CONFORMATIONAL CHANGES AND ACTIVATION OF PHOSPHOLIPASE A_2 ENZYMES

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List of abbreviations

APS:	Ammonium persulphate.
p-BPB:	Para-Bromophenacyl bromide.
BSA:	Bovine serum albumin.
CD:	Circular dichroism.
CMC:	Critical micelle concentration.
DiC8PC:	Dioctanoylphosphatidylcholine.
DCC:	Dicyclohexylcarbodiimide.
DMF:	Dimethyl formamide.
EDTA:	Ethylenediaminetetraacetic acid.
FA:	Fatty acid.
FT-IR:	Fourier transform infrared.
GPC:	Glycerophosphorylcholine.
^{3}[H]:	Tritium.
IRS:	Interface recognition site.
LPC:	Lysophosphatidylcholine.
MOPS:	Morpholinopropane sulphonic acid.
Nmm:	*Naja mossambica mossambica.*
PAGE:	Polyacrylamide gel electrophoresis.
PC:	Phosphatidylcholine.
PE:	Phosphatidylethanolamine.
PLA_2:	Phospholipase A_2.
PS:	Phosphatidylserine.
SDS:	Sodium dodecyl sulphate.
TEMED:	N,N,N,N,-Tetramethylethylenediamine.
TLC:	Thin layer chromatography.

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1. **Ahmad, T.** and Lawrence, A. J. (1993) Purification and activation Phospholipase A_2 isoforms from *Naja mossambica mossambica* venom. *Toxicon* **31** 1279-1291.

2. Mezna, M., **Ahmad, T.,** Chettibi, S., Drainas, D. and Lawrence, A. J. (1994) Zinc and Barium inhibit phospholipase A_2 from *Naja naja atra* venom by different mechanisms. *Biochemical J.* **301**: 503-508.

3. **Ahmad, T.**, Mezna, M., Chettibi, S. and Lawrence, A. J. (1994) Addition-order dependent modulation of the sensitivity of rabbit erythrocyte membrane to bee venom phospholipase A_2 by oleic acid, lysophosphatidyl choline and albumin. *Toxicon* (In press).

4. **Ahmad, T.** Lawrence, A. J. and Moores, G. (1994) High resolution two-part basic urea gels in the analysis of venom phospholipase A_2 isoforms. *Toxicon.* (in press).

5. **Ahmad, T.,** Lawrence, A. J., Mezna, M., Kelly, S. M. and Price, N. C. (1994) Conformational changes associated with activation bee venom of Phospholipase A_2. *Biochemical J.* Submitted.

6. **Ahmad, T.** et al. Activation of PLA_2 by long chain fatty acid involves novel unstable linkage (in preparation)

7. **Ahmad, T.** Lawrence, A. J., Price, N. C. and Kelly, S. M. (1994) Activation of acidic isoform of phospholipase A_2 from *Naja mossambica mossambica* by oleoyl imidazolide requires the co-operative action of two ionising groups. (Manuscript in preparation)

8. **Ahmad, T**, Mezna, M., Chettibi, S. and Lawrence, A. J. (1993) Stable Changes in the erythrocytes cytoskeleton. Supplement to *Molecular Biology of The Cell* **4** Oct. p 169a.

9. **Ahmad, T.**, Moores, G., Price, N. C., Kelly, S. M. and Lawrence, A. J. (1994) Analysis of conformational change model for PLA_2 activation (accepted for poster in the congress of IUBMB, September, 94 in India.

SUMMARY

Multiple forms of PLA_2 have been isolated from almost all snake venoms but suitable analytical technique have not been available for purification and characterisation. Sequence data showed that most PLA_2 isoforms did not differ in molecular weight or in net positive charge and the major differences lay in the content of negatively charged residues. We therefore concentrated on the development urea PAGE as analytical technique particularly basic urea gels. The performance of the acidic and basic urea gels has been improved to match and even exceed the resolution provided by SDS-PAGE. This has been achieved by the design of spacer gels, by the use of 8M urea and by the selection of polymerisation conditions and choice of electrolyte buffer. The basic urea gels were shown to be ideal for resolution of phospholipase A_2 isoforms. Phospholipase A_2 activity was recovered from both acidic and basic urea gels with high yield. The chromogenic PLA_2 detection method of Shier and Trotter (1978) has been modified for use with basic urea PAGE. By using basic urea gels along and basic urea gels in chromogenic PLA_2 detection method, a number of new PLA_2 isoforms were identified. The basic urea-PAGE (BG) method is proposed as the basis of a simple and rapid method for the classification of PLA_2 isoforms which should allow unambiguous identification of isoforms by referring bands for purified material to the isoform content of whole venoms.

All phospholipase A_2 enzymes tested were shown to be activated by long-chain free fatty acid and only by sub-group of acyl imidazolide. There are two major models for PLA_2 activation, the first favoured by the Verger and DeHaas (1976) is the hydrophobic anchor model and second,

favoured by this laboratory is the conformational change model. To distinguish between these models, we reinvestigated the phenomenon of activation of PLA_2 by glutaraldehyde in the presence of long chain fatty acid. Preliminary tests showed that 15-20% 1-propanol was required for this activation. Activation was transient and continued action of glutaraldehyde progressively inactivated the enzyme but the activated state was shown to be stabilised by borohydride reduction. Enzyme activated by glutaraldehyde in the presence of ^{3}[H]-oleic acid and stabilised with sodium borohydride lost the fatty acid on gel filtration in 20% 1-propanol but the non-radioactive enzyme remained activated. This was the first demonstration that the enzyme could be activated by conformational change. The possibility of conformational change during activation of enzyme was examined by CD and fluorescence spectroscopy. Comparison of the CD spectra of native and oleoyl imidazolide activated enzyme shows a change in secondary structure with apparent increase in both α-helix and β-sheet content. During reaction of the enzyme with oleoyl imidazolide , the protein fluorescence shows a biphasic response with initial fall attributed to occupation of the binding site followed by a progressive decrease with a shift of emission maximum from 341 to 348 nm.

Treatment of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* with oleoyl imidazolide gives a 60-fold increase in the lytic activity that can be attributed to covalent linkage of an oleoyl group to the protein. Radiolabelling experiments confirm that the bound fatty acyl chain is resistant to extraction by organic solvent or albumin

during gel filtration at pH 8.0, but is rapidly eliminated under the conditions of acidic/urea , basic urea and SDS-PAGE. This indicates that the modification is stable in the absence of denaturants and must involve unstable linkage. Fluorescence and kinetic studies of activated enzyme at different pHs indicate that two groups are involve. Evidence is presented to suggest that a histidine at position 10 or 22 in sequence WWHF side-chain is the most probable site of acylation.

Chapter One

Introduction

1.1.) General Review.

Phospholipase A_2 enzymes are found at low activity levels in association with most membranes and they are believed to play a role in lipid turnover and the modulation of the overall lipid composition of the membrane. In contrast phospholipase A_2 enzymes are almost ubiquitous components of digestive secretions and of animal venoms where they are found in extremely high concentrations. In both the cases their role is considered to be one of lipid/membrane destruction. Because of their potentially membrane lytic action there is a need to regulate the enzymic activity and to minimise the possibility of accidental membrane damage. In some cases the physiological regulatory mechanisms have been identified. Thus the activity of the digestive enzymes is normally masked in a pro enzyme which is activated at an appropriate location by partial proteolysis. Venom producing apparatus may be protected by the absence of susceptible phospholipids in the appropriate membranes of the secretary cells lack phosphatidyl phospholipids and therefore would not be affected by this activity. Almost all of these enzymes require calcium for activity, but there is little evidence that this ion can act as a physiological regulator. Many venom enzymes are inhibited by zinc and there is reason to believe that venoms contain high levels of this ion which may therefore act as an important regulator. Similarly the enzymes are very sensitive to the physico chemical; quality of the membrane surface and in particular to changes induced in it by detergents. This property is probably exploited by digestive enzymes that are activated by bile salts. One very dramatic example of the regulation of PLA_2 activity in membranes has been observed in erythrocytes where very low levels of lysophospholipids

are potent inhibitors of the lytic action. This is an especially intriguing regulatory mechanism because lysophospholipids are normally considered to be the major lytic effectors of PLA_2 induced membrane damaged. In general, however, there is little indication that specific regulators of PLA_2 activity exist. The exception to this comes from the present laboratory where evidence has been produced that the majority of PLA_2 enzymes are activated by long chain fatty acids. More interestingly some of these enzymes are specifically activated by long-chain fatty acids derivatives in a reaction that apparently attaches the acyl chain to a highly susceptible site in the protein. This evidence suggested that fatty acid activation and acyl group activation are equivalent and that all of these enzymes could therefore contain a specific fatty acid binding and regulatory site. The present work was therefore undertaken to characterise this site and to determine the mechanism of activation.

1.2.) Phospholipases.

Phospholipases A_2 (EC 3.1.1.4) (PLA_2) catalyse the hydrolysis of the 2-acyl ester linkage of 3-sn-phosphoglycerides and release lysophospholipids and fatty acids (de Haas *et al.*, 1968, Kini and Evans, 1989). Many PLA_2 enzymes, including those that form the basis of the present study, are extremely non-specific both with respect to the phospholipid head-group type and the acyl chain components. One of the most important actions of intracellular phospholipases is the release of arachidonic acid from membrane phospholipids which is then converted into a range of eicosanoids, most of which have significant roles in the inflammatory process. Most results suggest that PLA_2 activity is the rate limiting factor in

arachidonate release making it a vital regulator of inflammatory processes. Therefore there is very considerable interest in such enzymes that have high specificity for phospholipids that have the arachidonyl residue in the 2-position. Based on their molecular weight and substrate specificity, phospholipases are classified (table 1.2.) into subclasses (Glaser, *et al.*, 1993; Mayer and Marshall, 1993; Dennis, 1994). Secretory forms of PLA_2, such as those found in pancreatic or inflammatory fluids demonstrate no particular preference for arachidonate over other sn-2 fatty acids, and contain disulphide bonds that are essential for enzymic activity. The intracellular phospholipases have higher molecular mass and are generally localised in cytosol, prefer arachidonate over other sn-2 acyl groups, and are resistant to inactivation by reducing agents. Some intracellular phospholipases A_2 (Ca^{2+}-dependent PLA_2) require Ca^{2+} (100 to 1000 nM) others (Ca^{2+}-independent PLA_2) do not (Yang, *et al.*, 1994; Ackermann *et al.*, 1994).

The extra-cellular phospholipases are abundant in pancreatic juice and in the venoms of arthropods and snakes (Shipolini *et al.*, 1971; Tu, 1977; Habermann and Breithaupt, 1978; Glein and Straight, 1982; van Eijk *et al.*, 1983) and have been characterised in great detail both with regard to structure and function. These enzymes are closely related family of small proteins with a molecular mass of about 14 to 18 kDa, highly cross-linked and stabilised by as many as 7 disulphide bridges in a polypeptide chain containing about 120 amino acids and serve a variety of functions. Mammalian pancreatic phospholipase A_2 enzymes clearly have a digestive function, whereas the venom phospholipases which share

TABLE 1.2

Classification of phospholipase A_2 enzymes

Source	Location	Size *kDa*	Ca^{2+}	Other features
Group I A. Cobras and Kraits B. Porcine/human pancreas	Secreted	13-15	m M	14 Cys; Cys 11-Cys 85; no Cys 58-Cys 143
Group II A. Rattlesnakes and vipers; human synovial/platelets B.Gaboon viper	Secreted	13-15	m M	14 Cys; Cys 58-Cys 143; no Cys 11-Cys 85; COOH-terminal extension ending in Cys
Group III Bee	Secreted	16-18	m M	10 Cys; dissimilar sequence, but similar three-dimensional structure
Group IV Raw 264.7/rat kidney; human U937/platelets	Cytosolic	85	µ M	9 Cys; no disulphides; unrelated to I-III; Ca^{2+} binding domain homologous to PKC
Group ? Canine/human myocardium	Cytosolic	40	None	--

common catalytic properties and structural homology with the mammalian PLA_2's show a range of toxic actions and the ability to induce pathological symptoms such as; neurotoxic, myotoxic, cardiotoxic, haemolytic, anti-coagulant, convulsant and hypotensive effects in the experimental animals (Karlsson, 1979; Howard and Gundersen, 1980)

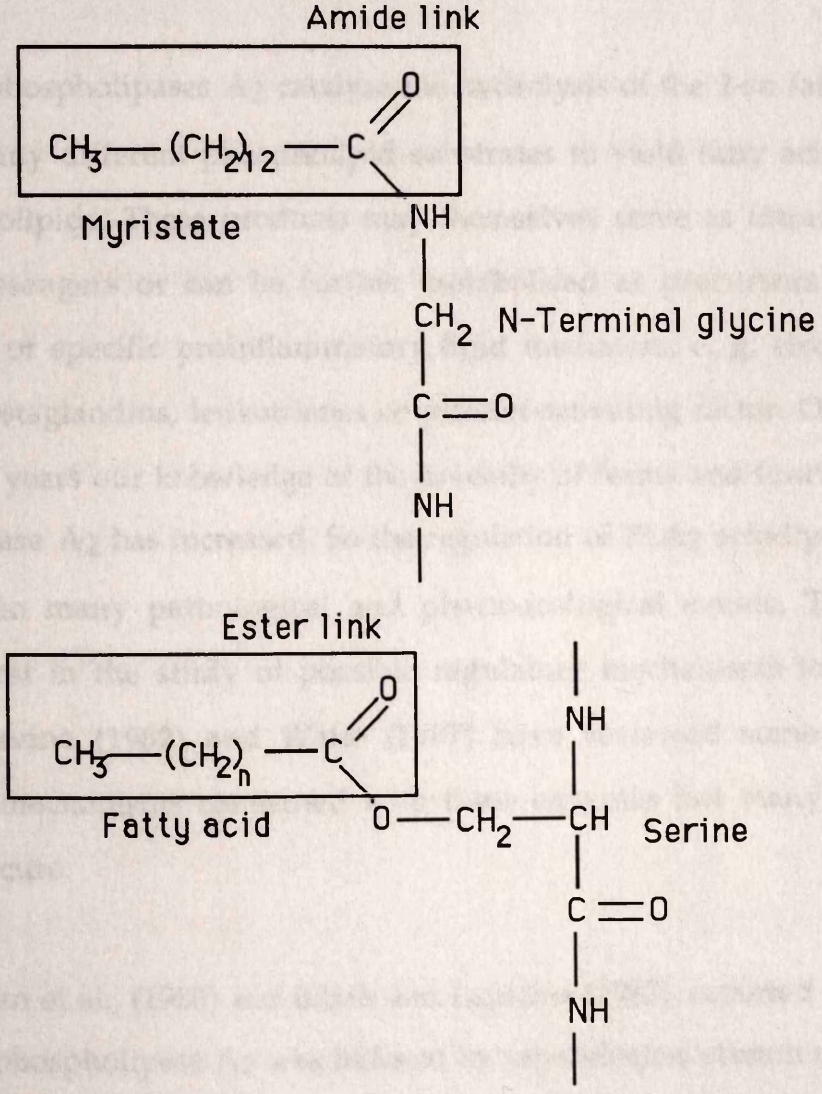
1.3.) Postranslational modification of proteins.

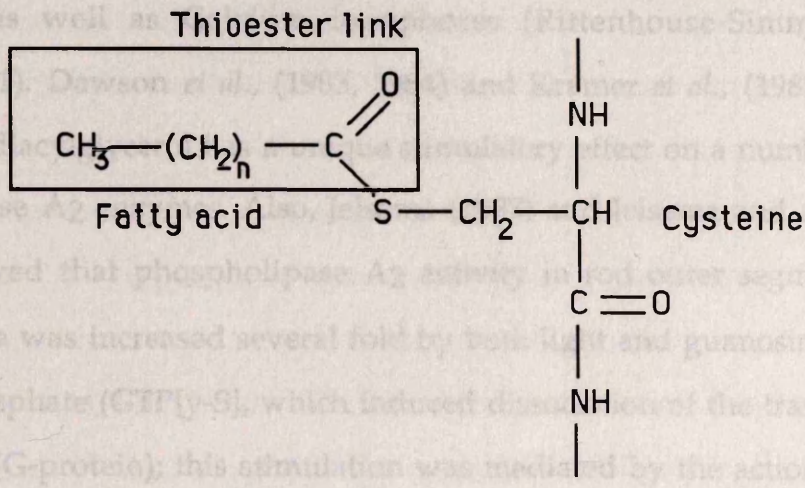
Many proteins are modified during or after their synthesis. Some of these modifications have been known for many years, as for instance glycosylation, phosphorylation and proteolytic cleavage of precursor polypeptides. These examples have been extensively covered in a number of review articles and in biochemical books over the years (Grand, 1989; Magee, 1990; Magee and Courtneidge 1985; Schmidt, 1989).

Another type of protein modification discovered more recently is the covalent attachment of lipid molecules like phospholipid, diacylglycerol and various species of long chain fatty acids. Such binding of lipid molecules are expected to change the physical properties of the respective entity quite dramatically, because largely hydrophilic residues are converted into very hydrophobic ones. This will, of course influence the interactions between such modified proteins and other molecules present in their vicinity, be it other proteins, lipids or even nucleic acid. This modification yields what could be regarded as a new class of proteins, which by analogy with glyco- or phosphoproteins, are termed as "acylproteins". Two types of acylproteins are presently distinguished in the literature, those which contain exclusively the fourteen carbon myristic acid (tetradecanoic acid) in an amide-linkage, and those which are pre-

dominantly modified with palmitic-, stearic- and oleic acid (hexa- and octadecanoic acids) in ester- or thioester-type linkage. (Branton, *et al.*, 1993; Resh, 1994; Veit, *et al.*, 1994)

When myristate has been detected covalently bound to protein molecules it is almost always linked through an amide bound to an N-terminal glycine residue, whereas palmitate is linked through an ester or thioester bond to serine (or threonine) or cysteine respectively. The ester and thioester linkages are labile in the presence of base (e.g. 0.1 M KOH in methanol) and neutral 1M hydroxyl amine) (Magee *et al.*, 1984) whereas the amide bonds are much more stable.





1.4.) Regulation of phospholipase A_2 activities.

As the phospholipases A_2 catalyse the hydrolysis of the 2-sn fatty acyl chain of many different phospholipid substrates to yield fatty acids and lysophospholipids. These products may themselves serve as intracellular second messengers or can be further metabolised as precursors in the production of specific proinflammatory lipid mediators, e. g. eicosanoid such as prostaglandins, leukotrienes or platelet-activating factor. Over the last several years our knowledge of the diversity of forms and functions of phospholipase A_2 has increased. So the regulation of PLA_2 activity is very important in many pathological and pharmacological events. There is great interest in the study of possible regulatory mechanisms for these proteins. Irvine (1982) and Waite (1987) have reviewed some of the regulation mechanisms concerned with these enzymes but many details remain obscure.

Broekman et al., (1980) and Billah and 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 diacylglycerol has a unique stimulatory effect on a number of phospholipase A_2 enzymes. Also, Jelsema (1987) and Jelsema and 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'-[y-thiol] triphosphate (GTP[y-S], which induced dissociation of the transducin subunits of (G-protein); this stimulation was mediated by the action of the β subunits. 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. The activity of PLA_2 enzymes under physiological condition is regulated by two dominant factors; the physico-chemical form of the substrate, and the presence of divalent metal ions. In addition, some of these enzymes are activated by the covalent addition of long chain fatty acyl residues. All three factors have been studied separately by many workers (De Haas *et al.*, 1970; Verger *et al.*, 1973; Drainas *et al.*, 1978), but the effect of each of these factors on the other and modulation of the enzymic activity is not well understood.

1.4.1.) The activation of phospholipase A_2 isoforms by long chain fatty acids.

Many venom phospholipase A_2 enzymes are susceptible to activation by long-chain fatty acylating agents (Drainas and Lawrence, 1978; Camero-Diaz *et al.*, 1985; Chettibi *et al.*, 1990). Phospholipase A_2 enzymes exhibit very complex kinetic behaviour, which can be attributed to the extreme

variety of chemical and physico-chemical properties presented by their substrates and to the fact that the enzymes act at a lipid/water interface. There is evidence that different aspects of the enzymic behaviour can be regulated independently and Rosenberg (1988) has reviewed the factors which determine enzymic and toxic activity in closely related venom enzymes. A good example of differential regulation is provided by the phospholipase A2 from honey bee (*Apis mellifera*) venom which can be activated toward some, but not all substrates either by free long chain fatty acids or else by weakly activated derivatives of these acids, of which the best example is oleoyl imidazolide. Whilst the free fatty acids act directly, the derivatives modify the protein to produce an adduct which is stable to a wide variety of conditions including the presence of the powerful fatty acid sequestering agent, serum albumin. The enzyme is fully activated by a single molar equivalent of the acylating agent suggesting that a highly specific site is involved. Van der Weile *et al.*, (1988a, b) have devised methods for long-chain fatty acylation of mammalian pancreatic phospholipase A2 enzymes at specific lysine residues which do not take advantage of the intrinsic selectivity of the site and have demonstrated that this modification also activates the enzyme towards erythrocyte lysis.

Drainas and Lawrence (1978) proposed that acylation of bee venom phospholipase A2 occurred at a specific fatty acid binding site, and that the target was also the residue which interacted with the carboxylate group of the free fatty acids. The high selectivity shown by this site towards long-chain fatty acid derivatives in comparison with their short chain analogues, indicated that hydrophobic interactions were the primary

determinant of reactivity. There is no clear indication that fatty acid binding sites of other proteins can be selectively acylated by such derivatives and therefore this appears to be an idiosyncratic property of this enzyme.

Although the activated enzymes were stable under non-denaturing conditions (Camero-Diaz *et al.*, 1985), all attempts to characterise the acylation sites have failed, principally because the linkage is labile upon partial denaturation and acyl residue is rapidly lost during acidic, basic or SDS-PAGE conditions. These results suggested that a novel, highly labile acylation could be involved: this might form the basis of a reversible regulation mechanism that would escape the detection by conventional labelling experiments. One of the attractive models for activation of phospholipase A_2 enzymes by long chain fatty acylation is that the modified side chain acts as a surface anchor at the lipid interface (Van der Weile *et al*, 1988a, b) However , a number of pieces of evidence, including the conformational stability of the activated enzyme, the lability of the linkage on denaturation and the fact that a bound acyl group would have very different anchoring properties from free fatty acids support the earlier model (Lawrence and Moores, 1975) that activation results from a conformation change in the protein.

1.4.2.) Substrate specificity.

Phospholipase A_2 enzymes have very broad specificity for different head groups and acyl chain substituents, but the most interesting effect of structure on susceptibility is related to change in the physico-chemical

form. In general, these enzymes act weakly on the free monomer form, strongly on the micellar form and very weakly on the physiologically significant bilamellar form.

1.4.2.1.) Monomeric substrates.

De Haas *et al.*, (1970) have reported that phospholipase A_2 enzymes act poorly on substrates below the critical micelle concentration (CMC) but the pancreatic phospholipase A_2 as well as the pro-enzyme act on short-chain lecithins in the monomeric state (De Haas *et al.*, 1971; Pieterson *et al.*, 1974). Well (1972) working on the action of the dimeric phospholipase A_2 from *Crotalus adamanteus* on the monomeric substrate dibutyryl-phosphatidylcholine gave the first detailed kinetic analysis and showed that the enzyme act at an optimum pH of 8-8.5 with Ca^{2+} as the only cation able to support activity. `Viljoen and Botes (1979) confirmed the results of Wells (1972) by using the pure phospholipase A_2 from *Bitis gabonica* to study the kinetics of hydrolysis of the monomeric derivative dihexanoylphosphatidylcholine (DiC_6PC). On the other hand, Volwerk *et al.*, (1979) studying the action of porcine pancreatic PLA_2 showed that the addition order of Ca^{2+} and the substrate was independent, in contrast to the model proposed by Wells (1972) for snake venom phospholipases.

1.4.2.2.) Monomer/ Micelle transition.

The action of some phospholipase A_2 enzymes on short/medium chain length substrates below and above their critical micelle concentration has been investigated, and the results showed a large

enhancement in the enzymic activity when the substrate concentration exceeded the CMC, but there are exceptions which are not yet fully understood. The use of monomeric short chain PC as substrate for the pancreatic PLA_2 and its zymogen, showed that both forms of the protein catalysed the hydrolysis of the substrate at a low rate, but the active form of the enzyme showed a dramatic enhancement of activity on the substrates above the CMC (Peiterson *et al.*, 1974). These results suggested that the active form of the pancreatic PLA_2 in contrast to its zymogen, contains a hydrophobic region which is involved in the recognition of the lipid-water interface.

An increase in the hydrolytic activity was also observed at high salt concentrations and this was thought to be enforced hydrophobic interactions between the enzyme and substrate. Van Dam Mieras *et al.*, (1975) studied the tryptic cleavage of the active pancreatic PLA_2 in the presence of non-hydrolysable substrate analogues, and proposed that a hydrophobic N-terminal sequence was strongly involved in interfacial binding, and named thus the Interface Recognition Site (IRS).

Wells (1974), examined the kinetics of venom PLA_2 from Crotalus adamanteus on different short-chain phosphatidylcholine substrates below and above the CMC. The results showed a similar increase in the enzymic activity with substrate concentrations above CMC and reported that the V_{max} of the enzyme acting on monomeric dibutyrylphosphatidylcholine (DiC_4PC) was about 3000 times lower than that observed on micellar form of dioctanoylphosphatidylcholine (Di_8PC). Although these observations showed a clear specificity for the substrate form, they

cannot be generalised for all phospholipase A_2 enzymes. In particular bee venom enzyme does not show similar properties (Shipolini *et al.*, 1974).

1.4.2.3.) Micellar substrates.

The micelle is now acknowledged to be a complex state with many sub forms. It is clearly the most susceptible to phospholipase A_2 attack, and almost all phospholipase A_2 enzymes show peak sensitivity against the micelle-forming substrate, dioctanoylphosphatidylcholine. These enzymes showed similar calcium dependence against both monomeric and micellar forms and in neither case is attack promoted by detergents. It is of interest that for the monomeric substrate DiC_6PC, PLA_2 attack is concentration dependent well above the CMC, whilst for the higher forms it is not, suggesting a structure-dependent effect on binding affinity (Lawrence, A. J., unpublished results).

De Haas *et al.*, (1971) using porcine pancreatic phospholipase A_2 with different short-chain micellar substrates differing in their side chain length between C_6 and C_{10} reported that the reaction progress curve followed simple Michaelis-Menten analysis, but the rates of hydrolysis of these substrates were very different. For example, it was found that under the same ionic strength conditions enzyme hydrolysed dioctanoyl-phosphatidylcholine with specific activity of 6 mmoles/min/mg, whereas didecanoyllecithin was not hydrolysed at all (Verger *et al.*, 1973). In contrast, by using a monolayer technique it was found that rates of hydrolysis of all lecithins with acyl chain varying C_8 to C_{12} were quite similar (Zografi *et al.*, 1971).

1.4.2.4.) Bilayer structure.

It has long been found that long-chain phospholipids which tend to form aggregated bilayer structures in aqueous solution are very poor substrates for phospholipase A_2 and in particular for the pancreatic enzyme (De Haas *et al.*, 1968). The action of porcine pancreatic PLA_2 on fully saturated long-chain phosphatidylcholine derivative was studied by Opden Kamp and coworkers (1974, 75). At the thermotrophic phase transition where these compound become susceptible to the enzyme, it was observed that the tighter packing of the phospholipid molecules at high surface pressure prevented the penetration of the enzyme into the interface and strongly reduced the enzymic activity. Sonication of the uni-lamellar or multi-lamellar vesicles was also found to increase their susceptibility to PLA_2 hydrolysis (Wilschut, *et al.*, 1976, 78).

To overcome the problem of the solubility, Jain and Cordes (1973 a, b) proposed the use of short-chain alcohols in the reaction medium and they showed that the bilayer structure remains closed under these conditions. At an optimum concentration of alcohol, the vesicles become excellent substrates and the reaction curves followed normal Michaelis kinetics. It was believed that this might be due to the incorporation of alcohol chains in the vesicles facilitating the penetration by the enzyme. This formed the basis of the assay method used by Drainas and Lawrence (1978) to study the activation of bee venom phospholipase A2 by long chain fatty acylation. They include 20 % 1-propanol in the assay buffer and used dioleoylphosphatidylcholine as the substrate. Under these conditions a very clear product activation by fatty acid was observed that could be

replaced by acylation of the enzyme. In addition, detergents of all types changed susceptibility to those characteristic of the micelle state, presumably by inducing micellar morphology.

1.4.2.5.) Erythrocytes membranes as substrates for phospholipase A_2.

Phospholipase A_2 enzymes purified from snake venoms have been widely used as tools to study the disposition of phospholipids in biological membranes (Op den Kamp, 1979; Roelofsen *et al.*, 1980). These enzymes have been particularly useful in elucidating the localisation of phospholipids in the erythrocyte membrane.

Gul and Smith (1972) using *Naja naja* phospholipase A_2 showed that extensive cleavage of phosphatidylcholine in red blood cells did not greatly increase susceptibility to hypotonic lysis. However, addition of serum albumin to the medium caused the enzyme to be highly lytic (Gul and Smith, 1974). The action of albumin was outlined by Deuticke *et al.*, (1981) who showed that more than 95 % of the free fatty acid and up to 80 % of the lysophospholipid resulting from the hydrolysis by *Naja naja* PLA_2 could be extracted by albumin without causing haemolysis. These results support those obtained by Vaysse *et al.*, 1986, 87) using bee venom phospholipase A_2 on rabbit and human red blood cell. The authors showed that the addition of the enzyme to intact rabbit erythrocytes caused about 65 % cleavage of PC with no haemolysis, whilst, PE and PC were hardly attacked at all.

Lawrence, studying the synergism of PLA_2 by oleic acid showed that

lysophospholipids had a profoundly inhibitory action, in contrast to its detergent-like activation in all other systems involving the catalytic action of these enzymes. He proposed that the generation of traces of lysophospholipid inhibited the enzyme very strongly and was the reason why PLA_2 enzymes are normally non-lytic to washed erythrocytes.

Drainas and Lawrence (1978) and Drainas and Lawrence (1981) studying the effect of acylation on the action of bee venom PLA_2 on rabbit erythrocytes showed that acylated enzyme caused a very small increase in the sub-lytic leakage in the absence of albumin, this was thought to be due to the inhibitory effect of lysophospholipid generated by the hydrolysis. Removal of the reaction products by albumin gave a dramatic increase in the haemolysis. However the addition of exogenous LPC and/ or Fatty acid in the presence of albumin showed different effects on the leakage rates determined partly by addition order of lysophospholipid and fatty acid.

1.4.3.) The regulation of PLA_2 activity by divalent metal ions.

Venom phospholipase A_2 enzymes are Ca^{2+} dependent enzymes, in which the Ca^{2+} ion contributes to the formation of the active site. Crystallographic analysis (Dijikstra, *et al.*, 1981a; Dijikstra *et al.*, 1981b; Scott et al., 1990; White et al., 1990) has shown that the Ca2+ ion interacts with oxygen atoms belonging to an aspartate (Asp-49) and carbonyl groups of tyr-28 and Gly-30 and Gly-32 residues in the enzyme and also to the phosphate oxygen in the substrate.

Long and Penny (1957) were the first to show that phospholipase A_2 was activated by calcium and inhibited by zinc. Roholt and 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.

