined with the C15:0 and C16:0 saturated fatty acids (pentadecyclic and palmitic acids), **(fig. 21)**. These last two fatty acids (pentadecyclic and palmitic ) were less soluble in acetone and required a high temperature $\geq 55^{o}C$ to dissolve, this may have affected their reaction with the carbonyldiimidazole during the synthesis of the activator and the low solubility of the imidazolide derivative may have reduced its rate of reaction with the enzyme.

Other long-chain fatty acids such as $CH_3(CH_2)_{11}$-S-S-$(CH_2)_2COOH$; 11-bromoundecanoic acid $Br(CH_2)_{11}CO_2H$; 2-bromohexadecanoic acid $CH_3(CH_2)_{13}CH$ $(Br)CO_2H$ and the unsaturated fatty acid (oleic acid) were also powerful activators for the bee venom phospholipase $A_2$,.**(fig. 22)**. These results suggested that the degree of activation of bee venom phospholipase $A_2$ was very dependent on the acyl chain length, but not very sensitive to its exact chemical composition.

### 3.3.3 The rate of activation of bee venom $PLA_2$

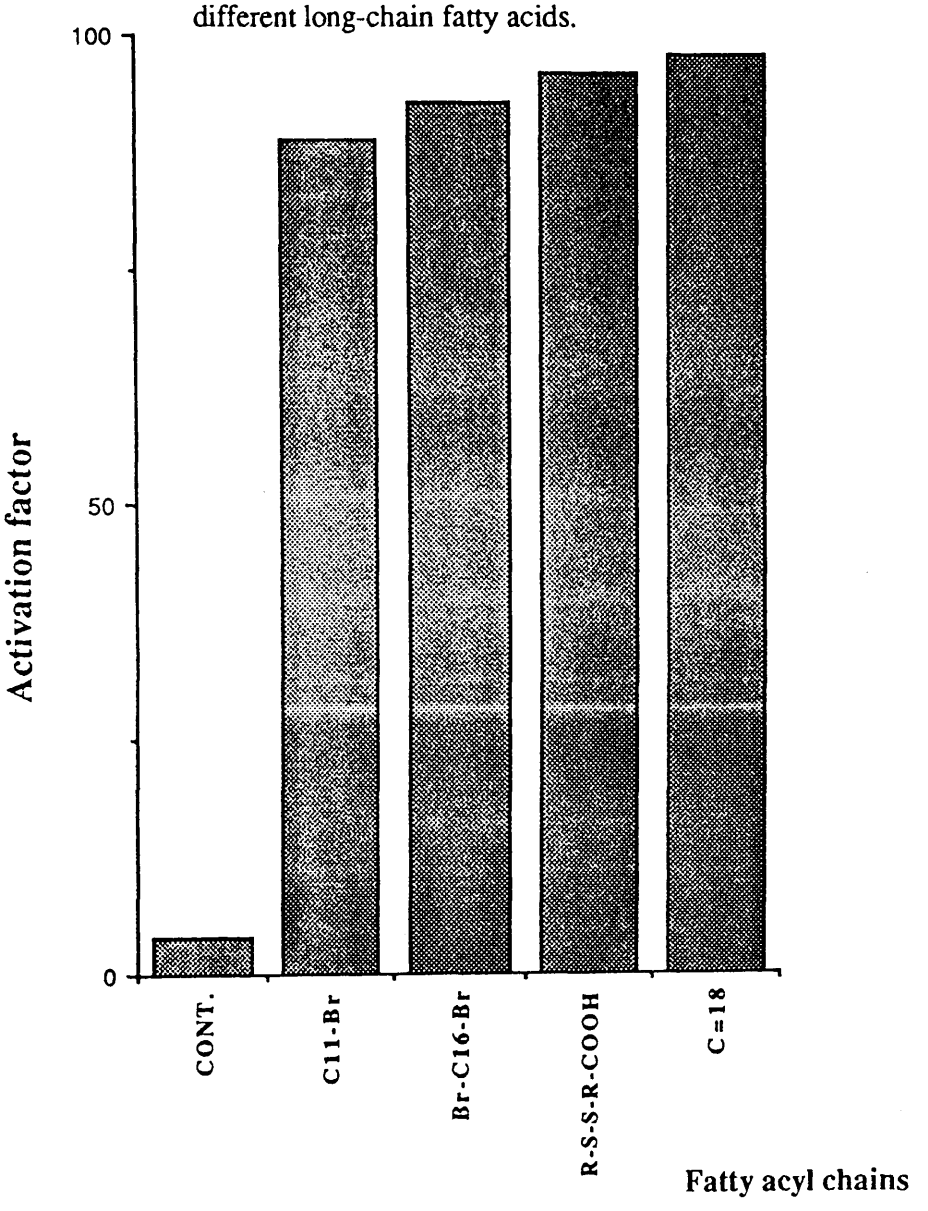
At a convenient protein concentration of ca. 1 mg/ml and at 37°C even most reactive activator (myristoyl imidazolide), used in 1:1 molar equivalent with the enzyme gave a half time of activation of ca. 30 min. Progress curves for the activation of bee venom phospholipase $A_2$ by the imidazolide derivatives of the saturated

Fig.(21) the activation factors obtained after the incubation of bee venom PLA2 with different saturated fatty acyl imidazolides.




Enzyme was activated by the incubation of 100 µl aliquots of solution of purified venom phospholipase $A_2$ enzyme (1mg per ml in 10 mM triethanolamine buffer, pH 8.0) with 2µl of an 0.2 % w/v solution of saturated fatty acyl imidazolide in acetone for two hours at 37°C. 1µg sample was tested for the ability to promote leakage from rabbit erythrocytes suspended in isotonic sucrose medium buffered at pH 7.4 with 10 mM MOPS/$Na^+$ and in the presence of 10mM albumin.

Fig.(22) The activation factors of bee venom PLA2 after its incubation with different long-chain fatty acids.




Enzyme was activated by the incubation of 100 µl aliquots of solution of purified venom phospholipase $A_2$ enzyme (1mg per ml in 10 mM triethanolamine buffer, pH 8.0) with 2µl of an 0.2 % w/v solution of unsaturated fatty acyl imidazolide in acetone for two hours at 37°C. 1µg sample was tested for the ability to promote leakage from rabbit erythrocytes suspended in isotonic sucrose medium buffered at pH 7.4 with 10 mM MOPS/$Na^+$ and in the presence of 10mM albumin.

acids of chain lengths $C_8$; $C_9$; $C_{10}$; $C_{12}$; $C_{13}$; $C_{14}$ and $C_{15}$ are shown in **Fig. 23** together with a progress curve for the unsaturated oleoyl derivative. These curves ratther unexpectedly showed that the reactions of many of the shorter chain compounds were as fast as those given by the more powerful activators. For example the reaction with the nonanoyl derivative appeared to reach completion sooner than that of the myristoyl derivative. At this stage it seemed probable that the short chain compounds might hydrolyse rapidly in the buffer and that this behaviour might reflect the fact that the activator had been destroyed by hydrolysis. To investigate whether or not the early phase of this curve corresponded to complete reaction of the enzyme with the shorter chain reagents, the treated enzymes were then incubated with oleoyl imidazolide and tested for further activation. The results also in **figure 23**, show clearly that the nonanoyl decanoyl and lauroyl derivatives all act in the same way. When they have raised the enzyme activity to a plateau level, they all block subsequent reaction with oleoyl imidazolide. This shows that the shorter chain ,'weak activators' react at the activation site at a very similar rate to the 'strong activators' , but give much lower activation. It therefore appears that the results obtained by Drainas do not hold for the purely aqueous system.

This result demonstrates very clearly that the rate of reaction at the activation site and the degree of activation produced by site occupation are determined by different factors. Full activation seems to require an acyl chain length > $C_{13}$, but maximum activation rates

Fig (23)

Solutions of purified bee venom phospholipase $A_2$ enzyme prepared as in (fig.21), were treated with fatty acyl imidazolide derivatives of different fatty acids, samples withdrawn at timed intervals and activity was estimated using rabbit erythrocyte leakage assay.

A=(a), $PLA_2$ +octanoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

B= (b), $PLA_2$ +nonanoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

C= (c), $PLA_2$ +decanoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

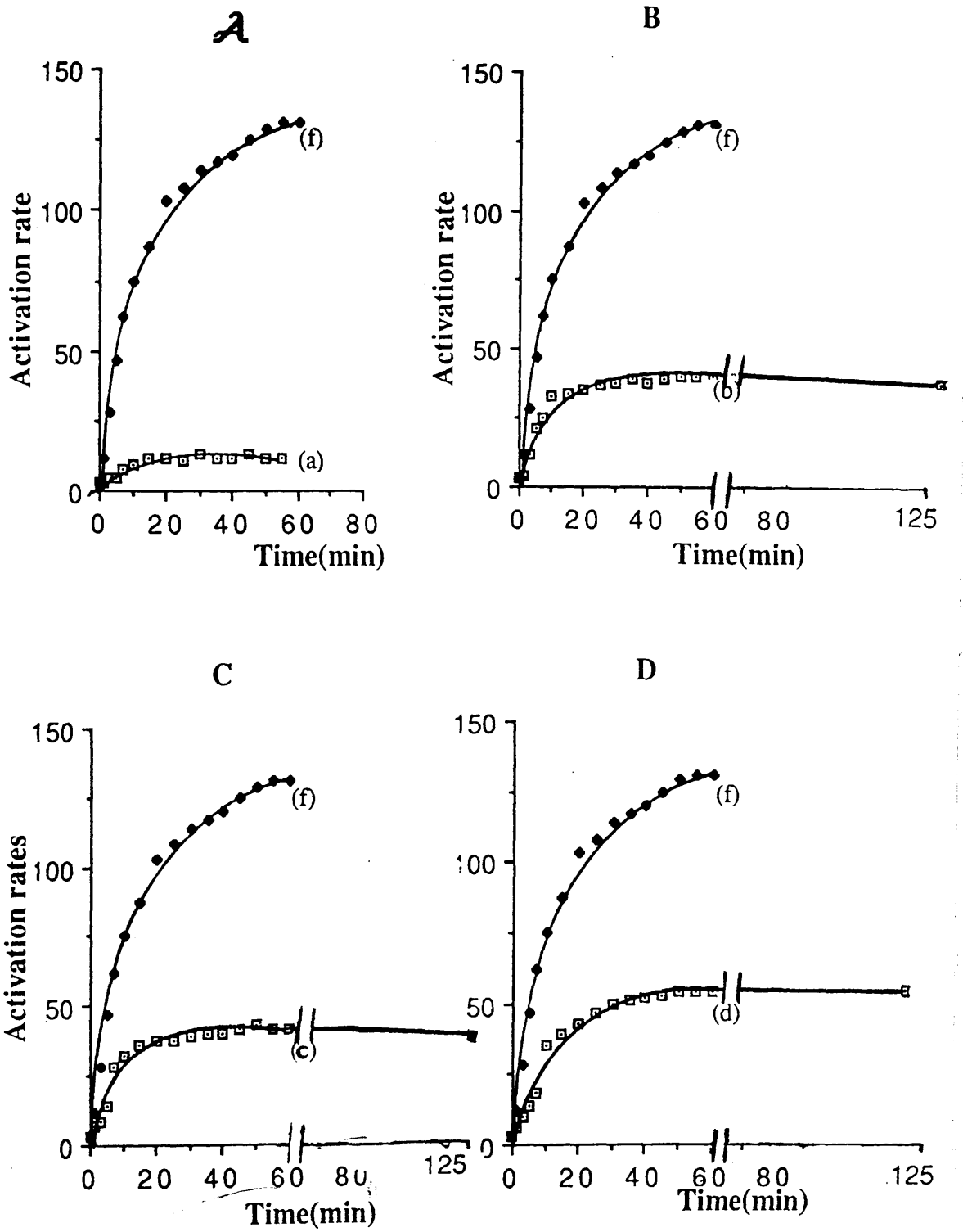
D= (d), $PLA_2$ +lauroyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

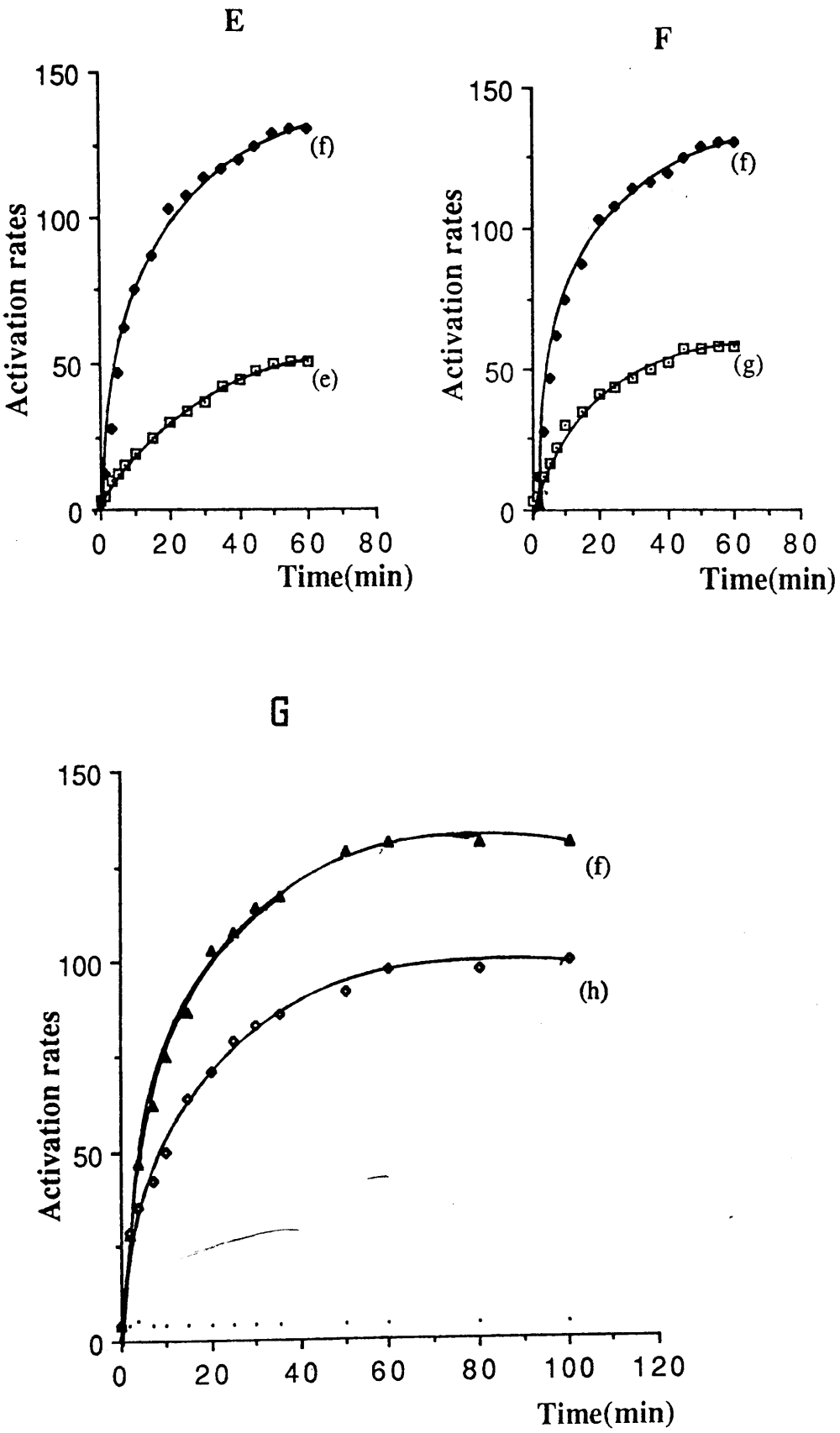
E= (e), $PLA_2$ +tridecanoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

F= (g), $PLA_2$ +pentadecanoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

G= (h), $PLA_2$ +oleoyl imidazolide; (f), $PLA_2$ + myristoyl imidazolide

After one hour from the incubation of the samples (b), (c) and (d) 0.2 % oleoyl imidazolide was added to each sample and assayed at different time intervals (5, 15, 30, 60, 125 minutes)(—/ /—).


A
B
C
D
Activation rate
Activation rates
Time(min)
150
100
50
0
0 20 40 60 80
125
(a)
(b)
(c)
(d)
(f)


E
F
G
150
100
50
0
Activation rates
(f)
(e)
(g)
(h)
0 20 40 60 80
Time(min)
0 20 40 60 80 100 120
Time(min)

require a chain length $\geq C_8$. These reaction rates fell sharply below a chain length of $C_7$ and shorter derivatives appeared not to react. These results are different from those shown by (Drainas, 1978). It seems probable that the difference could be due to the fact that reactions were carried out in 20 % n-propanol medium, whereas in the present work a purely aqueous system was used. A further difference between the two sets of experiments was that Drainas used a fixed high concentration (1 mM) of activator to obtain fast activation rates. When this was done in the present system it was found that high activator concentrations produced progressive inhibition of the enzyme and this was reflected in the time courses where activation reached an optimum level and then started to fall. This suggests that the activator binds relatively slowly to non-specific sites on the enzyme and that the effect of such binding is inhibitory.

### 3.3.4 Phospholipase $A_2$ protection by free fatty acids and their acyl derivatives against proteases and thiols: Evidence for an allosteric binding site.