Recently Mezna *et al* (1994) demonstrated the inhibition of phospholipase A_2 from the Chinese cobra (*Naja naja atra*) by zinc and barium by different mechanism. As according to crystal structure (Scott *et al.*, 1990) it is clear that the enzyme has two binding sites. According to our recent results (Mezna et al., 1994), it is demonstrated that inhibition by Ba^{2+} shows the kinetic characteristics of a conventional competitive inhibitor acting to displace Ca^{2+} from a single essential site, but Zn^{2+} has the paradoxical property of being more inhibitory at high than at low Ca^{2+} concentration. Kinetic analysis of the Ca^{2+}-dependence of enzyme activity shows a bimodal response, indicating the presence of two Ca^{2+}- binding sites with affinities of 2.7 μM and 125 μM respectively. The results are consistent with the model that the enzyme is activated by two Ca^{2+} ions, one that is essential and can be displaced by Ba^{2+}, and one that modulates the activity by a further 5-10 fold and which can be displaced by Zn^{2+}. An alternative model is also presented in which the Zn^{2+}- binding site is a phenomenon of the lipid/water interface.

1.5.) Purification of venom Phospholipase A_2 enzymes.

The purification of venom phospholipases was initially attempted by Slotta in the late 1930s (Slotta and Frankel-Conrat, 1938) It was until nearly

three decades later, however, that the venom phospholipases were purified on a large-scale basis, which permitted detailed kinetic and structural analysis (Waite, 1987).

Since the PLA_2 are small, stable enzymes, they are usually easy to isolate, and purity has not been a critical factor in sequence analysis. There are some things to look out for, however. The pancreatic PLA_2 must be activated by trypsin from proPLA precursors, and this step opens the possibility of proteolytic fragmentation. Moreover, the porcine pancreatic enzyme has been shown to consist of a mixture of isozymes that are not usually separated in commercial sources. Snake venoms are interesting in that they usually contain multiple enzymes, sometimes with quite distinct structures and functions. In fact, it is the exception to find a venom source with only one PLA_2 present. One example is *Crotalus atrox*, the western diamondback rattlesnake, which has a single, dimeric PLA_2 (Shen *et al.*, 1975). The acidic p*I* of this enzyme facilitates its purification simply by filtration and anion-exchange chromatography. The closely related eastern diamondback rattlesnake, Crotalus adamanteus has two dimeric PLA_2 which differ only in residue 117 which is Glu in α and Gln in β (Heinrikson *et al.*, 1977). The venom of *Agkistrodon piscivorus piscivorus* is more typical in having an acidic dimer and two basic monomeric PLA_2 (Maraganore *et al.*, 1984; Maraganore and Heinrikson, 1986). One of the latter is a "typical" PLA_2 in having an Asp at position 49, and the other has a Lys at this position, as well as some changes in calcium binding loop (Yoshizumi *et al.*, 1990). These Lys-49 phospholipase A_2 have been identified thus far only in crotalid venoms, including Agkistrodon, Bothrops, and Trimesurus (Yoshizumi *et al.*, 1990).

Another distinction that must be kept in mind when purifying PLA_2 from venom sources has to do with the possible presence in a single enzyme of both esterolytic and toxic activities. Notexin is a potent neurotoxin from the venom of the Australian tiger snake that has weak PLA_2 activity (Halpert and Eaker, 1975). The venom of Chinese water moccasin, Agkistrodon halys pallas, containing an acidic and a basic PLA_2, together with a neutral neurotoxin with PLA_2 activity (Chen *et al.*, 1987). Venoms of more elapid snakes have often been shown to consist of multiple PLA_2 forms, often with varying degrees of toxic activity. It is safe to say that much of the work reported on venom PLA_2 sequences has been done with major forms that are easily resolved from contaminants and other PLA_2 species. The rule, however, is that venoms will most likely present a variety of PLA_2, and this must be borne in mind when formulating purification protocols (Heinrikson, 1991).

Some further generalisations may be useful here. First, the PLA_2 are usually small and stable as compared to most proteins, and withstand rather harsh conditions of pH and temperature. Their low molecular weight (14, 000) makes them easily separable from the majority of contaminating proteins by simple gel-exclusion chromatography. Dimeric PLA_2 present a different problem in that they tend to move with proteases on such columns. However, if the columns are run in 5 % (v/v) acetic acid, dimers dissociate and run with monomeric PLA_2 on gel filtration (Welches, *et al.*, 1985). These acidic conditions, therefore, help to minimise proteolysis, although the seven disulphide bridges of PLA make these enzymes highly resistant to proteases. This first step usually provides

enzyme in high yield and, if the source is snake venom, in substantial state of purity. In fact, the only contaminants may be other PLA_2 species. Knowledge of the p*I* of the PLA_2 of interest will dictate use of an anion- or cation-exchange column procedure for the second step of purification (Heinrikson, 1991).

The primary structure of over 100 venom phospholipase A_2 enzymes is known (see appendix as well). Snake venoms contain multiple forms of PLA_2. Indian cobra (*Naja naja naja*) venom has been reported to contain as many as 14 isoenzyme forms of PLA_2. In the venom of Australian king brown snake (Pseudechis australis) 15 phospholipase A_2 isoforms have been reported. (Dufton *et al.*, 1983; Lu and Lo, 1981; Nishida *et al.*, 1982; Nishida et al, 1985a; Nishida et al., 1985b; Joubert et al., 1977, 1983, 1987; Verheij et *al.*, 1983; Aird *et al.*, 1985, 1986; Forest *et al.*, 1986; Kini *et al.*, 1986; Maraganore and Heinrikson, 1986; Ohara *et al.*, 1986; Ritonja *et al.*, 1986; Tanaka *et al.*, 1986, 1987; Frelat *et al.*, 1987; Mancheva *et al.*, 1987; Bouchier *et al.*, 1988; Ducancel *et al.*, 1988a, b; Takasaki *et al.*, 1988, 1990; Chewetzoff *et al.*, 1989; Kondo *et al.*, 1989; Schmidt and Middlebrook 1989; Yoshizumi *et al.*, 1989; Liu *et al.*, 1990; Harvey, 1991; Heinrikson, 1991)

Although the composition of venom is relatively simple, few workers have produced gel electrophoresis data to illustrate the overall venom composition. The components of venom typically range from small peptides to medium sized proteins and in most cases the bulk of the venom is in the low molecular weight range.

1.5.1.) *Naja mossambica mossambica* (spitting cobra) phospholipase A_2 isoforms.

A number of phospholipases A_2 have been purified, and their amino acid sequences determined, from the venoms of various snakes, including those belonging to the four families Elapidae, Hydrophiidae, Crotalidae and Viperidae. All the sequences show a high degree of homology. Some of the basic PLA_2s do not show this type of toxicity (Eaker, 1978). The structure-function relationship in PLA_2 enzymes has been discussed (Dufton and Hider, 1983; Kini and Iwanaga, 1986; Tsai *et al.*, 1987; Kondo *et al.*, 1989). The three forms of phospholipase A_2, CM-I, CM-II and CM-III, were purified by gel filtration on Sephadex G-50 followed by ion exchange chromatography on CM-cellulose (Joubert, 1977), They comprise each 118 amino acid residues and are cross-linked by seven intrachain disulphide bridges. The complete primary structure of three phospholipases A_2 has been elucidated and they closely resemble those from other snake venom and pancreatic phospholipase A_2. This is true for the sequence of the invariant amino acid residues. The amino acid sequence of three phospholipase A_2 enzymes from *Naja mossambica mossambica* venom were compared among themselves and also to some of known phospholipase A_2 sequences (Dufton and Hider, 1983; Heinrikson, 1991). The high degree of homology within the group CM-I, Cm-II and CM-III (97.2 %) is quite apparent. The sequence of CM-I differs of that of Cm-II in only 3 amino acids at position 95, 113 and 122 and the difference between CM-I and CM-III was found to be in 13 amino acids whereas, CM-II differs from CM-III in 11 amino acids. Overall there are 113 invariant amino acids out of 118 in the three forms of PLA_2 (Joubert, 1977).

The alignment of phospholipase A_2 from *Naja mossambica mossambica* with the other snake venom and pancreatic phospholipases showed a very high degree of homology especially in the residues which are reported to be involved in both the active site and the Ca^{2+} binding loop. For example the comparison of the sequence of CM-III with basic form PLA_2 from *Naja nigricollis* showed a replacement of only one amino acid (Leu) in position 67 for Phe in CM-III which is also a highly basic protein (Joubert, 1977; Dufton and Hider, 1983a; Van den Bergh *et al.*, 1989).

Like most snake venoms, the venom of *Naja mossambica mossambica* was found to be highly toxic to arthropods (Menashe *et al.*, 1981). The toxicity of this venom was suggested to be due to the presence of a basic phospholipase A_2 which was called component P3 (Zlotken *et al.*, 1975; Menashe *et al.*, 1980) and CM-III (Joubert, 1977). This isoform of the enzyme was purified and shown to be highly basic (p*I* 9.6) and also to be the main toxin of the venom (Menashe, 1981).

The inactivation of the enzyme by p-bromophenacylbromide resulted in an identical decrease in both toxicity and enzymatic activity and it was concluded that the toxicity is directly associated with the phospholipase activity. All the three phospholipases showed an absolute requirement for calcium (Menashe, 1980).

Chettibi *et al.*, (1990) using the two basic forms of PLA_2 from *Naja mossambica mossambica* for activation assays against erythrocyte membranes showed that the non-toxic form (p*I* 8.8) was activated by long-

chain fatty acylation, whereas the toxic form was not activated by it. The acidic form is highly susceptible to activation by long chain fatty acylation. The aim of this work was to find most suitable pair of isoforms for comparative studies of an activating and non-activating form. All the three forms of *Naja mossambica mossambica* are available commercially: they have similar molecular weight and are not resolved by SDS-PAGE methods, but the charge differences enabled the acidic and the basic non-toxic isoforms to be resolved from the toxic isoform by acidic urea gel electrophoresis. Calculations of the net charge (Joubert, 1977) at high pH indicated that basic urea gel electrophoresis should resolve all of the isoforms. We therefore attempted to purify the isoforms from the whole venom using both acidic and basic urea gel electrophoresis to monitor purification. Results indicated that phospholipase A_2 activity could be recovered from both basic and as well as acidic urea gels, despite the high and low running pH, making this a very powerful technique for monitoring the purification, identification and characterising new isoforms of PLA_2.

Our analysis showed that the acidic isoform was the most susceptible of the major isoforms to activation by oleoyl imidazolide and a purification method was devised to give greatest separating power for the more acidic venom components. In the established protocol the venom was chromatographed using CM resins which do not retain the acidic form and therefore cannot separate it from other non-absorbed acidic components. To overcome this problem, ion exchange separations were carried out using DE resins. The combination of new separation techniques and analytical methods resulted in the identification of at least three new phospholipase A_2 isoforms from the venom.

1.5.2.) Bee venom phospholipase A_2.

The venom of the common European honey bee (*Apis mellifera*) was found to be the richest source for phospholipase A_2 enzyme (Shipolini *et al.*, 1971; Banks and Shipolini, 1986). 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 p*I* 10.5. The amino acid analysis of bee venom PLA_2 Shipolini *et al.*, (1974a), was revised by Kuchler *et al.*, (1989) and corrections were made. The bee venom phospholipase A_2 was reported to have 14 carbohydrate moieties in (1:1:8:4) fructose: galactose: mannose and glucosamine respectively.

1.6.) Gel electrophoresis.

Separation of proteins and peptides with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is widely used analytical method for determination of purity and approximate molecular weight. Mobility of proteins on a well-defined system should correlate with mass of SDS-protein complexes. Most SDS-PAGE systems are capable of resolving proteins with molecular masses between 10,000 and 300,000, but become ineffective for applications in which resolution is required below 10,000. This system also become ineffective where proteins have similar molecular weights i.e. isoforms.

Snake venoms are a rich source of phospholipase A_2 enzymes, but each venom may contain multiple isoforms (Braganca and Sambray, 1967; Salach, *et al.*, 1971a; Salach, *et al.*, 1971b; Kini and Gowda, 1983; Hazlett and

Dennis, 1985; Bhat and Gowda, 1989; Bhat *et al.*, 1991; Heinrikson, 1991) which generally have very similar structures, but are normally separate gene products (Pungercar, *et al.*, 1991; Oda *et al.*, 1990; Ogawa *et al.*, 1992 Nakashima *et al.*, 1993). These isoforms may differ in a variety of ways, including toxicity, membrane lytic activity, metal ion dependence and reactivity with substrate and because the primary sequences of a very large number of isoforms are known, they provide a very diverse system to study the relationships between structure and function. Although phospholipase A_2 enzymes from different venoms may vary in size, the isoforms in a given venom do not normally differ greatly in length and therefore they are not resolved by SDS-PAGE (Evans *et al.*, 1980; Van den Bergh *et al.*, 1989). Although many isoforms differ by variation in hydrophobic amino acids, the majority vary in the content of charged residues and can therefore be separated by ion-exchange chromatography and identified by isoelectric focusing or by urea-based gel electrophoresis. Urea-gels have a very considerable cost advantage over isoelectric focusing methods and are well suited for running large slab gels which are ideal for comparative studies.

We have presented improved acidic and basic urea-page methods and demonstrated that electrophoresis at high pH is more a powerful method for separating isoforms (Ahmad and Lawrence, 1993). This clearly reflects the fact that isoforms tend to differ more by the number of acidic than basic residues. Basic urea-PAGE enabled at least three new isoforms to be identified from the venom of the spitting cobra *Naja mossambica mossambica*, one of which was a previously unresolved major component, but the resolution was still not adequate to allow minor

isoforms to be observed in whole venom samples. Because urea gels normally use a single electrolyte system which is either a dilute acid or a dilute base, there is no obvious stacking or focusing procedure and protein bands retain the shape of the gel boundary. However for high concentration gels, residual solution tends to polymerise in the wells and thus distort band shape. To overcome this we investigated the use of a dilute gel overlay and, at the same time, also examined the effect of increasing the urea concentration to 8M which is close to the maximum possible.

PLA_2 activity can be recovered from gel slices in acid or basic urea-PAGE despite the extreme pH values and a further aim was to use the histochemical method of Shier and Trotter (1978) to detect PLA_2 activity in high resolution electrophorograms of whole venoms as the basis of a simple method for monitoring purification.

1.7.) Conformation.

Circular dichroism studies of number of phospholipase A_2 enzymes were carried by number of groups. The μ-helical content of phospholipase A_2 enzymes vary from 27 to about 70 %. Well (1971a) studying *Crotalus adamanteus* phospholipase A_2 indicated that its α-helical content is nearly 70 %. Similar results were obtained for two phospholipase A_2 enzymes obtained from the venom of *Agkistrodon halys blomhoffii* (Kawauchi *et al.*, 1971). The α-helical contents of the two phospholipases A_2 from *Vipera ammodytes* venom were estimated to be 24 and 23 % respectively and the amounts of α-helix, calculated by the method of Bannister *et al.*, (1973) were 28, 27 and 22 % for DE-I, DE-II and DE-III

respectively while β-structure content was considered to be smaller in phospholipase A_2. (Gubensek and Lapanje, 1974; Joubert and Van der Walt, 1975).

Dufton *et al.*, (1983) carried out a circular dichroism study of phospholipase A_2 enzymes from different venoms and has shown that their CD curves are qualitatively similar, showing extrema at 223 nm, 210 nm and 193 nm. These bands normally indicate predominant helical structure form in these enzymes. The major variations between individual spectra are concerned with band intensity and this may be connected with different percentage contents of helix and ß structure in each case. Although some of the CD spectra appear to show considerable variation in this respect, the supplementary information suggests otherwise. In particular, the close sequential homology between *Crotalus atrox* and *Crotalus adamanteus* enzymes implies that their CD spectra are likely to be very similar (e.g. the bovine and porcine pancreatic enzymes). This being the case, the *C. atrox* and bovine pancreatic enzyme crystal structures can be compared to show that a large difference in CD intensity seems to arise from relatively small change in tertiary structure (i.e. the lose of helix D in *Crotalus* enzyme). Furthermore, since the bovine pancreatic enzyme and the C. atrox enzyme sequences are amongst the most dissimilar in the data set, it is not expected that conformational differences will substantially exceed those highlighted by the two crystal structures.

According to Provencher (1981) analysis of the CD curves, the average content of α-helix is about 40 %, which compares to observed contents of 50 % in the bovine pancreatic enzyme and a similar amount in the Crotalus enzyme.

Davidson and Dennis (1990) carried out sequence analysis and circular dichroism studies of Indian cobra (*Naja naja naja*) venom acidic phospholipase A_2. The PLA_2 from *Naja naja naja* gives a CD spectrum similar to spectra of other phospholipases A_2 (Dufton *et al.*, 1983), showing extrema at about 194, 204 and 220 nm. As expected by comparison to other venom phospholipases A_2, the CD spectra of non-denatured protein are dominated μ-helical components. The content of α-helix in *Naja naja naja* phospholipase A_2 was calculated by computer curve-fitting to be between 42 % (in H_2O, 20° C) and 50 % (in 10 mM $CaCl_2$, 20° C).

Dijkstra *et al.*, (1978, 81) gave a detailed crystal structure of bovine pancreatic phospholipase A_2 at 2.4Å and at 1.7Å resolution in which they showed a clear picture of the position of the Ca^{2+} ion binding site. This ion was located in the active site pocket and surrounded by seven oxygen ligands, including possible interactions of the N-terminus and H_2O molecule with the active site. The involvement of the N-terminal region in the catalytic activity was discussed in detail (Dijkstra *et al.*, 1981). This region was reported to have a very important role in the formation of a specific site called 'Interface Recognition Site' (IRS) (Verger *et al.*, 1973; Van).

They proposed that the induction of this site requires a very exact juxtaposition of the amino group with other atoms in the protein (Slotboom and De Haas, 1975). The 2.4Å resolution structure has established the presence of seven disulphide bridges, of which two had not been defined chemically, and at the same time they have shown that the secondary structure contains about 10 % β structure and ca. 50 % α-helix.

Other phospholipase A_2 enzymes from snake venoms have been crystallised and showed a close structural resemblance to the pancreatic enzyme (Pasek *et al.*, 1975; Keith *et al.*, 1981). Burnie *et al.*, (1985) proposed the existence of ionic bridges which form an intra-molecule linkage between the monomers in dimeric enzymes such as *Crotalus atrox*. This sort of linkage involves the aspartate in position 49 which is now believed to be very important in Ca^{2+} binding and catalytic activity of the majority of phospholipase A_2 enzymes (Van der Bergh *et al.*, 1989). The occupation of Asp 49 by this linkage prevents the binding of the substrate at the active site in the absence of Ca^{2+}, but in the presence of Ca^{2+} a conformation change in enzyme facilitates the substrate binding.

White *et al.*, (1990) and Scott et al., (1990) have determined the crystal structure of the Chinese cobra venom phospholipase A_2 (*Naja naja atra*) in a complex with a transition state analogue $DiC_8(2Ph)Ph$ acting an inhibitor. Which was found to bind firmly to the active site and the results showed a considerable similarity with the bovine class I phospholipases, however, they also reported the presence of two Ca^{2+} binding sites in each of the molecules of the asymmetric unit where the primary Ca^{2+} is in the same location as the one reported for pancreatic enzyme (Dijkstra *et al.*, 1981) which serves a catalytic activity. The secondary site was found about 6.6Å away from the first site. Unlike the primary site, this site was weakly penta-coordinated when the enzyme was inhibited by the transition analogue, but hepta-coordinated in the inhibited form. Recently Pan et al., (1994) corrected and resolve some discrepancy between the sequences determined by conventional protein sequencing and X-ray crystallography. They reported that the mature enzyme is consist of 119 amino acids and a

27 amino acid segment of signal peptide. The sequenced major PLA_2 with p*I* 4.991 shows a high degree of sequence homology to those PLA_2 of the same or closely-related genus.

Bee venom phospholipase A_2 shows very puzzling differences from the vertebrate secreted enzymes and it is structurally distinct from the class I/II super-families. The amino acid sequence was elucidated by Shipolini *et al.*, (1971), Shipolini, (1974) and Maraganore *et al.*, (1986) and sequence deduced from cDNA clone showed some difference from the chemically determined one. Nevertheless the segments which contain the residues involved in the Ca^{2+} binding and catalysis were found to be the same (Maraganore *et al.*, 1987; Kuchler *et al.*, 1989). Bee venom PLA_2 has also been crystallised by Scott *et al.*, (1990) in a complex with the phosphonate transition state analogue, and it has been shown that the functional sequences in class I/II PLA_2's were highly conserved in the bee venom enzyme, but in a different architecture.

Recently Fremont *et al*, (1993) have crystallised phospholipase A_2 from the venom of Indian cobra (*Naja naja naja*). The x-ray crystal structure was determined to 2.3Å resolution by molecular replacement techniques using a theoretical model constructed from homologous segment of the bovine pancreatic, porcine pancreatic and rattlesnake venom crystal structures. The 119 amino acid enzyme has an overall architecture strickingly similar to the other known PLA_2 structures with region implicated in catalysis showing the greatest structural conservation. In the crystal structure of *Naja naja naja* PLA_2, it was found unexpectedly that three monomers

occupy the asymmetric unit and are oriented with their catalytic sites facing the pseudo-threefold axis with ≈15 % of the solvent accessible surface of each monomer buried in trimer contacts.

1.8.) Phospholipase A_2 assays.

Numerous methods have been described for assaying the action of phospholipase A_2 on different forms of phospholipid substrates, 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. De Haas *et al.*, (1971) and Wells (1972) used titration methods to study the kinetics of pancreatic and some venom phospholipase A_2 enzymes. They reported that the activity of 1µg protein could be detected. Wells (1972), Canziani *et al.*, (1982) Bon and Saliou (1983) described colorimetric assay for phospholipase A_2 based on the fact that the proton release due to the ester hydrolysis changed the spectrum of phospholipase A_2 by some of the dyes used. Radio-labelling and thin layer chromatography were also used to determine the activity of the phospholipase A_2 enzymes. Van den Bosch and Aarsman (1979), Grossman *et al.*, (1974), Shakir (1981), Dey *et al.*, (1982) and Katsumata et al., (1986) have reported the use of this method for the detection of intracellular phospholipase A_2 by using either a labelled fatty acyl chain or labelled lysophospholipids.

Spectrophotometric assays were introduced for the measurement of phospholipase A_2 activity by Aarsman (1976) based on the use of thio-ester substrates which can be detected spectrophotometrically after reaction with Ellmans reagent. This method was applied for measurement of pancreatic phospholipase A_2 enzyme activity on monomeric lecithins by Volwerk (1979) and the degree of sensitivity was found to be 100-fold greater than the titrimetric method.

Lawrence (1971), Lawrence and Moores (1972) and Lawrence (1979) developed an assay system based on the change in solution conductance when a neutral ester is hydrolysed to yield a fatty acid anion and a buffer cation. This is a general method for many enzymes and has been used to measure Phospholipases A_2, C and D activities in addition to protease activities (Drainas and Drainas, 1985; Mezna and Lawrence, 1994). It is also used to measure erythrocyte leakage (Chettibi, 1990, Chettibi *et al.*, 1990) as the basis of indirect assays for PLA_2 and also for lytic peptides.

The main advantages of this apparatus over the conductimetric assay methods described by other workers is the ability to follow very early changes and so to obtain accurate initial rates and also to run simultaneous control reactions that can be subtracted to leave only the conductance change due to the reaction. Applying this method it was possible to detect the activity of 1ng of protein with a high degree of accuracy.

1.9. Aims of project.

Many proteins are post-translationally modified by covalent addition of long-chain fatty acyl groups either to -SH groups or to the terminal-NH_2 group (Schmidt, 1989; Magee, A. and Courtneidge, 1985; Magee, 1990). The acyl chain is believed to play a significant role in targeting proteins to their cellular designation, but there is no convincing evidence that in vivo fatty acylation is responsible for the modulation of any enzymic activity. In the case of phospholipase enzymes the acyl chain could stabilise the interaction of the enzyme with the substrate surface and thus act indirectly to increase the catalytic activity.

Earlier work had shown that venom phospholipase A_2 enzymes are susceptible to activation by long chain fatty acylating agents (Drainas and Lawrence, 1978; Camero-Diaz *et al.*, 1985; Chettibi *et al.*, 1990), but the nature of the chemical modification was unknown. Kinetic evidence showed that this activation was not determined by increased substrate affinity (Drainas, *et al.*, 1978), but by an increase in the stability of an active conformation (Camero-Diaz *et al.*, 1985). Two models for activation have been suggested:-

1) That the acyl-chain acts as a hydrophobic anchor to the lipid surface.
2) That the acyl-chain is buried within the protein where it cannot act as an anchor, but increases the catalytic activity of the enzyme by forcing a conformational change.

The aims of present study were:-

1) To develop electrophoretic method (s) to monitor phospholipase A_2 isoform purification.

1) To find and purify an abundant isoform that could be activated by acylation.

2) To find and purify an abundant isoform that was resistant to activation by acylation.

3) Try to characterise the site of acylation

4) To study the mechanism of activation of bee venom phospholipase A_2 and acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* and to distinguish between the hydrophobic anchor model and conformational change model.

Chapter Two

Materials and Methods

2.1.) Gel Electrophoresis.

Three types of gel electrophoresis were used in this study

2.1.1.) Basic/Urea polyacrylamide gel electrophoresis (Basic gels).

In this study basic urea gels were prepared in two different ways. The first type was developed as described by Ahmad and Lawrence (1993) and second type was a further modification of method of Chettibi *et al.*, (1989). In the first type, the stock 20 % urea gel mixture was made by adding 20 g acrylamide monomer (NBS Biologicals, North Mymms, Hatfield, Herts, England) 0.8 g of N,N^{-}-Methylenebis-acrylamide (Fisons scientific equipment, Loughborough, England) and 36 g of urea (BDH laboratory supplies, Poole, England) in distilled water to a total volume of 100 ml. The 18 % and 16 % acrylamide gels, were prepared by diluting with 6 M (i.e. 36 g per 100 ml) urea solution. The basic urea gels were then prepared by addition of 200 µl of ethanolamine (2 % v/v, Aldrich chemical Co. Ltd., Dorset, England) to 10 ml of these solutions and polymerised by addition of 50 µl of 10 % ammonium persulphate (Sigma Chemical Co. Ltd.) and 10µl TEMED (N,N,N^{-},N^{-}-tetramethylethylenediamine, 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 gel was polymerised at 37 ^{0}C in an incubator in order to obtain uniform setting. The gel dimensions were either 1 x 140 x 110 mm or 1 x 190 x 160 mm. Ethanolamine (2 % v/v) was used as electrolyte in both tanks. Samples were prepared by 1:1 v:v addition to a solution of 0.005 % bromophenol blue in 50 % sucrose and migration was toward the anode.

The second type of basic urea gel was modification form of first type (Ahmad *et al.*, 1994). In this type of gel a number of changes were made. Urea concentration was increased from 6 to 8 M and a 7% spacer gel was cast on top of the running gel. To 15 ml mixture of 20 % urea gel mixture (20 g acrylamide, 0.8 g bisacrylamide and 48 g urea in distilled water to a total volume of 100 ml) 300 µl of ethanolamine was added and polymerised by addition of 15 µl of TEMED and 75 µl of 10% ammonium persulphate. The final mixture was mixed and poured into the gel plates and about 300 µl of ethanol was layered on the running gel in order to n sharp boundry. .. The running gel was set in about 15 minutes at 37 ^{o}C. The ethanol layer was poured off and running gel was rinsed with spacer gel solution(7 %) twice, prepared by diluting 20 % gel urea mixture with 8 M urea To 10 ml of 7 % spacer gel mixture 200 µl of ethanolamine was added and polymerised by the addition of 10 µl of TEMED and 50 µl of 10 % ammonium persulphate and poured into gel plates with the comb positioned. The spacer gel took about 20-30 minutes to set.

2.1.2.) Propionic acid/urea polyacrylamide gel electrophoresis (Acidic gels).

These types of gel were based on the method of Panyin and Chalkley (1969) and were developed by Chettibi and Lawrence (1989) for the study of bee venom peptides. In this study the acidic gels were also of two types, the first based on the method of Chettibi and Lawrence and second type which is modified form of first type (Ahmad *et al.*, 1994). The Acidic urea gels were then prepared by addition of 200 µl of propionic acid (2 % v/v, Aldrich chemical Co. Ltd., Dorset, England) to 10 ml of 20 % acrylamide solutions containing 6M urea and polymerised by addition of 50 µl of 10 %

ammonium persulphate (Sigma Chemical Co. Ltd.) and 10 µl TEMED (N,N,N',N'-tetramethylethylenediamine, 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 polymerised at 37 ^{0}C in an incubator in order to obtain uniform setting. Acetic acid (2 % v/v) was used as electrolyte in both tanks. The second type of gel was prepared similarly to the second type of basic urea gels except that propionic acid was used instead of ethanolamine and 2 % acetic acid was used as electrolyte in both tanks. The acidic urea gel took a longer time to set compared to basic urea gels. Samples were prepared by mixing at 1:1 v/v with neutral red dye solution (0.1 % neutral red and 50 % glycerol). Gels were run with 2 % acetic acid in both anode and cathode compartments at 20 mA until the red dye approached the bottom of the gel (about 2 hours).

2.1.3.) SDS gel electrophoresis.

Details of methodology vary with the type of gel and buffer system selected but certain chemicals and solutions are common to many methods.

Acrylamide-bisacrylamide. 30 %

Acrylamide	28.5 g
Bisacrylamide	1.5 g

To distilled water to make final volume of 100 ml

Upper tank buffer x 5

Trizma base (Tris [Hydroxymethyl]aminomethane)	31.6 g
Glycine	20.0 g
Sodium dodecyl sulphate (SDS)	5.0 g

To distilled water to make final volume of 1 litre.

Lower tank buffer x 5

Trizma base	60.5 g
SDS	5.0 g

To 1 litre distilled water and pH was adjusted to 8.1 with 5 M HCl

Running gel buffer

Trizma base	18.15 g
SDS	0.4 g

pH 8.9 with 5 M HCl and make the volume to 100 ml with distilled water

Stacker gel buffer

Trizma base	5.9 g
SDS	0.4 g

pH 6.7 with 5 M HCl and total volume of 100 ml with distilled water.

Boiling mixture

Stacker gel buffer	2.5 ml
Merceptoethanol	2.5 ml
SDS	1.0 g

The mixture was warmed until SDS dissolved completely then about 0.5 ml of glycerol and of 1 % bromophenol blue were added. The volume was

made to 10 ml with distilled water. Samples were prepared by mixing 1:1 v/v with boiling mixture.

The gels were prepared according to following protocol

	Final acrylamide concentration (%)					
	5	**6**	**7.5**	**10**	**12.5**	**15**
30 % acrylamide (ml)	4	9.6	12	16	20	24
R. G. B. (ml)	-	12	12	12	12	12
S. G. B. (ml)	6	-	-	-	-	-
Dis. water (ml)	14	26.4	24	20	12.4	8.4
Glycerol (ml)	-	-	-	-	3.6	3.6
TEMED (µl)	20	20	20	20	20	20
APS (µl)	200	150	150	150	150	150

2.1.4.) Gel staining and destaining.

Gels were stained for 30 minutes with 0.1 % Coomassie brilliant blue G (Sigma Chemical Co. Ltd.) prepared with methanol 50%/water 50 %/acetic acid 7% and destained with destaining solution prepared with 50 ml methanol, 70 ml acetic acid to total volume of 1 litre with distilled water.

2.1.5.) Rhodamine 6G gel electrophoresis.

This type of gel was developed based on the principle of Shier and Trotter (1978). According to this method the phospholipase A_2 activity was detected following electrophoresis in polyacrylamide gels. A suspension of lecithin trapped in the gel matrix is used as the substrate. Enzyme activity

was detected by using rhodamine 6G to stain unsaturated free fatty acids released by enzyme action. This type of electrophoresis was carried out on basic urea gels. To 15 ml of 20 % acrylamide/urea mixture purified egg lecithin was added to make a final lecithin concentration of 5mg/ml. The mixture was sonicated and 300 µl of ethanolamine was added and then polymerised with the addition of 75 µl of APS and 15 µl of TEMED. The solution was poured into gel plates and about 300 µl of ethanol was layered on. The running gel set in about 15 minutes. And 7 % spacer gel was prepared by diluting 20 % acrylamide/urea with 8 M urea. To 10 ml 0f 7% mixture EDTA was added to final concentration of 1 mM and 200 µl of ethanolamine was added and gel was polymerised by addition of 50 µl of 10 % APS and 10 µl of TEMED. The ethanol layer was poured off and gel was washed twice with spacer gel solution and poured into gel plates with the comb positioned. Ethanolamine (2 %) containing 2 mM EDTA was used as an electrolyte buffer in both tanks. Samples were prepared either in the presence or absence of bromophenol blue containing 2 mM EDTA. The phospholipase A_2 activity in the gel was localised by incubating the gel at 37 ^{o}C with gentle shaking in a bath containing 100 to 200 ml of 0.1 M triethanolamine buffer of pH 8.0 containing 20 mM $CaCl_2$, and melittin (5 µg/ml) and diluted with 10 % by volume of a 0.12 % aqueous solution of rhodamine 6G. When one or more unit of PLA_2 activity liberates 1 µmol of titratable fatty acid per min from a lecithin emulsion at pH 8.0 and 25 ^{o}C was used in the gel, dark-red bands indicating the presence of free unsaturated fatty acids became visible in about 10 minutes even without washing out excess dye. At the end of incubation period, the gels were washed with numerous changes of water, which ultimately removed all the dye from the cleared zone. However, when very high levels of

phospholipase A_2 activity were used, stained central spots that were resistant to extraction with water remained. The washing procedure did not stop the enzyme activity if EDTA was not added to the washing bath. The gels were photographed with reflected white light against a black background.

2.2.) Protein purification.

Lyophilised whole venoms of *Naja mossambica mossambica, Naja naja, Naja haje, Naja nivea, Naja hannah* (king cobra) , *Naja naja atra, Naja nigricollis nigricollis, Naja nigricollis pallida, Naja nigricollis crawshawii, Naja melanoleuca, Crotalus atrox, Crotalus adamanteus, Bungarus fasciatus, , , Vipera russelli* (Russell's viper), *Enhyrina schistosa* (common sea snake), *Agkistrodon piscivorus piscivorus* and Bee venom (*Apis mellifera*) were purchased from Sigma Chemical Co. Ltd., Dorset, England and dissolved in distilled water in the presence of protease inhibitor phenyl methyl sulphonyl fluoride (PMSF). All buffers used for protein purification were prepared from 20 mM ammonium acetate and the pH was adjusted by addition of ammonia solution. Bio-gel P-30 was purchased from BioRad, Hertfordshire, U. K., and prepared as a column (3.5x40 cm) in 20 mM ammonium acetate, pH 6.35, which was also used as eluting buffer. DE-52 purchased from Whatman Ltd column were prepared as described elsewhere for appropriate proteins and equilibrated in 20 mM ammonium acetate buffers (pH range 9.8 to 5.0). Column effluents were monitored by UV absorption either at 206 or 280 nm, by gel electrophoresis and by PLA_2 activity measurements using the conducti-

metric modified method as described elsewhere. Gradients were made using a simple sealed gradient former with pumped efflux with passive connection to a reservoir of the second buffer.

2.3.) Identification and recovery of enzyme activity.

The number of new isoforms of PLA_2 were identified in *Naja mossambica mossambica, Naja naja atra* and *Agkistrodon piscivorus piscivorus* venoms by basic urea gel electrophoresis, as phospholipase A_2 activity was recovered from basic and acid urea gels. Enzymes were subjected to basic urea gel electrophoresis such that dye front migrated > 135 mm. The purified phospholipase A_2 isoforms were run into two lanes, experimental and guide lane in basic urea gels. These were separated and the guide lane stained with Coomassie blue. The experimental lane was aligned with the guide lane, cut into 1-1.5 mm slices and extracted by macerating in 10 mM triethanolamine buffer of pH 8.0 in an ependorf tube using a thin glass rod, the suspension was then centrifuged for 15 minutes and filtered by means of 1 ml syringe connected to a filter chamber. The samples were assayed as described elsewhere but using 1 mM $CaCl_2$ to ensure maximum sensitivity. All assay were made in triplicate. Phospholipase A_2 activity was recovered with high yield. In order to recovered PLA_2 activity from acidic urea gels, the gels were prerun for about three hours. Both the upper and lower tanks buffer were changed and then enzymes were run in two lanes as experimental and guide lane. And enzymes were extracted as described above. PLA_2 activity was also recovered with high yield. For analysis of *Naja naja atra* PLA_2 isoforms,

different fractions from Biogel P-30 column of were run and a new minor isoform was identified by chromogenic assay as described in rhodamine 6G gel electrophoresis. Similarly new isoforms in the venom of *Agkistrodon piscivorus piscivorus* were identified by chromogenic assay method by using different Bio-gel P-30 fractions.

2.4.) Preparation of buffers.

2.4.1.) Isotonic saline.

Isotonic saline buffer was used for the preparation of mammalian erythrocytes, which was standard isotonic saline buffered with 10 mM MOPS, pH 7.4. Nine grams of sodium chloride (NaCl, Fisons scientific equipment, Loughborough, England) was dissolved in 900 ml of distilled water in the presence of 10 ml of 1 M NaOH. The pH was adjusted to 7.4 with MOPS (Sigma Chemical Co. Ltd.) and final volume was made upto 1 litre with distilled water.

2.4.2.) Erythrocyte assay buffer.

These assays required a low conductance, isotonic buffer which was prepared by dissolving 98 g of sucrose (Fisons scientific equipment, Loughborough, England) in 900 ml of distilled water containing 10 ml of 1 M NaOH. The pH was adjusted to 7.4 with MOPS and final volume was made up to one litre with distilled water.

2.4.3.) Triethanolamine buffer (cationic buffer.

The stock solution of 200 mM triethanolamine was prepared by mixing 40 ml of 5 M hydrochloric acid (HCl) in 900 ml of distilled water and pH was brought to 8.0 with triethanolamine (Sigma Chemical Co. Ltd). The final volume was made up to one litre with distilled water. The stock solution was diluted to 10 mM for use.

2.5.) Preparation of erythrocytes.

About 20 ml of blood was taken from New Zealand white rabbits (from animal house, Department of Physiology, Glasgow University) and collected into a universal containing heparin (Sigma Chemical Co. Ltd., 0.1-0.2 mg/ml of blood). The blood was centrifuged at 3000 rpm for 15 minutes, the serum and buffy coat were removed by aspiration with vacuum pump and the cells were resuspended in isotonic saline. The washing procedure was repeated for three times and finally the erythrocytes were made up to 33 % v:v with isotonic saline. The erythrocytes were stored in the fridge and used within five days.

2.6.) Preparation of substrates.

2.6.1.) Analysis and detection of phospholipids.

Phospholipids were routinely analysed by thin layer chromatography (TLC) as follows. A drop of solution of phospholipids were spotted onto the silica gel coated, plastic TLC sheets (Mark AG Darmstaf, Germany) and

allowed to dry. The chromatogram was developed by a solution of chloroform/methanol/acetic acid/water, 25/15/4/2 respectively. The chromatogram 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 blue/green spot which faded very rapidly. To detect amino phospholipids phosphatidyl ethanolamine and phosphatidyl choline, the chromatogram was sprayed with ninhydrin reagent and heated to develop the full colour tensity. After this, it was dipped in molybdenum blue to detect all phospholipids.

2.6.2.) Preparation of egg yolk lecithin.

Lecithin (1,2 acyl-3-sn glycerophosphatidylcholine) was prepared by the method of Brockerhoff and Yurkowski (1965). Three dozen egg yolks were separated, homogenised in 500 ml acetone and filtered to remove the acetone along with the yellow pigments 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 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 phospholipids corresponding to phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE).

2.6.3.) Purification of lecithin.

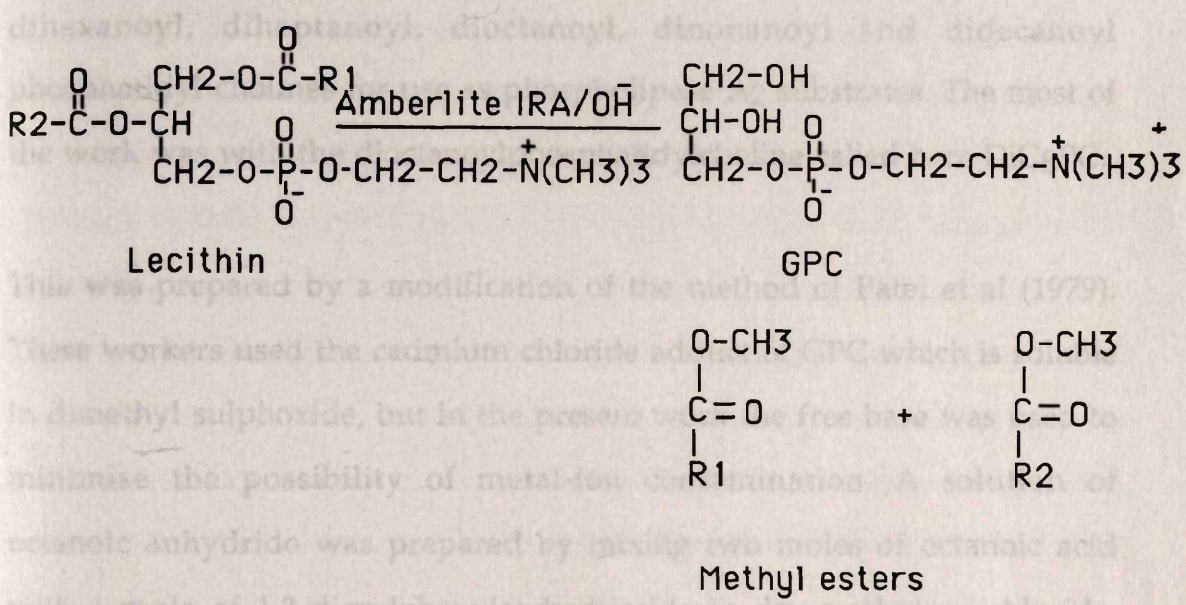
The egg yolk lecithin prepared above was further purified on an alumina column (aluminium oxide, activated, neutral, Aldrich Chemical Co. Ltd., Dorset, England). The main phospholipids of lecithin are phosphatidyl ethanolamine and phosphatidyl choline. The lecithin was dissolved in chloroform and applied to alumina column, prewashed with chloroform. The column was washed two or three times with chloroform until all phosphatidyl ethanolamine was eluted and there was no blue band on TLC plates. The phosphatidyl choline was then eluted with a mixture of chloroform/methanol in ratio of 10:1.

2.6.4.) Preparation of glycerophosphorylcholine (GPC).

GPC was prepared by a new method (Lawrence et al. unpublished results). 60 g of crude lecithin (PC+PE) was dissolved in 500 ml methanol and mixed with a strong base anion-exchanger resin (Amberlyst A26, BDH, Co. Ltd.) which was prepared in its hydroxide form by mixing 100 ml of 1 M NaOH solution with 50 g Amberlyst resin, stirred for 15 minutes and washed four times with distilled water and then four times with ethanol. The mixture of lecithin-resin was stirred at 55 ^{o}C.

The progress of the reaction was judged by spotting the solution on the TLC sheet and staining with molybdenum blue. Reaction progress was assessed from the colour and stability of the spot (lecithin and lysolecithin

gave stable blue spot but GPC gave a blue greenish spot which fades completely within 5 to 10 minutes). Using this test the reaction was found to reach completion in two to three hours.

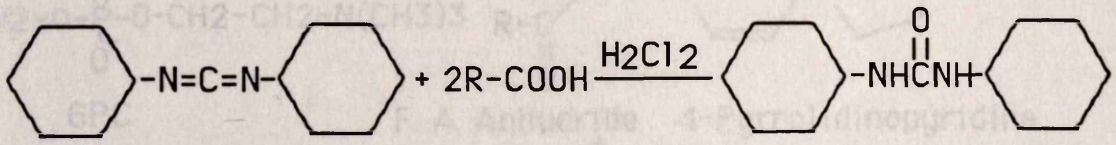


The resin was removed by filtration and the solvent evaporated off leaving an oil residue which we believed was GPC contaminated with glycerol. Tests showed that the only solvent which could dissolve glycerol, but not GPC was dimethyl-formamide (DMF). The residue was therefore washed with dimethyl formamide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

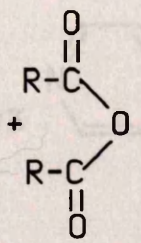
2.6.5.) Preparation of dioctanoylphosphatidylcholine.

Glycerophosphorylcholine (GPC) was used for the preparation of dihexanoyl, diheptanoyl, dioctanoyl, dinonanoyl and didecanoyl phosphatidyl cholines for use as phospholipase A_2 substrates. The most of the work was with the dioctanoylphosphatidylcholine 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-dicyclohexylcarbodiimide in dry methylene chloride. After the reaction was over the solution was filtered and evaporated. The reaction proceeds as follow.

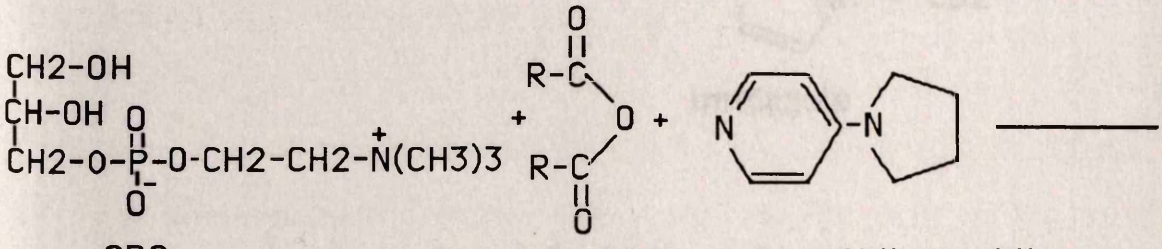


dicyclohexylcarbodiimide Fatty acid Dicyclohexylurea

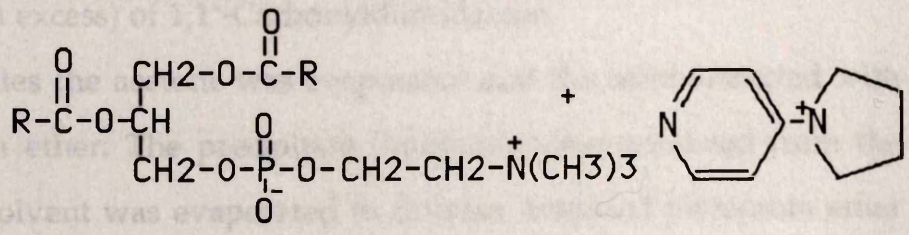


Anhydride

Two ml of the anhydride was dissolved in 10 ml of dry benzene containing 0.145 g (1 mmol) 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 alumina column which was washed with chloroform and product eluted with 1 to 8 v/v methanol/chloroform. The elute 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 (DiC8PC), was weighed, dissolved in methanol and deionised with Amberlite, monobed resin MB-3 (BDH Chemical Ltd. Poole, England)



GPC F. A. Anhydride 4-Pyrrolidinopyridine

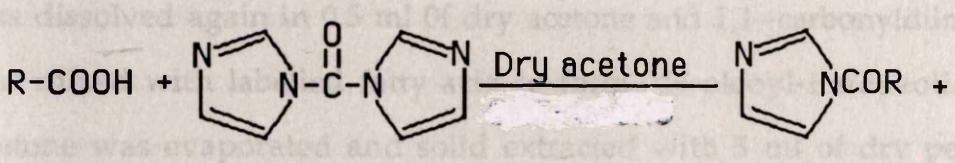


Diacylphosphatidyl Choline

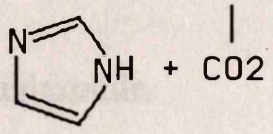
2.7.) Preparation of activators.

2.7.1.) Preparation of fatty acylimidazolide.

The stock solution of 200 mM fatty-acyl imidazolide of different fatty acids were prepared by mixing 1:1 of free fatty acid (Sigma Chemical Co. Ltd.) and 1,1'-Carbonyldiimidazole (slightly excess)(Aldrich Chemical Co. Ltd.) in 1 ml of dry acetone and used without further purification.



Fatty acids 1,1-carbonyldiimidazole Fatty acid imidazolide



Imidazole

The pure acylimidazolide was prepared by mixing one molar equivalent of free fatty acid dissolved in acetone with two molar equivalents (in excess) of 1,1'-Carbonyldiimidazole.

After 10 minutes 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 traces of imidazole from the fatty

acylimidazolide, then the solvent was evaporated and the solid of pure fatty acylimidazolide (activator) was stored (A. J. Lawrence , unpublished result). The stock solution of 200 mM was diluted to 4 mM for use.

2.7.2.) Preparation of ^{3}[H]-oleoyl-imidazolide.

^{3}H-oleoyl-imidazolide was prepared by mixing 0.14 mg of ^{3}H-oleic acid with 0.56 mg of cold oleic acid in 1ml of toluene and about 150 µl of this diluted solution was stored as such. The solvent was evaporated and solid was dissolved again in 0.5 ml 0f dry acetone and 1,1--carbonyldiimidazole was mixed with labelled fatty acid to form ^{3}H-oleoyl-imidazolide. The acetone was evaporated and solid extracted with 5 ml of dry petroleum ether to remove any excess imidazolide. The solvent was then removed by freeze-drying and the solid was dissolved again in 0.5 ml of acetone.

2.7.3.) Activation of phospholipase A_2 by acyl-imidazolide.

This was carried out as described by Camero-Diaz et al (1985). Five µl of 200 mM triethanolamine buffer was added to 95 µl of 1 mg/ml phospholipase A_2 and then treated with 2 µl of 4 mM fatty acid imidazolide to give equimolar amounts of activator and enzyme. The reaction reached to completion after one to two hours incubation at 37 ^{o}C.

2.8.) The determination of protein concentration.

Several methods have been developed for the determination of

protein concentration. Over the years many indirect and direct techniques have been described for the measurement of protein concentration (Gaspar, 1980). Of the former procedures, the most important technique is the determination of nitrogen by Kjeldahl method. The latter group includes procedures such as infrared spectrophotometry, turbidimetry, fluorimetry, refractometry and polarography. If the primary sequence of the protein under investigation has been established, then analysis of amino acids composition can yield accurate quantitative data. It should be remembered that certain amino acids , especially tryptophan, cystine and cysteine, are particularly sensitive to acid hydrolysis, making data on these amino acids unreliable. In addition, glutamine and asparagine are converted to their respective acids by acid hydrolysis from they therefore indistinguishable. In practice, of course, the requisite amino acid sequence data will often not be available to the purification scientist. The methods most commonly used today for the measurement of protein concentration are based either on ultraviolet (UV) absorption of the protein solution or visible region spectrophotometry after reaction of the protein with a chemical to generate chromophores.

Here in order to measure the concentration of the bee venom phospholipase A_2, three different methods were used, ultraviolet spectrophotometry, visible spectrophotometry using bradford reagent and the most accurate amino acids analysis. Most protein exhibit an absorption maximum at 280 nm which is attributable to the phenolic group of tyrosine

and the indolic group of tryptophan. The extinction coefficient, usually expressed either as $E_{280}^{1\%}$ or $E_{280}^{1mg/ml}$, varies significantly from protein to protein depending on the precise amino acid composition. Values of $E_{280}^{1mg/ml}$ for most proteins lie in the range 0.4-1.5, but extremes include some parvalbumins and related Ca^{2+}-binding proteins(0.0) and lysozyme (2.65). The method is consequently rather inaccurate unless the protein is relatively pure and its extinction coefficient is known and calibrated against dry weight. We used bee venom phospholipase A_2 enzyme sample, and other sample was 1mg/ml albumin was taken as a standard. Their absorptions at 280 nm were measured.

Absorption of sample	0.326
Absorption of 1mg/ml albumin	0.494

And the concentration of sample 1 was calculated on the basis of albumin was 0.65 mg/ml.

When the concentration of the same sample was determined using Bradford reagent and their absorption was measured at 595 nm. The concentration of the bee venom phospholipase A_2 was calculated (using 1mg/ml albumin as a standard), it was this 0.33 mg/ml. The spectrum of the same sample is shown in the figure 2.8.I.

Here measurement of the concentration of phospholipase A_2 was done by amino acid analysis using high performance liquid chromatography (HPLC) on 820-FP spectrofluorometer with taurine as internal standard for analysis. The table 2.8. shows the

Figure. 2.8.I. Ultraviolet spectrum of the bee venom phospholipase A_2. Purified bee venom phospholipase A_2 was dissolved in distilled water and ultraviolet(UV) absorption spectrum was recorded between 220 and 320 nm using Shimadazu UV-160A, UV-Visible recording spectrophotometer.

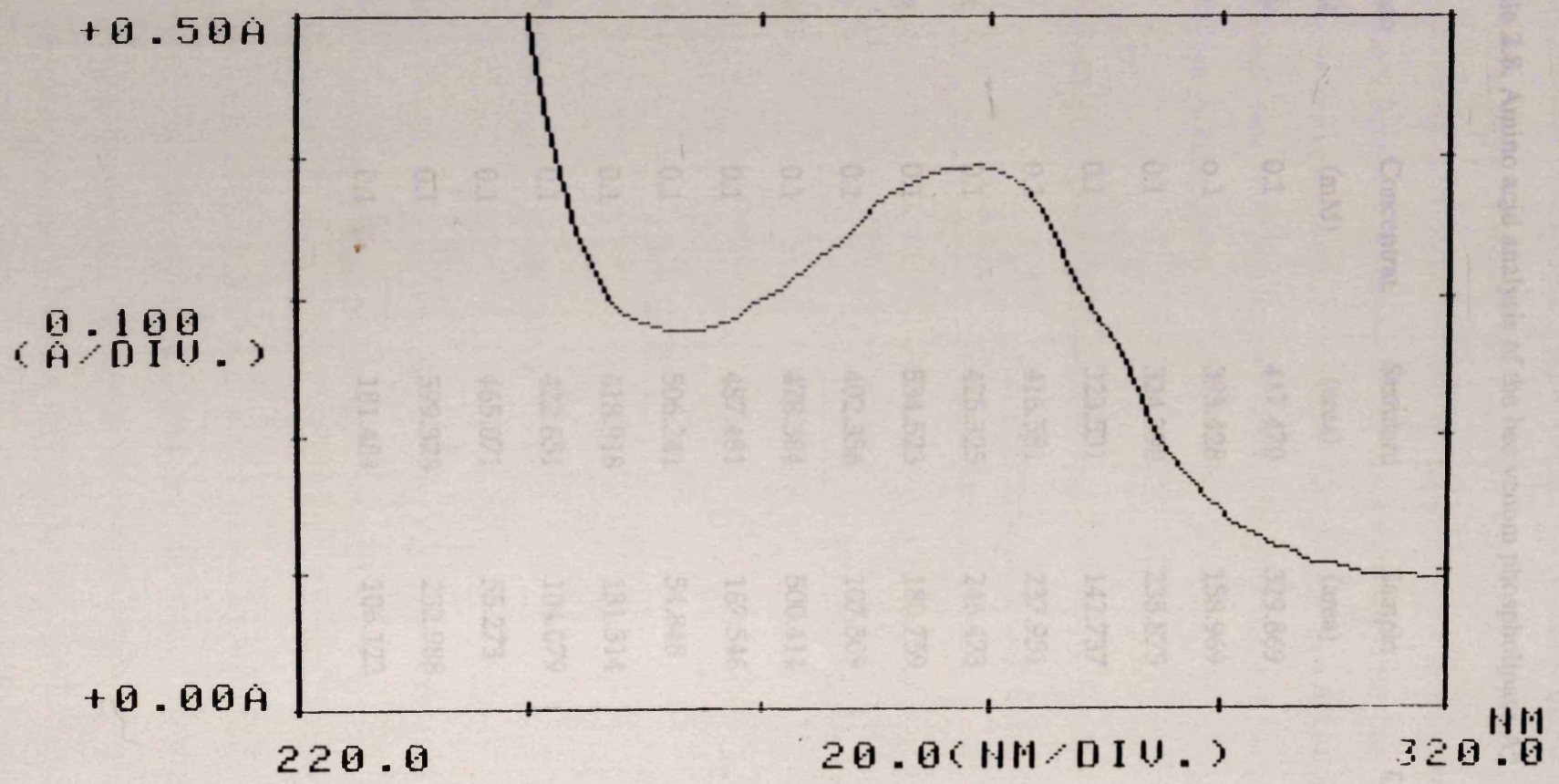

+0.50A
0.100
(A/DIV.)
+0.00A
220.0
20.0(NM/DIV.)
320.0
NM

Table 2.8. Amino acid analysis of the bee venom phospholipase A_2

Amino acids	Concentrat. (mM)	Standard (area)	Sample (area)	Corrected (mM)
Asp	0.1	417.470	329.869	0.15
Glu	o.1	393.428	158.969	0.08
Ser	0.1	324.390	235.875	0.14
His	0.1	323.531	142.737	0.08
Gly	0.1	416.581	237.951	0.11
Thr	0.1	425.325	246.473	0.11
Arg	0.1	534.523	180.759	0.06
Ala	0.1	402.356	107.509	0.05
Tau	0.1	478.384	500.414	
Tyr	0.1	487.481	167.546	0.07
Met	0.1	506.241	54.848	0.02
Val	0.1	618.918	131.314	0.04
Phe	0.1	422.851	104.079	0.05
Ile	0.1	465.071	55.273	0.02
Leu	0.1	599.328	232.988	0.07
Lys	0.1	181.484	106.122	0.11

concentrations of standard amino acids and concentrations sample amino acid. The area was calculated from figure 2.8.II. by computerised programming. The corrected concentration of each amino acids (as shown in table 2.8.) were calculated by the following formula and from the corrected concentrations of amino acids the concentration of sample was calculated, this time the concentration was 0.12 mg/ml. This concentration was taken as standard.

Calculation Of Amino acid concentration

$$= \frac{\text{Area of sample A. A.}}{\text{Area of std. A. A.}} \times 0.1 \times DF = mM$$

0.1= Concentration of std run

DF=Dilution factor

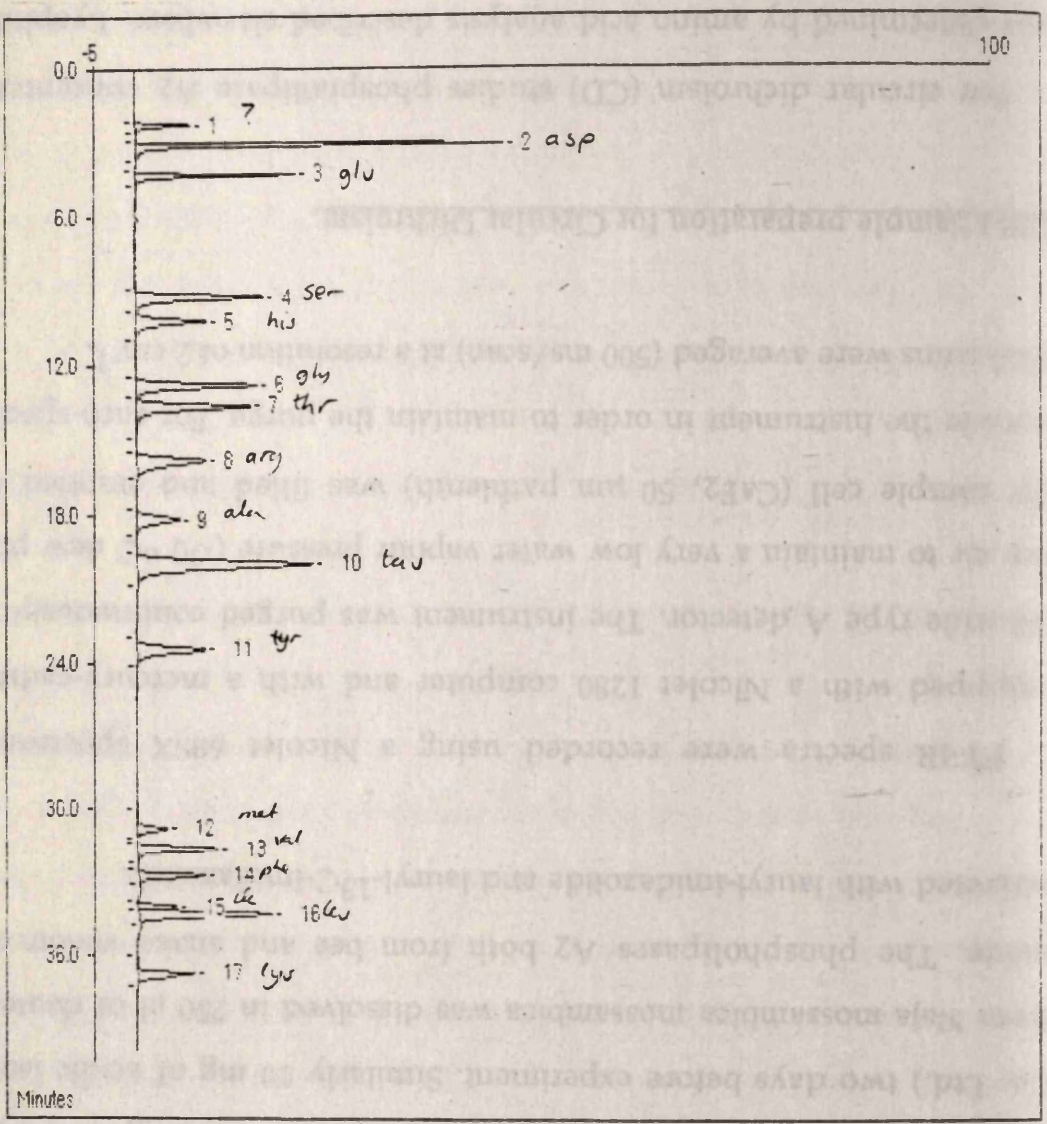
2.9.) Sample preparation for FT-IR.

2.9.1.) Preparation of activator.

For FT-IR studies phospholipase A_2 was activated with lauryl-imidazolide, which was prepared as follow.

One molar equivalent of lauric-1-^{13}C acid (99 atom % ^{13}C, Sigma Chemical Co. Ltd.) was mixed with 1 molar equivalent (used in slightly excess) of 1,1--carbonyldiimidazole in 1 ml of dry acetone. The solvent acetone was evaporated to dryness and solid was dissolved in acetonitrile. The activator was used as such without further purification. Similarly 200 mM lauryl-imidazolide was prepared in dry acetone and acetone was evaporated to dryness and solid was dissolved in acetonitrile.

Figure 2.8.II. Amino acid analysis of the bee venom phospholipase A_2. The bee venom PLA_2 sample (500 µl) was added to thurnberg tube and 500 µl Aristar HCl and 10 µl of 10 mM taurine (internal standard) was also added. The tube was sealed under vacuum and hydrolyse in oven for 24 hours at 106°C. The tube was removed, cooled and placed in a dessicator and lyophilised to dryness and the sample was washed twicely with 500 µl of water and lyophilised and was dissolved in 1 ml distilled water. From 1 ml of sample 500 µl was diluted to 1 ml (1:2 dilution) with dis. water. and ready for analysis. Twenty µl sample was injected onto HPLC for analysis using Phenomenex ULTRACARB C8 5µm column and 820-FP spectrofluorometer.


-5
100
Peak
0.0
6.0
12.0
18.0
24.0
30.0
36.0
1 ?
2 asp
3 glu
4 ser
5 his
6 gly
7 thr
8 arg
9 ala
10 leu
11 tyr
12 met
13 val
14 phe
15 ile
16 leu
17 lys
Minutes

2.9.2.) Phospholipase A_2 preparation.