Drainas and Lawrence proposed that the site on bee venom phospholipase $A_2$ that reacted with acyl imidazolides was also an allosteric binding site for free fatty acids. The work of Camero-Diaz et al., (1985), showing that activation by acyl imidazolides protected bee venom phospholipase $A_2$ against thiols and proteases, provides an ideal means to test this hypothesis.

Because there was no unambiguous assay for fatty acid activation of bee venom phospholipase $A_2$, protection studies provided the only possible alternative approach. This allowed the changes in the structure of the enzyme produced by the activator or its analogue to be studied in the absence of activation. The experimental advantage was that protection could be determined using the hydrolysis of short chain substrates as the assay method. This was both more convenient and more sensitive than methods that assayed activation directly. If fatty acids and acyl groups occupy the same allosteric site on the enzyme, then both should produce the same conformation change and confer protection against thiol and proteases by the same mechanism. Protection by free fatty acids should be determined by the fractional occupation of the binding site and it was expected that this would correlate with the affinity of the corresponding acyl imidazolide for the same site.

The rate of inactivation of bee venom phospholipase $A_2$ by thiols was compared for enzyme pretreated with a molar equivalent of the an acyl imidazolide or incubated with an equimolar concentration of free fatty acid. Cleland's reagent Dithiothreitol (DTT) was used in these studies at a concentration which give a large differential sensitivity between native enzyme and that activated with myristoyl imidazolide, whilst giving relatively fast reaction rates. The results in **Table 2** showed that fatty acids always provided less protection than did the corresponding imidazolide, indicating weaker binding to the enzyme. All free fatty acids, which gave rise to activating

Table 2. The degree of protection of bee venom phospholipase $A_2$ against thiol (DTT) after treatment with short-chain and long-chain free fatty acids or their acyl derivative.

| Time (min) | control | Octanoic acid $C_8$ | Myristic acid $C_{14}$ | Octanoyl imidazolide | myristoyl imidazolide |
|---|---|---|---|---|---|
| 0 | 89.0 | 86.0 | 124.0 | 86.0 | 126.0 |
| 2 | 77.0 | 81.0 | 84.6 | 81.3 | 122.2 |
| 10 | 56.2 | 73.2 | 75.0 | 77.9 | 83.5 |
| 18 | 28.0 | 58.1 | 66.1 | 65.0 | 79.8 |
| 25 | 21.3 | 39.5 | 51.0 | 44.2 | 53.8 |
| 33 | 17.9 | 23.5 | 34.6 | 27.9 | 42.8 |
| 40 | 6.7 | 11.6 | 26.9 | 20.9 | 37.3 |
| 50 | 3.3 | 5.8 | 20.6 | 12.7 | 33.3 |
| 65 | 1 | 3.4 | 14.5 | 11.6 | 28.5 |
| 180 | 0 | 2.9 | 7.8 | 10.4 | 20.6 |
| over night | 0 | 2.9 | 7.5 | 5.8 | 19.5 |

100µl of native and native bee venom phospholipase $A_2$ incubated with an equimolar concentration of free fatty acid or its acyl derivative for two hours was treated with 4 µl of 10mg/ml thiol dithiotheriotol (DTT) and 0.5 µl samples were withdrawn at different time interval and assayed for activity towards the hydrolysis of dioctanoyl phosphatidyl choline in 2 ml of 10 mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C.

imidazolide derivatives protected the enzyme to some degree.

These results also showed that protection was proportional with the degree of activation and were consistent with the model that protection was conferred by a conformation change with little contribution from direct blocking by the acyl group.

**Bee venom $PLA_2$ protection against proteases.**

A comparison was also made of the stability in the activity of native and activated bee venom phospholipase $A_2$ towards proteases [trypsin, chymotrypsin and thermolysin type X (from Bacillus thermo-proteolyticus rokko)

The data obtained from the incubation of 100 μg protein with 10μg proteases showed that in all cases the native enzyme was inactivated much faster than the activated enzyme. It was of interest that although trypsin attacked both forms of the enzyme much more rapidly than either chymotrypsin or thermolysin, it failed to produce the same degree of inactivation for the activated enzyme after a long term incubation (**Table 3**).

### 3.3.5 The identification of the acylation site on $PLA_2$.

Two techniques were used in this work in order to understand the acylation mechanism of bee venom phospholipase $A_2$ and to identify the site of acylation. The first technique was to activate the enzyme using fatty acyl imidazolides carrying tritium label in the acyl chain.

Table (3) The susceptibility of native and activated bee venom phospholipase $A_2$ to different proteases.

| | Native $PLA_2$ activity remaining(%xmin) | | | Activated $PLA_2$ activity remaining(%xmin) | | |
|---|---|---|---|---|---|---|
| TIME (min) | trypsin | chymotrypsin | thermolysin | trypsin | chymotrypsin | thermolysin |
| 0 | 100 | 100 | 100 | 100 | 100 | 100 |
| 5 | 8.3 | 17.1 | 66.6 | 35.5 | 61.2 | 85.3 |
| 20 | 0 | 3.5 | 9.5 | 27.0 | 25.8 | 35.3 |
| 40 | 0 | 1.3 | 5.9 | 25.8 | 16.3 | 19.8 |
| 60 | 0 | 0 | 3.5 | 22.4 | 13.7 | 12.0 |
| over night | 0 | 0 | 1.3 | 12.0 | 5.1 | 1.70 |

100 µl samples of native and activated bee venom phospholipase $A_2$ were incubated with 10 µg of each protease; 0.5 µl samples were withdrawn at different time interval and assayed for activity towards the hydrolysis of dioctanoyl phosphatidyl choline in 2 ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 at 37°C.

The second technique was the chemical modification of some essential amino acid residues of the enzyme which are thought to be involved in the acylation mechanism.

**a. The use of the $^{3}H$ radiolabelled activator.**

The full activation of bee venom phospholipase $A_2$ was achieved after incubation for two hours with a calculated molar equivalent of $^{3}H$ oleoyl imidazolide or myristoyl imidazolide both of which had been synthesised from pure radiolabelled fatty acid which had not been diluted with cold carrier. Enzyme activation were confirmed using either rat or rabbit red blood cells leakage assay system. When this highly radioactive protein was subjected to electrophoresis on a propionic acid/urea polyacrylamide gel, very little radioactivity was found with the major protein band. The major fraction (> 99 %) appeared at the top of the gel track. Although Lyall had shown incorporation of radioactivity into the enzyme using this SDS gel electrophoresis, it was clear that the level of label uptake into the enzyme was extremely low. Therefore despite the very high stability of the activated enzyme and the fact that albumin cannot reverse the activation by acyl imidazolides, although it can remove free fatty acids from the enzyme very effectively, these results now indicate that the chemical modification with imidazolide involved is not very stable.

Despite this result, protein degradation studies were undertaken

using the radiolabelled enzyme to see if any radiolabelled fragments could be obtained. The radiolabelled activated enzyme was incubated overnight with an excess of either n-mercaptoethanol or DTT to reduce the four disulfide bridges. The reduced enzyme was checked for activity using the short-chain phospholipid substrate dioctanoyl phosphatidyl choline ( $DiC_8PC$) assay system. After the enzyme had been completely inactivated by this treatment, trypsin was added to the mixture and incubation was continued out for a further two hours at 37°C. The mixture was subject to electrophoresis on a 22.5 % propionic acid/urea polyacrylamide gel and peptide fragments were detected using coomassie blue staining, **(fig. 24a)**. After two days of the gel destaining the tracks which contain the fragments were sliced to 1mm pieces and dissolved in 1ml hydrogen peroxide ($H_2O_2$) for 24 hours at 37°C. Ecoscint scintillation fluid was added to each sample and counted. Unfortunately, and in confirmation of earlier results, 95 % of the radioactive counts were found at the top of the gel track and no peak of radioactivity was found to coincide with any of the detectable fragments of the protein, **(fig. 24b)**. This negative result provided some confirmation that the acyl imidazolides did not work by non-specifically labelling lysine residues, because the bond formed (a an amide linkage) would be stable on the gel. Nevertheless the possibility remained that any specific labelling was of a small peptide fragment that was not retained on the gel during destain.

Fig.(24a) Gel electrophoresis of activated bee venom phospholipase $A_2$ after tryptic hydrolysis.

200 µl of 10 mg per ml fully activated bee venom $PLA_2$ with $^3H$ oleoyl imidazolide activator was incubated with 4 µl of 10 mg/ml trypsin for two hours, the hydrolysed enzyme was applied to 22.5% propionic acid/urea polyacrylamide gel electrophoresis.

Channels a and b, 30 and 40 µl activated $PLA_2$ respectively.

Channels c and d, 40 and 30 µl activated $PLA_2$ + trypsin respectively.

Channels e and f, 40 and 30µl trypsin only respectively.

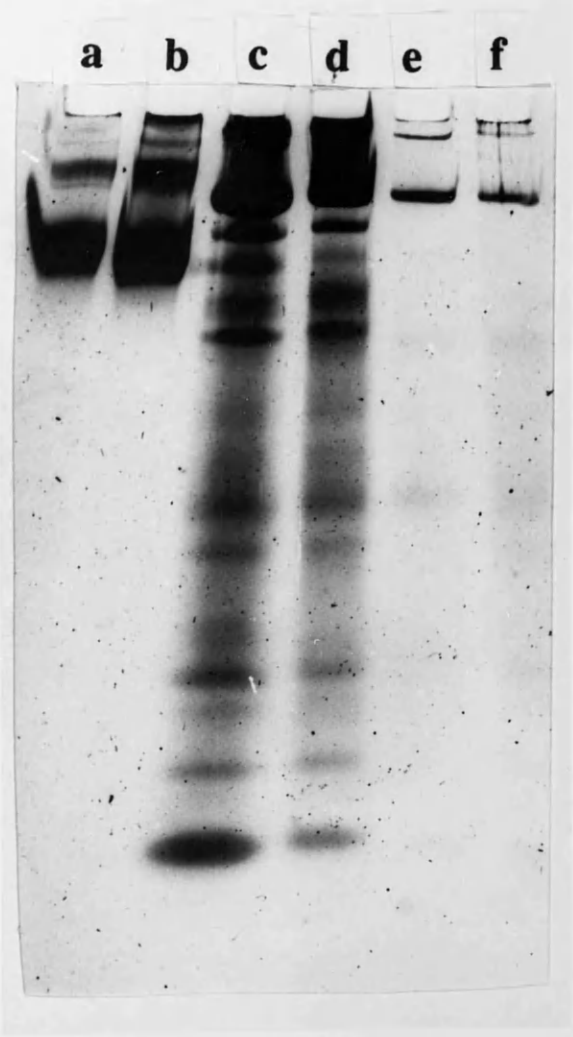
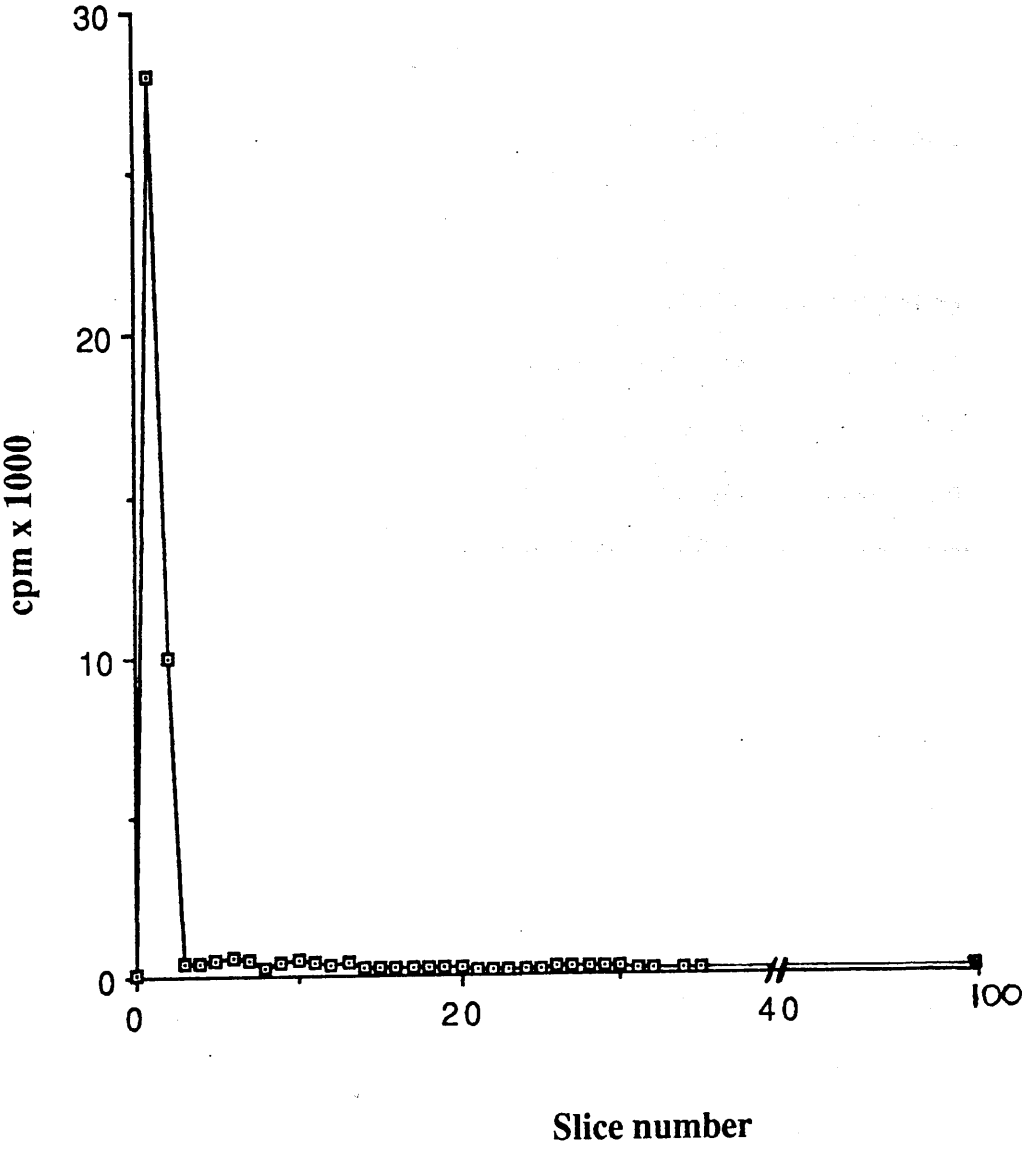

a
b
c
d
e
f

Fig.(24)b The radioactivity counts from activated bee venom PLA2 after trypsin cleavage.



**Cyanogen bromide cleavage of native and activated bee venom phospholipase $A_2$**

Because some of the fragments resulting from protease digestion of the radiolabelled activated enzyme are too small to be detected on propionic acid/urea/polyacrylamide gel, it was important to obtain larger fragments from this enzyme to facilitate the detection of any possible acyl residue on the protein.

The sequence data for bee venom $PLA_2$ published by Shipolini et al., (1974), showed the presence of three methionine residues at the positions 36, 41, 81, and an oligosaccharide chain linked to N-13. Therefore, cyanogen bromide cleavage of the reduced and carboxymethylated protein should give three large peptide fragments and one pentapeptide. When this was carried out and the products separated by electrophoresis on 22.5 % propionic acid / urea / polyacrylamide gel it was possible to detect three major fragments although there was a possibility that one of these was an unresolved doublet. The sequence predicted that the three large peptides were the N-terminus with 35 amino acids and 8 positive charges, the C-terminal peptide with 48 amino acids and 11 positive charges and a middle peptide with 39 amino acids and 5 positive charges, **(fig. 25a)**. Predictions from size and charges suggested that the migration order should be N-terminus > C-terminus > middle peptide. However, the N-terminal peptide, which carries a large carbohydrate moiety, was detected directly by the sugar staining technique and corresponded to

Fig.(25a) Cyanogen bromide cleavage of bee venom phospholipase $A_2$.

The reduced and carboxymethylated bee venom phospholipase $A_2$ was ethanol precipitated, dissolved in 500 µl of distilled water. 50 µl was removed from the sample and the remaining 450 µl was cleaved with cyanogen bromide and ethanol precipitated. the samples were mixed with 1:1 v/v of protein / 50% glycerol containing neutral red dye and applied to 22.5 % propionic acid/urea polyacrylamide gel electrophoresis.

A; 20 µl bee venom phospholipase $A_2$

B; 20µl bee venom phospholipase $A_2$ after reduction and carboxymethylation

1, 2 and 3 fragments from the cleaved protein by cyanogen bromide.

Fig.(25b-c) The identification of sugar containing peptide of bee venom phospholipase $A_2$ after cyanogen bromide cleavage.

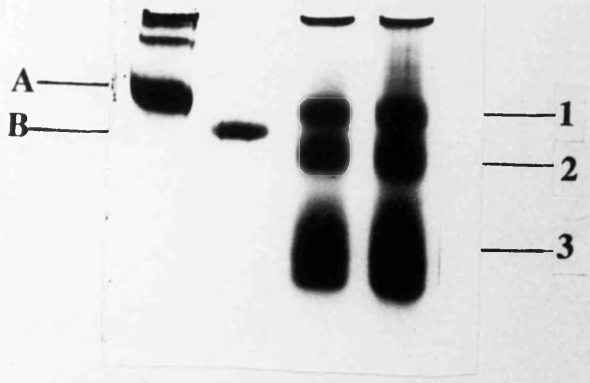
Plate (b), 22.5% propionic acid / urea / polyacrylamide gel electrophoresis stained with coomassie blue

Plate (c), 22.5 % propionic acid/urea polyacrylamide gel electrophoresis stained with sugar staining.