Bee venom phospholipase A_2 was purified as described elsewhere. The lyophilised 20 mg (on weight basis) bee venom phospholipase A_2 was dissolved into 1 ml of deuterium oxide (99.9 atom % D, Sigma Chemical Co. Ltd.) two days before experiment. Similarly 10 mg of acidic isoform from Naja mossambica mossambica was dissolved in 750 µl of deuterium oxide. The phospholipases A_2 both from bee and snake venom were activated with lauryl-imidazolide and lauryl-^{13}C-imidazolide.

FT-IR spectra were recorded using a Nicolet 60SX spectrometer equipped with a Nicolet 1280 computer and with a mercury-cadmium telluride type A detector. The instrument was purged continuously with dry air to maintain a very low water vapour pressure (-70 ^{o}C dew point); the sample cell (CaF_2, 50 µm pathlenth) was filled and emptied from outside the instrument in order to maintain the purge. For each spectrum 1000 scans were averaged (500 ms/scan) at a resolution of 2 cm^{-1}.

2.10.) Sample preparation for Circular Dichroism.

For circular dichroism (CD) studies phospholipase A_2 concentration was determined by amino acid analysis described elsewhere. Lyophilised acidic isoform of PLA_2 from venom of *Naja mossambica mossambicca* and bee venom PLA_2 were dissolved in 10 mM Borate buffer of pH 8.0. In both cases the solution was divided into two parts and one part was activated with oleoyl-imidazolide. C. D. spectra of native and activated bee and acidic form of snake venom phospholipases A_2 were recorded over

the range of 260 to 190 nm using a JASCO J-600 spectropolarimeter with IBM PS2 control and data acquisition. All spectra were recorded at 25 ^{o}C using cell path length of 0.1 cm.

2.11.) Sample preparation for fluorescence.

Bee venom PLA_2 and acidic isoform of PLA_2 from *Naja mossambica mossambica* were dissolved in 10 mM borate buffer of pH 8.0. Fluorescence spectra of native and activated forms of PLA_2 (with oleoyl-imidazolide) from bee and snake venom were recorded at 25 ^{o}C using Perkin Elmer Luminescence spectrometer LS 50B.

2.12.) The conductimetric apparatus.

The conductimetric apparatus was developed by Lawrence (1971). The original apparatus was six cell system, which was further modified by Lawrence (1975) and Chettibi et al (1990). The reaction cell was a glass tube of 1 cm diameter with 2 platinum electrodes of ca 2 mm diameter fused into the wall of the cell and sealed from the outside by magnetic pellet.
The cells were mounted in a water bath, temperature controlled by a high precision thermoster-operated relay working as a high gain on/off switch.
Results were presented in either numerical or graphical form as conductance values or differences between successive readings. Automatic subtraction facilities were provided for any cell selected as a blank (control).

2.12.1.) The circuit.

The measuring circuit is an AC bridge with reference (control) and cell arms. In the original design, a diode network was used to obtain a linear difference that was sign-dependent. The output from each of eight bridges was fed into an analogue selector switch.

The present circuit was redesigned using an ARC-PCB programme and built with low-noise operational amplifiers. The AC component of each arm of the bridge was fed through an analogue selector switch and then rectified in an active precision rectifier network followed by a voltage compactor. This circuit was more compact than the original and had lower noise characteristics, but the main source of noise appeared to be in the cells themselves.

2.12.2.) Calibration.

Because of 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 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 by numerical data processing. To obtain the correction values the following procedure was adopted. Two ml of 10 mM triethanolamine buffer of pH 8.0 was added to each cell and when the conductance reading 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 and the gradients used to determine the correction factors for each cell. These were simply multiplying factors which made all of the readings identical.

2.13.) Phospholipase A_2 assays.

Two different conductimetric assay methods were used to study the kinetics of phospholipases A_2 isoforms. The first assay was used in order to study the catalytic activity of phospholipase A_2 enzymes directly by following the hydrolysis of various phospholipid substrates, whereas the second assay was applied on erythrocytes by following the progress of leakage of electrolytes from intact cells, this was used for study of activation mechanism of phospholipases.

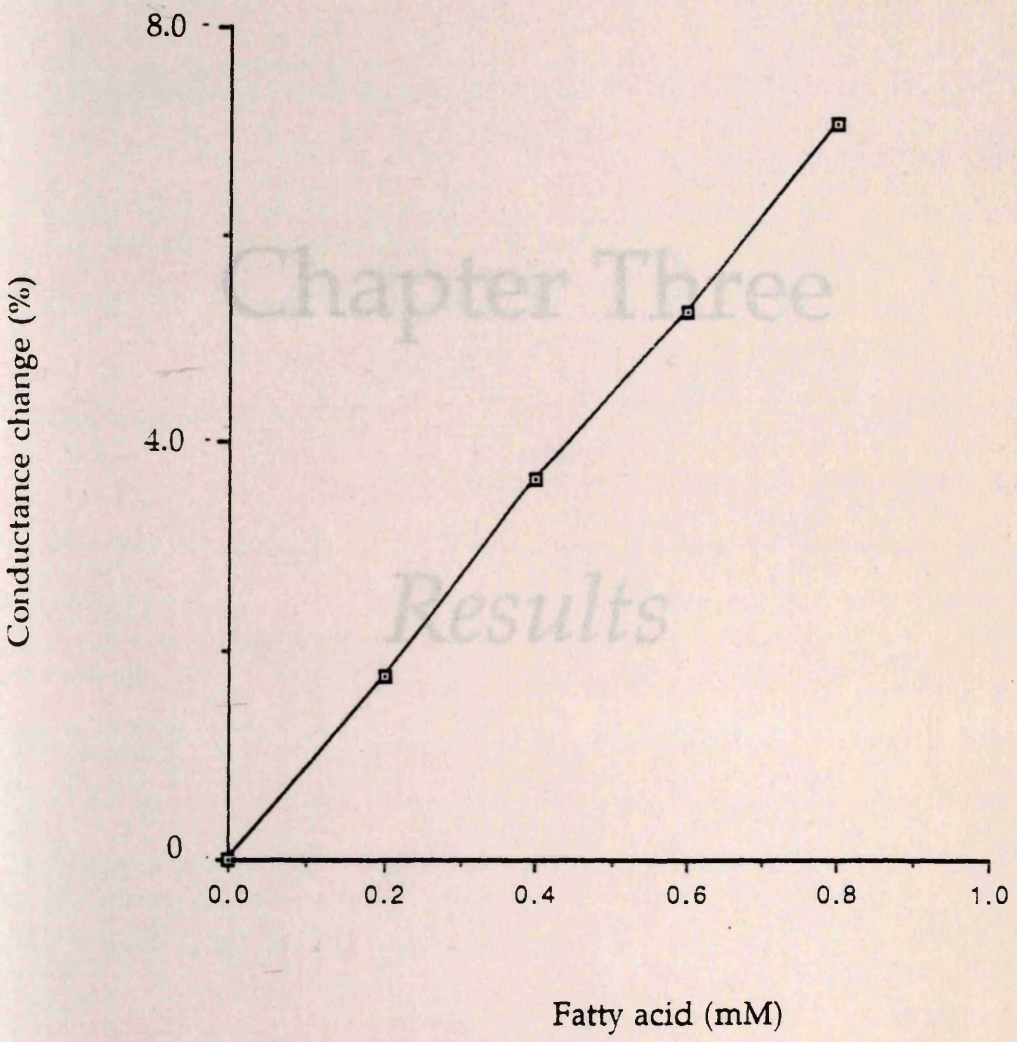
2.13.1.) Calibration of the phospholipase A_2 assay.

Calibration of phospholipase A_2 assay was carried out by repeated addition of 2 µl of 100 mM octanoic acid to 2 ml of 10 mM triethanolamine/Cl^- buffer of pH 8.0 to mimic the ionic change caused by phospholipid hydrolysis;

$$(CH_2\text{-}CH_2\text{-}OH)_3N + R\text{-}COOH \text{------} > (CH_2\text{-}CH_2\text{-}OH)_3N^+H + R\text{-}COO^-$$

The conductance change was corrected for dilution by carrier solvent and plotted against the fatty acid concentration. Fig. 2.12.1.

Figure 2.13.1. Calibration of the conductimetric assay with fatty acid.



The calibration was carried out by the addition of 2 µl aliquotes of 100 mM octanoic acid to conductivity cells containing 2 ml of 10 mM triethanolamine/HCl buffer of pH 8.0 at 37 °C.

Chapter Three

Results

Part I

3.1.1.) The development of basic/acidic-urea polyacrylamide gel electrophoresis.

Within the fields of protein and nucleic acid chemistry the majority of separation methods fall into three broad categories, namely those based on size differences, those based on the differences in the electrical charge carried by the molecules, and those based on some specific biological or chemical properties of the molecule under investigation. (Andrews, 1986).

Electrophoretic separations of proteins and peptides based on the single criterion of the size do not exploit the power of the technique to the full. Many samples that appear to be homogenous by size-based separation reveal heterogeneity of charge based mobility and this is the basis of the very high power of 2-dimensional electrophoresis. Experience has shown that electrophoresis based on native charge does not give high resolution unless a focusing method is employed, for reasons that remain obscure, if the separation is carried out in the presence of high concentration of urea and where electrolyte is either a weak acid or a single weak base. Urea PAGE is therefore is carried out under the acidic (pH 3.5), or the basic (pH 10.6) conditions.

The basic urea PAGE has not found a niche for protein separations although it is extensively used for nucleotide analysis. In part this is because the resolution given by existing methods does not approach that of SDS-PAGE or even acid urea PAGE and in part because there is no clearly perceived field of application.

Although PLA_2 enzymes from different venoms may vary in size, the isoforms in a given venom do not normally differ greatly in length and therefore they are not well resolved by SDS-PAGE (Evans, et al., 1980; Van Den Bergh et al., 1989). Many isoforms differ by variation in hydrophobic amino acids, but the majority vary in the content of charge residues and can therefore be separated by ion-exchange chromatography and identified by isoelectric focusing or by urea-based gel electrophoresis. Urea gels have a very considerable cost advantage over isoelectric focusing methods and are well suited for running large slab gels which are ideal for comparative studies.

The study of venom peptides had been carried out in this laboratory using acid urea PAGE with 6M urea and with propionic acid substituted for the more conventional acetic acid to improve gel setting and resolution. Although this proved to be a powerful method for analysing the venom components, when applied to one of the venom of interest here, *Naja mossambica mossambica*, it could not resolve two of the major isoforms. Inspection of the known sequences indicated that all isoforms should be separable on gels run at high pH. The acid/urea PAGE recipe was therefore adapted for the basic conditions using the minimum possible modifications. Initially the urea concentration was maintained at 6M and the setting reagents APS and TEMED used as in the acidic system. Early results confirmed that the basic urea PAGE could achieve high resolution of PLA_2 isoforms and experiments were carried out to improve the performance.

3.1.1.1.) The role of urea.

In contrast to SDS, the role of urea is completely obscure. There are no convincing explanations of the reason why it increases band sharpness in a dramatic fashion. Earlier workers have used varied concentrations, but in general higher concentrations give better resolution. However in the acidic urea PAGE there has been a tendency to use concentrations higher than 6M, Investigation of the use of 8M urea for both acidic and basic gels showed that this gave a substantial improvement in both cases.

3.1.1.2.) The role of the electrolyte.

The acidic or basic urea gels use a single weak acid or weak base electrolyte so that there is no counterion other than H^+ for acidic gels and OH^- for basic gels. Attempts to devise more complex buffer systems tend to decrease the quality of resolution. Thus the simple electrolytes are limited to weak acids and weak bases.

Thus the choice of acid or base determines the running pH which can be slightly modified by varying the electrolyte concentration. Experience has shown that a concentration of ca 2% is optimal. The running pH affects the molecular basis of separation because it determines the net charge of the protein. Table 3.1.1.2. shows the typical pKa values of protein side-chain residues and indicates that for acidic gels running below pH 4 K, H and R and the N-terminal group alone will be ionised whilst for basic gels running at pH [a]10.6 D+E+Y-R and the C-terminal group will be ionised. At intermediate pHs the ionisation state will be less readily

defined and there are therefore advantages in using pH extremes. However the most important factor was the quality of resolution and this was determined by investigation. Subjectively the results for basic gels indicated that ethanolamine gave significantly better results than ammonia which was significantly better than triethanolamine, and therefore ethanolamine was used in all subsequent work. Investigation of the effect of concentration indicated that 2% was significantly better than lower concentrations.

Table 3.1.1.2.

pK values of ionisable groups in proteins

Group	Typical pK
Aspartic acid	4.4
Glutamic acid	4.4
Histidine	6.5
Cysteine	8.5
Tyrosine	10.0
Lysine	10.0
Arginine	12.0

3.1.1.3.) Setting conditions.

Investigation showed that the basic gels could be set by APS in the absence of TEMED, however optimum results appear to be obtained with the use of TEMED. It was clear that the use of higher concentrations degraded resolution. For the acidic gels setting times decreased rapidly

with TEMED concentration but optimal results were obtained under the conditions described by Chettibi et at., (1989)

3.1.1.4.) Gel concentration.

Once the basic gel system had been established it was found possible to vary the concentration of acrylamide/bis acrylamide over a range from 10-22.5% with no other modifications.

3.1.1.5.) Stacking gels.

Because the basic or the acidic urea PAGE contain a single electrolyte and also because significant variation in pH might cause some components to change their migration direction, there was no simple stacking buffer programme possible. For this reason most such gels had been run as single gel systems. However it was clear that casting a single slab gel lead to very inhomgenous zones near the top surface. This could not be avoided as long as the well-forming combs were in place during setting. Thus it seemed desirable to cast the gel in two stages using an overlay for the main running stage. This presented the opportunity to test the use of a low concentration gel to act as a stacking gel where stacking was simply produced when the proteins encountered a gel density discontinuity. Thus the main running gel was cast with an ethanol overlay and this produced a very sharp upper boundary. On to top of this the well-forming stacking gel was cast using the same electrolyte concentration but with a gel concentration of 7%.

The earlier gels using s single slab with 6M urea produced rather varied quality results, which could be of high quality but the results of these modifications was to produce gels that ran consistently well. In particular the use if the stacking system removed the tendency for bands to show trailing edges especially at high loading.

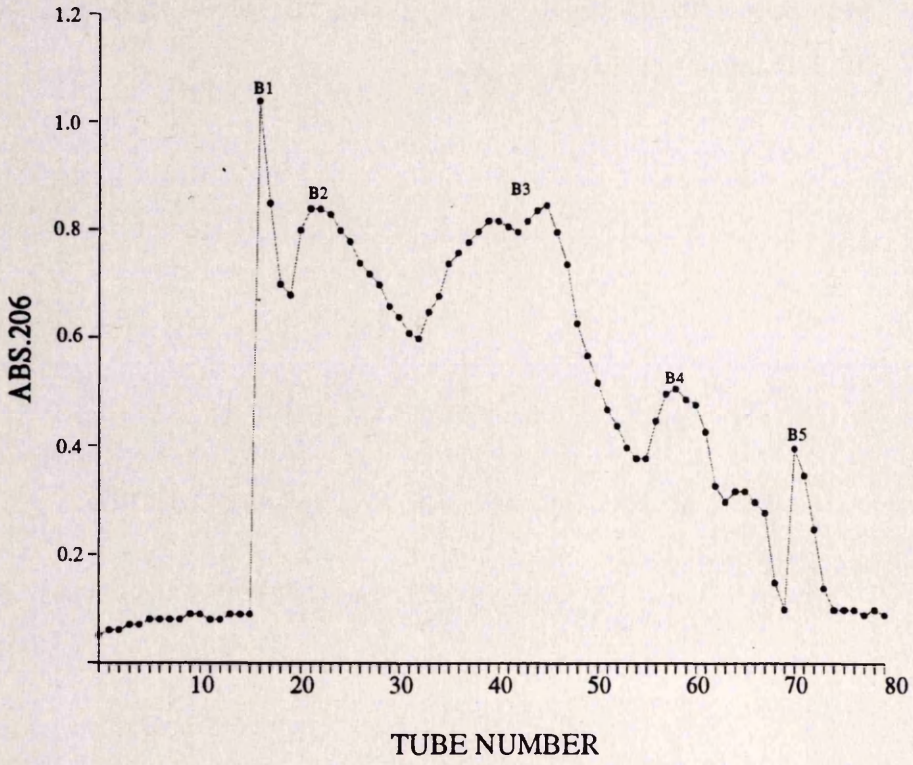
3.1.2.) Purification of phospholipase A_2 from the venom of *Naja mossambica mossambica.*

This work started with an attempt to purify the isoforms of PLA_2 from the venom of *Naja mossambica mossambica* in bulk. At this point it was believed that the venom contained three major isoforms of the enzyme and that two of these could be resolved on the acid/urea PAGE.

The purification procedure was carried out as shown below. Active fractions were then subject to analysis by acidic, basic urea and SDS polyacrylamide gel electrophoresis. The results show very clearly the superior resolution of the basic over the acidic urea method and of either over SDS-PAGE.

Whole *Naja mossambica mossambica* lyophilised venom (500 mg) was dissolved in distilled water at 250 mg per ml in the presence of the protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) and venom was fractionated on Bio-gel P-30 column (3.5 x 40 cm) in 20 mM ammonium acetate (fig.3.1.2.I.). Phospholipase A_2 activity was highest in peak B2, but about 10 % of the activity was present in peak B3. The acidic acid urea gel electrophoresis (fig. 3.1.2.II.) showed that peak B2 was largely

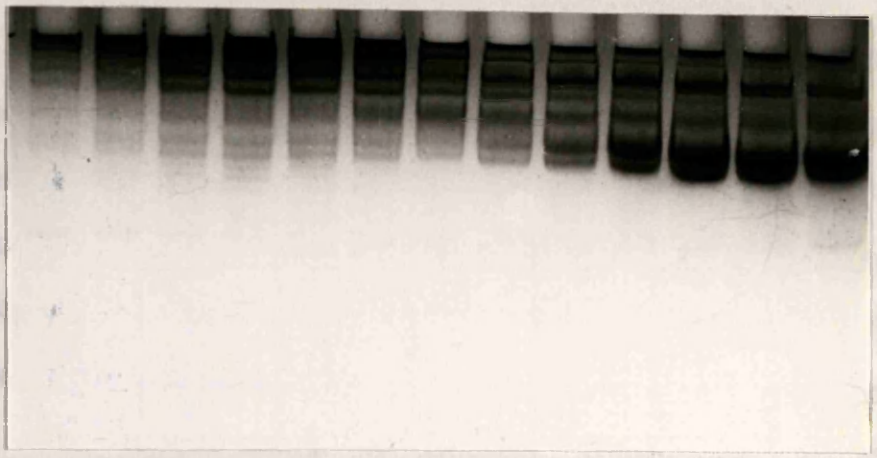
Figure. 3.1.2.I. Gel filtration column chromatography of *Naja mossambica mossambica* venom.



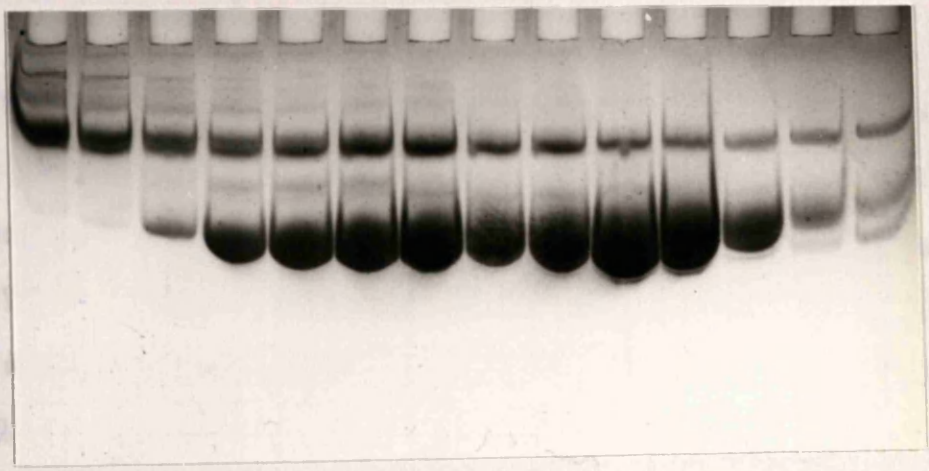
The lyophilised whole venom (500 mg) was dissolved in 2 ml of distilled water in the presence of protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF) and 2 ml venom solution was then applied to a column of Bio-Gel P-30 (3.5 X 40 cm) pre-equilibrated with 20 mM ammonium acetate at pH 6.35. The column was eluted with the equilibration buffer (same buffer) at 40 ml/hr. and the absorption was monitored at 206 nm. Five ml samples were collected, analysed by the acid/urea gel electrophoresis and pooled as samples B1-B5 corresponding to tubes 16-19, 20-32, 33-55, 56-69 and 70-80 respectively and lyophilised to remove ammonium acetate.

Figure. 3.1.2.II. a, b & c. Analysis of different fractions from gel filtration Bio-Gel P-30 column of *Naja mossambica mossambica* on unmodified 20 % acidic/urea gel electrophoresis. Gels a, b and c show selective fractions from gel filtvation Chromatography.

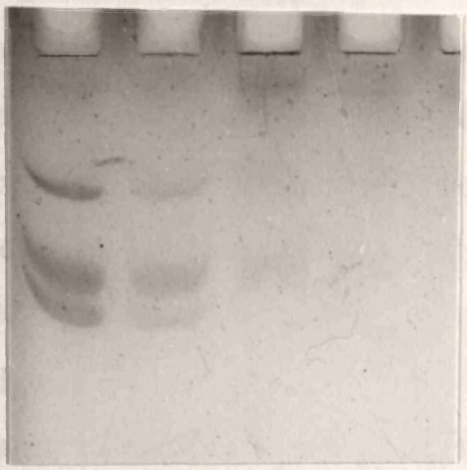
a



b



c



free (< 5 %) of species that had higher mobility than that of PLA_2 and which were known by gel filtration results to be lower molecular weight peptides. These peptides were the predominants of peak B3, which contained the bulk of the haemolytic activity of the venom. This peak also contained a PLA_2 species that tended to spread very widely and which migrated at the same rate as the CM-III isoform. The active fractions were pooled and freeze-dried to remove ammonium acetate and chromatgraphed on DE-52 cellulose.

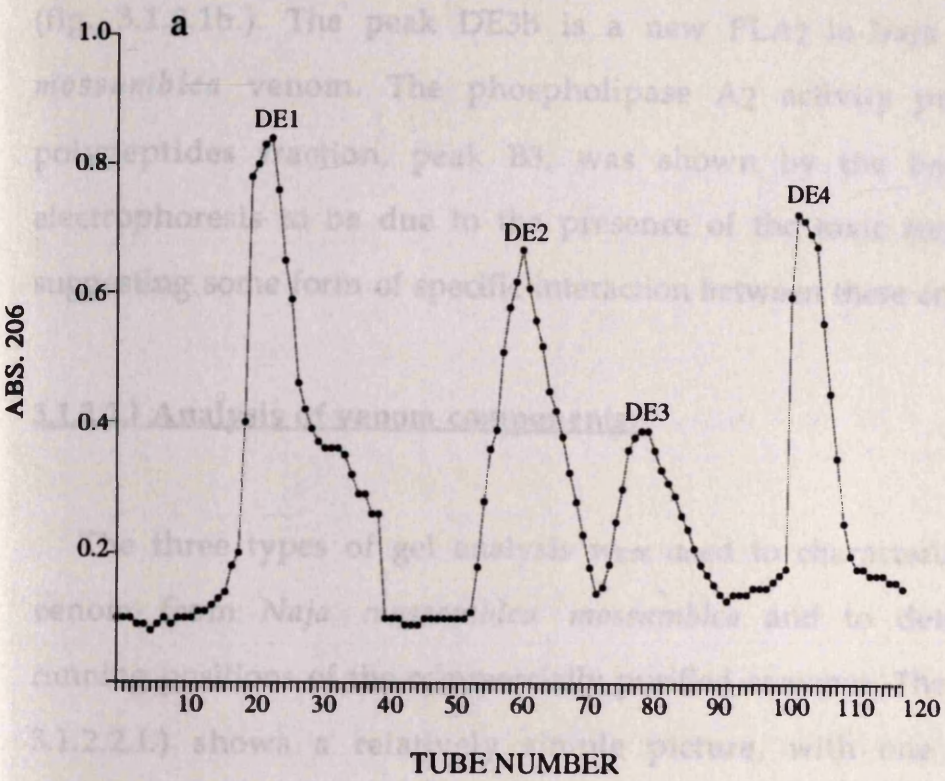
3.1.2.1.) DE-52 ion exchange chromatography.

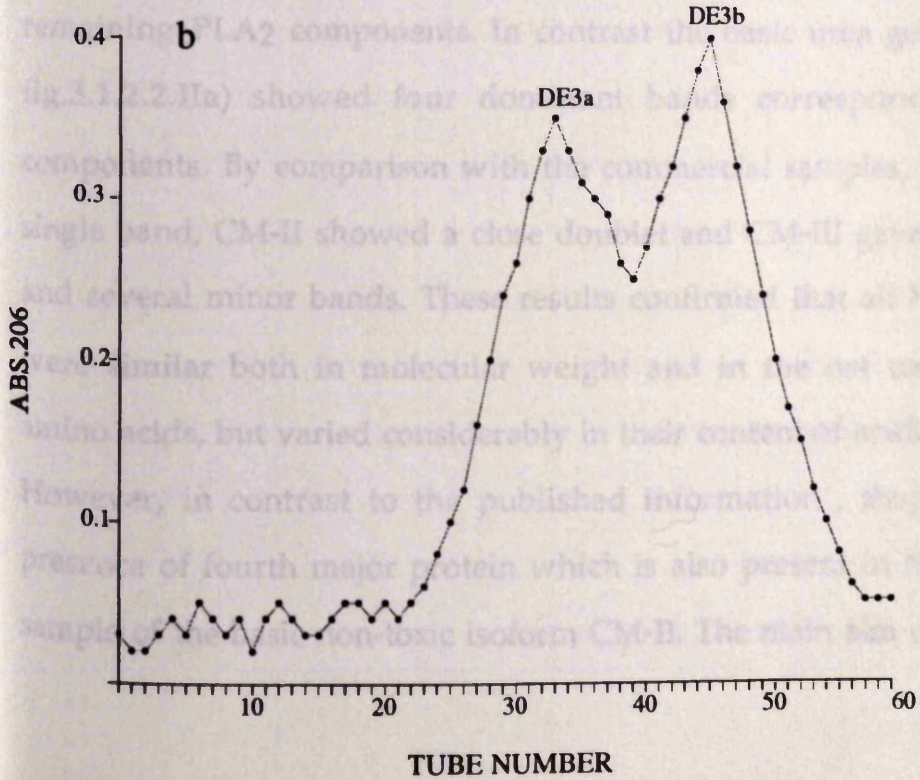
The freeze-dried protein from peak B2 was dissolved in distilled water and the pH adjusted to 9.8 with ammonia solution. It was then applied to a column (2 x 7 cm) of DE-52, equilibrated in 20 mM ammonium acetate at pH 9.8. When the initial run-through peak had been collected the absorbed protein was eluted with gradient of 20 mM ammonium acetate of pH 5.2 (fig.3.1.2.1a). Gel electrophoresis and activity assay showed that non-absorbed activity (DE1) corresponded to the toxic basic isoform contaminated with high molecular weight proteins. The gradient eluted three different active forms, the first being the basic non-toxic (DE2), followed by minor component (DE3) that was electrophoretically distinct, and then by a major peak of activity (DE4) corresponding to the acidic isoform. DE3 was rechromatographed on a smaller DE-52 column (2 x 3 cm) but using 20 mM ammonium acetate at pH 9.4 as the equilibration buffer and eluted with gradient of 20 mM ammonium acetate, pH 5.0. Two peaks were obtained and both exhibited phospholipase A_2 activity

Figure. 3.1.2.1. Ion exchange chromatography on DEAE cellulose

a) The lyophilised sample B2 (figure 3.1.4.3.) was dissolved in 1 ml of distilled water and the pH was adjusted to 9.8 with ammonia solution and applied to a column of DE-52 cellulose (2 X 7 cm) equilibrated with 20 mM ammonium acetate at pH 9.8. The column was eluted at 20 ml per hour, initially with 60 ml of the equilibration buffer, followed by 20 mM ammonium acetate, pH 5.2, the pH of the eluant measured and the absorption monitored at 206 nm. one ml fractions were collected, analysed by the acid/urea gel electrophoresis, pooled as fractions DE1, DE2, DE3 and DE4 representing the tube numbers 17-41, 54-74, 75-94 and 103-114 and corresponding to an elution pH of 9.8, 8.8-8.0, 8.0-7.5 and <7.0 respectively. The pooled samples were lyophilised to remove ammonium acetate.

b) The lyophilised sample DE3 was dissolved in 1 ml of distilled water and pH was adjusted to 9.4 with ammonia solution and was rechromatographed on a DE-52 cellulose column (2 X 3 cm) as above, but using a starting pH of 9.4. Elution was with 30 ml of equilibration buffer followed by a 20 mM ammonium acetate at pH 5.0 as described above and 1 ml samples were collected. Samples were pooled as DE3a and DE3b representing tubes 24-40 and 41-58 and corresponding to a pH range of 8.8-8.0 and <8.0 respectively.


a
DE1
DE2
DE3
DE4
ABS. 206
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
90
100
110
120
TUBE NUMBER


b
DE3a
DE3b
ABS.206
0.4
0.3
0.2
0.1
10
20
30
40
50
60
TUBE NUMBER

(fig. 3.1.2.1b.). The peak DE3b is a new PLA_2 in *Naja mossambica mossambica* venom. The phospholipase A_2 activity present in the polypeptides fraction, peak B3, was shown by the basic urea gel electrophoresis to be due to the presence of the toxic isoform CM-III, suggesting some form of specific interaction between these components.

3.1.2.2.) Analysis of venom components.

The three types of gel analysis were used to characterise the whole venom from *Naja mossambica mossambica* and to determined the running positions of the commercially purified enzymes. The SDS gel (fig. 3.1.2.2.I.) shows a relatively simple picture, with one major band corresponding to the various PLA_2 isoforms and at a running position consistent with a molecular weight of 14,000 and a single band presumed to correspond to the major lytic peptide components. The acid/urea PAGE (Unmodified, Fig.3.1.2.2.IIb.) resolved the toxic basic isoform from the remaining PLA_2 components. In contrast the basic urea gel (unmodified, fig.3.1.2.2.IIa) showed four dominant bands corresponding to PLA_2 components. By comparison with the commercial samples, CM-I ran as a single band, CM-II showed a close doublet and CM-III gave a major band and several minor bands. These results confirmed that all PLA_2 isoforms were similar both in molecular weight and in the net content of basic amino acids, but varied considerably in their content of acidic amino acids. However, in contrast to the published information , they revealed the presence of fourth major protein which is also present in the commercial sample of the basic non-toxic isoform CM-II. The main aim of this work

Figure. 3.1.2.2.I. Analysis of phospholipase A_2 enzymes from *Naja mossambica mossambica* venom on 12 % SDS-PAGE

The SDS gel electrophoresis prepared as described in materials and methods. Lane 1: whole venom. Lane 2: toxic PLA_2 (BG-1 & BG-2) from Sigma Chemical Co. Ltd. Lane 3: toxic PLA_2 (BG-1 & BG-2) purified from whole venom. Lane 4: basic non-toxic PLA_2 (BG-3 & BG-4) from Sigma Chemical Co. Ltd. Lane 5: basic non-toxic PLA_2 (BG-3 & BG-4) purified from whole venom. Lane 6: newly purified PLA_2 (BG-5) from whole venom. Lane 7: acidic PLA_2 (BG-6) from Sigma Chemical Co. Ltd. Lane 8: acidic PLA_2 (BG-6) purified from whole venom. Ten μg sample was loaded in each case.