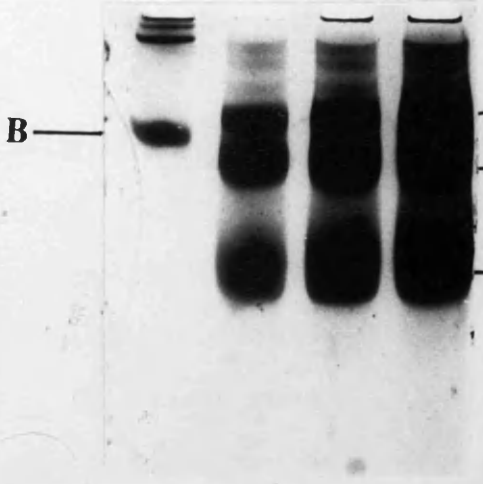
B= in both plates is the bee venom $PLA_2$ after reduction and carboxymethylation

1, 2 and 3 fragments from the cleaved protein by cyanogen bromide.

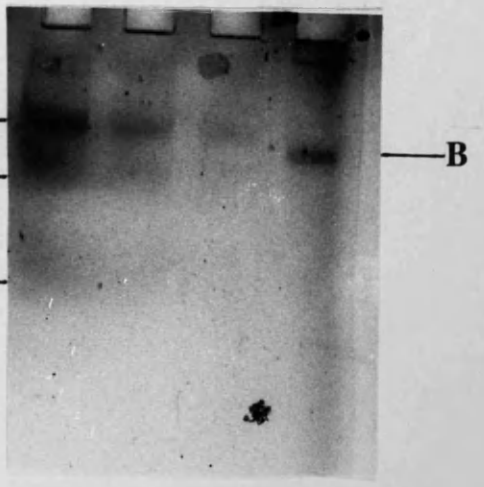
a




b




c



the band with lowest mobility (**fig.25b, c**). This slow migration could be attributed to the presence of a very large oligosaccharide located at the amino acid residue number 13 which increased the size of this peptide and affected its running position. These results are in agreement with the results obtained by Shipolini and coworkers (1974) where they found that the sugar group is responsible for increasing the molecular weight of the protein from 14555 to 15800 Da.

A further attempt to identify the acylation site was made by preparing cyanogen bromide fragments from enzyme activated by $^{3}H$ labelled activator (oleoyl imidazolide). The fully activated enzyme was reduced and carboxymethylated for the cyanogen bromide cleavage and precipitated with ethanol, dissolved in the appropriate amount of distilled water, and incubated with cyanogen bromide as described in Materials and Methods, then applied to a 22.5% propionic acid / urea / polyacrylamide gel. It was quite clear at this stage that the bulk of the radiolabel was recovered in the ethanolic supernatant. After the staining and destaining the gel, 1 mm slices from the tracks containing the fragments were dissolved in 1ml hydrogen peroxide ($H_2O_2$) (100 volumes) for 24 hours at 37°C. 10 ml Ecoscint Scintillation liquid was added to each sample and counted for radioactivity. The result obtained were the same as the result obtained with tryptic study.

The inescapable conclusion from all of these results is that the

activator dissociated from the enzyme very easily under the acid conditions of the gel. Because carboxymethylation was also done under acidic conditions it seemed unlikely that the bound label would survive this stage.

**Ammonia(basic)/urea/polyacrylamide gel and activated bee venom $PLA_2$.**

All of the above results indicated that the identical mobility observed for native and activated bee venom phospholipase $A_2$ on acid/urea acrylamide gels was due to the rapid dissociation of the acyl residue (the same result was found for many snake venom enzymes, see below). The simplest way to change the gel conditions in order to prevent this dissociation was to substitute a weak base for the weak acid to obtain a similar gel composition but running at an alkaline pH. Using 2 % of 0.880 ammonia as the electrolyte, gels were obtained that set readily and had a running pH of ca. 11. (see materials and methods).

Native, 25 %, 50 %, 75 % and fully activated bee venom phospholipase $A_2$ were applied to 22.5 % ammonia/ urea/ polyacrylamide gel using bromophenol blue as marker dye and then run at 20 mA for three hours. After the standard coomassie brilliant blue G staining / destaining procedure, a migration pattern was found that indicated that the activated enzyme had not lost the acyl group under these conditions. **Figure. 26a, b**, shows that the fully activated enzyme ran with very low mobility and tended to smear near the

Fig.(26) Gel electrophoresis of native and activated bee venom phospholipase $A_2$ using ammonia(basic)/urea polyacrylamide gel.

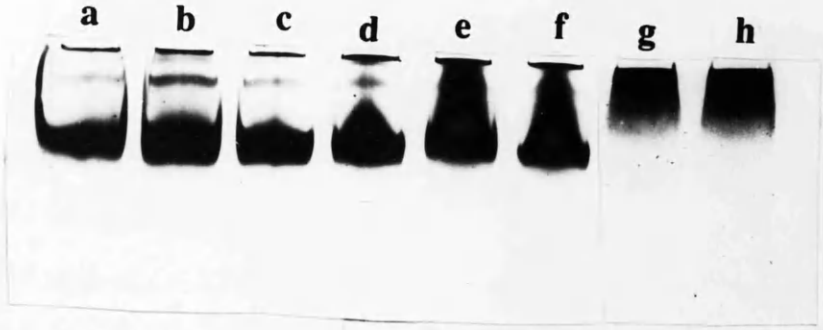
Plate (A). 22.5% ammonia (basic) /urea polyacrylamide gel showing in Channels a, b and c the native bee venom phospholipase $A_2$.

Channels d, e and f activated $PLA_2$ with a degree of activation of 25%, 50 % and 75 % respectively.

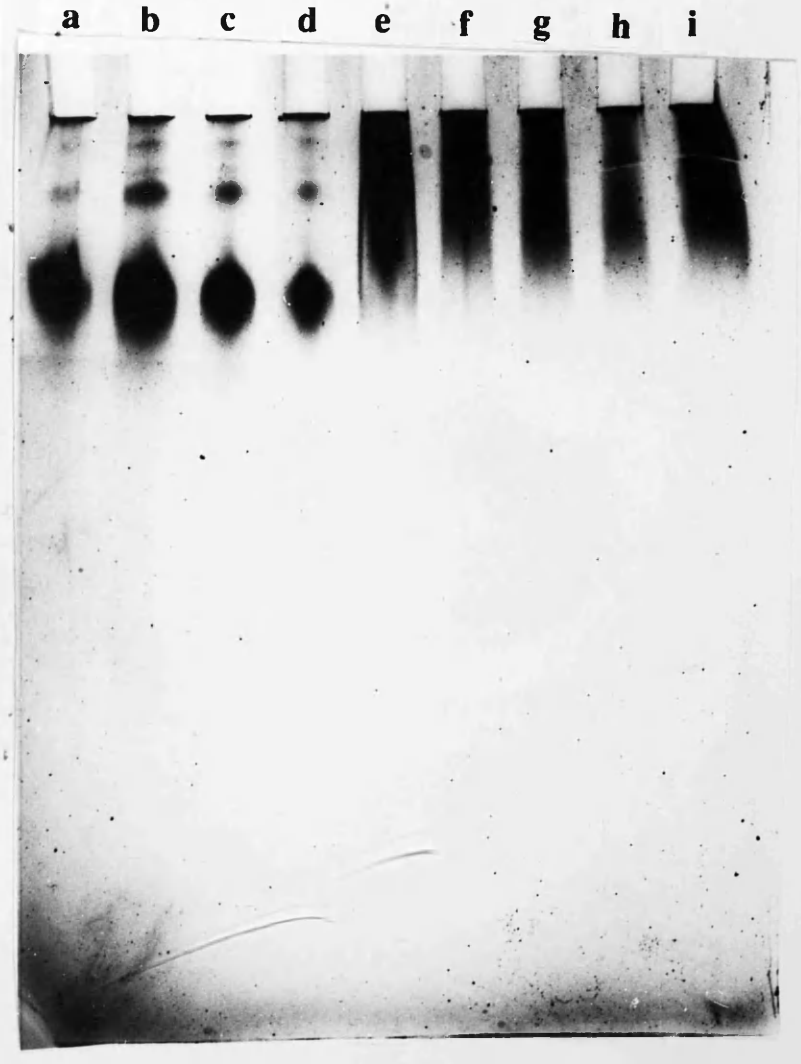
Channels g and h fully activated phospholipase $A_2$.

Plate B. 12.5% ammonia (basic) /urea polyacrylamide gel showing in Channels a, b, c and d, 80 µl, 40 µl, 20 µl and 10 µl of native bee venom phospholipase $A_2$.

Channels e, f, g, h, i, 80 µl, 40 µl, 20 µl 10 µl and 80 µl of the fully activated bee venom phospholipase $A_2$.




A




B

origin. Where the activated enzyme was present with inactivated enzyme there was no separation into separate bands, but the normal enzyme showed a smeared 'tail'. These results suggest that the activated enzyme aggregated under this basic pH conditions and also tended to bind to the native enzyme. This data also supported the original model that activation was due to a chemical modification of the protein by the acylating agent. Taken together with previous results it did, however, suggest that the linkage between the acyl residue and the protein was highly labile.

**b. Chemical modification.**

Acylation of amino groups **C**; **K**; **H**; **Y** in proteins have been discribed by (Lundblad & Noyes, 1984). However phospholipase $A_2$ enzyme could also be activated, although with lower specificity, by less selective acylating agents that would be expected to show highest reactivity towards primary amino-groups. These groups therefore remained as the most probable targets. Clearly the N-terminal residue should be the most susceptible residue towards acylating agents. It was highly probable that this residue, which is in a very hydrophobic part of the protein, might be buried within the structure, however this could confer the property of high specificity towards hydrophobic reagents.

The aim of this part of the investigation was to use reagents and procedures that would enable the chemical nature of the target group to be determined.

**1.Primary amino-group modification.**

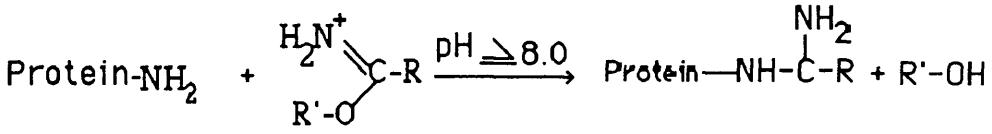
Conductimetric studies of acylation reaction provided a simple experimental method for examining the reactivity of the reagents used under the reaction conditions. Such experiments confirmed the strong dependence of the reactivity of amines on the $pK_a$ of the amino-group. Hence for all of these studies, the N-terminal group should be the most susceptible group present in the enzyme. It was therefore very important to see whether or not the activation phenomena were most strongly affected by modification of the most sensitive group present to distinguish between the N-terminal residue and the **K** side-chain residues.

Previous studies had shown lysine residues to be involved in the catalytic activity of phospholipase $A_2$ enzymes. Yang et al., (1981) were used cyanate at pH 8.0 to study the involvement of **K** residues in the association between enzymatic activity and lethal toxicity of *Naja naja atra* venom, also Maraganore & Heinrikson, (1985) used the chemical modification of the **K** residues of phospholipase $A_2$ from *Agkistrodon piscivorus piscivorus* by trinitrobenzene-sulfonic acid (TNBS) to show that lysyl residues of this enzyme are strongly involved in the binding of phospholipids in the absence of calcium.

**The effect of methyl acetimidate hydrochloride (M.A-HCl ) on the activation of bee venom $PLA_2$.**

Coggins, (1978) reported that the imido esters react specifically

with the amino groups of proteins at pH above 8.0 to form amidines as follows:




The mechanism of this reaction involves nucleophilic attack of the unprotonated form of the amino group on the protonated amido ester. The incubation of native and activated bee venom phospholipase $A_2$ with an excess of methyl acetimidate hydrochloride at pH ≥ 8.0 at 37°C shows a 40 % decrease in the activity of the native form and 16 % of the activated enzyme towards the hydrolysis of the short-chain substrate ($DiC_8PC$) **(fig. 27a).** After 24 hours incubation, the amidinated native enzyme was passed through an Ultrogel AcA202 medium size column (0.5 cmx 10 cm) to separate the amidinated protein from small molecular reaction products

The pure amidinated native enzyme was than incubated with 2 % myristoyl imidazolide to test whether or not the enzyme still has the potential for activation. Using rabbit erythrocyte leakage assay system, it was clear that the pure amidinated native enzyme was still; capable of reacting with this activator and gave an activation factor of about 40 fold, **(fig. 27b)**. In addition, the activity of the activated, amidinated enzyme towards the hydrolysis of the short-chain substrate ($DiC_8PC$) was increased by about 40 %, **(Fig. 27a).**

Fig.(27)a

100 µl of 1mg/ml solutions of native $PLA_2$ and $PLA_2$ treated with 0.2 % myristoyl imidazolide were incubated with two molar excess of methyl acetimedate-HCl and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5 µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100µM o-phenanthroline.

- Native $PLA_2$.
- Native $PLA_2$+ M.A-HCl .
- Native $PLA_2$+ M.A-HCl + activator.
- Activated $PLA_2$.
- Activated $PLA_2$ + M.A-HCl .

Fig.(27)b

1µg sample of native $PLA_2$ treated with M.A-HCl and then the oleoyl imidazolide was assayed for activity in the rabbit erythrocyte leakage assay.

- Native $PLA_2$.
- Native $PLA_2$+ M.A-HCl + activator.
- Activated $PLA_2$.

Fig.(27)a The effect of methyl acetimidate-HCl on the activity and activation of bee venom PLA2.

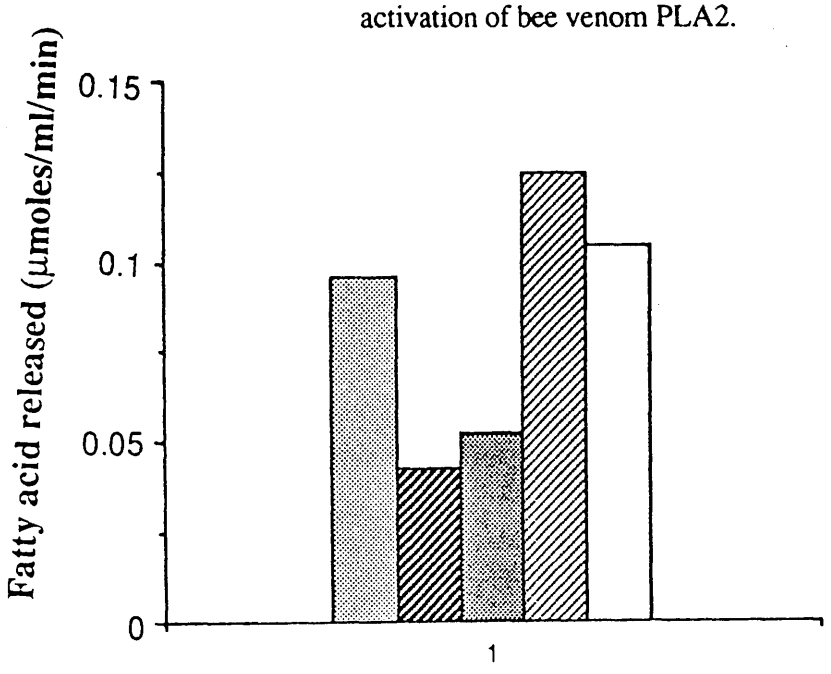
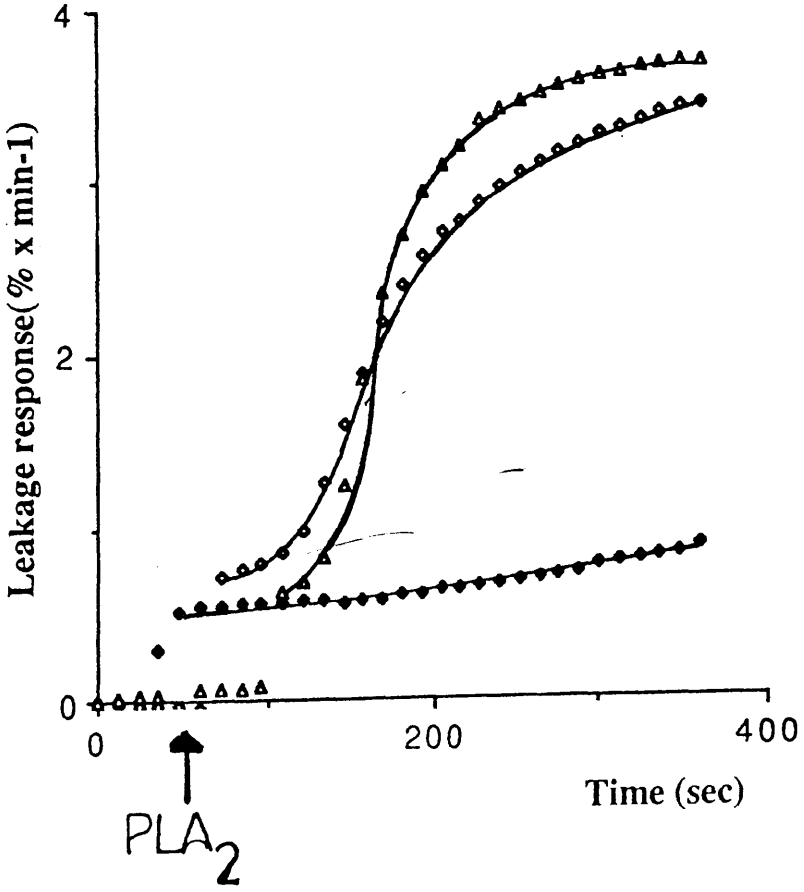



Fig.(27)b The effect of methyl acetimidate-HCl on the activation of bee venom PLA2.