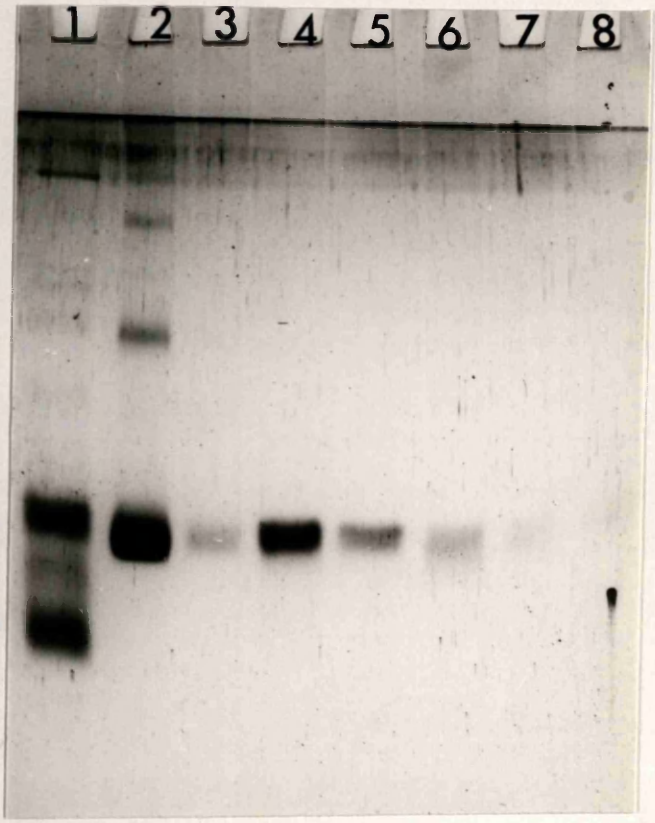
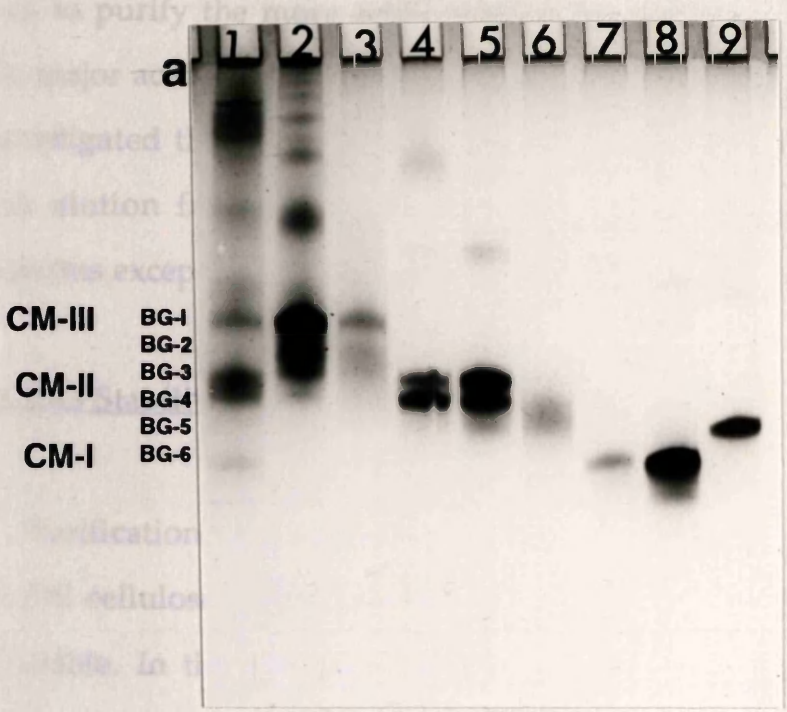
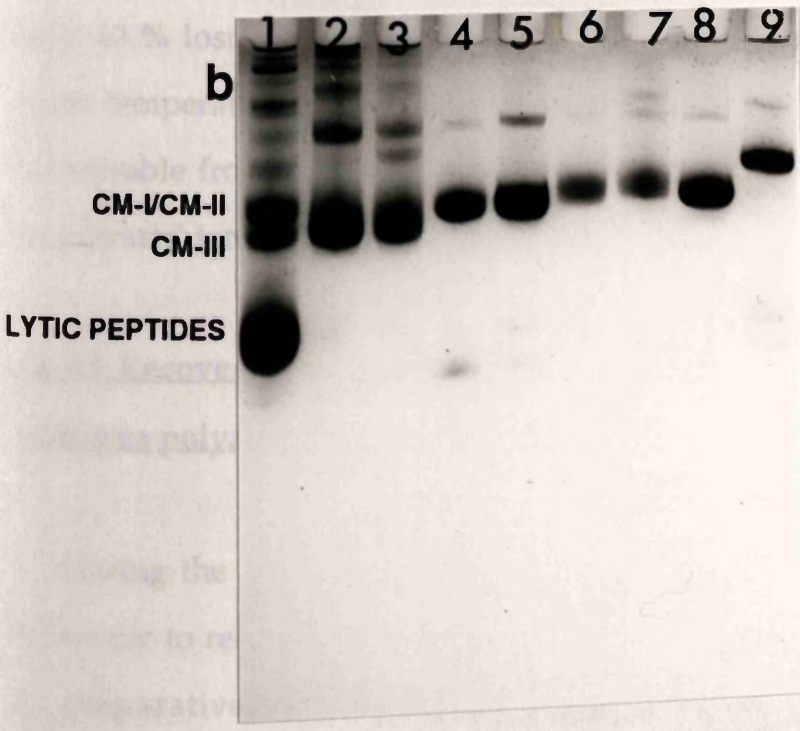

1
2
3
4
5
6
7
8
PLA2 ISOFORMS
LYTIC PEPTIDES

Figure. 3.1.2.2.II. Analysis of phospholipase A2 enzymes from *Naja mossambica mossambica* venom on a) Basic/urea and b) acidic urea gel electrophoresis.

The unmodified basic urea and unmodified acidic urea gel electrophoresis were prepared as described in materials and methods.

Samples of commercially purified PLA2 isoforms from *Naja mossambica mossambica* and isoforms purified according to the procedures described were subject to electrophoresis on the basic and the acidic urea gel electrophoresis. Lane 1: whole venom. Lane 2: toxic PLA2 (BG-1 & BG-2) from Sigma Chemical Co. Ltd. Lane 3: toxic PLA2 (BG-1 & BG-2) purified from whole venom. Lane 4: basic non-toxic PLA2 (BG-3 & BG-4) from Sigma Chemical Co. Ltd. Lane 5: basic non-toxic PLA2 (BG-3 & BG-4) purified from whole venom. Lane 6: newly purified PLA2 (BG-5) from whole venom. Lane 7: acidic PLA2 (BG-6) from Sigma Chemical Co. Ltd. Lane 8: acidic PLA2 (BG-6) purified from whole venom Lane 9: PLA2 purified from *Naja naja atra* whole venom. 10 µg sample was loaded in each case.


a
1 2 3 4 5 6 7 8 9
CM-III
CM-II
CM-I
BG-1
BG-2
BG-3
BG-4
BG-5
BG-6


b
1 2 3 4 5 6 7 8 9
CM-I/CM-II
CM-III
LYTIC PEPTIDES

was to purify the more acidic isoform for activation studies and because the major acidic isoform is not absorbed by CM cellulose at neutral pH we investigated the possibility of obtaining higher purification by absorption and elution from DE resins. Preliminary investigation showed that all isoforms except CM-III were absorbed by DE cellulose at pH< 9.5.

3.1.2.3.) Stability of PLA_2 enzymes to high pH.

Purification of the more acidic isoform (BG-6) could be accomplished on DE cellulose within the pH range where the enzymes were known to be stable. In the case of the CM-III isoform, however, absorption to DE resins required a pH>10. The stability of this enzyme was therefore examined by sequential assay of samples incubated at high pH and at either room temperature or 37 ^{o}C. The results showed that no activity was lost for incubations carried out below pH 11.5 at temperature <37 ^{o}C, with only 40 % lost when the enzyme was incubated at pH 12 for 24 hours at room temperature (fig. 3.1.2.3.). This result indicated that activity might be recoverable from the basic/urea gels despite the fact that these gels run at an elevated temperature, i.e. 35-45 ^{o}C.

3.1.3.) Recovery of phospholipase A_2 activity from the basic/urea and acid/urea polyacrylamide gel electrophoresis.

During the application of polyacrylamide gel electrophoresis it is often necessary to recover the separated proteins. This can be achieved by means of preparative electrophoresis using continuous elution or after the localisation of the protein band by means of homogenisation of the gel

slice and subsequent elution, by chemical depolymerisation of the gel, or by electrophoretic methods (Hiroshi and Snell, 1972; Bray and Brownee, 1973; Bernebeu, et al 1980). Electrophoretic elution from gel slices requires a means for supporting the gel slices in an electric field and means for collecting the eluted material.

Attempts to recover phospholipase A_2 activity from the basic and the acidic urea gels were successful and PLA_2 activity from both types of gel electrophoresis was recovered with high yield. A simple method was developed for recovery of phospholipase A_2 activity from the basic and the acidic urea gel electrophoresis. Analysis of activity recovered from basic and acidic gels was carried out for purified phospholipase A_2 isoforms from a variety of sources. The gels were pre-run for 3 to 4 hours to removed the oxidising agents. Both upper and lower tank buffers were refilled with fresh buffer. The purified paired proteins sample were subject to the basic and the acidic urea gel electrophoresis on parallel tracks using guide and test track for each sample. The guide track was stained with Coomassie blue and aligned with test track and slices of 1-1.5 mm were cut from region corresponding to bands on the guide track. The slices were put into ependorf tubes containing 100 µl of 10 mM triethanolamine buffer of pH 8.0. The enzymes were eluted simply by macerating the slices and after centrifugation the samples filtered as described in materials and methods. The results Table 3.1.3. shows that all enzymes could be recovered from both the basic and acidic urea gels with high yields.

Table 3.1.3. Recovery of phospholipase A_2 activity after electrophoresis on the basic or the acidic urea gels.

Enzymes and source	Activity recovered (%) Basic-urea gels	Acidic-urea gels
Non-toxic PLA_2 from *Naja mossambica mossambica* venom	95±4	91 ± 4
Toxic PLA_2 from *Naja mossambica mossambica* venom	94 ± 4	89 ± 5
Newly purified PLA_2 from *Naja mossambica mossambica* venom	81±6	76 ± 5
Acidic PLA_2 from *Naja mossambica mossambica* venom	89±3	86 ± 2
PLA_2 from *Naja naja atra* venom	89±4	86 ± 3
PLA_2 from Honey bee venom	85±3	82 ± 2

Paired purified protein samples were electrophoresed on the basic and the acidic urea gels in the presence of 8 M urea in a guide and test lane. Protein bands were detected in the guide lane by coomassie blue staining and the corresponding region of the test lanes cut into 1.5 mm slices which were macerated with 100 µl of 10 mM triethanolamine buffer, pH 8.0. Five µl samples were assayed for PLA_2 activity as described in materials and methods. Each value is a mean of three determinations with ± S. D.

3.1.3.1.) Recovery of phospholipase A_2 activity from whole venoms of four major venomous families of snake from basic/urea gel electrophoresis.

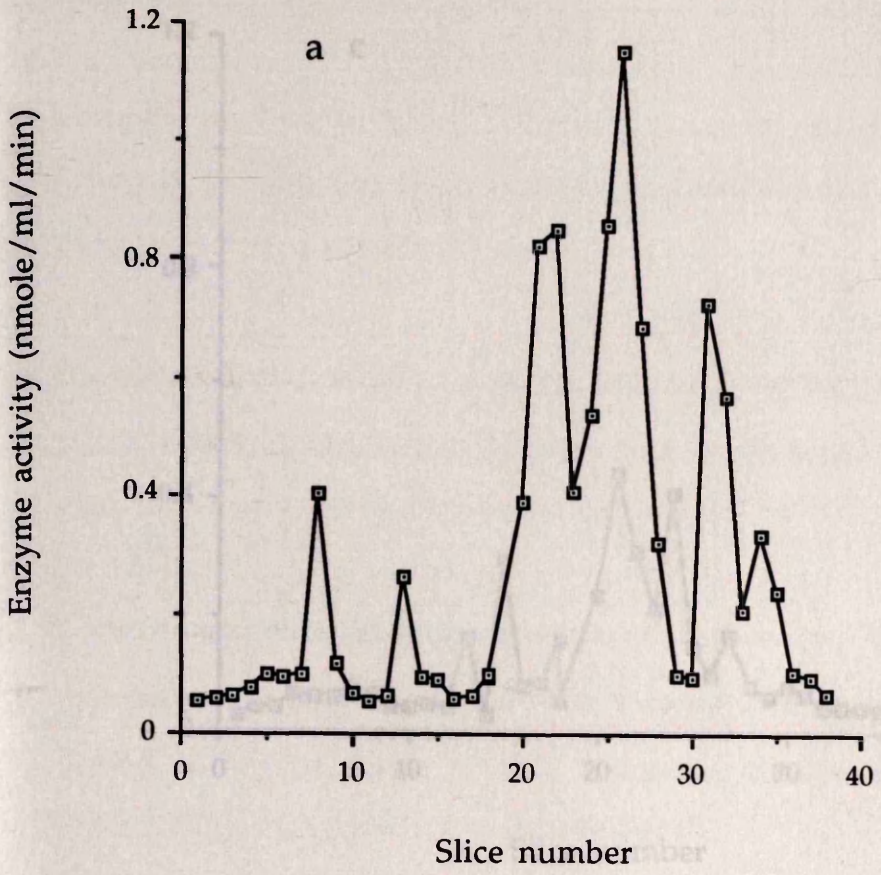
The systematic classification of snakes still presents many problems, but most authorities (according to Harvey, 1991) would now recognise between 11 and 13 distinct families. Venomous snakes are identified in only five families: *Elapidae*, *Hydrophiidae*, *Viperidae*, *Crotalidae* and *Colubridae*. Family *Elapidae* is a large family that includes the kraits, cobras, mambas and coral snakes. Family *Hydrophiidae*, which comprises sea snakes, is considered by some to be a subfamily of *Elapidae*. Family *Viperidae* (true vipers) is the most widespread family of venomous snakes. It is represented throughout Europe, Africa, Asia and the Americas. Family *Crotalidae* is considered by some a subfamily of the *Viperidae*. Family Colubridae is the largest of all families. It is dominant family of snakes in all parts of the world. Most snakes in this family are harmless, but some are venomous. (Harvey, 1991)

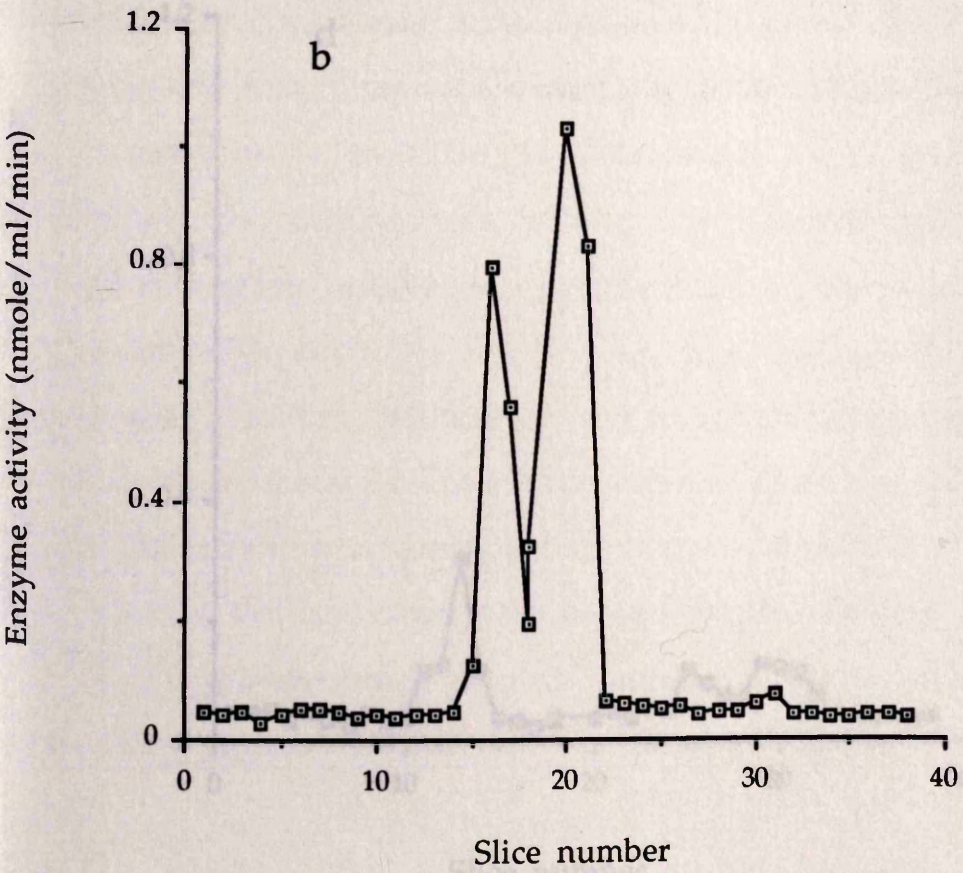
We have tried to recover phospholipase A_2 activity from each of the different families. The whole venoms from the major four families were run on the basic urea gels in two lanes, guide and experimental lane, the guide lane was stained with Coomassie blue and aligned with experimental lane and slices of 1-1.5 mm were cut and enzymes were extracted and phospholipase A_2 assay were performed to identify enzymes. Figure 3.1.3.1. a, b, c, and d shows that the enzymatic activity could be recovered from four major snake families on basic urea gels. The peaks in the figures indicate the presence of PLA_2. The multiple and broad peaks

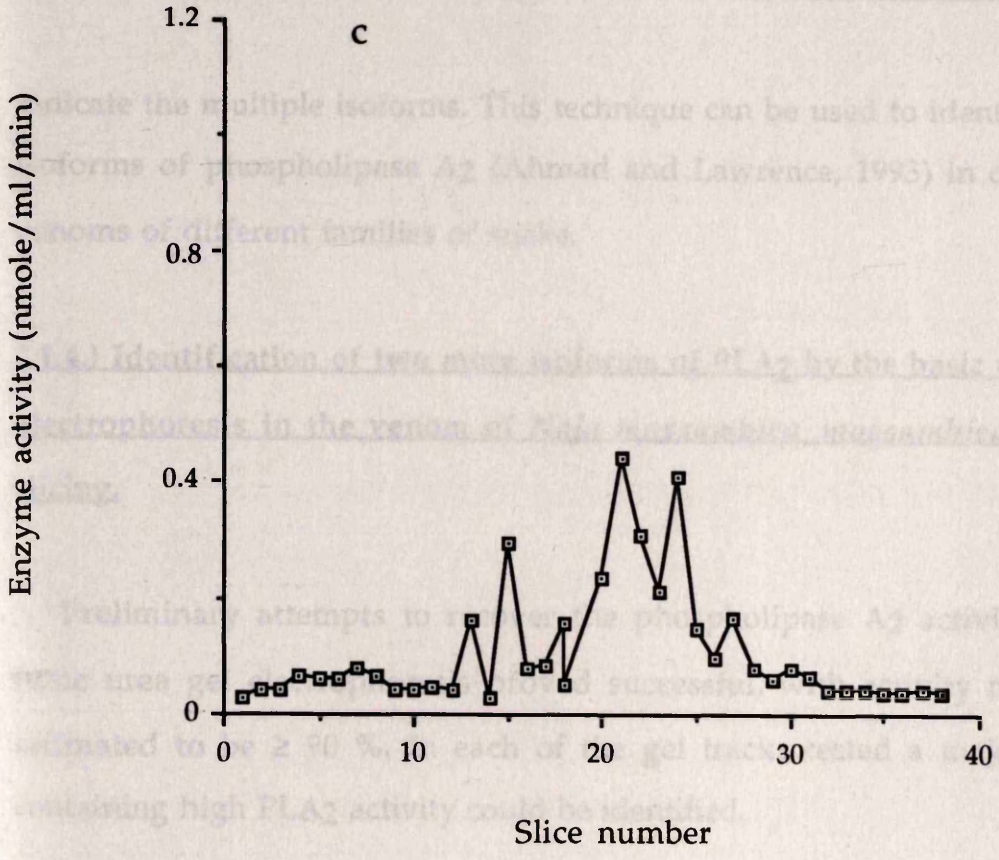
Figure 3.1.3.1. Recovery of activity and identification of PLA_2 isoforms by the basic urea gel electrophoresis.

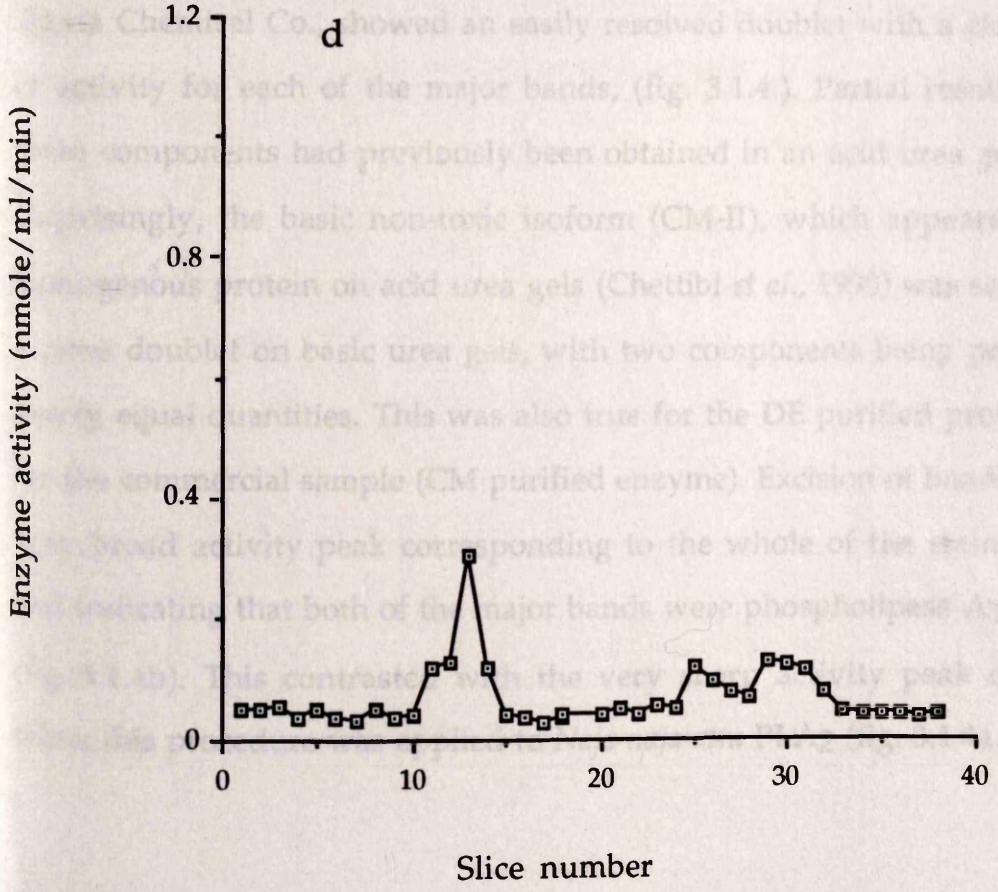
Paired whole venom samples were electrophoresed on the basic urea gel in a guide and test lane. Protein bands were detected in the guide lane by Coomassie blue staining and the corresponding region of the test lanes cut into 1-1.5 mm slices which were macerated with 75 µl of 100 mM triethanolamine buffer, pH 8.0. Five µl samples were assayed for PLA_2 activity using 0.6 mM dioctanoylphosphatidylcholine in the presence of 1 mM $CaCl_2$.

(a) *Naja mossambica mossambica* whole venom (family *Elapidae*) (b) *Agkistrodon piscivorus piscivorus* whole venom (family *Crotalidae*).: Next page, (c) *Russell's viper* whole venom (family *Viperidae*), (d) *Enhydrina schistos*a whole venom (family *Hydrophiidae*).


a
1.2
0.8
0.4
0
0
10
20
30
40
Enzyme activity (nmole/ml/min)
Slice number


b
1.2
0.8
0.4
0
0
10
20
30
40
Enzyme activity (nmole/ml/min)
Slice number


c
1.2
0.8
0.4
0
0
10
20
30
40
Enzyme activity (nmole/ml/min)
Slice number


d
1.2
0.8
0.4
0
0
10
20
30
40
Enzyme activity (nmole/ml/min)
Slice number

indicate the multiple isoforms. This technique can be used to identify new isoforms of phospholipase A_2 (Ahmad and Lawrence, 1993) in different venoms of different families of snake.

3.1.4.) Identification of two more isoforms of PLA_2 by the basic urea gel electrophoresis in the venom of *Naja mossambica mossambica* by gel slicing.

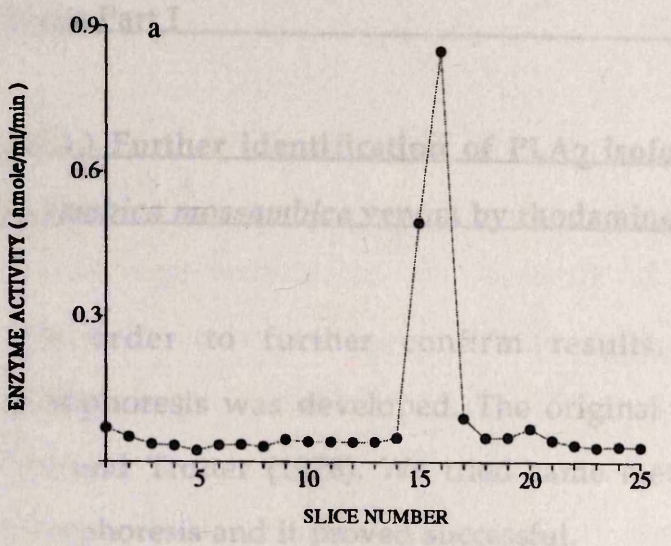
Preliminary attempts to recover the phospholipase A_2 activity from basic urea gel electrophoresis proved successful, with activity recovery estimated to be $\geq$ 90 %. In each of the gel tracks tested a major band containing high PLA_2 activity could be identified.

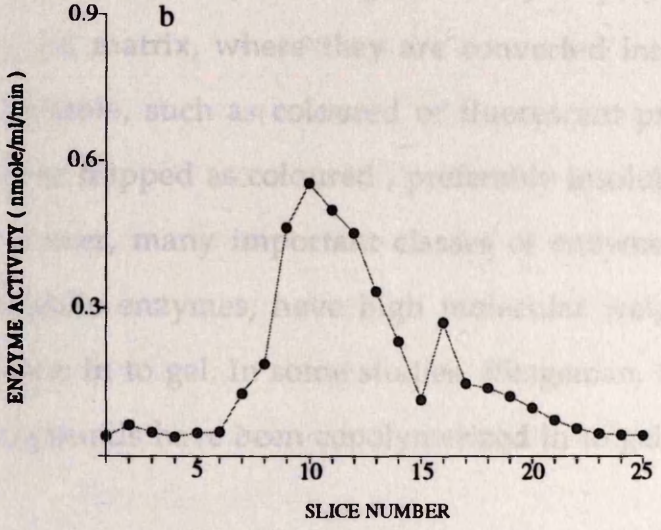
The basic toxic isoform, either purified here or as purchased from Sigma Chemical Co., showed an easily resolved doublet with a clear peak of activity for each of the major bands, (fig. 3.1.4.). Partial resolution of these components had previously been obtained in an acid urea gel. More surprisingly, the basic non-toxic isoform (CM-II), which appeared to be homogenous protein on acid urea gels (Chettibi *et al.*, 1990) was seen to be a close doublet on basic urea gels, with two components being present in nearly equal quantities. This was also true for the DE purified protein and for the commercial sample (CM purified enzyme). Excision of bands gave a very broad activity peak corresponding to the whole of the stained zone and indicating that both of the major bands were phospholipase A_2 species (fig..3.1.4b). This contrasted with the very sharp activity peak obtained when this procedure was applied to *Naja naja atra* PLA_2 (fig. 3.1.4a.)

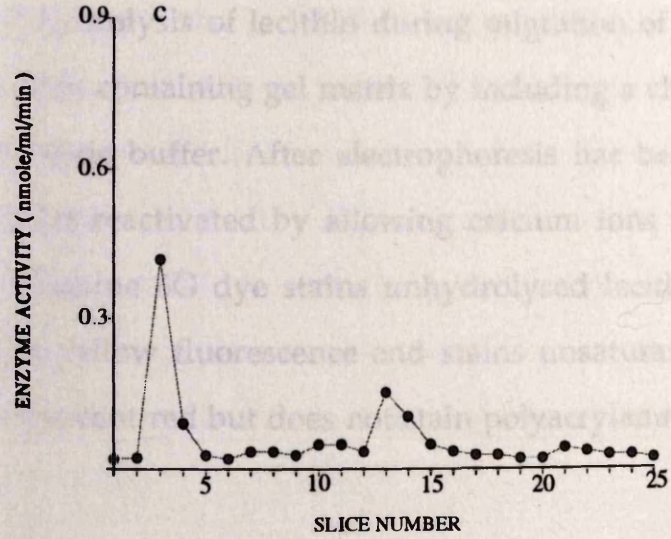
Figure. 3.1.4. Identification of new isoforms of PLA_2 by the basic urea gel electrophoresis.

Paired protein samples were electrophoresed on a basic urea gel in a guide and test lane. Protein bands were detected in the guide lane by Coomassie blue staining and the corresponding region of the test lanes cut into 1-1.5 mm slices which were macerated with 75 µl of 100 mM triethanolamine buffer, pH 8.0. Five µl samples were assayed for PLA_2 activity using 0.6 mM dioctanoylphosphatidylcholine in the presence of 1 mM $CaCl_2$.

(a) PLA_2 from *Naja naja atra* venom. (b) Non-toxic PLA_2 from *Naja mossambica mossambica* venom. (c) Toxic PLA_2 from *Naja mossambica mossambica* venom.


a
0.9
0.6
0.3
ENZYME ACTIVITY (nmole/ml/min)
5
10
15
20
25
SLICE NUMBER


b
0.9
0.6
0.3
ENZYME ACTIVITY (nmole/ml/min)
5
10
15
20
25
SLICE NUMBER


c
0.9
0.6
0.3
ENZYME ACTIVITY (nmole/ml/min)
5
10
15
20
25
SLICE NUMBER

3.1.4.1.) Further identification of PLA2 isoforms in the venom of *Naja mossambica mossambica* venom by rhodamine 6G gel electrophoresis.

In order to further confirm results, the rhodamine 6G gel electrophoresis was developed. The original method was developed by Shier and Trotter (1978). We tried same method on our basic urea gel electrophoresis and it proved successful.

Assays of enzymes in gels usually employ substrates that diffuse into the gel matrix, where they are converted into products that are readily detectable, such as coloured or fluorescent products, or compounds that can be trapped as coloured , preferably insoluble materials (Gabriel, 1971). However, many important classes of enzymes, such as transferases and catabolic enzymes, have high molecular weight substrates that will not diffuse in to gel. In some studies (Stegeman, 1968) high molecular weight compounds have been copolymerized in to gel matrix.

Because the best-characterised phospholipase A_2 enzymes require calcium ions for catalysis (van den Bosch, 1974), it is possible to prevent the hydrolysis of lecithin during migration of these enzymes through the lecithin containing gel matrix by including a chelating agent (EDTA) in the electrode buffer. After electrophoresis has been completed, the enzymes can be reactivated by allowing calcium ions to diffuse into the gel. The rhodamine 6G dye stains unhydrolysed lecithin in the gels a faint pink with yellow fluorescence and stains unsaturated fatty acids a dark, non-fluorescent red but does not stain polyacrylamide.

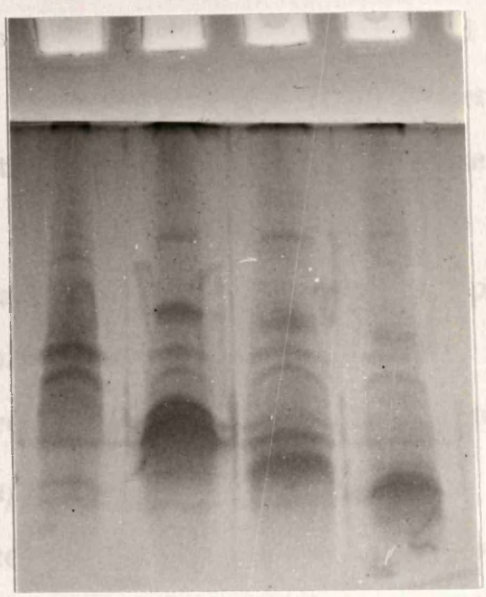
The different fractions of phospholipase A_2 isoforms from *Naja mossambica mossambica* were ran on the basic urea gel containing purified egg lecithin. all six isoforms of phospholipase A_2 in *Naja mossambica mossambica* were confirmed by incubating gels in rhodamine 6G containing 20 mM $CaCl_2$ at 37 ^oC as described in materials and methods. In addition to six isoforms identified earlier (Ahmad and Lawrence, 1993) further two more isoforms were identified (fig.3.1.4.1.).

3.1.5.) analysis of whole venoms.

The acidic and the basic urea PAGE was used together with the SDS-PAGE to resolve and identify venom components from 14 different venoms including bee venom and from all major snake venom families (four) including two *Crotalus* species, sea snake, *Russell's viper* and five *elapid* species including four closely related cobras, *Naja mossambica mossambica*, *Naja nigricollis nigricollis*, *Naja nigricollis crawshawii* and *Naja nigricollis pallida*.

The first observations were that for these low molecular weight components bands obtained with SDS-PAGE were not as sharp as those seen for high MW proteins. Overall in terms of band sharpness both of the urea methods gave better performances than SDS-PAGE, The specific feature of acidic PAGE was that it resolved the highly basic peptide components of venoms. In contrast these components tended to show cathodic migration even at the high pH of basic gels and were therefore lost. Thus the basic gels were only suitable for examination of the higher MW venom components.

Figure. 3.1.4.1. Identification of phospholipase A_2 isoforms in the venom of *Naja mossambica mossambica.* The basic urea gels were prepared as described in materials and methods but incorporating 5 mg/ml of purified egg phosphatidylcholine in the running gel with 1 mM EDTA in the stacking gel and the tank electrolyte. Samples of purified enzymes were prepared by mixing 1:1 with bromophenol blue, but containing 1mM EDTA. After electrophoresis the gel was rinsed twice with distilled water and immersed in a solution containing 20 mM $CaCl_2$ and 0.012 % rhodamine 6G buffered with 0.1 M triethanolamine/Cl^-, pH 8.0 and incubated at 37 °C for 5-10 minutes. The reaction was stopped by washing with a solution containing 20 mM EDTA. The gels were photographed against black background and reflected white light. Lane a: basic toxic isoform, Lane b: basic nontoxic isoform, Lane c: acidic newly purified isoform, and Lane d: acidic isoform.


a
b
c
d

A comparison of venom components particularly venom phospholipase A_2 isoforms was made on three types of gel electrophoresis, i. e. SDS, the acidic urea and the basic urea polyacrylamide gels.

SDS-PAGE of purified PLA_2 enzymes confirmed that this method would not resolve isoforms of similar MW and as most of the known isoforms within given venoms do not differ greatly in MW little resolution was expected. In contrast the basic and acidic PAGE methods, which have rather similar overall resolving power were able to separate isoforms and results were largely in accord with the sequence predictions.

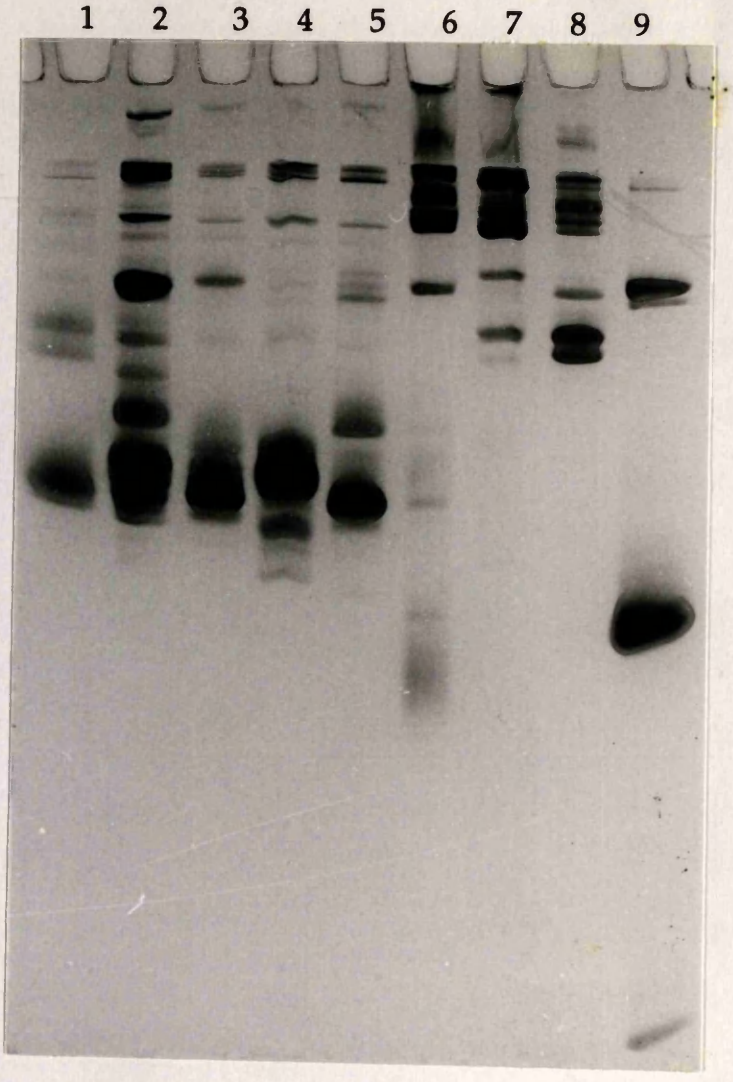
This acidic urea gel is ideal for resolving polypeptides (figure 3.1.5.I.), but as regards phospholipase A_2 enzymes, is not good in resolving isoforms. It resolved the basic toxic isoforms from PLA_2 components in the venom of *Naja mossambica mossambica*. It can also resolved bee venom glycosylated and non-glycosylated components. The SDS gels showed one major band corresponding to various phospholipase A_2 isoforms running position consistent with a molecular weight of 14,000 and a single band presumed to correspond to the major polypeptide components of the venoms (figure 3.1.5.II.). In contrast, the basic urea gel electrophoresis is ideal for resolving PLA_2 isoforms. The mobility of a basic urea gel is determined by the ratio of net negative charge (i.e. N-ter+Asp+Glu+Tyr-Arg) to total mass. Many major polypeptides are virtually devoid of carboxylate side chains and remain cationic even at high pH. The gels therefore tend to display only the high molecular weight proteins of the venoms. Snake venoms contain multiple forms of phospholipase A_2.

Figure. 3.1.5.I. Analysis of whole venoms on 20 % modified acidic/urea gel electrophoresis having 7 % spacer gel and 8 M urea. 20 µg of each venom was loaded.

a) Lane 1: *Naja mossambica mossambica*, Lane 2: *Naja melanoleuca*, Lane 3: *Naja naja atra*, Lane 4: *Naja nivea*, Lane 5: *Naja naja*, Lane 6: *Crotalus adamanteus*, Lane 7: *Crotalus atrox*, Lane 8: *Agkistrodon piscivorus piscivorus*, Lane 9: Bee venom

b) Lane 10: *Enhydrina schistosa* (sea snake), Lane 11: *Bungarus fasciatus*, Lane 12: *Naja hannah*, Lane 13: *Naja nigricollis nigricollis*, Lane 14: *Naja nigricollis crawshawii*, Lane 15: *Naja nigricollis pallida*, Lane 16: *Naja haje*, Lane 17: *Russell's viper*

a



b

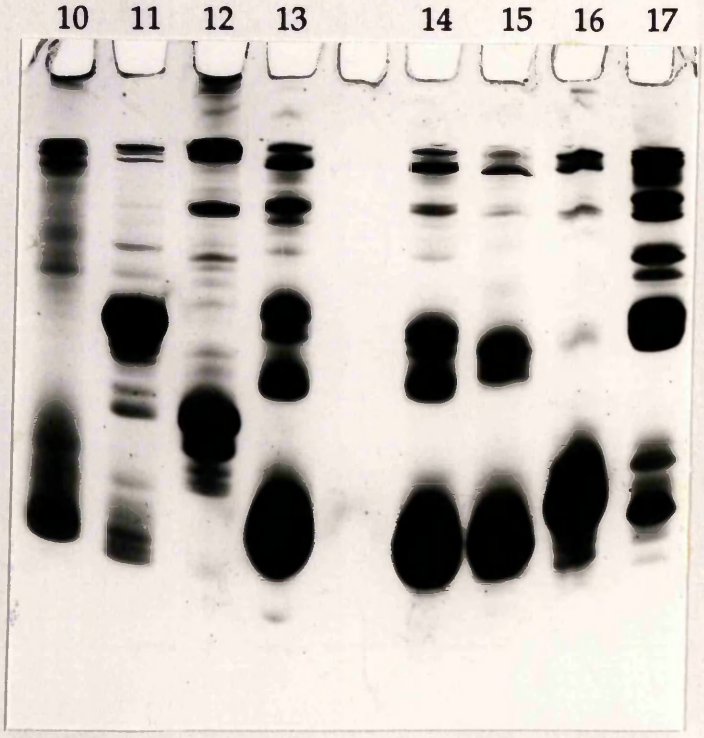


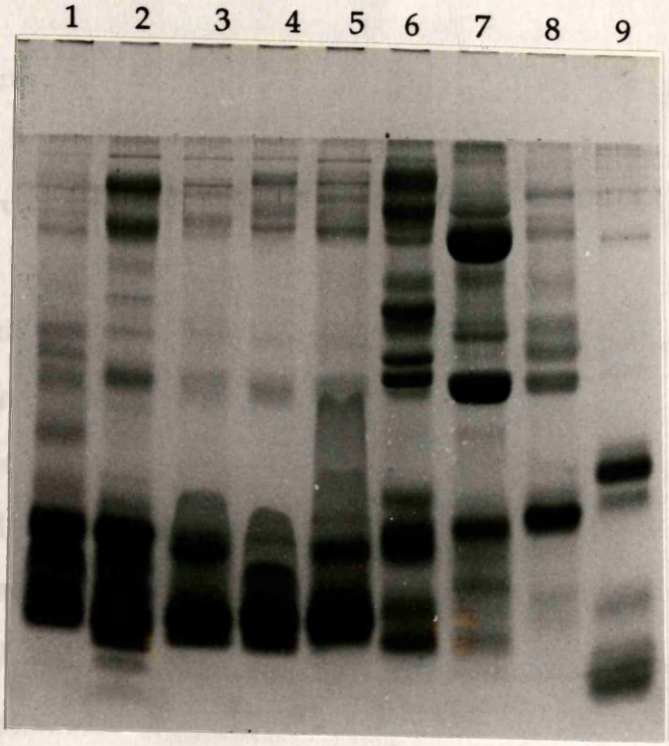
Figure. 3.1.5.II. Analysis of whole venoms on 12 % SDS gel electrophoresis.

The SDS-PAGE 12 % was prepared as described in materials and methods and 20 µg of each venom was loaded.

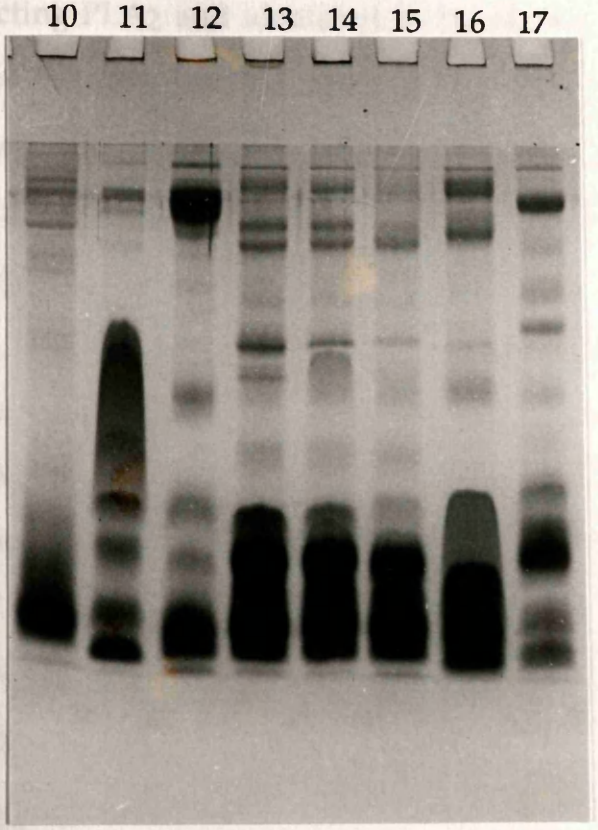
a) Lane 1: *Naja mossambica mossambica*, Lane 2: *Naja melanoleuca*, Lane 3: *Naja naja atra*, Lane 4: *Naja nivea*, Lane 5: *Naja naja*, Lane 6: *Crotalus adamanteus*, Lane 7: *Crotalus atrox*, Lane 8: *Agkistrodon piscivorus piscivorus*, Lane 9: Bee venom

b) Lane 10: *Enhydrina schistosa* (sea snake), Lane 11: *Bungarus fasciatus*, Lane 12: *Naja hannah*, Lane 13: *Naja nigricollis nigricollis*, Lane 14: *Naja nigricollis crawshawii*, Lane 15: *Naja nigricollis pallida*, Lane 16: *Naja haje*, Lane 17: *Russell's viper*.

a



b



Indian cobra (*Naja naja naja*) venom has been reported to contain as many as 14 isoforms of PLA_2 (Shiloach *et al.*, 1973, Bhat and Gowda, 1989) and the venom of *Naja mossambica mossambica* was reported to contain 3 isoforms (Joubert, 1977); an acidic (CM-I), a basic non-toxic (CM-II) and basic toxic (CM-III). The presence of several isoenzyme forms of PLA_2 in cobra venom is not due to subspecies polymorphism but exists in a venom sample collected from a single snake. (Hazlett and Dennis, 1985). The basic urea gel electrophoresis is ideal for monitoring purification and identification of phospholipase A_2 isoforms. Figure 3.1.5.III. shows that phospholipase A_2 isoforms can be resolved into six bands in the venom of *Naja mossambica mossambica*. Enzymatic activity can be recovered with high yield from basic gel so that new isoforms of phospholipase A_2 were identified by running enzymes on basic urea gels and then cutting slices and extracting PLA_2 and identified by doing normal conductimetric assay as described earlier. The venom of *Agkistrodon piscivorus piscivorus* was fractionated on Bio-gel P-30 column and then ran on basic urea gel, it resolved in to a number of bands, including at least four major bands of phospholipase A_2 isoforms. A number of acidic proteins were identified. The basic urea gel has resolved dimeric phospholipase A_2 in the venoms *Crotalus atrox* and *Crotalus adamanteus* nicely into two bands presumed to be phospholipase A_2 isoforms. A number of bands in the venom of Viper russel were identified as phospholipase A_2 isoforms.

In summary, comparison of the gel results for the three methods showed the following.

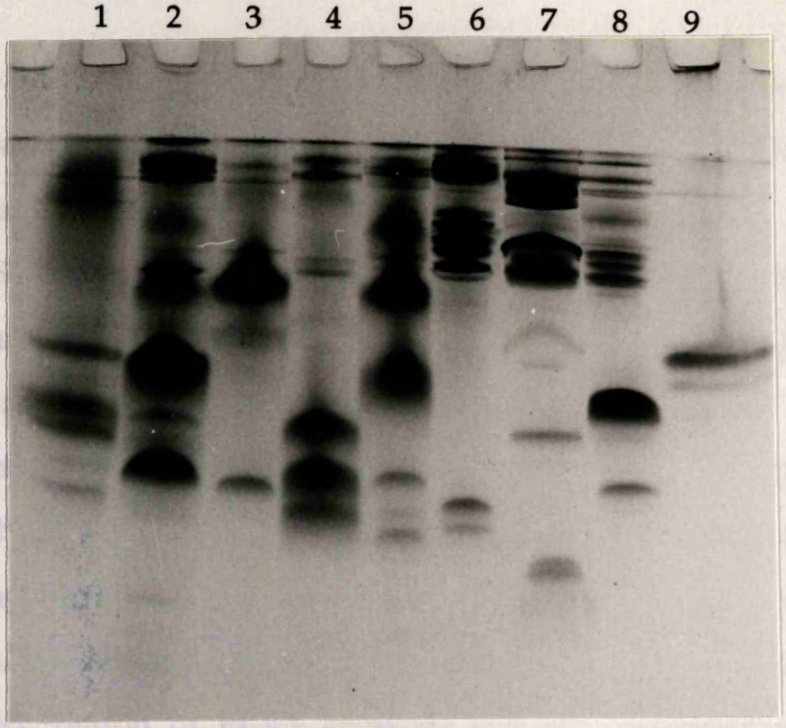
1) All gave high resolution.

Figure. 3.1.5.III. Analysis of whole venoms on 20 % modified basic urea gel electrophoresis having 7 % spacer gel and 8 M urea. Twenty µg of each venom was loaded in each lane.

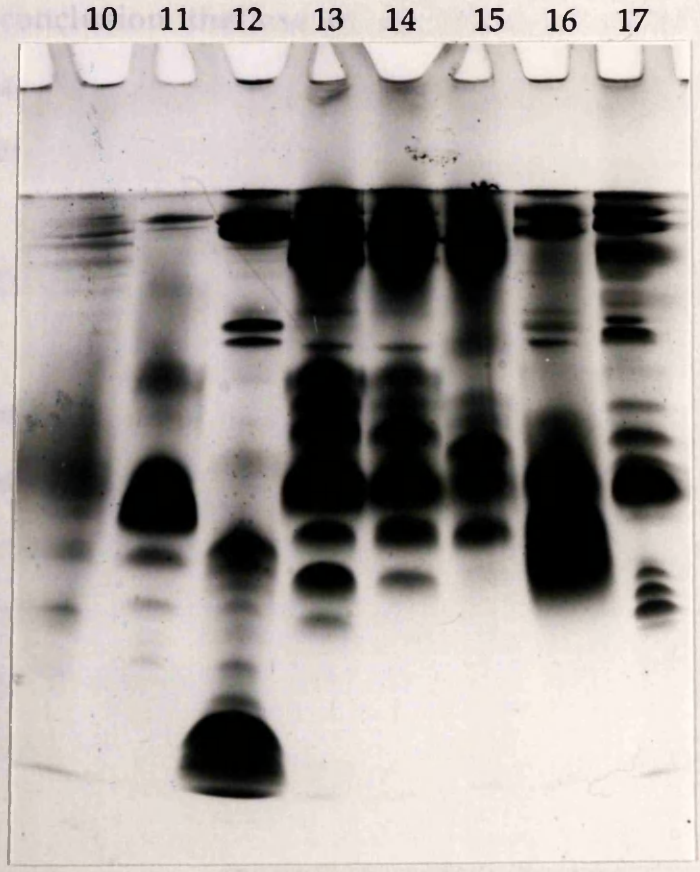
a) Lane 1: *Naja mossambica mossambica*, Lane 2: *Naja melanoleuca*, Lane 3: *Naja naja atra*, Lane 4: *Naja nivea*, Lane 5: *Naja naja*, Lane 6: *Crotalus adamanteus*, Lane 7: *Crotalus atrox*, Lane 8: *Agkistrodon piscivorus piscivorus*, Lane 9: Bee venom

b) Lane 10: *Enhydrina schistosa* (sea snake), Lane 11: *Bungarus fasciatus*, Lane 12: *Naja hannah*, Lane 13: *Naja nigricollis nigricollis*, Lane 14: *Naja nigricollis crawshawii*, Lane 15: *Naja nigricollis pallida*, Lane 16: *Naja haje*, Lane 17: *Russell's viper*.

a



b



2) The acid/urea PAGE resolves small peptide components where present. For example bee venom shows a very clear band due to the major peptide melittin (MW 2.6 kD).

3) SDS-PAGE tends to emphasise similarities between different venoms, but the acid/urea PAGE and to a greater extent basic/urea PAGE give very much more distinct patterns for the different venoms.

4) Activity staining carried out on the basic/urea PAGE indicates that almost all of the higher mobility bands are PLA_2 isoforms. This is most strikingly demonstrated with the venoms from the sub species *Naja nigricollis* where almost all of the bands that have been resolved are in the mobility region of PLA_2 enzymes and activity staining confirms this where carried out.

In conclusion the use of all three gel types give very valuable information about venom purification, but monitoring of isoforms is most successfully done with the urea gels, especially basic/urea PAGE.

3.1.6.) Purification of phospholipase A_2 from the venom of *Naja naja atra.*

Phospholipase A_2 of *Naja naja atra* venom has been isolated and purified by Lo *et al* (1972) and its chemical, enzymatic and pharmacological characterisations have also been reported (Lo and Chang, 1976; Chiang *et al*, 1973; Lee and Ho, 1978; Yang *et al*, 1981). It is an acidic single chain polypeptide and consists of 120 amino acid residues with seven disulphide bonds (Tsai et al, 1981).

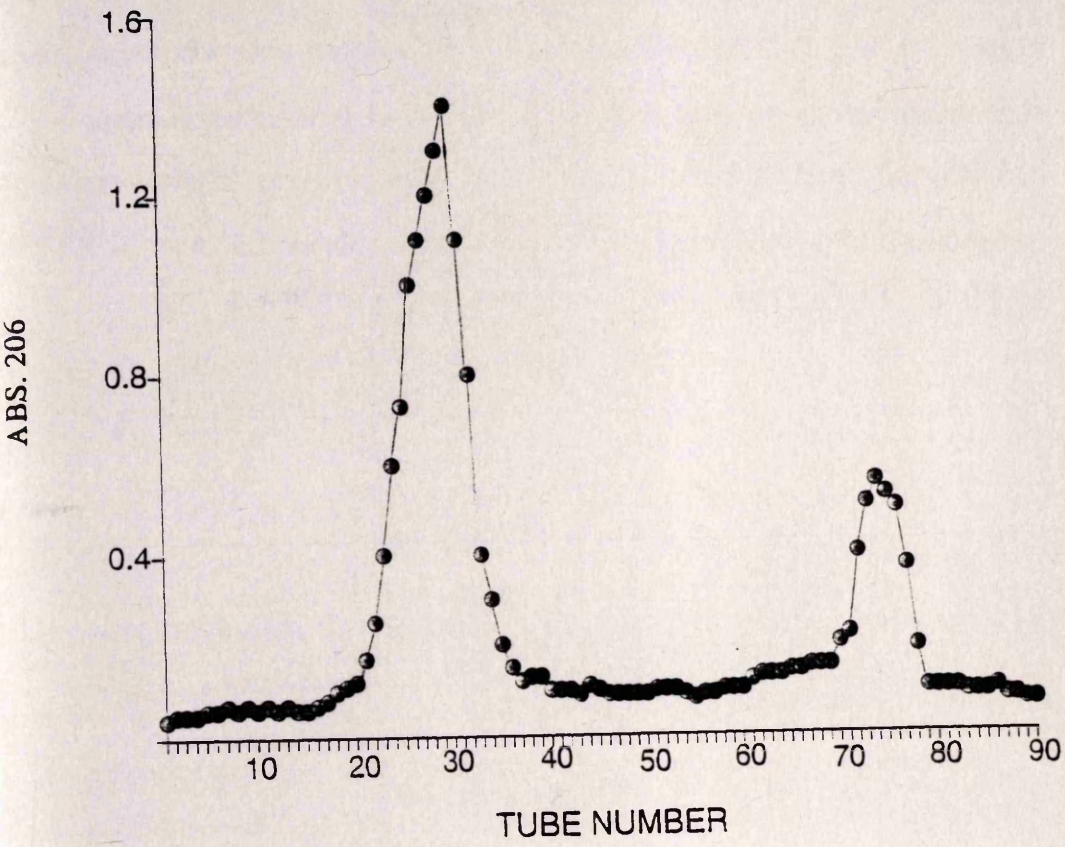
In most cases, purification involves a number of steps (Braganca *et al*,

1969; Salach *et al.*, 1968; Jayanthi and Gowda, 1983) . We have purified phospholipase A_2 from *Naja naja atra* in a single step. The lyophilised venom 200 mg (Sigma Chemical Co. Ltd.) was dissolved in 1 ml of distilled water in the presence of the protease inhibitor PMSF and the pH was adjusted to 9.4 with ammonia solution The protein sample was then applied to column (2 x 5 cm) of DE-52, equilibrated in 20 mM ammonium acetate at pH 9.4, when the initial run-through peak had been collected the absorbed protein was eluted with linear gradient of 20 mM ammonium acetate of pH 5.0 (fig. 3.1.6.I.). The gel electrophoresis and enzymatic assay showed that non-absorbed peak corresponded to phospholipase A_2. The purity of phospholipase A_2 was checked on the basic/urea (fig.3.1.6.II.a) and the acidic/urea (fig. 3.1.6.II.b) gel electrophoresis. The single highly purified phospholipase A_2 was obtained in a single step. As at high pH the polypeptides and other proteins did not stick to the DE-52 column.

3.1.6.1.) Identification of new phospholipase A_2 in the venom of *Naja naja atra* by rhodamine 6G gel electrophoresis.

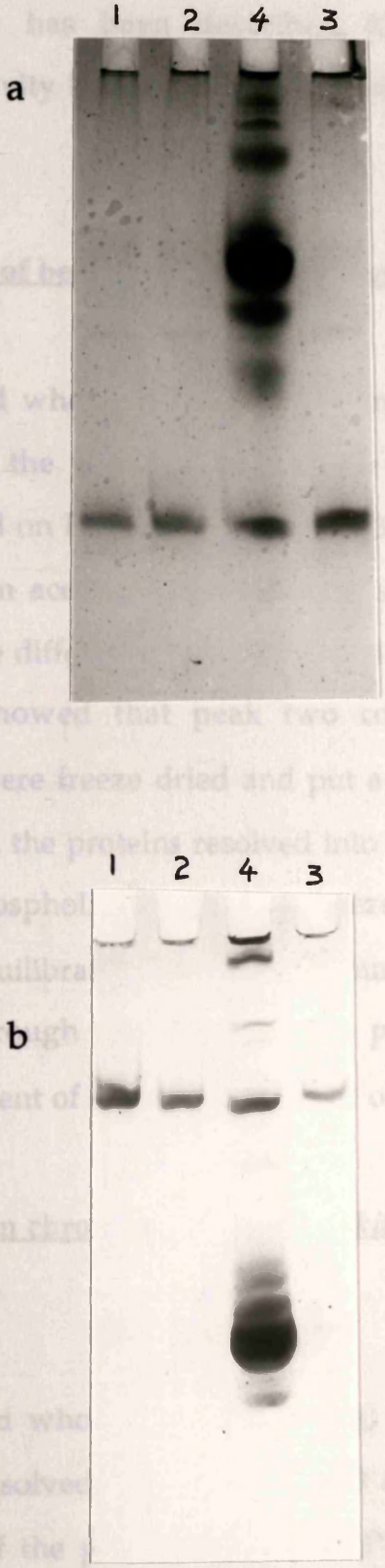
In another preparation of phospholipase A_2 from the whole venom, we loaded about 100 mg (200 mg/ml) of *Naja naja atra* venom to Bio-gel P-30 (3.5 x 30 cm) column using a 20 mM ammonium acetate buffer. The different fractions from the Bio-gel P-30 column were run on a basic urea gel in the presence of egg lecithin. The proteins sample were ran and incubated with rhodamine 6G dye as described previously and surprisingly three new minor isoforms were identified in the *Naja naja atra* venom, which was known to contain a single isoform of PLA_2 for a long time, but

Figure. 3.1.6.I. Ion exchange column chromatography on DEAE cellulose of *Naja naja atra* venom.



The lyophilised 200 mg *Naja naja atra* venom was dissolved in 1ml of distilled water in the presence of protease inhibitor, phenyl methyl sulphonyl fluoride (PMSF) and pH of venom solution was adjusted to 9.4 with ammonia solution. The venom sample was then applied to column (2 X 5 cm) of DE-52, equilibrated with 20 mM ammonium acetate at pH 9.4. The column was eluted at 25 ml per hour, initially with 40 ml of the equilibration buffer and the absorbed protein was eluted with linear gradient of 20 mM ammonium acetate at pH 5.0. The absorption was monitored at 280 nm and one ml fractions were collected.

Figure 3.1.6.II. a) Different purified PLA2 fractions and whole *Naja naja atra* whole venom on unmodified 20% basic urea gel electrophoresis.
b) Different purified PLA2 fractions and *Naja naja atra* whole venom on unmodified 20% acidic urea gel electrophoresis. Lanes 1, 2 and 3 show purified PLA_2 and Lane 4 shows whole venom.


1
2
4
3
a
1
2
4
3
b

a minor isoform has been described (Chang, *et al.*, 1975) and a corresponding activity band was clearly visible on basic urea-PAGE (fig. 3.1.6.1.)

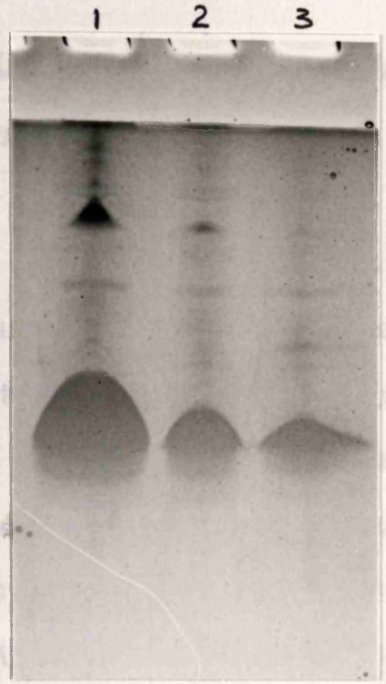
3.1.7.) Purification of bee venom phospholipase A_2.

The lyophilised whole bee venom 500 mg was dissolved in 2 ml of distilled water in the presence of protease inhibitor PMSF. The whole sample was loaded on Bio-gel P-30 column (3.5 x 40 cm) equilibrated with 20 mM ammonium acetate of pH 6.5. The same buffer was used as the eluting buffer. Five different peaks were obtained. Enzymatic assay and gel electrophoresis showed that peak two contained phospholipase A_2 fractions, which were freeze dried and put again on Bio-gel P-30 column, this time on FPLC, the proteins resolved into seven different peaks, Peak 2 which was of phospholipase A_2, was freeze dried and this time put on CM-52 column, equilibrated with 20 mM ammonium acetate of pH 5.2 and when first run through was collected, the phospholipase A_2 was eluted with a linear gradient of ammonium acetate of 0.6 M of pH 5.2.

3.1.8.) Gel filtration chromatography of *Agkistrodon piscivorus piscivorus* snake venom.