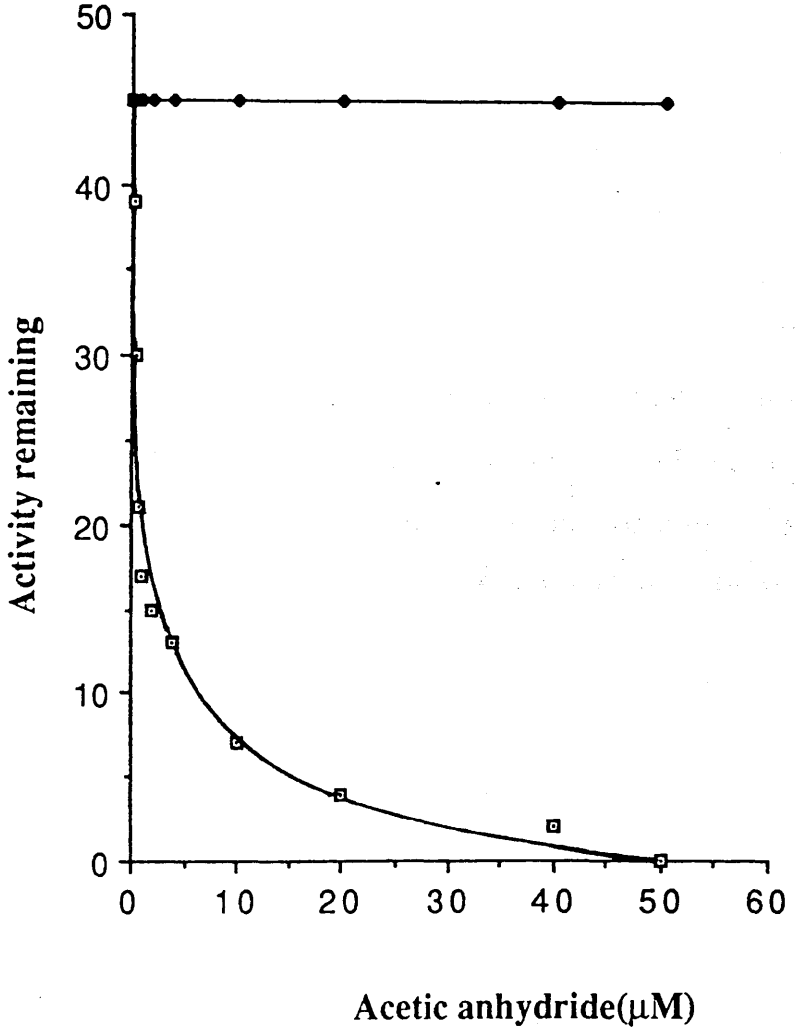
**The effect of acid anhydrides.**

Acylation of amino groups in proteins by reaction with carboxylic acid anhydrides has been extensively used to study the importance of the **K** residues in proteins. It is generally carried out with acetic anhydride at alkaline pH in either a pH-stat or in saturated sodium acetate solution.

Modification of the N-terminal amino group and the amino groups of the **K** residues of phospholipase $A_2$ from different sources by acetic and succinic anhydrides has been carried out by many workers in conjunction with x-ray crystallographic studies. This has not been possible with the bee venom enzyme which is a glycoprotein and has proved to be difficult to crystalise. Therefore, in this work chemical modification was studied using the newly developed method of propionic acid / urea / polyacrylamide gel electrophoresis (see chapter three) to determine degree of the modification obtained.

Native and activated bee venom phospholipase $A_2$ were incubated with different concentrations of acetic anhydride at pH 8.0. The two forms of enzyme were then assayed for activity using short-chain phospholipid substrate ($DiC_8PC$) in triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100 μM o-phenanthroline and assayed for activation using either egg lecithin substrate in 20 % n-propanol buffer in the presence of 100 μM o-phenanthroline or rat red blood cells in the presence of 10 μM albumin. The effect of a range of concentrations of acetic anhydride, **(fig. 28)** was correlated with the

Fig.(28) The effect of acetic anhydride on the activity of the native PLA2 from Naja mossambica mossambica.




10 mg of pure snake venom phospholipase $A_2$ was dissolved in 1ml of 200mM triethanolamine/$Cl^-$ buffer pH 8.0. 100μl of 1mg/ml solutions of this sample were treated with different acetic anhydride concentrations and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30 mg/ml) by 0.5μl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100 μM o-phenanthroline.

⊙——⊙ $PLA_2$ + acetic anhydride.

◆——◆ The ratio of the loss in the enzyme activity to the loss of activation.

degree of modification shown by electrophoresis. The results for bee venom phospholipase $A_2$ gave a consistent pattern, although the bands were not well separated. Similar results were obtained with the treatment of the phospholipase $A_2$ (CMII) isoform from the snake venom of *Naja mossambica mossambica* with acetic anhydride in a molar ratios 1:1 , 1:10 and 1:100. Here the electrophoretic pattern was quite clear and the higher concentrations produced a step pattern from which it could be deduced that a maximum of 8 groups were available for modification, **(fig. 29)**. From these results it was clear that the activated enzyme, as seen on the gel, showed very little difference in mobility from the native species and certainly less than the minimum step seen with acetic anhydride. Measurement of the residual activation of the modified protein and its ability to be activated by oleoyl imidazolide showed that activity fell very rapidly with the extent of acetylation, but that the degree of activation obtainable fell to the same extent. When the degree of activation was plotted as a proportion of the level of activity, the results indicated that activation, as such, was not affected by the treatment **(fig. 28)**. There was no evidence that the band pattern observed was due to sequential acylation of protein residues, but it seems most probable that there would be a spectrum of reactivities and that the certain groups, probably including the N-terminus, would be acylated more rapidly than others. Because the residual active enzyme present after a treatment that completely modified two amino-groups, was activatable in proportion to its activity, it was clear that these highly

Fig.(29) Gel electrophoresis of the native and modified snake venom phospholipase $A_2$.

100µl of 1mg/ml solutions of native phospholipase $A_2$ and native treated with acetic anhydride of the non-toxic pI 8.8 isoform from the venom of *Naja mossambica mossambica* was mixed with 2:1 v:v of water containing 50% glycerol with neutral red marker dye and 20 µl was applied to 22.5% propionic acid/urea polyacrylamide gel.

Channel 2, native $PLA_2$.

Channels 1, 3 and 4, native $PLA_2$ + acetic anhydride in the molar ratios of 1:1, 1:10, 1:100 respectively.

Channel 5, myoglobin chymotryptic fragments.


1
2
3
4
5

reactive residues played no role in the activation phenomenon.

It was of further interest that enzyme activated by oleoyl imidazolide was protected against the complete inhibition by the anhydrides in comparison with the native enzyme, (**fig.30**). However the activity of native enzyme, inhibited by acetic anhydride could not be restored by the addition of oleoyl imidazolide.

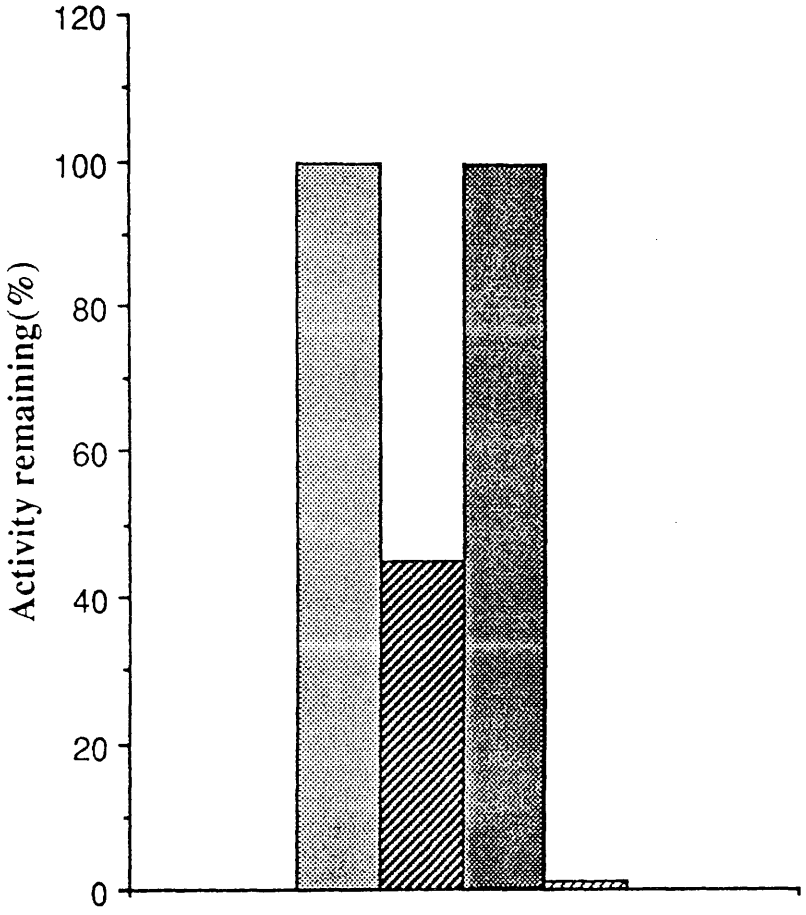
The next experiments were designed to see whether or not primary amino-groups played an indirect part in the activation reaction. This was done using the reagent dimethyl maleic anhydride, which forms a stable adduct at neutral-high pH, but can be rapidly removed at mild acid pH.

Special care was taken to ensure that the pH during this, and subsequent procedures remained above 8.0 by using a high concentration of triethanolamine buffer (200 mM). The sample was then treated with oleoyl imidazolide for five hours, the excess reagent removed by precipitating the protein with ethanol. The protective groups (dimethyl maleoyl residues) were then removed by lowering the pH below 6.5 with propionic acid.

Reaction condition for the modification of **K** residues in the protein with this reagent are usually obtained at pH $\geq$ 8.0 and the dissociation can be occurred under acid pH (pH $\leq$ 6.5.) or with the treatment of the enzyme with 1 M of hydroxylamine pH 10.0.

Incubation of the native bee venom phospholipase $A_2$ dissolved in

Fig.(30) The inhibition of native and activated bee venom PLA2 by acetic anhydride.




100 µl of 1mg/ml solutions of native and activated snake venom phospholipase $A_2$ were treated with 10 molar excess of acetic anhydride and assayed by conductimetric estimation of the hydrolysis of egg lecithin substrate in 20% n-propanol in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 1mM calcium and 100µM o-phenanthroline.

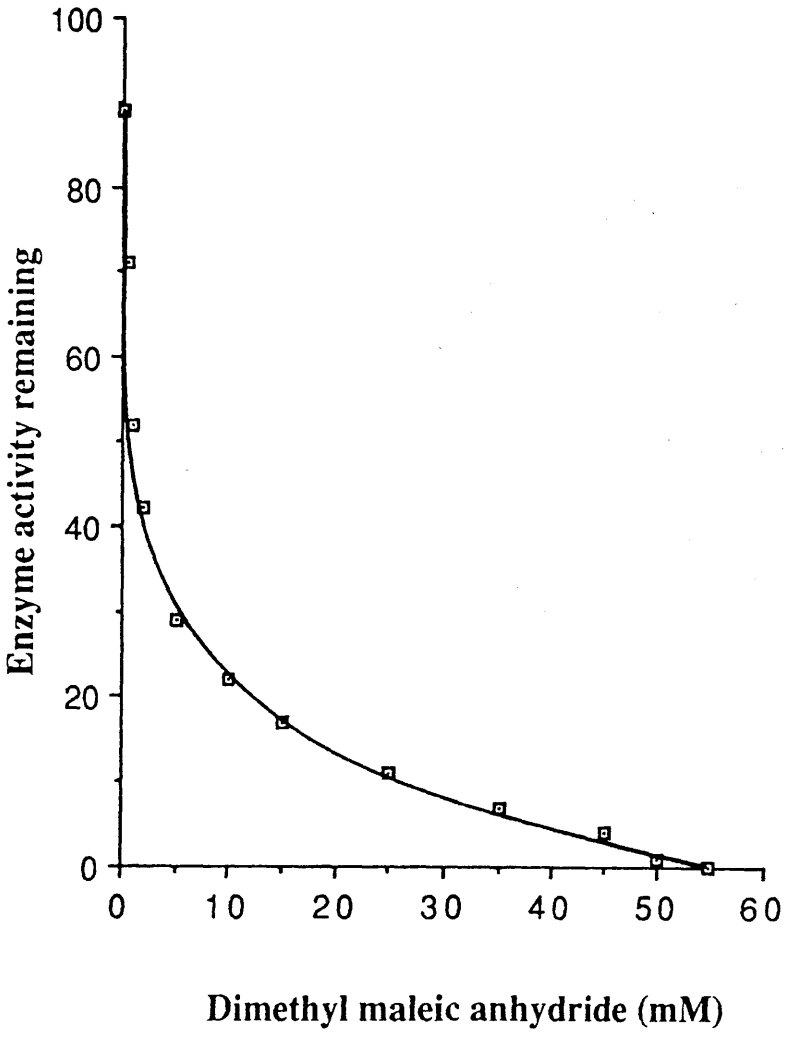
- Activated $PLA_2$.
- Activated $PLA_2$ + anhydride.
- Native $PLA_2$.
- Native $PLA_2$ + anhydride

200 mM triethanolamine/HCl buffer at pH 8.0 with different concentrations of dimethyl maleic anhydride resulted in a concentration dependent decrease in the enzyme activity. At 50 mM dimethyl maleic anhydride concentration, the enzyme activity was abolished completely, **(fig. 31)**.

The inactivated enzyme was now incubated with oleoyl imidazolide for 5 hours, then assayed for activity using short-chain phospholipid substrate ($DiC_8PC$) in the presence of 100 μM o-phenanthroline and for activation using rat or rabbit red blood cell assay in the presence of 10 μM albumin. The addition of the activator had no effect on the inactivated enzyme in any of these assays. The inactivated enzyme was then treated with 1 % propionic acid to lower the pH of the mixture and cause dissociation of the dimethyl maleoyl residues from the enzyme.

The enzyme was assayed for activity and activation and the results obtained showed that about 70 % of the enzymic activity was recovered after this treatment, showing that the protective groups had been removed. In addition, the response in the activation assay corresponded to 25 % of the value obtained for the original sample. Based on the amount of activity recovered, this indicated that ca. 36 % of the enzyme could be activated after this procedure, **(fig.32a,b)**. These results provide a very clear indication that the receptor site for the acyl residue is not a primary amino-group. However it is still possible that such residues are involved in facilitating the acylation

Fig.(31) The inhibition of the native bee venom PLA2 by dimethyl maleic anhydride.




10mg bee venom phospholipase $A_2$ was dissolved in 1ml of 200 mM triethanolamine/$Cl^+$ buffer pH 8.0. 100µl samples were treated with different dimethyl maleic anhydride concentrations and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100 µM o-phenanthroline.

reaction. This might explain why the degree of activation was lower than expected. The possible weakness of the experimental design was that oleoyl imidazolide could react with the enzyme after the acid treatment had removed the dimethyl maleoyl groups and before the ethanol precipitation step. However this appeared to be very unlikely because the activation reaction was known to be very slow below pH 6.0.

**2. Arginine modification.**

Until approximately ten years ago the specific chemical modification of **R** was relatively difficult to achieve. The high $pK_a$ of the guanidine functional group ($pK_a \approx 12$ to 13) necessitated fairly drastic reaction conditions ($pH \geq 12$) to generated an effective nucleophile and most proteins are not stable to extreme alkine pH. Phenylglyoxal is a moderately specific reagent for modifying **R** residues although it can react rather more rapidly with the N-terminal residue. Vensel & Kantrwitz, (1980) used this reagent to study the involvement of the **R** residues in the binding of the substrate to porcine phospholipase $A_2$ enzyme and showed that incubation of the enzyme with 13.2 mM phenylglyoxal at pH 8.5 abolished the enzymic activity within 80 minutes. This reagent was therefore used here to study the possible role of arginine residues in the acylation/activation mechanism of bee venom phospholipase $A_2$.

Fig.(32)a Two samples of 100 µl of 1mg/ml solution of native bee venom phospholipase $A_2$ prepared (as in Fig.31) were incubated with 50 mM dimethyl maleic anhydride for two hours. One of the samples was treated with 1% propionic acid. In all cases samples were assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5 µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100 µM o-phenanthroline.

- Native $PLA_2$.
- Native $PLA_2$ + DMMA.
- Native $PLA_2$ + DMMA + propionic acid.

Fig.(32)b Two samples of 100 µl of 1mg/ml solution of native bee venom phospholipase $A_2$ prepared (as in fig.31), were incubated with 50 mM dimethyl maleic anhydride for two hours and than treated with 2% oleoyl imidazolide for one hour at 37°C. One of the sample was treated with 1% propionic acid, activity measured in the rabbit erythrocyte leakage assay.

- Native $PLA_2$ + Oleoyl imidazolide.
- Native $PLA_2$ + DMMA + Oleoyl imidazolide.
- Native $PLA_2$ + DMMA + Oleoyl imidazolide + propionic acid.

Fig.(32)a Inhibition of native bee venom PLA2 by dimethyl maleic anhydride and reversal by propionic acid .

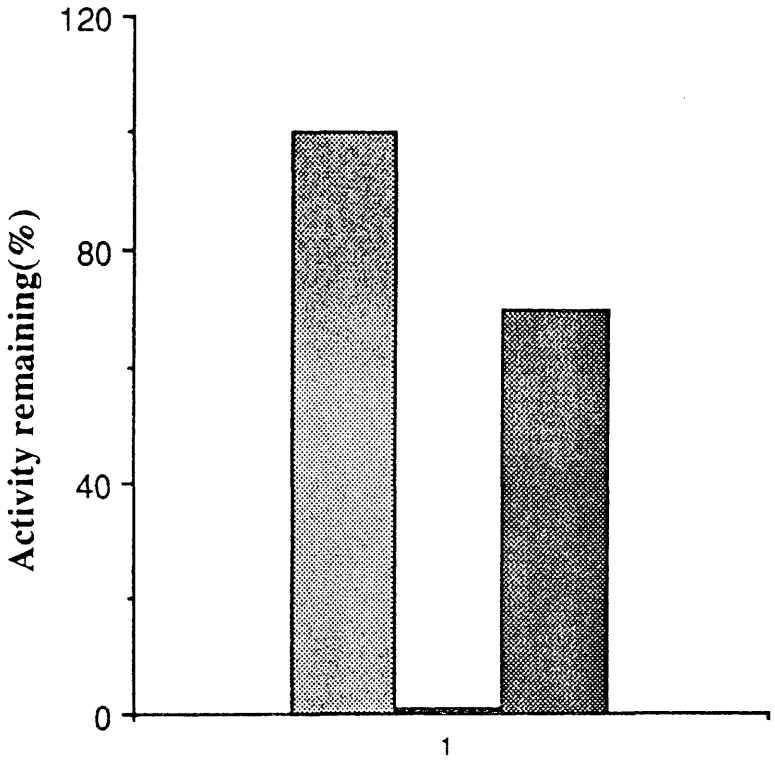
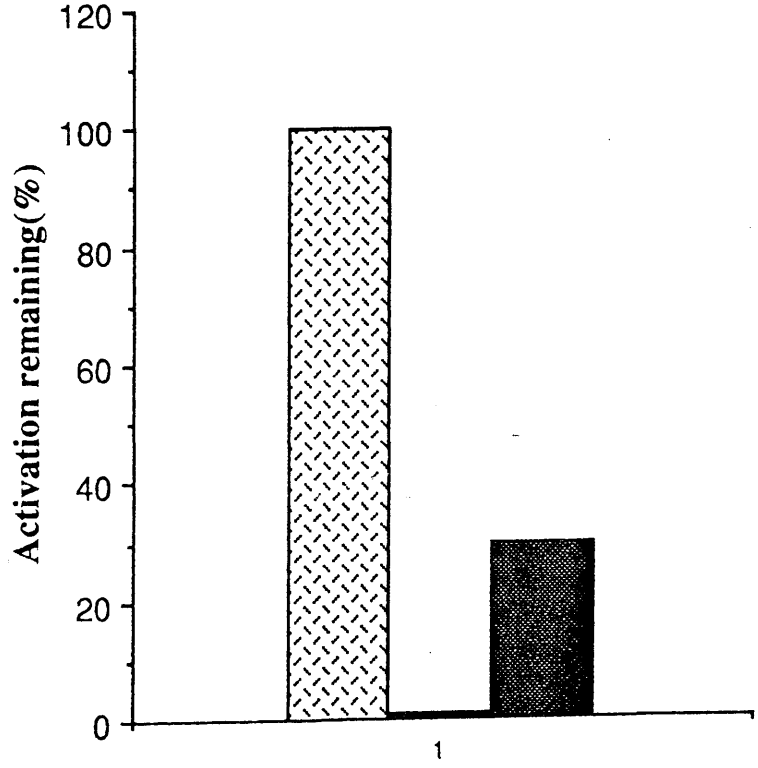



Fig.(32)b The effect of oleoyl imidazolide on the native and inhibited bee venom PLA2 by dimethyl maleic anhydride in the absence and presence of proiponic acid.

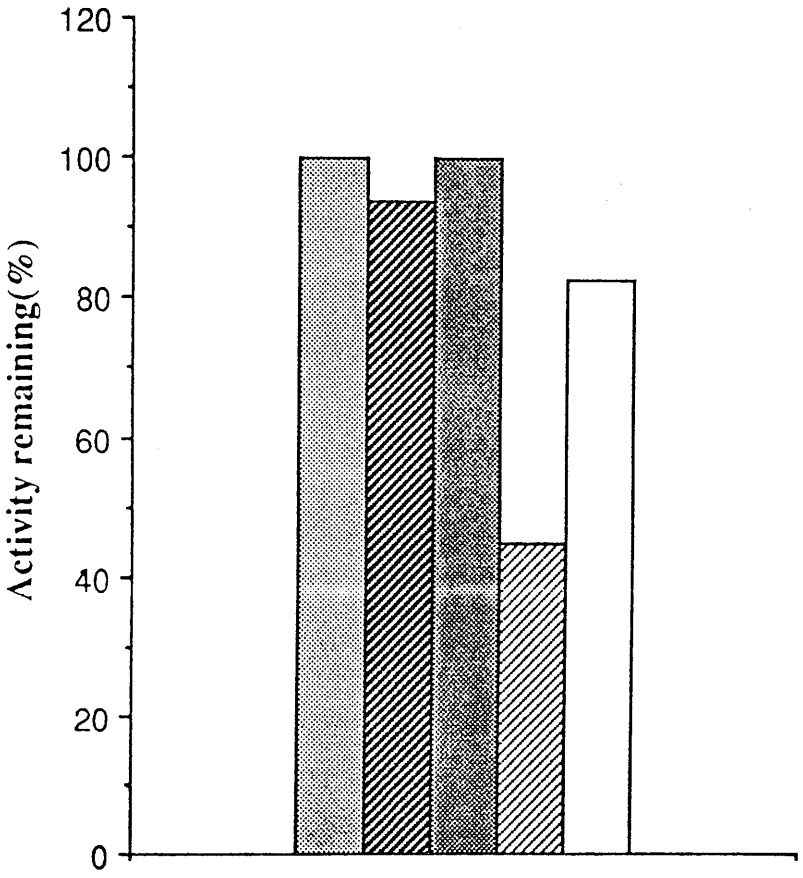


**The effect of phenylglyoxal on bee venom $PLA_2$ activity.**

Native and activated bee venom phospholipase $A_2$ were treated with phenylglyoxal and then passed through a small Bio-Gel P6 column to remove any excess reagent. The purified enzymes were freeze dried, dissolved in the appropriate amounts of 10 mM triethanolamine buffer pH 8.0 to give the same concentration as the starting materials and assayed against the hydrolysis of short-chain phospholipid substrate ($DiC_8PC$) in the presence of 100 μM o-phenanthroline. Preliminary results showed that this gave a 58% loss of activity for the native enzyme and a 5% loss of activity for the activated enzyme. The addition of 2% oleoyl imidazolide activator native phenylglyoxal treated enzyme almost completely restored the catalytic activity towards the hydrolysis of short-chain phospholipid substrate ($DiC_8PC$), **(fig. 33)**. This was the first example in which the activator was able to reverse an inhibitory action rather than just to prevent it occurring.

The phenylglyoxal treated native enzyme was applied to 22.5% propionic acid/urea/ polyacrylamide gel to determine the number of arginine residues to have been modified. **Figure 34**, showed that the migration of the protein was ca. 2/3 that of the untreated enzyme, corresponding to a loss of about seven positive charges from a total of 23 in each molecule of enzyme.

Fig.(33) The effect of phenylglyoxal on the activity of native and activated bee venom PLA2.




100 µl of 1mg/ml solutions of native and activated bee venom phospholipase $A_2$ were treated with 25mM phenylglyoxal and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) with 0.5 µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100µM o-phenanthroline. ▩ Activated $PLA_2$; ▨ activated $PLA_2$ + phenylglyoxal; ▩ native $PLA_2$; ▨ Native $PLA_2$ + phenylglyoxal; □ Native $PLA_2$ + phenylglyoxal + Oleoyl imidazolide.

Fig.(34) Gel electrophoresis of purified bee venom phospholipase $A_2$ after phenylglyoxal treatment

50 µl of 1 mg/ml solutions of native phospholipase $A_2$ and native treated with phenylglyoxal were mixed with 2:1 v:v of water containing 50% glycerol with neutral red marker dye and to 22.5% propionic acid/urea polyacrylamide gel.

Channel 1, 20 µl, native $PLA_2$.

Channels 2 and 3, 20, 10 µl of native $PLA_2$ + phenylglyoxal respectively.


1
2
3

**The effect of phenylglyoxal on bee venom $PLA_2$ activation.**

The effect of phenylglyoxal on the activation of bee venom phospholipase $A_2$ was tested using rabbit red blood cells in the presence of 10 μM bovine serum albumin. Incubation of the native enzyme with phenylglyoxal greatly reduced the degree of activation by oleoyl imidazolide seen in the erythrocyte leakage assay, however, treatment of the fully activated enzyme with this reagent resulted in 60 % decrease in the enzyme activation **(fig. 35)**.

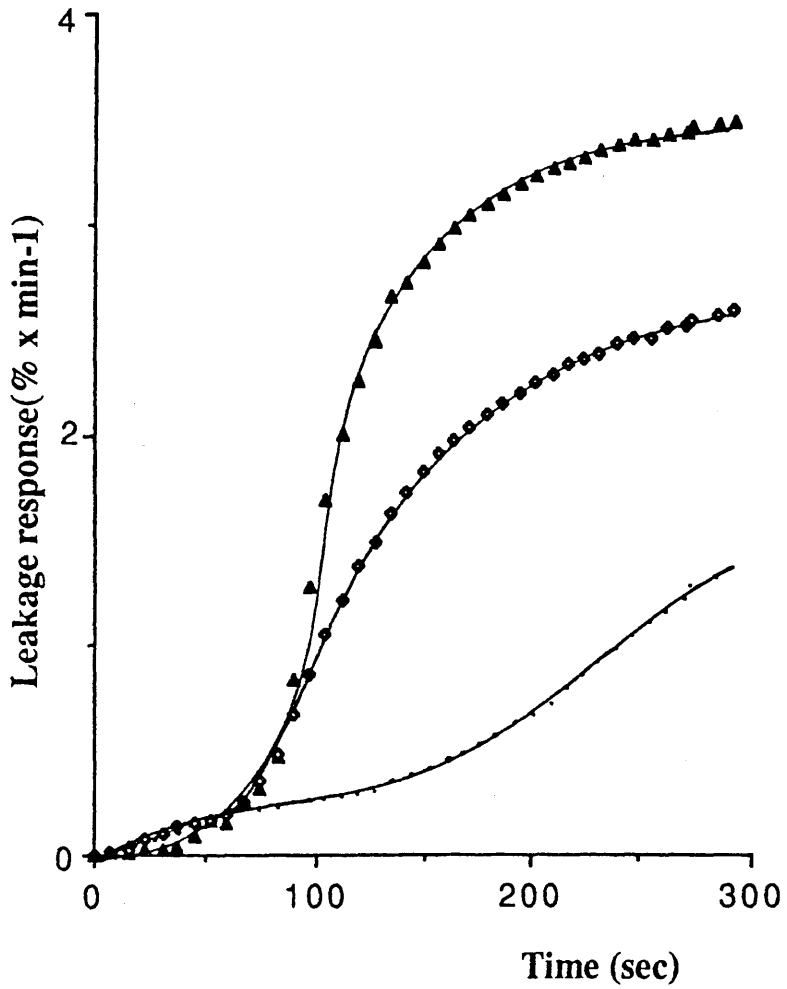
These results showed that the treated enzyme can possess almost full catalytic activity, but show only ca. 20 % of its degree of activation. Even when the control reduction in activation for activated enzyme treated with phenylglyoxal is taken into account, this relative loss of activation appears to be significant.

In part these results could be attributed to modification of the N-terminal amino group, but some of the observations do not correspond to anything seen with the acylating reagents (acetic anhydride etc) and therefore must be a consequence of the modification of arginine residues.

**3. Tyrosine modification.**

Nitration of **Y** residue(s) in the native bee venom phospholipase $A_2$ with 25 mM tetranitromethane (TNM) caused a rapid 50 % increase in

Fig.(35) The effect of phenylglyoxal on the activation of bee venom PLA2 before and after the addition of the oleoyl imidazolide.




100 µl of 1mg/ml solutions of native bee venom phospholipase $A_2$ were incubated with 2% oleoyl imidazolide and 50 mM phenylglyoxal. The activity was measured using rabbit erythrocyte leakage assay. ▲—▲ Native $PLA_2$ + Oleoyl imidazolide; o—o Native $PLA_2$ + Oleoyl imidazolide + phenylglyoxal; ·····• Native $PLA_2$ + phenylglyoxal + Oleoyl imidazolide.

the enzyme activity towards short-chain phospholipid substrate ($DiC_8PC$ ). This modified enzyme was strongly affected by the addition of oleoyl imidazolide, which caused inhibition rather than enhancement of activity **(fig.36a)**.

Treatment of activated bee venom phospholipase $A_2$ with this reagent partially inhibited both the catalytic activity and the activation shown in the standard rat or rabbit red blood cell leakage assays., **(fig.36b)**. This was the exact opposite of the behaviour found for the native enzyme.

**4. Histidine modification.**

*p*-Bromophenacyl bromide was reported to inactivate phospholipase $A_2$ enzymes by occupying the **H** residue at the active site (Renetseder et al., 1988). The following experiments were designed to study any possible involvement of either this or other **H** residues in the putative activation site.

**Protection against *p*-bromophenacyl bromide inactivation by acylation.**

Using the standard short-chain substrate assay for phospholipase $A_2$ activity, 100 mM *p*-bromophenacyl bromide (p-BPB) was found to inactivate the native enzyme at a convenient rate. Time courses of inactivation were obtained for both native and acylated/activated enzyme and interestingly both showed an increase of about 20 % in the activity within the first 5 minutes. After this time the activity of

Fig.(36)a

100 µl of 1mg/ml solution of purified native bee venom $PLA_2$ was incubated with 50µg tetranitromethane(TNM) and than treated with 2% oleoyl imidazolide and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5 µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100µM o-phenanthroline.

- Native $PLA_2$.
- Native $PLA_2$+ tetranitromethane(TNM).
- Native $PLA_2$+ TNM + oleoyl imidazolide.

Fig.(36)b

100 µl of 1mg/ml solution of fully activated bee venom $PLA_2$ was incubated with 50µg tetranitromethane(TNM) and activity measured in the rabbit erythrocyte leakage assay.

- Activated $PLA_2$.
- Activated $PLA_2$ + TNM.

Fig.(36)a. The effect of tetranitromethane(TNM)on the activity and activation of native bee venom PLA2.

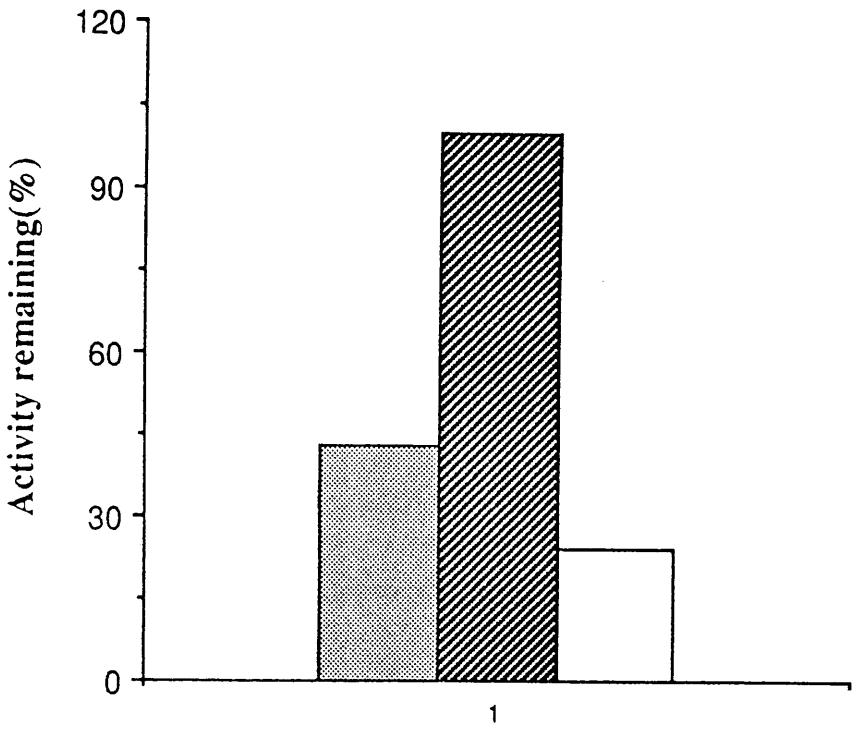
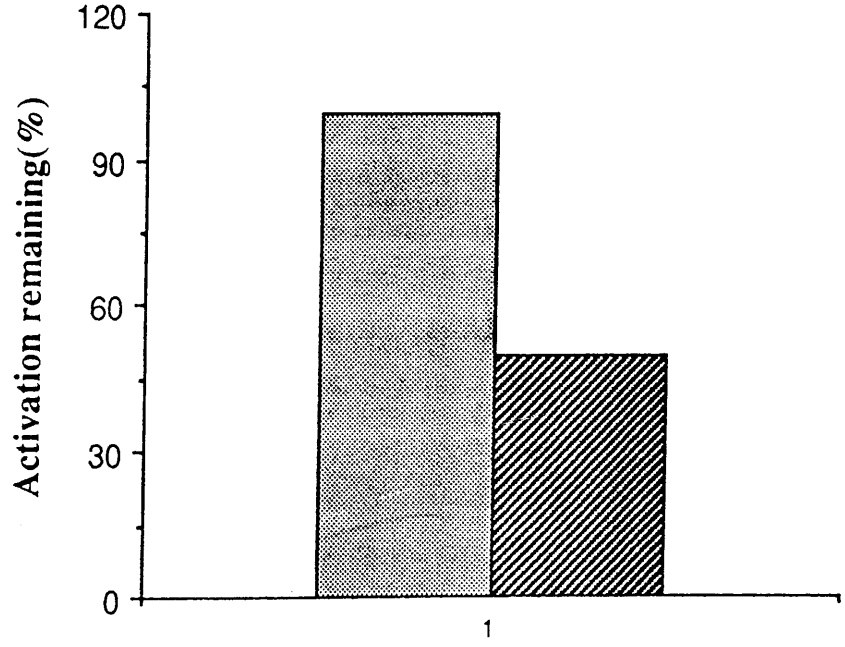



Fig.(36)b. The effect of tetranitromethane (TNM) on the activated bee venom phospholipase A2.



the native enzyme decreased at a rather faster rate than that of the activated enzyme. After 90 minutes incubation the activity of the native enzyme was abolished completely whereas the acylated enzyme retained about 65 % of its initial activity. These results were in qualitative agreement with previous data (Lyall, 1984), but the degree of protection of the activated enzyme was considerably lower in the present case. It seemed likely that this was due to the fact that a lower relative concentration of activator was used in the present case. Excess activator should hydrolyse to produce free fatty acid which might confer protection by binding at the active site (as postulated above). To test this possibility the enzyme was incubated with excess oleoyl imidazolide (2%) and after two hours of incubation, the fully activated enzyme was ethanol precipitated to remove any excess of free fatty acid or imidazolide before preincubation with *p*-bromophenacyl bromide.