The lyophilised whole venom (100 mg) from *Agkistrodon piscivorus piscivorus* was dissolved in 1 ml of 20 mM ammonium acetate of pH 8.0 in the presence of the protease inhibitor, PMSF. The whole venom was fractionated on Bio-gel P-30 column (3.5 x 30 cm) using 20 mM ammonium acetate of pH 8.0 as an eluting buffer. Five different peaks

Figure. 3.1.6.1. Identification of phospholipase A_2 isoforms in the venom of *Naja naja atra.* The basic urea gels were prepared as described in materials and methods but incorporating 5 mg/ml of purified egg phosphatidylcholine in the running gel with 1 mM EDTA in the stacking gel and the tank electrolyte. Samples of ~~purified~~ enzymes were prepared by mixing 1:1 with bromophenol blue, but containing 1mM EDTA. After electrophoresis the gel was rinsed twice with distilled water and immersed in a solution containing 20 mM $CaCl_2$ and 0.012 % rhodamine 6G buffered with 0.1 M triethanolamine/Cl^-, pH 8.0 and incubated at 37 °C for 5-10 minutes. The reaction was stopped by washing with a solution containing 20 mM EDTA. The gels were photographed against black background and reflected white light. Lanes 1, 2 and 3 show different gel filtration fractions.


1
2
3

were obtained. Gel electrophoresis and PLA_2 assay showed that peak numbers 2 and 3 have phospholipase A_2 activity. As the venom of *Agkistrodon piscivorus piscivorus* contained the dimer PLA_2 and monomeric PLA_2, so at pH 8.0 dimeric ran as dimeric and monomer ran as monomeric PLA_2. The basic urea gel electrophoresis showed a number of unidentified acidic proteins (fig. 3.1.8.).

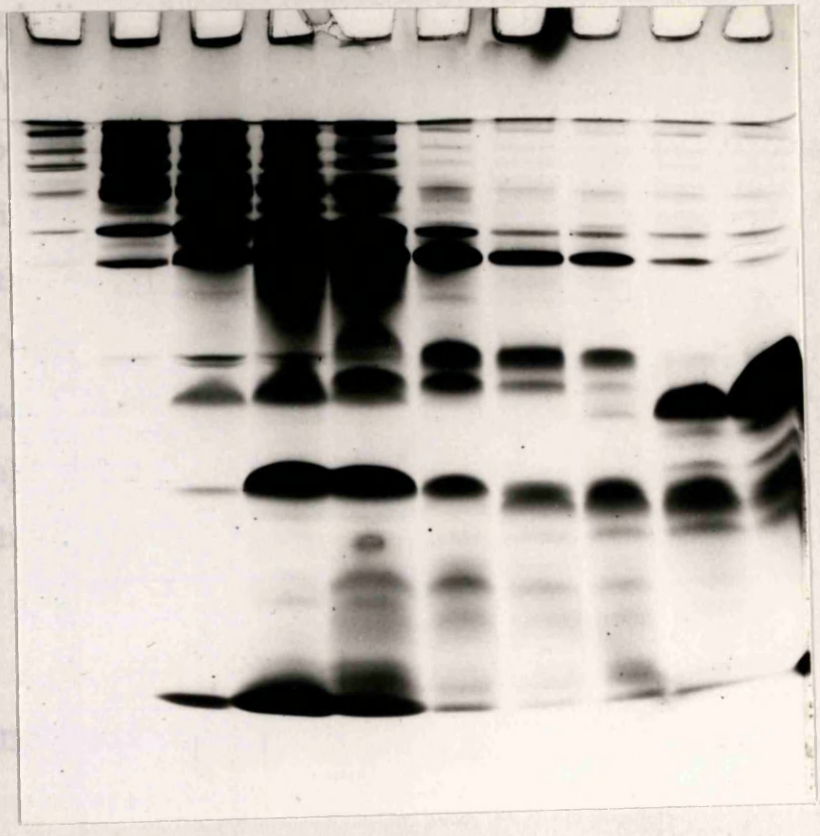
3.1.8.1.) Identification of phospholipase A_2 isoforms in the venom of *Agkistrodon piscivorus piscivorus.*

Snake venoms often contain multiple forms of phospholipase A_2 which differ in sequence, p*I* and in some cases their state of aggregation. It is not uncommon to find as many as four different enzymes in a single venom source, The gel filtration step at neutral pH permits size separation of dimeric and monomeric phospholipases; the dimeric enzymes have been observed thus far to be acidic molecules with $pI \approx 4$. The designation of phospholipase A_2 as dimeric enzymes based upon their behaviour on gel filtration columns is purely operational and does not imply that these enzymes are functional only as dimers or monomers (Maraganore *et al.*, 1984).

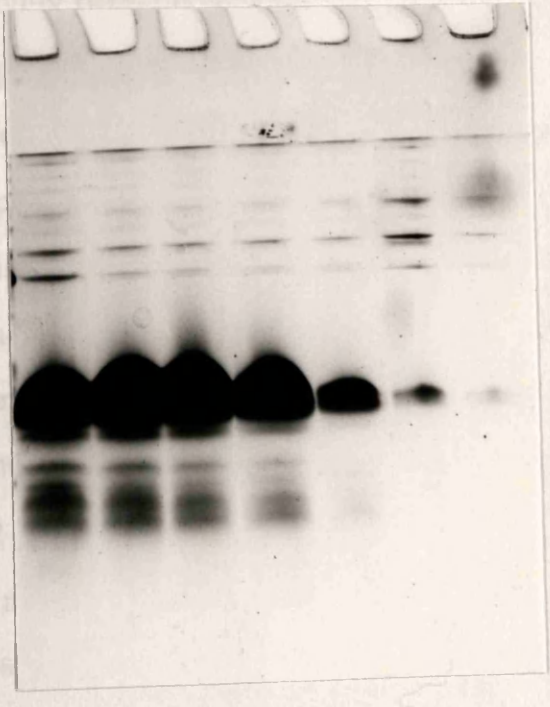
There were three known phospholipases A_2 in the venom of *Agkistrodon piscivorus piscivorus*, an acidic dimer, and two basic monomeric enzymes. The separation of the dimeric from the monomeric was achieved by gel filtration on Bio-gel P-30 as shown on basic urea gel electrophoresis (fig. 3.1.8.) Of the two basic monomeric enzymes, one was designated as Asp-D-49 and other was designated as Asp-K-49. The Asp-K-

Figure. 3.1.8. a & b. Different fractions from gel filtration Bio-Gel P-30 column chromatography on modified 20% basic urea gel electrophoresis, indicating presence of a number of acidic proteins in *Agkistrodon piscivorus piscivorus* venom. Gel a and b show selective gel filtration fractions.

a



b

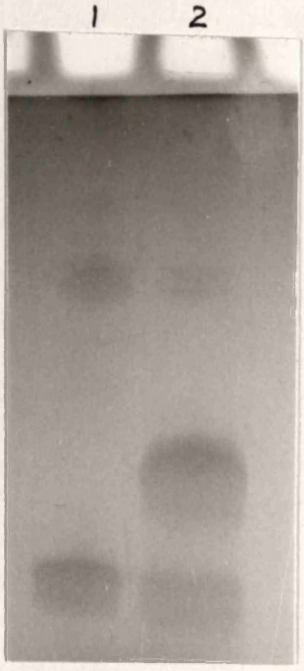


49 phospholipase A_2 was reported to be inactive (Van Den Bergh et al., 1989). We have identified new isoforms of phospholipase A_2 in this venom by rhodamine 6G gel electrophoresis. The different fractions from the gel filtration column were run on the basic urea gel in the presence of egg lecithin as described earlier. The dimeric acidic form ran as a close doublet with one major and one minor components and the monomeric form also ran as doublet, both are active components. Apart from these four major phospholipases A_2, there were at least two or three more minor phospholipases A_2 (fig. 3.1.8.1.).

3.1.9.) Arginine modification of PLA_2 isoforms.

Until approximately ten years ago the specific chemical modification of arginine was relatively difficult to achieve. The high pKa of the guanidine functional group (pKa≈12-13) necessitated fairly drastic reaction conditions to generate an effective nucleophile and most proteins are not stable to extreme alkaline pH. Phenylglyoxal is a moderately specific reagent for modifying R residues although it can react rather more rapidly with the N-terminal residue. Vensel and Kantrwitz (1980) used this reagent to study the involvement of arginine 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 tried here to see modification of arginine in phospholipase A_2 isoforms on basic urea gel

Figure. 3.1.8.1. Identification of phospholipase A_2 isoforms in the venom of *Agkistrodon piscivorus piscivorus*. The basic urea gels were prepared as described in materials and methods but incorporating 5 mg/ml of purified egg phosphatidylcholine in the running gel with 1 mM EDTA in the stacking gel and the tank electrolyte. Samples of ~~purified~~ enzymes were prepared by mixing 1:1 with bromophenol blue, but containing 1mM EDTA. After electrophoresis the gel was rinsed twice with distilled water and immersed in a solution containing 20 mM $CaCl_2$ and 0.012 % rhodamine 6G buffered with 0.1 M triethanolamine/Cl^-, pH 8.0 and incubated at 37 °C for 5-10 minutes. The reaction was stopped by washing with a solution containing 20 mM EDTA. The gels were photographed against black background and reflected white light. Lanes 1, and 2 show gel filtration fractions.


1
2

electrophoresis. Basic urea gel electrophoresis has the advantage that you can see arginine modification. As the mobility of basic urea gel is determined on basis of the ratio of net negative charge (i.e. N-ter+Asp+Glu+Tyr-Arg) to total mass, so arginine modified proteins run faster on these gels as shown. Modification of arginine by this reagent causesthe protein to form dimer, tetramer, octamer and so on in presence of excess monomer (fig. 3.1.9.).

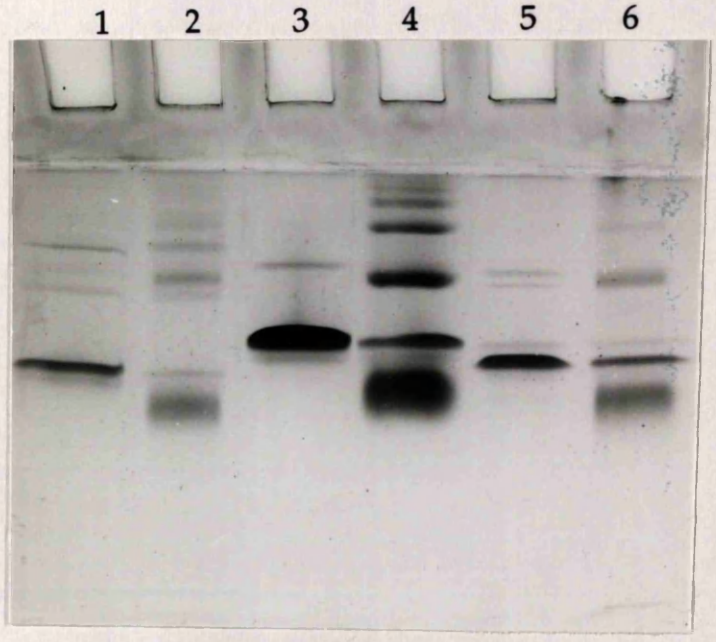

1
2
3
4
5
6

Figure. 3.1.9. Arginine modification of phospholipase A_2 isoforms.
The phospholipase A_2 isoforms were treated with phenylglyoxal and the native and phenylglyoxal treated enzymes were ran on modified 20% basic urea gels.
Lane 1: acidic PLA_2 from *Naja mossambica mossambica*, Lane 2: acidic PLA_2 from *Naja mossambica mossambica* treated with phenylglyoxal, Lane 3: basic non-toxic PLA_2 from *Naja mossambica mossambica*, Lane 4: basic non-toxic PLA_2 from *Naja mossambica mossambica* treated with phenylglyoxal, Lane 5: PLA_2 from *Naja naja atra*, Lane 6: PLA_2 from *Naja naja atra* treated with phenylglyoxal.

Part II

3.2.1.) Activation of bee venom phospholipase A_2 in 1-propanol by long-chain fatty acid.

There is considerable interest in using enzymes in organic or mixed water/organic solvents where they can sometimes exhibit interesting properties not displayed in aqueous medium (Klibanov, 1989; Dordick, 1991; Gupta, 1992). In the case of phospholipase enzymes the major use of organic solvents has been to dissolve the water insoluble substrates to make a homogenous reaction medium. This has frequently been achieved by the use of emulsifiers to disperse long-chain lecithins (De Haas *et al.*, 1971) and this tends to increase the catalytic activity of the enzymes. Others have used a variety of alcohols for this purpose and in contrast, this seems to lower the activity of the enzymes. Warwicker *et al.*, (1994) used 20 % alcohol (methanol or ethanol) and have shown that the activity of porcine pancreatic phospholipase A_2 measured at pH 8.0, is reduced when methanol or ethanol is added to the aqueous solution.

Drainas (1978) used a conductimetric modification of the titrimetric assay where the substrate long-chain phosphatidylcholine species was dissolved in 20 % 1-propanol and have shown that when the substrate was highly purified by deionisation, reaction progress curves catalysed by bee venom PLA_2 were biphasic due to activation by the fatty acid reaction product and that exogenous fatty acids with chain length > C12 could remove the biphasic nature of the reaction progress curves. Mezna *et al.*, (1994) has shown that 1-propanol is highly inhibitory for the PLA_2 catalysed hydrolysis of short/medium chain length phosphatidylcholine

derivatives. The aim of this study was to investigate the effect of 1-propanol concentration on product activation and to extend the results to investigate fatty acid binding by other enzymes.

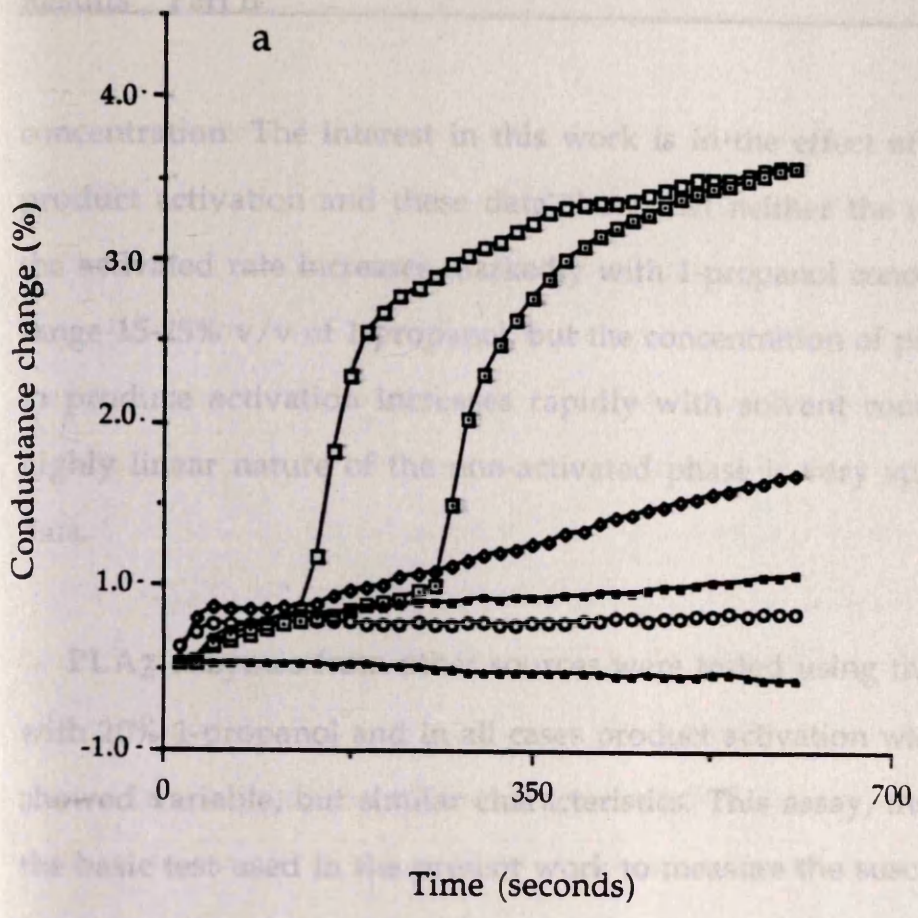
3.2.1.1.) The effect of 1-propanol concentration on product activation of phospholipases.

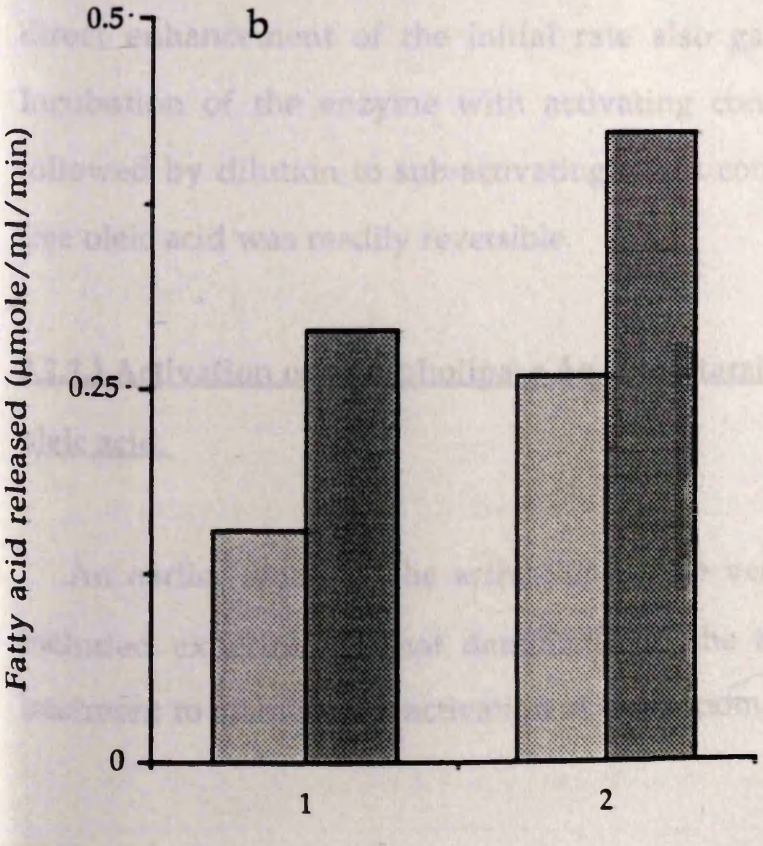
Egg phosphatidylcholine was used as a substrate for bee venom PLA_2 using the standard conductimetric assay at 20% v/v of 1-propanol. It became clear that deionisation of the substrate resulted in the production of highly biphasic curves and that these became monophasic in the presence of excess long-chain fatty acids. The effect of 1-propanol concentration was also investigated over the range 0-25% v/v and figure 3.2.1.1.Ia. shows that no reaction could be detected below 15% 1-propanol concentration presumably because the reaction products are non-conducting under these conditions. Above 15% propanol the biphasic curves showed a slow initial phase which did not change greatly in rate, but extended very considerably with increased 1-propanol concentration.

These results confirm that the release of long-chain fatty acids of chain length >C12 cannot be followed by conductimetric analysis in pure aqueous medium and that with egg phosphatidyl choline the limit lies between 10 and 15% 1-propanol. More interestingly they show that where rates can be measured they are not very strongly affected by 1-propanol concentration. This is in marked contrast to the data of Mezna who showed that the activity of the bee venom enzyme against 1-palmitoyl, 2-nonanoyl phosphatidylcholine fell between 15 and 20% 1-propanol

Figure. 3.2.1.1.I. Activation of the bee venom phospholipase A_2 in 1-propanol.

The purified phospholipase A_2 (1mg/ml) from the bee venom was dissolved in 10 mM triethanolamine/HCl buffer of pH 8.0. The different triethanolamine/HCl buffers of pH 8.0 containing 1-propanol concentration of 0.0 ( ), 5.0 ( ), 10.0 ( ), 15.0 ( ), 20.0 ( ) and 25.0 % ( ) were prepared. Phospholipase A_2 (2 µl) conductimetric assays were carried out by using egg phosphatidylcholine as a substrate and 0.1 mM $CaCl_2$ in 2 ml of respective buffer (a). Figure (b) shows comparison of native and activated phospholipase A_2 with oleoyl imidazolide on 15 and 20% 1-propanol concentrations. The enzyme assays were done as in (a). (1) Native enzyme (2) activated enzyme. ( ) 15 % 1-propanol ( ) 20 % 1-propanol.


a
4.0
3.0
2.0
1.0
-1.0
Conductance change (%)
0
350
700
Time (seconds)


b
0.5
0.25
0
Fatty acid released (µmole/ml/min)
1
2

concentration. The interest in this work is in the effect of 1-propanol on product activation and these data show that neither the unactivated nor the activated rate increases markedly with 1-propanol concentration in the range 15-25% v/v of 1-propanol, but the concentration of product required to produce activation increases rapidly with solvent concentration. The highly linear nature of the non-activated phase is very apparent in these data.

PLA_2 enzymes from other sources were tested using the egg PC assay with 20% 1-propanol and in all cases product activation was apparent and showed variable, but similar characteristics. This assay, therefore, formed the basic test used in the present work to measure the susceptibility of the enzyme to free fatty acids. As a standard assay for fatty acid activation the ratio of the initial rate to the maximum product activated rate gives a value of ca 50-fold. Activation by inclusion of free oleic acid, measured as direct enhancement of the initial rate also gave values of ca 50-fold. Incubation of the enzyme with activating concentrations of fatty acid followed by dilution to sub-activating levels confirmed that activation by free oleic acid was readily reversible.

3.2.2.) Activation of phospholipase A_2 by glutaraldehyde in the presence of oleic acid.

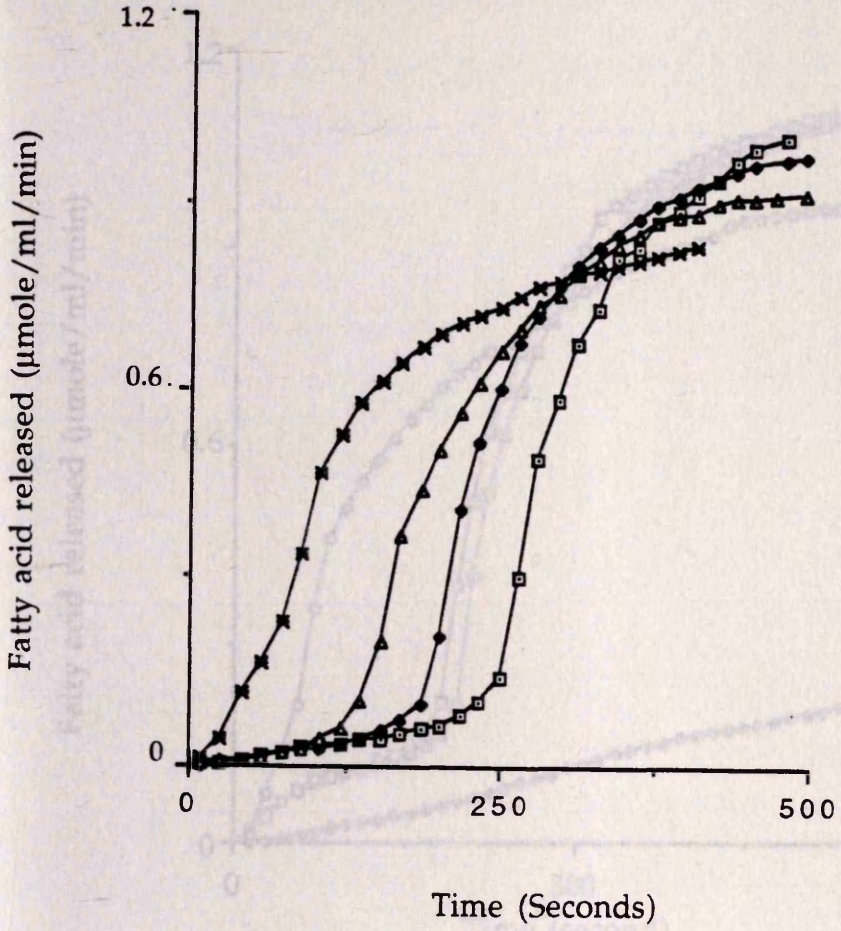
An earlier study of the activation of bee venom PLA_2 by fatty acids included experiments that demonstrated the ability of glutaraldehyde treatment to stabilise the activation of bee venom PLA_2 by oleic acid

against dilution. In these experiments no tests were carried out to see whether or not fatty acid remained associated with the enzyme as a consequence of the glutaraldehyde treatment. The original work showed that enzyme activated in the presence of glutaraldehyde was quite unstable and the aim of this work was to find conditions necessary to stabilise the activity and then to see whether the fatty acid could be removed. Figure 3.2.2.I. shows the time-dependent activation of bee venom phospholipase A_2 by 0.25 % glutaraldehyde in the presence of 0.1 mM oleic acid. Bee venom phospholipase A_2 (1 mg/ml in 10 mM triethanolamine buffer of pH 8.0 containing 20 % v/v 1-propanol) was incubated with 0.1 mM of free oleic acid, an aqueous solution of glutaraldehyde was added to give a final concentration of 0.25% v/v. Two µl aliquots were withdrawn at measured time intervals and tested for activation using egg phosphatidylcholine as the substrate. The controls figure 3.2.2.II. included measurement of the effect of oleic acid and of glutaraldehyde alone. Using the increase in initial rate as a measure of activation, the maximum activation was induced by the combination of oleic acid and glutaraldehyde but this occurred with very considerable general inactivation as measured by the fall in maximum rate.

3.2.3.) Stabilising activated PLA_2 by borohydride reduction.

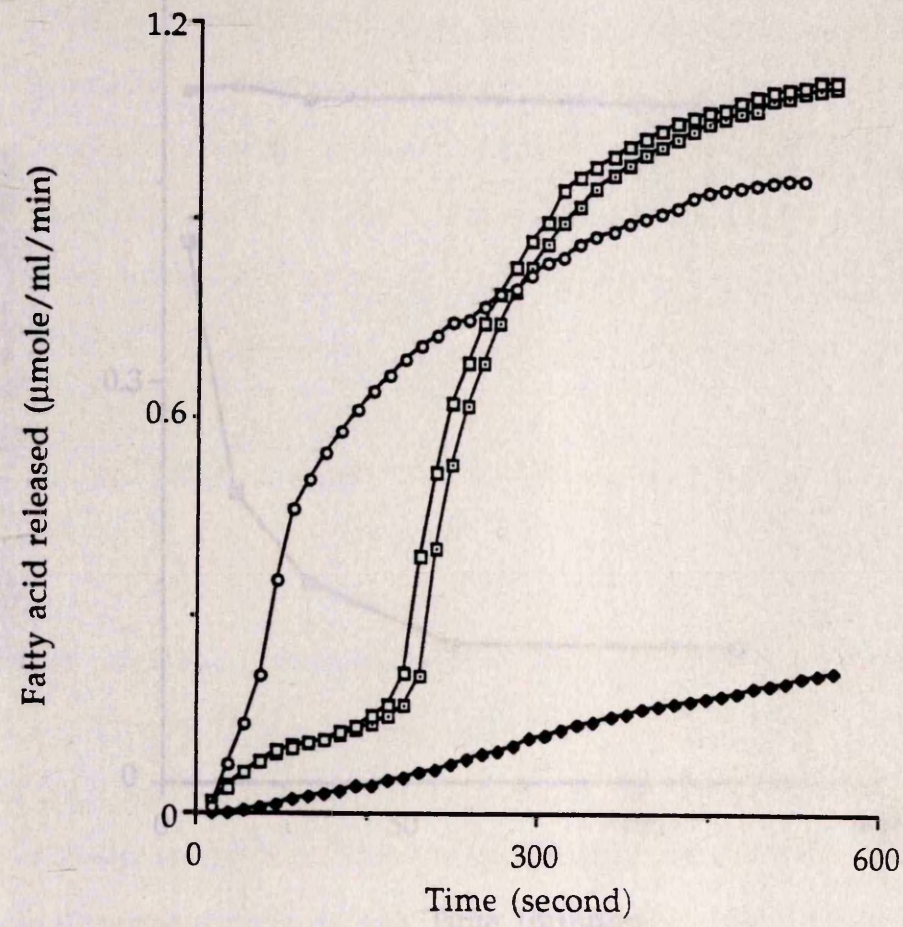
The simplest method to stabilise the enzyme against inactivation by glutaraldehyde was reduction using sodium borohydride. Preliminary tests showed that the enzyme was stable to this reagent at pH 8.0. Figure 3.2.3. shows that Borohydride reduction was very effective in stabilising the glutaraldehyde activated enzyme. On the assumption that

Figure. 3.2.2.I. Time dependent activation of bee venom phospholipase A_2 by glutaraldehyde in the presence of oleic acid.



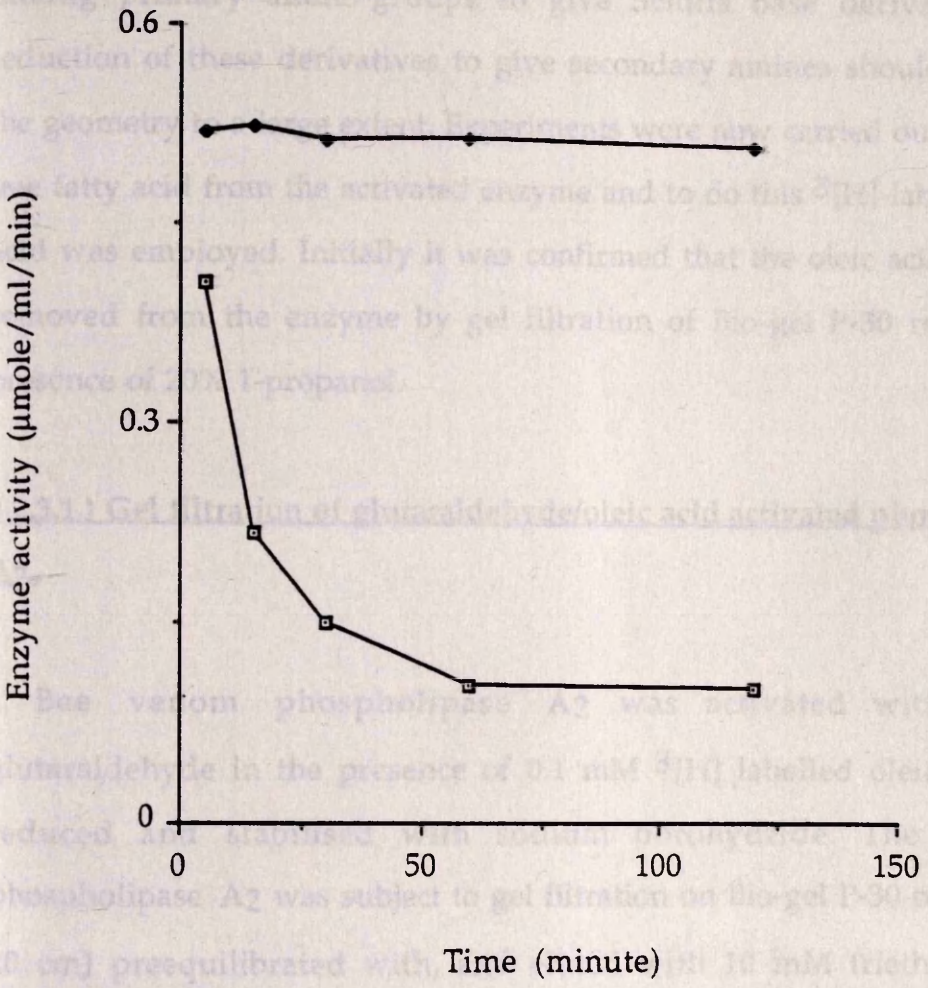
The purified bee venom phospholipase A_2 (1 mg/ml) was dissolved in 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % 1-propanol. The enzyme was then treated with 0.1 mM oleic acid and 0.25 % glutaraldehyde and the progress of activation was checked by taking 2 µl of sample at different time interval. The conductimetric assays were carried out by using 2 ml of 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol in conductimetric cells. The purified egg phosphatidylcholine was used as substrate and 0.1 mM $CaCl_2$ was also added to cells. (-□-) native enzyme, (-●-) after 1 minute of treatment, (-▲-) after 5 minute of treatment, (-✱-) after 10 minutes of treatment.