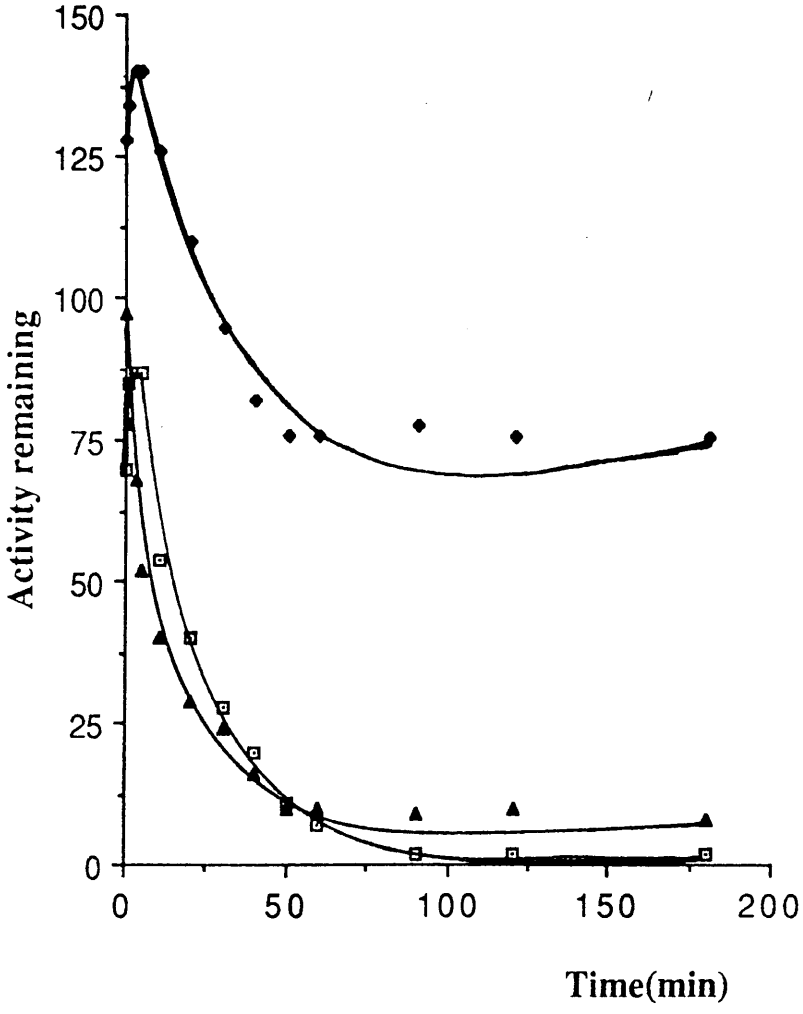
**Figure 37,** shows that the degree of inhibition for the precipitated enzyme was very similar to the native enzyme and that no protection was observed under these conditions.

**Protection against *p*-BPB. inactivation by free fatty acids.**

The incubation of bee venom phospholipase $A_2$ with 2% oleic acid for five hours gave very high protection against inhibition by *p*-bromophenacyl bromide (p-BPB) **(fig. 38a).**

These results confirmed that protection against inactivation by *p*-

Fig.(37) The prior effect of ethanol precipitation on the inhibition of native activated bee venom PLA2 with p-bromophenacyl bromide.




100 µl of 1mg/ml solutions of native $PLA_2$ and $PLA_2$ treated with 0.2% oleoyl imidazolide were incubated with 1.08 mg of p-bromophenacyl bromide before and after ethanol precipitation and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100µM o-phenanthroline. ▲——▲ Native $PLA_2$ + p-bromophenacyl bromide; ●——● Activated $PLA_2$ + p-bromophenacyl bromide; ▣——▣ Activated $PLA_2$ + ethanol precipitation + p-bromophenacyl bromide.

bromophenacyl bromide was entirely due to the free fatty acid. It seemed very unlikely that free fatty acid would protect by binding at the activation site if the bound acyl residue did not do so. Hence the free fatty acid probably acts as a product inhibitor, preventing access of the reagent to the true active site.

When the protection experiment was repeated using the standard rabbit erythrocyte leakage assay as a measure of activity, a very surprising result was obtained. The enzyme showed a progressive increase in its leakage-generating activity, reminiscent of activation by acyl imidazolides, **(fig.38b)**. A possible explanation of this result was that chemical reaction between p-bromophenacyl bromide and the fatty acid generated a weak long-chain acylating agent capable of modifying the activation site directly. This was tested by preincubating the reagents together before adding them to the enzyme, but this had no effect in either assay. An alternative hypothesis is that p-bromophenacyl bromide is itself capable of acylating the activation site, but that activation can only be seen if the active site is left unmodified. Thus the role of the free fatty acid here would be to protect the active site.

### 3.3.6 Activation of snake venom $PLA_2$ enzymes.

Drainas (1978), Lyall (1984) and Elansari (1986) had already shown that the activation of venom phospholipase $A_2$ by oleoyl imidazolide is a widespread phenomenon. There was, however, an indication that

Fig.(38)a

100 µl of 1mg/ml solutions of native and activated bee venom phospholipase $A_2$ with 2 % oleoyl imidazolide were incubated with 1.08 mg of p-bromophenacyl bromide and assayed by conductimetric estimation of the hydrolysis of dioctanoyl phosphatidyl choline (30mg/ml) by 0.5µl of enzyme in 2ml of 10mM triethanolamine/$Cl^-$ buffer pH 8.0 in the presence of 100µM o-phenanthroline.

▲——▲ Native $PLA_2$ + p-bromophenacyl bromide.

●——● Native $PLA_2$ + oleic acid + p-bromophenacyl bromide.

Fig.(38)b

1µg sample of the enzyme treated with oleic acid and the one treated with oleic acid and than incubated with p-bromophenacyl bromide(p-BPB) were assayed for activity using rabbit erythrocyte leakage assay.

□——□ Native $PLA_2$ + oleic acid.

◆——◆ Native $PLA_2$ + oleic acid + p-BPB

Fig.(38)a Oleic acid protection of native bee venom PLA2 from the inhibition by p-bromophenacyl bromide.

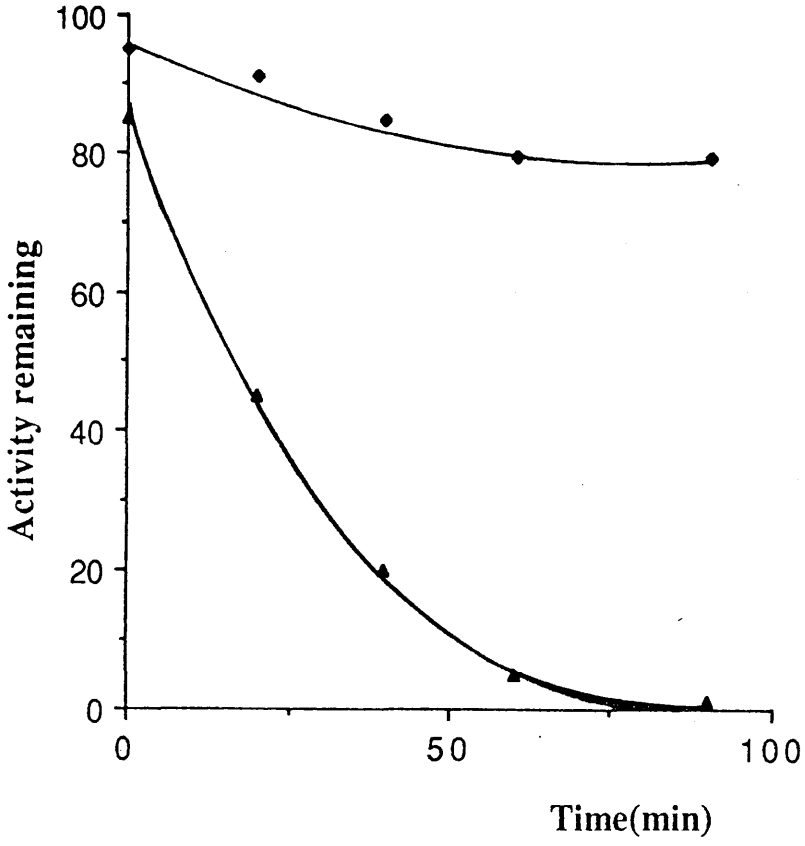
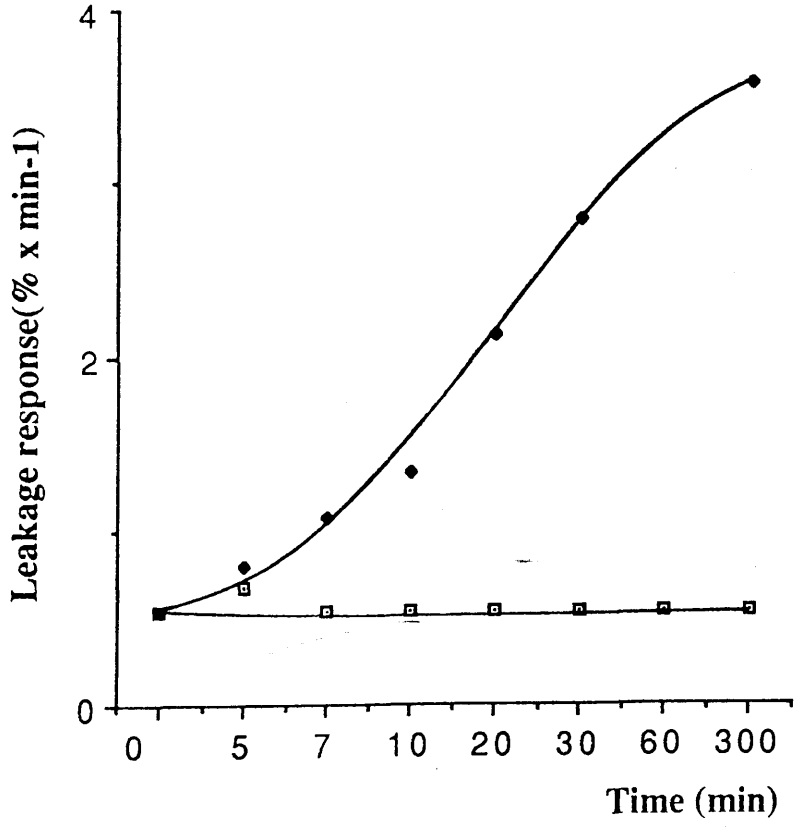



Fig.(38)b The activation of native bee venom PLA2 by p-bromophenacyl bromide in the presence of oleic acid .

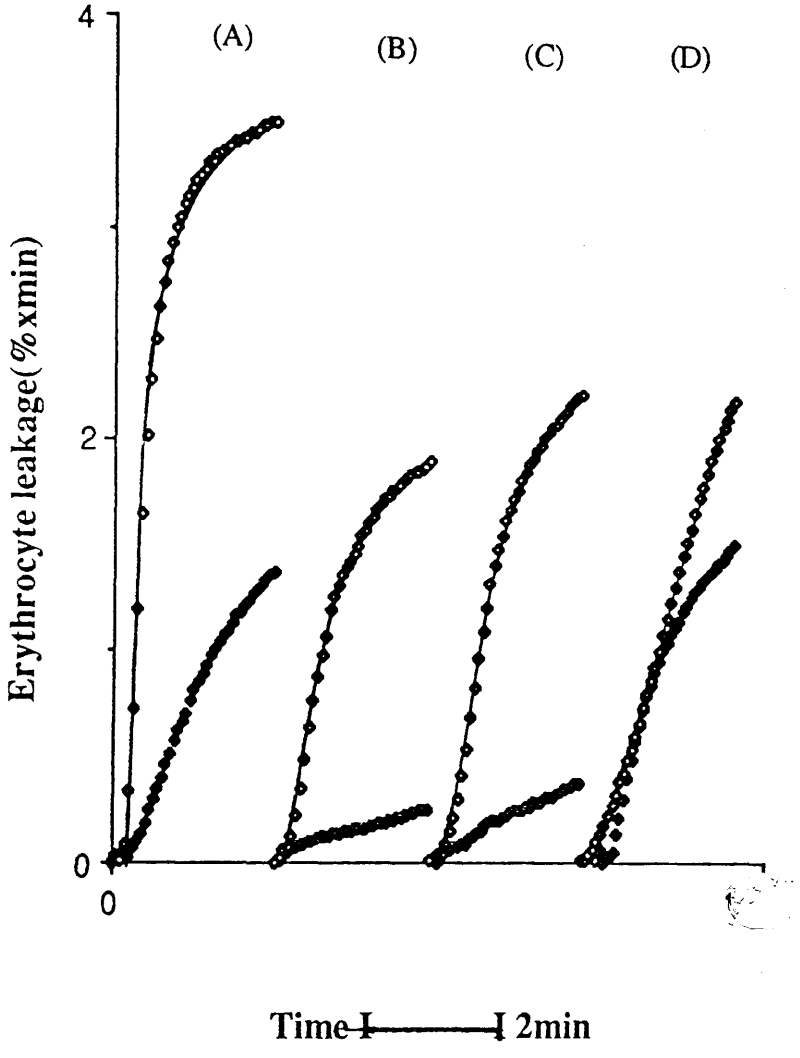


some of these enzymes, perhaps the majority, were insensitive to the activator. The following studies were undertaken to find out if the sensitive enzymes showed any common structural features and to see if any of them possessed useful features to enable the mechanism of activation to be elucidated.

Only a few of these enzymes were readily available in pure form. These included the three isoforms of phospholipase $A_2$ from *Naja mossambica mossambica* (CMI, CMII, CMIII) and the enzyme from *Naja naja* venom. Treatment of these enzyme with oleoyl imidazolide under the conditions used for activating the bee venom enzyme gave the results shown in **(fig. 39)**. The two least basic isoforms (CMI and CMII) of phospholipase $A_2$ from *Naja mossambica mossambica* and phospholipase $A_2$ from *Naja naja* venom showed very similar activation to bee venom phospholipase $A_2$ whereas the highly basic, toxic, isoform (CMIII) phospholipase $A_2$ from *Naja mossambica mossambica* was partially inhibited by this treatment. This was especially interesting because the toxic isoform contained three more lysine residues than the other forms and only this most basic form showed any detectable modification on acid/urea gel electrophoresis, **(fig. 40)**. This apparently stable modification, which did not produce activation, may well be an example of acylation of a lysine residue.

The ratio of the lytic and catalytic activities could be used as a simple measure of relative lytic potencies. By this test, the non-

Fig(39) The activation of different form of naja mossambica mossambica PLA2 and naja naja venom PLA2 by oleoyl imidazolide.




100 µl of phospholipase $A_2$ enzyme was incubated with oleoyl imidazolide for two hours at 37°C and activity measured using rabbit erythrocyte leakage assay

A,Non-toxic isoform of phospholipase $A_2$ (CMII) from *Naja mossambica mossambica* (pI 8.8)

B, Acidic isoform of phospholipase $A_2$ (CMI) from *Naja mossambica mossambica*

C,Phospholipase $A_2$ from *Naja naja* venom

D, Toxic isoform of phospholipase $A_2$ (CMIII) from *Naja mossambica mossambica* (pI 9.7)

toxic, isoform of *Naja mossambica mossambica* phospholipase $A_2$ (CMII) was seen to be a far more lytic enzyme than the bee venom phospholipase $A_2$. Nevertheless, it showed the same preference for cell type as the bee venom enzyme and gave similar response to the activation by oleoyl imidazolide. Comparison of.the time courses of activation of this enzyme with that of the bee venom enzyme showed that *Naja mossambica mossambica* phospholipase $A_2$ (CMII) activated at a rate more than 20 fold greater than the bee venom phospholipase $A_2$ enzyme, **(fig. 41)**, (it should be noted that in order to obtain measurable rates, *Naja mossambica mossambica* phospholipase $A_2$ (CMII) and oleoyl imidazolide were both diluted 2 fold with respect to the concentrations used for the bee venom). The results obtained by the activation of this isoform of the *Naja mossambica mossambica* phospholipase $A_2$ (CMII) by different saturated fatty acyl derivatives were very similar to that obtained with bee venom enzyme.

Other phospholipase $A_2$ enzymes such as phospholipase $A_2$ from *Naja naja atra*; porcine or bovine pancreas; *C. adamanteus* and the active (notoxin II-5) as well as the non-active (notoxin II-1) forms from the Australian tiger snake venom *Notechis scutatus scutatus* were also treated with oleoyl imidazolide activator and assayed for both activity and activation by the standard procedures. The results showed that all of these enzymes were highly active towards the short chain substrate and either only weakly active, or inactive in the leakage assay. Only notoxin II-5 from the crude venom of the

Fig.(40) Gel electrophoresis of native and activated phospholipase $A_2$ enzymes from different sources.

Channels 1 and 2, native and activated bee venom $PLA_2$

Channels 3 and 4, native and activated $PLA_2$ from the non-toxic isoform (CMII) of *Naja mossambica mossambica* (pI 8.8)

Channels 5 and 6, native and activated $PLA_2$ from the toxic isoform (CMII) of *Naja mossambica mossambica* (pI 9.7)

Channels 7 and 8, native and activated $PLA_2$ from *Naja naja* venom.

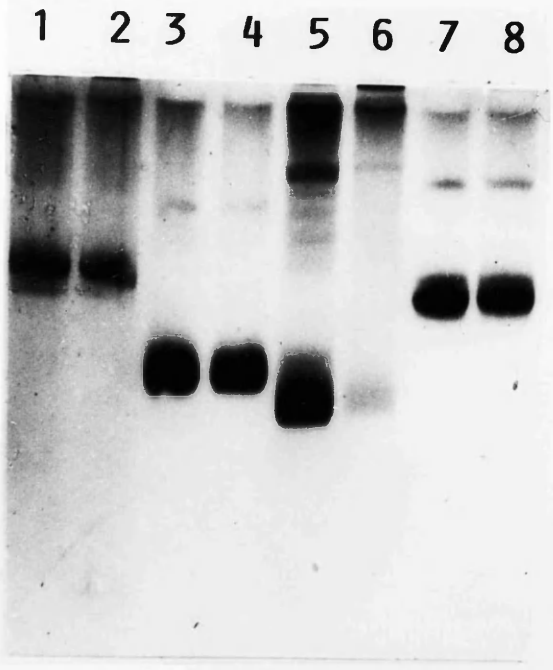
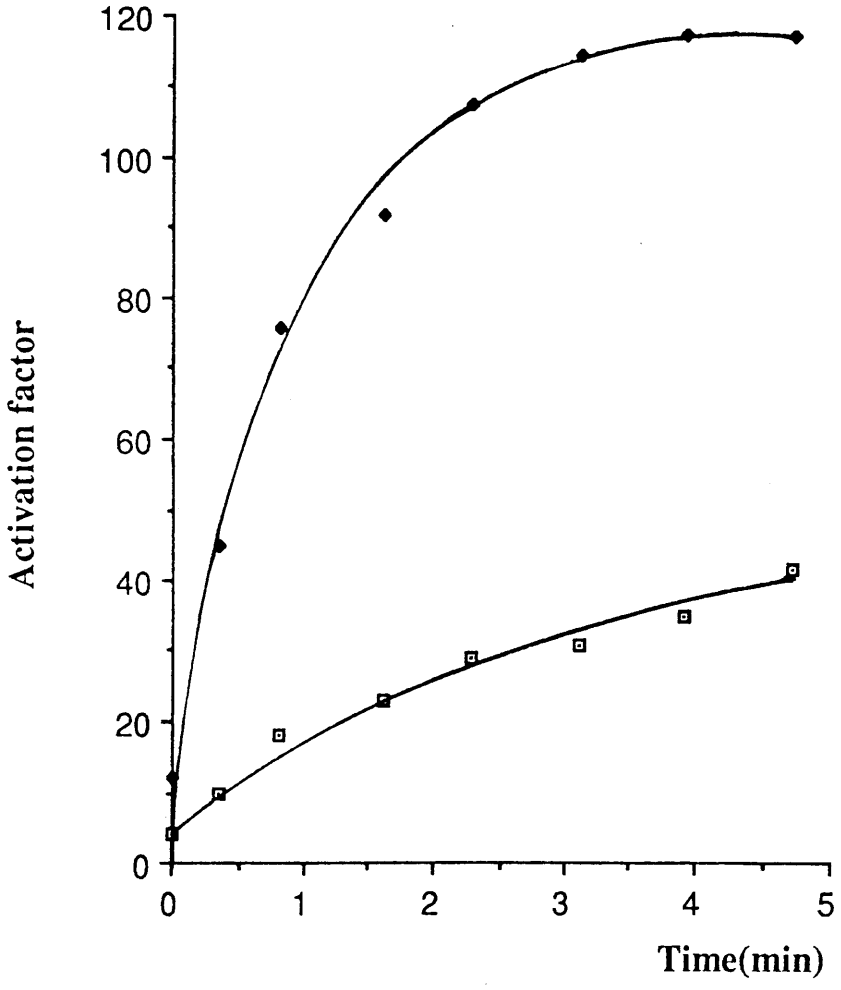

1 2 3 4 5 6 7 8

Fig. (41) The time course of activation of snake and bee venom PLA2 by oleoyl imidazolide.




Solutions of pure phospholipase $A_2$ enzymes were treated with oleoyl imidazolide, samples withdrawn at timed intervals and activity measured in the rabbit erythrocyte leakage assay.

●——● Non-toxic pI 8.8 isoform of $PLA_2$ from *Naja mossambica mossambica* at (20 µg in 100µl ) was treated with 0.4 µg of oleoyl imidazolide.

□——□ Bee venom (100µg in 100µl) treated with 2 µg of oleoyl imidazolide.

Australian tiger snake *Notechis scutatus scutatus* enzyme showed detectable activation in the erythrocytes leakage assay, **(Table 4).**

Table (4), The effect of oleoyl imidazolide on the a former group of highly homologue phospholipase $A_2$ enzymes from different sources.

| Enzyme source | short-chain substrate hydrolysis assay | rabbit erythrocytes leakage assay |
|---|---|---|
| *Naja naja atra* $PLA_2$ | very high activity | no activation |
| Pancreatic $PLA_2$ (Porcine and bovine) | low activity | no activation |
| *C. adamanteus* $PLA_2$ | high activity | no activation |
| *Notechis scutatus scutatus* II-5 | high activity | high activation |
| *Notechis scutatus scutatus* II-1 | no activity | no activation |

100 µl of 1 mg/ml protein was incubated overnight with 2 µl of 2% oleoyl imidazolide. Samples were withdrawn and assayed for activity using dioctanoyl phosphatidyl choline assay and activation using rat or rabbit erythrocytes leakage assay.

# Chapter Four
# Discussion

## 4.1 Kinetic analysis of bee venom $PLA_2$ activity.

Phospholipase $A_2$ was shown to be activated towards the hydrolysis of phospholipid substrates by calcium and to be inhibited by heavy metal ions (zinc or copper). However the nature of the enzyme/substrate interface is poorly understood and cannot be treated by the simple kinetic analyses used for water soluble enzymes. Attempts to simplify this using water soluble substrates have been only partially successful (Wells, 1972). Undoubtedly where short chain substrates, which are monomeric in aqueous solutions, have been used, these difficulties have been largely overcome. Most of the work, including that given here, has used the more sensitive condensed substrates where the interpretations of results are quite complex.

A detailed kinetic analysis of phospholipase $A_2$ enzymes was reported by (de Haas et al., 1971) for pancreatic phospholipase $A_2$ using different short chain substrates and by (Wells, 1972) for *Crotalus adamanteus* phospholipase $A_2$ using the short-chain substrate (dibutyryl-lecithin). The kinetic data obtained by both groups was consistent with the reaction sequence in which the calcium ion freely dissociated from the enzyme, but the phospholipid substrate bound to the enzyme/calcium complex only, thus giving the obligatory addition order in which calcium binding preceded substrate binding. Similar results were reported very recently by (Tsai et al., 1985) on the study of the metal-binding properties of bee

venom phospholipase $A_2$ using 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, showing that the binding of calcium to the enzyme caused a conformational change in the enzyme to the active form in which the Pro-S oxygen of the phosphate group of the substrate interacts with the enzyme bound calcium ion and oriented the substrate in the correct conformation.

The results of the present work support the simple mechanism for the kinetic equation which can be represented as

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EAB \xrightarrow{k_5} E + A + P \quad \text{.....(1)}$$

the total concentration of the enzyme is

$$E_t=[E]+[EA]+[EAB] \quad \text{.....(2)}$$

resolving this equation

$$E_t=[EAB]\{1+(k_4+k_5/k_1[A])+(k_4+k_5/k_3[B])+(k_4+k_5/k_3[B])(1+k_2/k_1[A])\} \quad \text{.....(3)}$$

the rate is $k_5[EAB]$ or

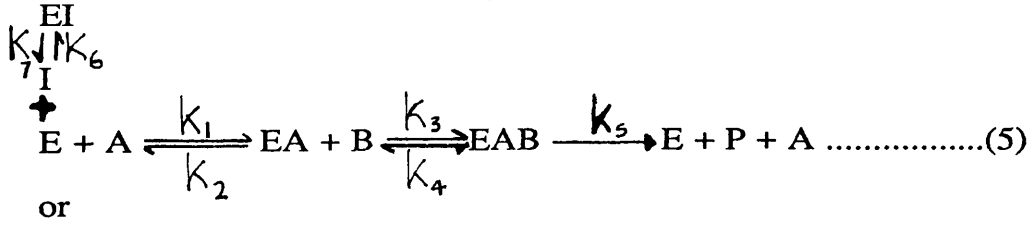
$$E_t=[v/k_5]\{1+(k_4+k_5/k_1[A])+(k_4+k_5/k_3[B])+(k_4+k_5/k_3[B])(1+k_2/k_1[A])\} \quad \text{.....(4)}$$

An equation of exactly the same form is obtained if the addition order of the reagents is reversed as can be seen from inspection of (4) addition of [A] to the complex [EB] (the only change obtained was in changing the constant $k_1$ with the constant $k_3$), this means that the

addition order could not be established by kinetic analysis of the enzyme in the presence of calcium and substrate alone. It is clear from the equation (4) that both $V_{max}$ and $K_m$ were affected by the addition of the moderators [A] and this is in total disagreement with the result presented by Wells, (1972) where he showed that at different calcium concentration the $K_m$ was changed as a function of dibutyryllecithin concentration at various fixed level of calcium, but there is no change in the initial velocity, whereas, at different calcium concentration at various fixed level of dibutyryllecithin the initial velocity was changed but no change observed in the $K_m$.

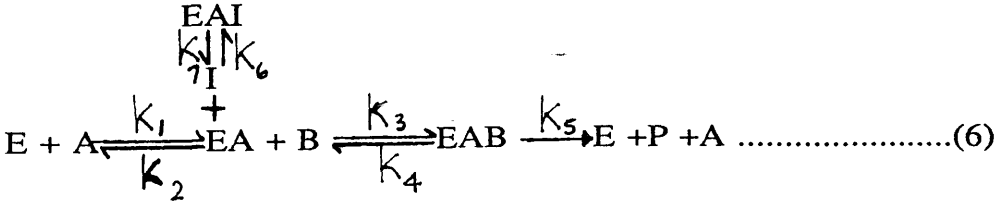
In the presence of the heavy metal the equation (1) can be represented in the following forms

1- if the heavy metal effect is on the enzyme the equation can be represented as;




or

2- if the heavy metal effect is on the complex [EA] the equation can be represented as;



from the equation (5) the total concentration of the enzyme can be represented as

$$Et = [E] + [EA] + [EAB] + [EI] \quad \text{.....(7)}$$

from (7):

$$E_t=[v/k_5]\{1+(k_4+k_5/k_1[A]) + (k_4+k_5/k_3[B]) +(k_4+k_5/k_3[B])(1+k_2/k_1[A]) + (k_4+k_5/k_3[B])(1+k_2/k_1[A])(k_6/k_7)([I]) + (k_4+k_5/k_1[A])(k_6/k_7)([I])\} \quad \text{.....(8)}$$

and from the equation (6) the total concentration of the enzyme will be represented as;

$$E_t = [E] + [EA] + [EAB] + [EAI] \quad \text{.....(9)}$$

therefore by resolving the equation (9)

$$E_t=[v/k_5]\{1+(k_4+k_5/k_1[A]) + (k_4+k_5/k_3[B]) + (k_4+k_5/k_3[B])(1+k_2/k_1[A]) + (k_6/k_7)([I])\} \quad \text{.....(10).}$$

result from the equation (8) indicate that both $K_m$ and the $V_{max}$ were affected by the addition of the heavy metal whereas the result from the equation (10) indicate that only $V_{max}$ was affected in presence of the heavy metal ion inhibitor; results presented in this work support the equation (8) which suggest that in the presence of the heavy metal ion inhibitor both the $K_m$ and the $V_{max}$ are effected.

## 4.2 Metal ion binding to bee venom $PLA_2$

The amino acid residues of pancreatic phospholipase $A_2$ which interact with calcium have been well characterized using X-ray crystallographic studies (Dijkstra et al.,1981). In addition a second low affinity binding site for calcium has also been characterized at alkaline pH in enzyme with an **E** residue at the position **71**, this site was reported to be involved in increasing the affinity of the enzyme for the lipid-water interfaces (Slotboom et al., 1983; Donne-Op den Kelder et al., 1983 and Drakenberg et al., 1984).

From the structure of bee venom phospholipase $A_2$ presented by (Shipolini et al., 1974) it appears that this enzyme has no **E** residue at the position **71** but it has been suggested that this enzyme may have two calcium-binding sites (Drainas, 1978). Tsai et al., (1985) proposed that a tyrosine residue on the protein is more likely to be involved in the calcium binding because of the shift of the peak at 287 nm which they assumed is consistent with an aromatic residue. Elansari, (1986) reported that this enzyme has two binding sites, one with high affinity and another with low affinity, but did not discuss the role of these two sites in modulating the activity of the enzyme.

The interaction of calcium with bee venom phospholipase $A_2$ has not been well characterized. However the present studies using the conductimetric assay system showed that calcium-binding of bee venom phospholipase $A_2$ is more complex than was proposed previously. The concentration of calcium required for half-maximum

activity is much lower than found by (Shipolini et al., 1971). It is of interest that many phospholipase $A_2$ enzymes show similar affinity for calcium, although there are others which have much lower affinity, resembling that originally proposed for the bee venom enzyme. It is possible that values obtained in the absence of transition metal chelators may generally be in error and it is clear that valid results can only be obtained where the concentrations of all divalent cations can be specified. This work shows that trinitriloacetic acid has an ideal calcium buffering range for work with this enzyme. It allowed an unambiguous determination of the calcium dissociation constant of ca. 5 μM which is rather less than the concentration of calcium found in our distilled water. In contrast, the buffering range for EDTA/EGTA is much too low to be useful in this system. These results are in marked contrast with those obtained by other workers who have consistently given much higher values (Shipolini et al., 1971; Tsai et al., 1985).

The role of calcium in the catalytic mechanism of bee venom phospholipase $A_2$ has not been thoroughly elucidated although there is no reason to suppose it would differ greatly among phospholipase $A_2$ types. The results presented in this work indicate that the enzyme has two different calcium-binding sites, one of which can also bind transition metal ions. The calcium ion acts as an activator and serves two different functional mechanisms for the protein, first it is involved in the catalytic activity ($V_{max}$) and, secondly it is responsible for increasing the affinity of the enzyme for the

substrate. In contrast, the transition metal ion acts as an inhibitor which decreases the affinity of the enzyme for the substrate, but has little effect on $V_{max}$

The results obtained from the addition of the chelators (o-phenanthroline; penicilamine or cyclam) to the assay mixtures showed a clear change in the shape of the hydrolysis curve of the short-chain substrate ($DiC_8PC$) by bee venom phospholipase $A_2$ , which became much more highly linear. This effect could not be obtained by increasing the calcium ion concentration in the absence of the transition metal ion chelator, suggesting that the putative metal ion was not a simple competitor for the calcium site. On the other hand, the addition of the chelator to the assay mixtures did not change the initial rate, suggesting that it had very little effect on the $V_{max}$ term, whilst calcium had its major effect on this term. These results strongly suggest that the heavy metal does not compete with the calcium for a single binding site

Although the phospholipase $A_2$ from bee venom has quite different amino acid sequence from phospholipase $A_2$ from other sources (Shipiloni et al., 1974; Slotboom, 1982) the mechanistic features of the metal binding described in this work may be applicable to phospholipase $A_2$ from other sources because calcium is commonly required by phospholipase $A_2$. The enzymes from bee venom, *Naja mossambica mossambica, Naja naja* venom, the ß-subunit from the venom of the South American rattlesnake *Crotalus durrissus terrificus*, notoxin (II5) of the crude venom of the Australian tiger

snake *Notechis scutatus scutatus* and bovine pancreas all show a very similar response to the treatment with the chelator (o-phenanthroline).

### 4.3 The activation mechanism of venom $PLA_2$. enzymes by fatty acyl derivatives.

The proposal by Drainas et al. (1981) that incubation of some venom phospholipase $A_2$ enzymes with long-chain fatty acyl derivatives results in a time dependent incorporation of the acyl residues into the enzyme, has been reexamined in this work. Direct activation of phospholipase $A_2$ by oleoyl imidazolide had been demonstrated for the enzyme from bee venom using the red blood cell/albumin assay. This assay was used in this work as the primary tool for further investigations and revealed many interesting features that should be pursued further, which are discussed below.

It has been pointed out (Drainas et al.,1981) that albumin may act by removing the lysophospholipid which is shown to be a potent inhibitor to phospholipase $A_2$ in this system. It also removes free fatty acids which are potent stimulators of phospholipase $A_2$ mediated leakage in rabbit, dog and guinea pig red blood cells, but not in rat and mouse red blood cells. Thus on balance the effect of lysophosphatides must outweigh that of the fatty acids. An additional phenomenon, electrolyte leakage associated with the extraction of free fatty acids from erythrocytes had been discussed by Drainas et

al.,1981), but the contribution of this effect towards the leakage response to phospholipase $A_2$ was thought to be negligeable. However the present work shows that this extraction process sensitised the cells to phospholipase $A_2$ action. It is therefore possible that the sensitisation forms part of the process whereby albumin synergises the action of phospholipase $A_2$ enzymes. As such it should be a self-stimulating process which might account for the biophasic nature of the leakage curves seen whenever albumin is present.

The present work showed that red blood cells from different animals had differing responses to albumin and fatty acid synergism of phospholipase $A_2$, but comparison of their phospholipids compositions showed no obvious structural basis for this (White, 1973). Moreover all these cells showed the same relative response to different phospholipases and, in so far as these studies has been carried out, to the activation of these enzymes.

Bee venom phospholipase $A_2$ has high activity towards short-chain phospholipid substrates which form micelles and very low activity towards long-chain phospholipids substrates which form bilayers. All of the studies of activated enzyme showed that the effect was very much greater for the bilayer substrates than for the micellar or monomeric substrates.

This can be interpreted by two different mechanisms. One, favored by Verger & de Haas (1976) is that activation increases the

interaction of the enzyme with the bilayer type surface by increasing the hydrophobicity of the enzyme. Kinetically this mechanism might be expected to affect the substrate binding term ($K_m$) rather than the catalytic term ($V_{max}$). The alternative mechanism is that activation changes the protein conformation which could act either on $V_{max}$ or on $K_m$. Drainas (1978), carried out a kinetic study of activated and native bee venom phospholipase $A_2$ and showed that activation was determined by the $V_{max}$ term and not by increased affinity of the enzyme for the substrate surface.

Van der Weile et al.(1988a,b), who favour the concept of interfacial modulation, have shown that pancreatic phospholipase $A_2$ can be made to attack erythrocytes by binding long-chain fatty acyl residues to lysine groups. This was achieved by modification after specific blocking steps and did not rely on the intrinsic reactivity of these groups towards the reagent as in the present case. Their evidence strongly supports the idea that the acyl groups interact directly with the substrate surface but in the present case no such supporting evidence has been obtained.

Covalent incorporation of a tritium labelled ($^3H$) myristoyl or oleoyl residue into bee venom phospholipase $A_2$, by incubation with the appropriate imidazolide derivatives was apparently demonstrated by its stability towards extraction with organic solvents, resistance to dissociation by albumin and to alkali and mildly acidic pH. However the dissociation of this acyl residue on acid / urea / polyacrylamide

gel electrophoresis and under acidic denaturing conditions cast some doubt on this interpretation. It was in any case clear that the bond type formed was not a stable one and therefore probably not of a kind described elsewhere in protein modification studies.

Because the bond is so labile in denatured enzyme, it now seems even more probable that the acyl group is covalently bound to an amino acid residue deeply buried in the enzyme, consistent with the original model that acylation activated the enzyme by producing a conformation change. The effect of this change would then be to make the enzyme more specific towards phospholipids in the bilayer forms as compared with the micelle or monomeric forms. Camero-Diaz et al., (1985) argued that the conformation of the enzyme was induced by the substrate surface and that the bilayer favoured a low activity state. Only when the enzyme was stabilised in the high activity state by the activator it was able to overcome this effect. This conclusion was based largely on results obtained by Lawrence et al., (1975) in study of the activation of the enzyme by glutaraldehyde in the presence of free fatty acid and extended by the work of Camero-Diaz et al., (1985) where fixation was shown to preferentially inhibit the high activity state shown towards micellar substrates and towards long-chain phospholipids in the presence of activating levels of fatty acids, but not in their absence.

## 4.4 Phospholipase $A_2$ enzymes activation by short-chain fatty acyl derivatives.

The reaction of phospholipase $A_2$ with short-chain acyl imidazolides which give relatively low activation factors appears to be slightly faster than its reaction with the long-chain compounds, which give maximal activation. Although the possibility was considered that the low level of activation was due to hydrolysis of the reagent, this appeared not to be the case. Subsequent treatment of enzyme weakly activated by a short-chain derivative with a long-chain derivative revealed that the site was fully modified by the short chain reagent after the short incubation period. Therefore the short chain derivatives appear to bind very rapidly at the activation site but do not produce the full activation response. This could be interpreted in two ways; either the chain is not bulky enough to produce the required conformation change in the protein or else it is not long enough to reach the lipid surface as the hydrophobic anchor model might require.

The most interesting feature found in my work is that despite the fact that reactivity of the longer compounds falls with increasing chain length, very short chain imidazolides do not block the activation site. This points to a critical role for methylene residues in the $C_5$-$C_7$ positions in determining binding of the activator to the enzyme before reaction takes place. The results of these studies also showed that excess of any of these compounds, especially the short

chain derivatives produced progressive inhibition of the enzyme, suggesting that they might bind non-specifically to other residues in the protein.

Activation of bee venom phospholipase $A_2$ also increased the stability of the enzyme towards inactivation by thiol and protease degradation. This protection correlated with the degree of acylation up to the addition of one acyl residue per protein molecule. Similar results were obtained by Silomiany et al. (1984-1985) with the acylated gastric mucus glycoprotein which protects the gastric mucosa from chemical and mechanical damage. However there did seem to be a contribution towards protection by the free fatty acids which had not been observed in the earlier studies and which could be due to direct binding of the fatty acid at the true active site.

Protection of enzymatic activity from an external inactivating reagent by substrate, products or an activator is generally regarded as evidence that the target residues for that reagent residue within the active site. However it is possible that binding of these compounds to an allosteric site causes a conformational change which renders a target residue outside the active site less reactive. In these studies the protection of activated bee venom phospholipase $A_2$ against proteases and thiols was strong evidence for a conformational change in the protein. In contrast the protection of the enzyme against inactivation by *p*-bromophenacyl bromide by activation was shown to be almost entirely due to the presence of an ethanol extractable

component, probably free fatty acid released by, or present in, the activator solution. This protection therefore appear to be a direct consequence of the fatty acid blocking the active site. One of the most interesting of the results came from this study, namely that the combination of free fatty acid and *p*-bromophenacyl bromide activated the enzyme with the progressive character of a chemical reaction. One interpretation for this is that *p*-bromophenacyl activates the enzyme directly by reaction at the activation site, but this can only be detected if the enzyme is protected against simultaneous inactivation by the reagent. The results obtained here show that free fatty acid would provide such protection.

### 4.5 The allosteric binding site of phospholipase $A_2$.

Up till now there has been very little information available concerning the nature of the amino acid residues essential for the chemical reaction of activator molecules with the protein. **K**, **H** and **Y** have been identified as being the most reactive groups in proteins other than sulphahydryl residues, (which are not present in phospholipase $A_2$ enzymes). The fact that acyl imidazolides were the most selective activators gave some reason to belive that a **Y** might be the target residue, but it is now clear that these reagents modify **Y**, **K** (and the N-terminal amino-group) and **H** residues. Lyall, (1984) showed that the reaction rate was controlled by a pKa of 6.5 indicating that a **H** residue might be involved, although the N-terminal group or an anomalous glutamate residue could easily

display such a pKa value.

Studies of the modification of phospholipase $A_2$ enzymes by different chemical reagents indicated that **K**, **H**, **R**, **Y** and the N-terminal residues were involved in the reaction. This hypothesis is supported by the results obtained from the treatment of the enzyme with dimethyl maleic anhydride which acts as a readily removable blocking agent for the modification of lysyl groups, tyrosyl groups and the N-terminal amino-group. It appeared that at least one free primary amino group was essential for the acylation to occur. The work with the snake venom enzyme indicated that the amino group required was not the most reactive residue towards acetic anhydride, indicating that it was not the N-terminal residue

It is clear from **figure 32a-b** that the primary amino groups, which react readily with anhydrides are important for the catalytic activity of the enzyme. However it is hard to determine whether or not these residues are also involved in activation, because the loss in the enzyme catalytic activity is proportional with the loss of its activation (**fig. 28**). The critical test was to see if the enzyme could bind functional activator after being inactivated by a reversible lysine blocking agent. This was observed to be the case although the inactivated enzyme did not achieve full activation by this means.

The fact that activated phospholipase $A_2$ ran quite differently from the native enzyme on alkali gels, showed that the modification was stable under these conditions, However the ready dissociation of the

radioactive activator ($^3$H oleoyl imidazolide) from the enzyme on acid / urea / polyacrylamide gel electrophoresis is powerful evidence that the bond is not stable under the acid conditions. This is in full agreement with the fact that activated enzymes from whatever source always ran with identical mobility to the native enzyme on these acid gels. Acylation should decrease the mobility of these proteins due to both charge and size modification and the only alternative explanation for the observations was that the enzyme might adopt a more compact form when activated. The results of Camero-Diaz et al. (1985) provided strong support for such an explanation, but it now appears to be irrelevant.

Because the amount of activator required to give full activation was far more less then the amount of acetic anhydride required to prevent activation, therefore it seems highly unlikely that the N-terminal or the **K** residue is the acylation site. Results obtained from the treatment of the non-toxic isoform of phospholipase $A_2$ enzyme from *Naja mossambica mossambica* with acetic anhydride showed that the modification of a single amino group could easily be detected by this method. All this evidence produces a consistent case that **K** residues are not the target for the acylation mechanism and if **K** residues have any role in the activation response, it must be an indirect one in which they act to position the activating agent for reaction, or facilitate the reaction by some other means. It is of interest that the reactive amino-groups essential for activity are protected from acylation when the enzyme is activated.

Treatment of native bee venom phospholipase $A_2$ with phenylglyoxal significantly reduced the catalytic activity by about 50%, but decreased the degree of activation by oleoyl imidazolide about 6-7 fold. It was especially interesting that this partially activated enzyme showed almost fully restored catalytic activity, indicating that each molecule of the enzyme had reacted with the activator. Thus it seems that phenylglyoxal treated enzyme can bind the activator, but show very little response to it. Despite the fact that this reagent can also react with the N-terminal amino-group, similar results were never obtained with simple acylating agents. It therefore seems that the present effects must be attributed to the modification of an arginine residue or residues. Furthermore the addition of phenylglyoxal to the fully activated enzyme hardly affected the catalytic activity, but reduced the degree of activation to ca. 40% of the control value. This data suggested that two different groups were modified on the native enzyme by the reagent, and that one of them was protected in the presence of the activator.

The results obtained from the nitration of **Y** residues by tetranitromethane (which does not block the phenol group) provides extremely interesting results. It is clear that the activator still binds to its target residue after this modification. The enzyme loses some activity against simple substrates only if the activator residue is present, but it is activated in proportion to its residual activity. Thus the conformation change produced by activation seems to affect the active site differently when **Y** residue are nitrated.

From the above results it can be concluded that, **H** residues are the most probable targets for acylation by the activators. Comparison of the amino acid sequences of the responsive and the non-responsive phospholipase $A_2$ enzymes to activation by long-chain fatty acyl residues shows that a **H** residue at the position **22** (**26** for the bee venom enzyme) is found in all susceptible proteins (**Table 5**). This residue is in an extremely hydrophobic pocket in snake venoms which should give it unusual properties. Unfortunately, this hydrophobic pocket was not present in bee venom phospholipase $A_2$ or in the notoxin II-5 from venom of the Australian tiger snake *Notechis scutatus scutatus* which also showed a high degree of activation. On the assumption that the nature of the environment would affect the rate of reaction at such a residue, it would be important to measure the kinetics of activation for many of these species. The available data is limited, but it shows that one of the snake venom enzymes reacts with the activator much faster than the bee venom enzyme does. It is possible that in these last mentioned cases the hydrophobic environment is given by the folded structure.

**Table 5** : Aligned amino acid sequences of highly responsive and non-responsive phospholipase $A_2$ enzymes to the acylation by oleoyl imidazolide. The original references for each sequence are given in (Dufton et al., 1983 and Kini & Evans, 1987).

```
   1                 10                  20                  30
(1) NLYQFKNMIHCTVP-SRP-WWHFADYGCYCGRGG
(2) NLYQFKNMIHCTVP-SRP-WWHFADYGCYCGRGG
(3) NLVQFSYLIQCANHGRRP-TRHYMDYGCYCGWGG
(4) NLYQFKNMIQCTVP-SRS-WWDFADYGCYCGRGG
(5) NLYQFKNMIHCTVP-SRP-SLAYADYGCYCSAGG
(6) ALWQFRSMIKCAVPGSHP-LMDFNNYGCYCGLGG
(7) NLYQFKNMIHCTVP-SRP-WWHFADYGCYCGRGG
                40                  50                  60                  70
(1') KGTAVDDLDRCCQVHDNCYGEAEKL-G-----WPYLT
(2') KGTAVDDLDRCCQVHDNCYGEAEKL-G-----WPYLT
(3') SGTPVDELDRCCKVHDDCYSDAEK-KG-----SPKMS
(4') SGTPVDDLDRCCQVHDNCYNEAEKISG-----WPYLT
(5') SGTPVDELDRCCKTHDDCYARATKSYS-----TPYWT
(6')SGTPVDKLDRCCETHDNCYRDAKNLDSKFLVDNPYTE
(7') KGTAPDDLDRCCQVHDNCYEKAGKM-G-----WPYFT
                80                  90                  100
(1") LYKYE-C-SQGKLT-CSGGNNKCAAAVCNCDLVAANC
(2") LYKYE-C-SQGKLT-CSGGNNKCEAAVCNCDLVAANC
(3") AYDYY-CGENQP-Y-CRNIKKKCLRFVCDCDVEAAFC
(4") LYKYE-C-SQGKLT-CSGGNNKCAAAVCNCDLVAANC
(5") LYSWQ-CIEKQP-T-C-DSKTGCQRFVCDCDATAAKC
(6") SYSYS-C-SNTEIT-CNSKNNACEAFICNCDRNAAIC
(7") LYKYK-C-SQGKLT-CSGGNNKCGAAVCNCDLVAANC
   110                 120                 130
(1''') FAGARYIDANYNINL-KQR-CQ.
```

(2''') F A G A P Y I D A N Y N V N L - K Q R - C Q.

(3''') F A K A P Y N N A N W N I D - T K K R - C Q.

(4''') F A G A R Y I D A N Y N I N L - K Q R - C Q.

(5''') F A K A P Y N K E N Y N I D - P K K R - C Q.

(6''') F S K A P Y N K E H K N L D - T K K - Y C.

(7''') F A G A R Y I D A N Y N I N - F K K R - C Q.

(1)=*Naja mossambica mossambica.* (CM-I)

(2)=*Naja mossambica mossambica* (CM-II) the acidic form.

(3)=*Notechis scutatus:* II-5

(4)=*Naja naja atra*

(5)=*Notechis scutatus:* II-1

(6)=Porcine pancreatic phospholipase $A_2$

(7)=*Naja mossambica mossambica* (CM-III) the toxic form.

Interestingly the toxic isoform of phospholipase $A_2$ from *Naja mossambica mossambica* bound the activator and showed an inhibition in both assays (short-chain substrate assay and erythrocyte. in the presence of 10 μM albumin) instead of activation. This was the only enzyme that showed clear evidence of forming a stable adduct with the activator under the conditions of the acid gel. This might be due to the presence of a **K** residue in a hydrophobic pocket allowing it to.react with the activator.

All the data in this thesis supports the conclusion that the activation of phospholipase $A_2$ enzymes by acyl imidazolides is due to covalent

modification of the protein. However, because of the instability of the adduct on acid/urea /polyacrylamide gel electrophoresis and under denaturing conditions, the tentative conclusions that a novel acylated histidine residue is responsible for the activation phenomena must be investigated further by different techniques. This work has also shown that snake venom enzymes, which are non-glycosylated and which react with the activators very rapidly, would be more suitable than the bee venom enzyme for study by other methods such as crystallography and magnetic resonance techniques. Finally the possibility of changing amino-acid residues by the techniques of 'protein engineering' opens up new means for identifying the relevant amino-acids and determining their contributions to the reaction mechanisms.

# Chapter Five
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