Figure. 3.2.2.II. Stabilisation of fatty acid activation of bee venom phospholipase A_2 by glutaraldehyde.



The purified bee venom phospholipase A_2 (1 mg/ml) was dissolved in 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol. The enzyme was then incubated at 25 °C either along (-□-) , with 0.1 mM oleic acid (-□-), with 0.25 % glutaraldehyde (-●-) or with 0.1 mM oleic acid followed by 0.25 % glutaraldehyde (-○-). After about 10 minutes, 2 µl samples were withdrawn for conductimetric measurement of the time course of hydrolysis of substrate. The conductimetric assays were carried out by using 2 ml of 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % 1-propanol in conductimetric cells. The purified egg phosphatidylcholine was used as substrate and 0.1 mM $CaCl_2$ was also added to cells.

Figure. 3.2.3. Borohydride reduction of glutaraldehyde treated bee venom phospholipase A_2.



Two samples of bee venom phospholipase A_2 (1 mg/ml) in 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol were treated with 0.25 % glutaraldehyde in the presence of 0.1 mM oleic acid at 25 °C. Enzyme assays were performed by using phosphatidylcholine as substrate and 0.1 mM $CaCl_2$ in 2 ml of triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol. One sample (-●-) was treated with sodium borohydride and 2 µl aliquots withdrawn for assay at measured times. The other sample (-□-) was not treated with sodium borohydride.

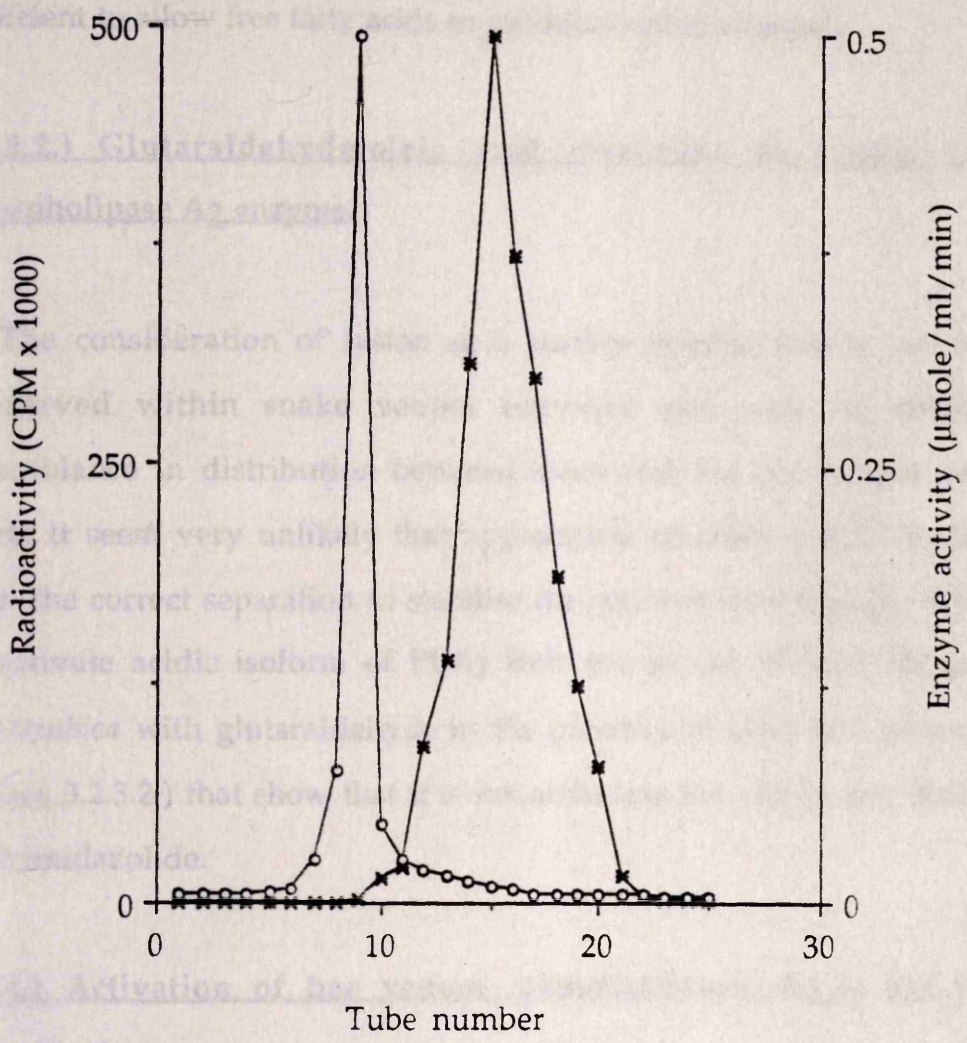
glutaraldehyde acts by trapping the enzyme in an activated state by cross linking primary amino-groups to give Schiffs base derivatives, the reduction of these derivatives to give secondary amines should not alter the geometry to a large extent. Experiments were now carried out to extract free fatty acid from the activated enzyme and to do this ^{3}[H]-labelled oleic acid was employed. Initially it was confirmed that the oleic acid could be removed from the enzyme by gel filtration of Bio-gel P-30 resin in the presence of 20% 1-propanol.

3.2.3.1.) Gel filtration of glutaraldehyde/oleic acid activated phospholipase A_2.

Bee venom phospholipase A_2 was activated with 0.25 % glutaraldehyde in the presence of 0.1 mM ^{3}[H] labelled oleic acid and reduced and stabilised with sodium borohydride. The activated phospholipase A_2 was subject to gel filtration on Bio-gel P-30 column (1 x 10 cm) preequilibrated with, and eluted with 10 mM triethanolamine buffer of pH 8.0 containing 20 % 1-propanol. Figure 3.2.3.1. shows that the enzyme could be separated from ^{3}[H] labelled oleic acid. The enzyme in the active peak was then demonstrated to be activated both on the criteria of total recovery of activation and on the ratio of the maximum to the initial rate.

These experiments show that of a total activation factor of 50-fold, a factor of 10-fold is due to some form of conformational stabilisation. The residual 5-fold can only be obtained if the free fatty acid is also present in

Figure. 3.2.3.1. Activation of bee venom phospholipase A_2 by glutaraldehyde does not require the presence of oleic acid.



Purified bee venom phospholipase A_2 (1 mg/ml) in 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol was activated with 0.25 % glutaraldehyde in the presence of 0.1 mM ^{3}[H] labelled oleic acid and then passed through a gel filtration Bio-gel P-30 column (10 x 1 cm) prepared in 10 mM triethanolamine/HCl buffer containing 20 % v/v 1-propanol and enzyme was tested for retention of activity. Enzyme activity (-o-) and radioactivity (-*-)

the enzyme. Thus fixation by glutaraldehyde stabilises a relatively activated form of the enzyme, but conformational flexibility is still sufficient to allow free fatty acids to produce further changes.

3.2.3.2.) Glutaraldehyde/oleic acid treatment of snake venom phospholipase A_2 enzyme.

The consideration of lysine as a surface residue that is not highly conserved within snake venom enzymes and with no detectable resemblance in distribution between these and the bee venom enzyme made it seem very unlikely that appropriate residues would be present with the correct separation to stabilise the conformation change. Attempts to activate acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* with glutaraldehyde in the presence of oleic acid give results (figure 3.2.3.2.) that show that it is not activating but can be activated with acyl imidazolide.

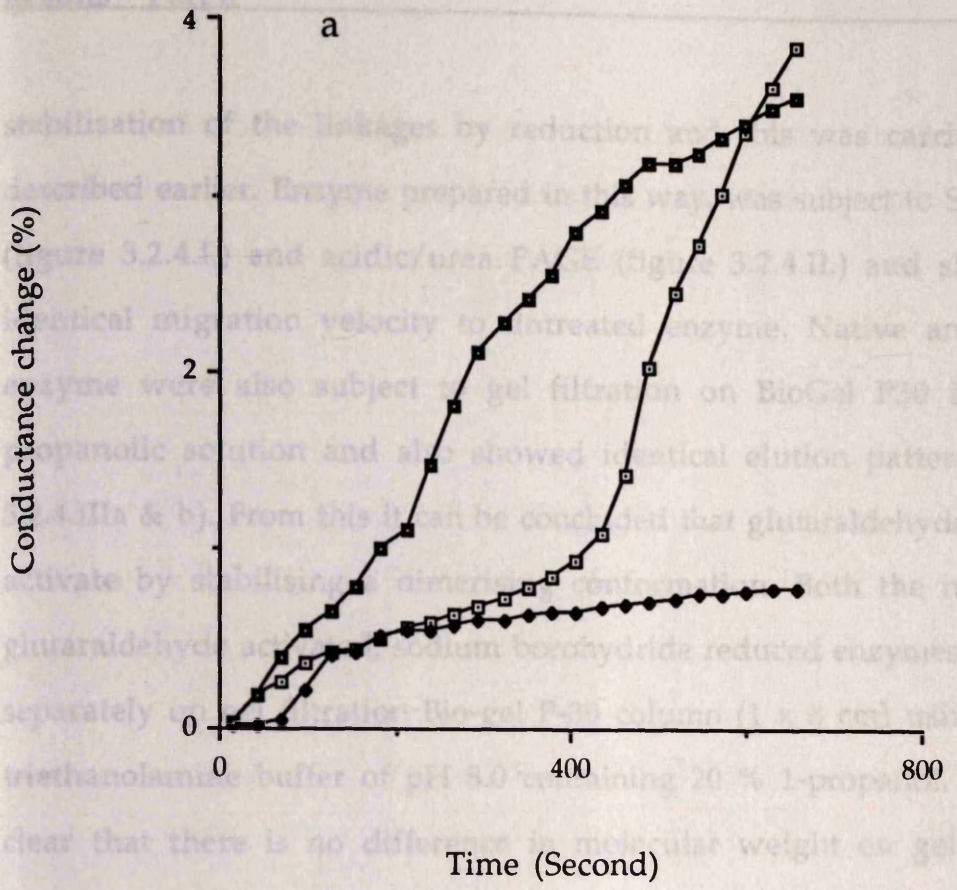
3.2.4.) Activation of bee venom phospholipase A_2 is not due to dimerisation.

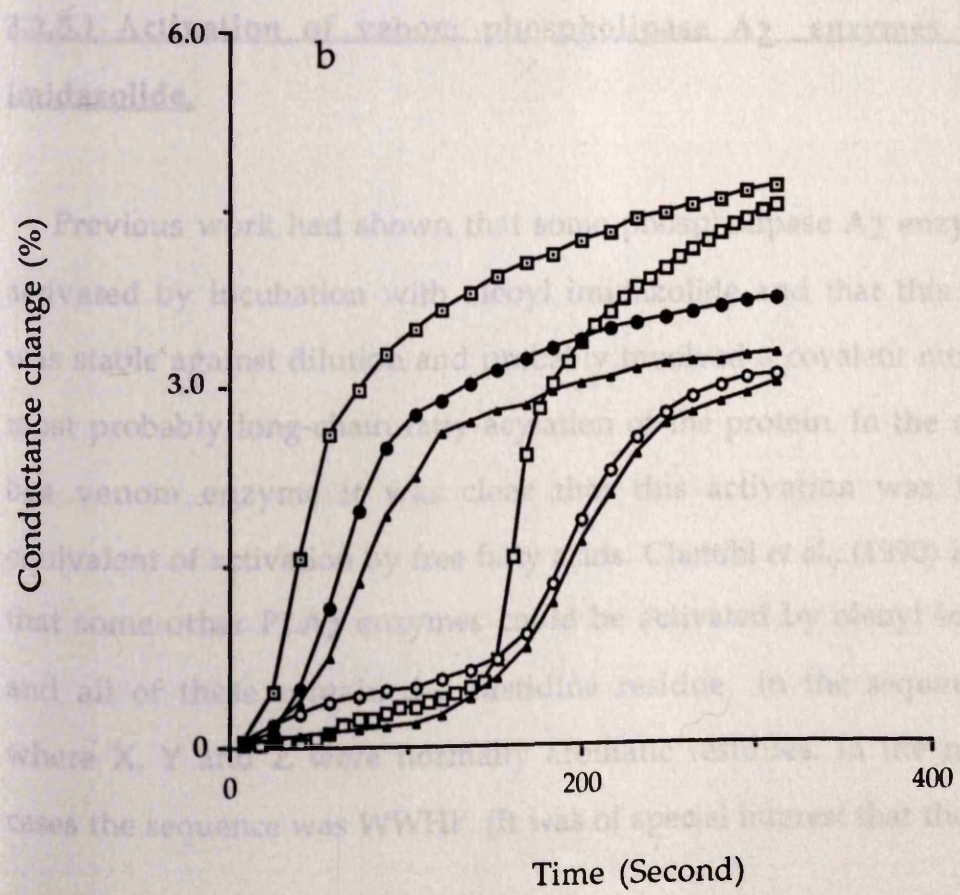
Because the best known property of glutaraldehyde is its ability to cross link proteins it was clear that the reagent could produce intramolecular as well as intermolecular bonds. Many studies have been carried out that suggest that PLA_2 enzymes dimerise at high concentrations and are most active in the dimer form. Experiments were therefore carried out to see if the enzyme activated by glutaraldehyde/oleic acid had changed in molecular size. The essential first step in any such investigation is

Figure. 3.2.3.2. Stabilisation of fatty acid activation of acidic isoform of phospholipase A_2 by glutaraldehyde.

a) The purified non-toxic acidic isoform (BG-6) of phospholipase A_2 (1 mg/ml) from the venom of *Naja mossambica mossambica* in 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol was incubated either along (—□—) or with 0.25 % glutaraldehyde (—●—) or with 0.1 mM oleic acid followed by 0.25 % glutaraldehyde (—■—). The enzyme assays were carried out in the presence of 0.1 mM $CaCl_2$ using 2 ml of 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol. Egg phosphatidylcholine was used as substrate.

b) The non-toxic acidic (BG-6) (1 mg/ml) and non-toxic basic (1 mg/ml) isoforms of PLA_2 from the venom of *Naja mossambica mossambica* and bee venom phospholipases A_2 (1 mg/ml) in 10 mM triethanolamine/HCl buffer of pH 8.0 were activated with molar equivalent of oleoyl imidazolide (activator) and enzyme assay were performed as in (a). native (—□—) and activated bee venom PLA_2 (—▣—), native (—○—) and activated acidic PLA_2 (—●—), native (—▬—) and activated non-toxic basic PLA_2 (—▬—).


a
4
2
0
Conductance change (%)
0
400
800
Time (Second)


b
6.0
3.0
0
Conductance change (%)
0
200
400
Time (Second)

stabilisation of the linkages by reduction and this was carried out as described earlier. Enzyme prepared in this way, was subject to SDS PAGE (figure 3.2.4.I.) and acidic/urea PAGE (figure 3.2.4.II.) and showed an identical migration velocity to untreated enzyme. Native and treated enzyme were also subject to gel filtration on BioGel P30 in 20% 1-propanolic solution and also showed identical elution patterns (figure 3.2.4.IIIa & b). From this it can be concluded that glutaraldehyde does not activate by stabilising a dimerising conformation. Both the native and glutaraldehyde activated, sodium borohydride reduced enzymes were run separately on gel filtration Bio-gel P-30 column (1 x 8 cm) using 10 mM triethanolamine buffer of pH 8.0 containing 20 % 1-propanol. It is very clear that there is no difference in molecular weight on gel filtration column.

3.2.5.) Activation of venom phospholipase A_2 enzymes by oleoyl imidazolide.

Previous work had shown that some phospholipase A_2 enzymes were activated by incubation with oleoyl imidazolide and that this activation was stable against dilution and probably involved a covalent modification, most probably long-chain fatty acylation of the protein. In the case of the bee venom enzyme it was clear that this activation was the stable equivalent of activation by free fatty acids. Chettibi *et al.*, (1990) had shown that some other PLA_2 enzymes could be activated by oleoyl imidazolide and all of these contained a histidine residue in the sequence XYHZ where X, Y and Z were normally aromatic residues. In the majority of cases the sequence was WWHF. (It was of special interest that the sequence

Figure. 3.2.4.I. SDS gel electrophoresis of native and activated bee venom phospholipase A_2 with glutaraldehyde in presence of oleic acid.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12.5 %) was prepared as described in material and methods. a) native bee venom PLA_2 and b) Bee venom PLA_2 treated with 0.25 % glutaraldehyde, 0.1 mM oleic acid and reduced with sodium borohydride.

Figure. 3.2.4.II. The acidic acid/urea gel electrophoresis of native and activated bee venom phospholipase A_2 with glutaraldehyde in presence of oleic acid.

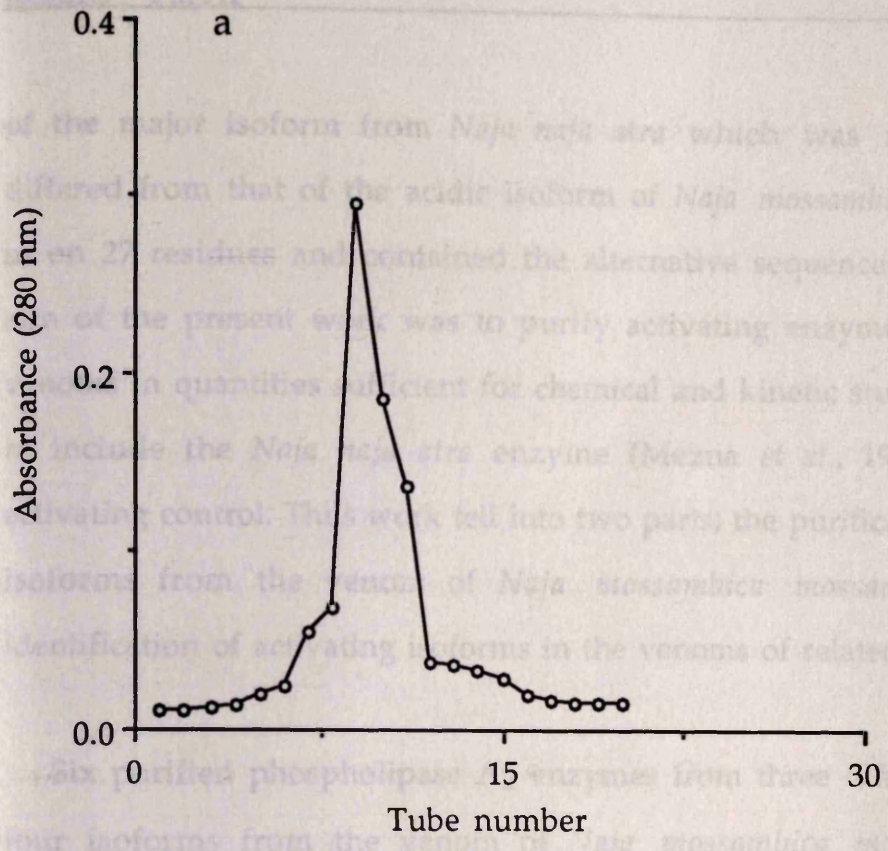
The acidic acid/urea (20 % containing 8M urea) gel electrophoresis was prepared as described in materials and methods. a) Native bee venom PLA_2 and b) treated bee venom PLA_2 with 0.25 % glutaraldehyde, 0.1 mM oleic acid and reduced with borohydride.

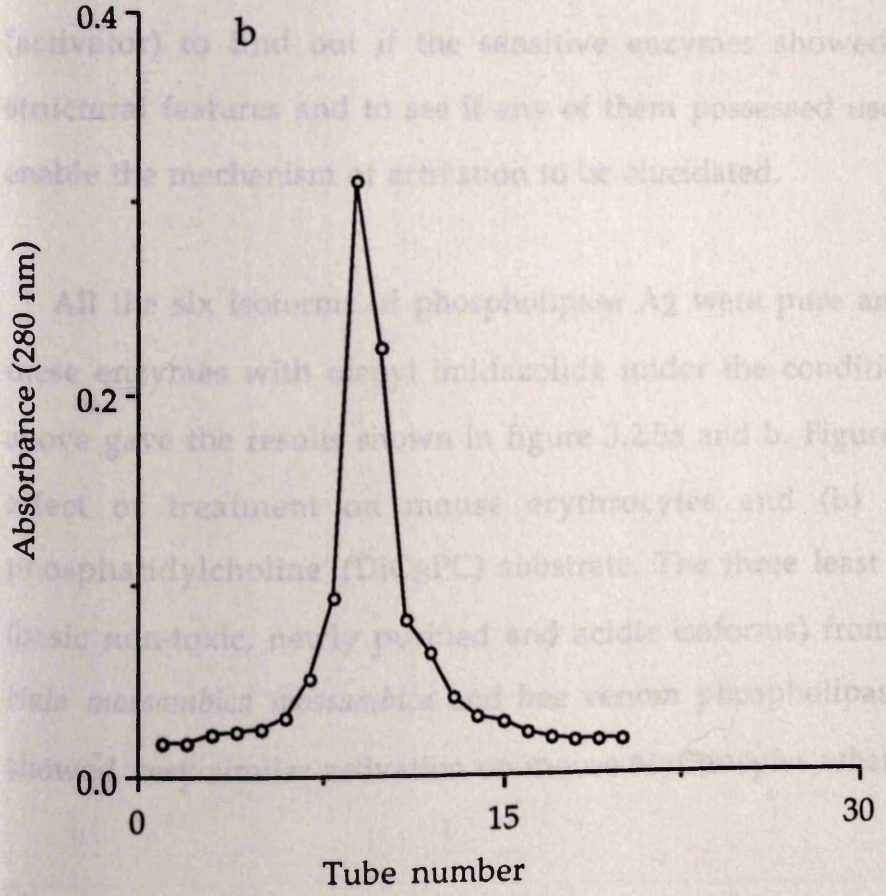
Figure. 3.2.4.III. Activation of the bee venom PLA_2 by glutaraldehyde in the presence of oleic acid is not due to dimerisation.

Gel filtration Bio-gel P-30 column chromatography of native and treated bee venom phospholipase A_2. The Bio-gel P-30 column (1 x 8 cm) was prepared and equilibrated in both cases with 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol and 200 µg of the bee venom phospholipase A_2 in 200 µl of 10 mM triethanolamine/HCl buffer of pH 8.0 containing 20 % v/v 1-propanol was applied to column and 1/2 ml fractions were collected.

a) Native bee venom PLA_2 (200 µg).

b) The bee venom PLA_2 (200µg) treated with 0.1 mM oleic acid, 0.25 % glutaraldehyde and reduced with sodium borohydride.


a
0.4
0.2
0.0
Absorbance (280 nm)
0
15
30
Tube number


b
0.4
0.2
0.0
Absorbance (280 nm)
0
15
30
Tube number

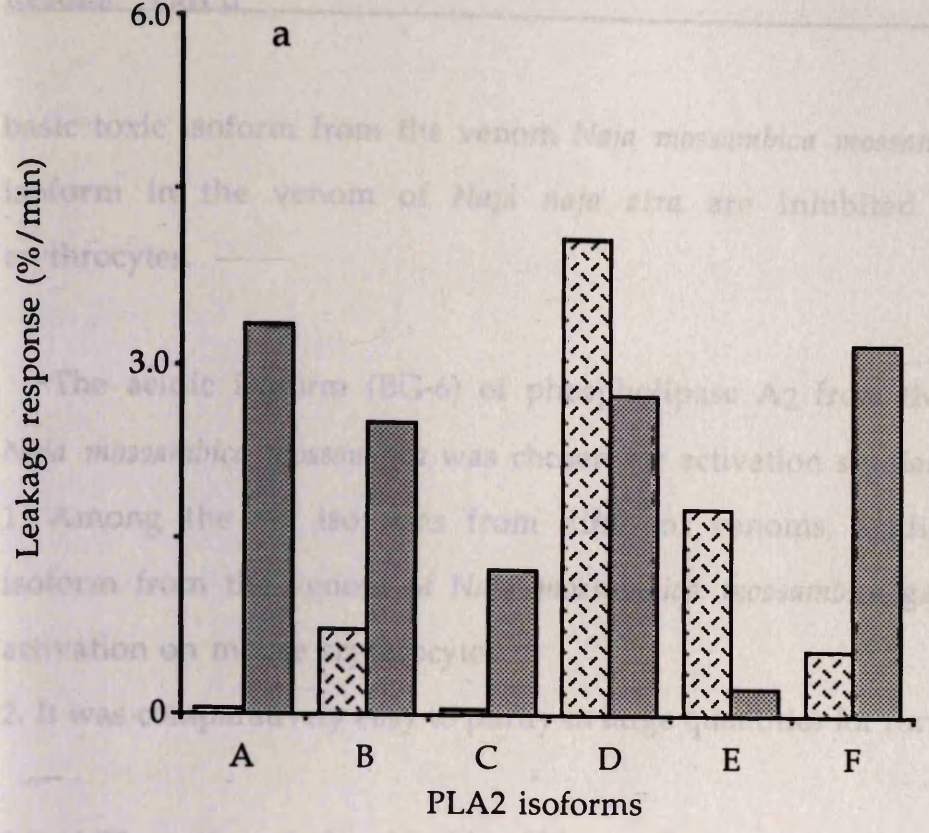
of the major isoform from *Naja naja atra* which was non-activating differed from that of the acidic isoform of *Naja mossambica mossambica* in on 27 residues and contained the alternative sequence WWDF). One aim of the present work was to purify activating enzymes from whole venoms in quantities sufficient for chemical and kinetic studies. This was to include the *Naja naja atra* enzyme (Mezna *et al.*, 1994) as a non-activating control. Thus work fell into two parts; the purification of isoforms from the venom of *Naja mossambica mossambica* and the identification of activating isoforms in the venoms of related species.

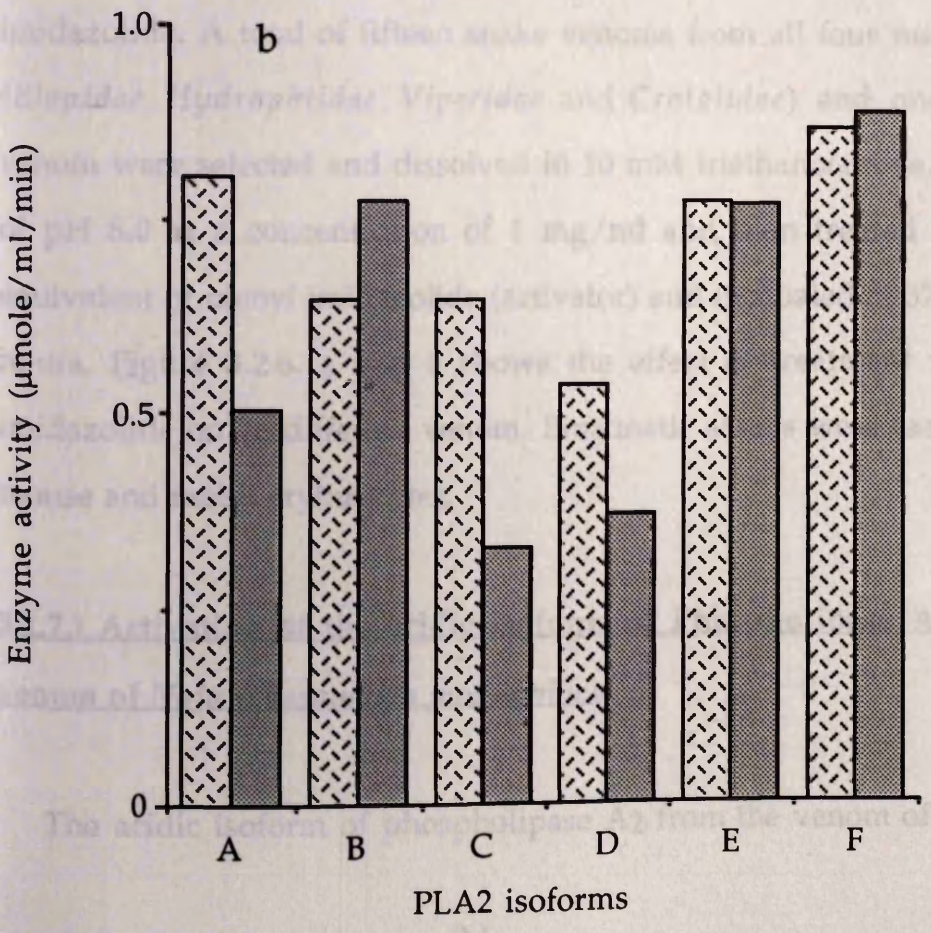
Six purified phospholipase A_2 enzymes from three different venoms, four isoforms from the venom of *Naja mossambica mossambica*, one major isoform from the *Naja naja atra* and one from bee venom (*Apis mellifera*) were treated with molar equivalent of oleoyl imidazolide (activator) to find out if the 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.

All the six isoforms of phospholipase A_2 were pure and treatment of these enzymes with oleoyl imidazolide under the conditions mentioned above gave the results shown in figure 3.2.5a and b. Figure (a) shows the effect of treatment on mouse erythrocytes and (b) on dioctanoyl phosphatidylcholine (DiC8PC) substrate. The three least basic isoforms (basic non-toxic, newly purified and acidic isoforms) from the venom of *Naja mossambica mossambica* and bee venom phospholipase A_2 isoforms showed very similar activation on mouse erythrocytes whereas the highly

Figure 3.2.5. Activation and inhibition of phospholipase A_2 isoforms. The purified phospholipase A_2 isoforms from different snake venoms and bee venom were treated with molar equivalent of oleoyl imidazolide and enzyme assays were carried out either a) on mouse erythrocytes or b) on substrate, dioctanoylphosphatidylcholine as described in materials and methods.

A) Acidic isoform (BG-6) from *Naja mossambica mossambica*. B) Basic non-toxic isoform (BG-3/4) from *Naja mossambica mossambica*. C) Newly purified isoform (BG-5) from *Naja mossambica mossambica*. D) Basic toxic isoform (BG-1/2) from *Naja mossambica mossambica*. E) Phospholipase A_2 from *Naja naja atra*. F) Bee venom phospholipase A_2. (⊡) native enzymes, (▩) activated enzymes.


a
6.0
3.0
0
Leakage response (%/min)
A
B
C
D
E
F
PLA2 isoforms


b
1.0
0.5
0
Enzyme activity (µmole/ml/min)
A
B
C
D
E
F
PLA2 isoforms

basic toxic isoform from the venom *Naja mossambica mossambica*, major isoform in the venom of *Naja naja atra* are inhibited on mouse erythrocytes.

The acidic isoform (BG-6) of phospholipase A_2 from the venom of *Naja mossambica mossambica* was chosen for activation studies, because

1. Among the six isoforms from different venoms, acidic non-toxic isoform from the venom of *Naja mossambica mossambica* gave the best activation on mouse erythrocytes.
2. It was comparatively easy to purify in large quantities for further studies.

3.2.6.) The action of oleoyl imidazolide on whole venoms.

Whole venoms of the various snakes and bee were treated with oleoyl imidazolide. A total of fifteen snake venoms from all four major families (*Elapidae*, *Hydrophiidae*, *Viperidae* and *Crotalidae*) and one from bee venom were selected and dissolved in 10 mM triethanolamine/HCl buffer of pH 8.0 at a concentration of 1 mg/ml and then treated with molar equivalent of oleoyl imidazolide (activator) and incubated at 37 °C for two hours. Figure 3.2.6. a and b shows the effect of treatment with oleoyl imidazolide on 16 different venom. Enzymatic assays were carried out on mouse and rabbit erythrocytes.

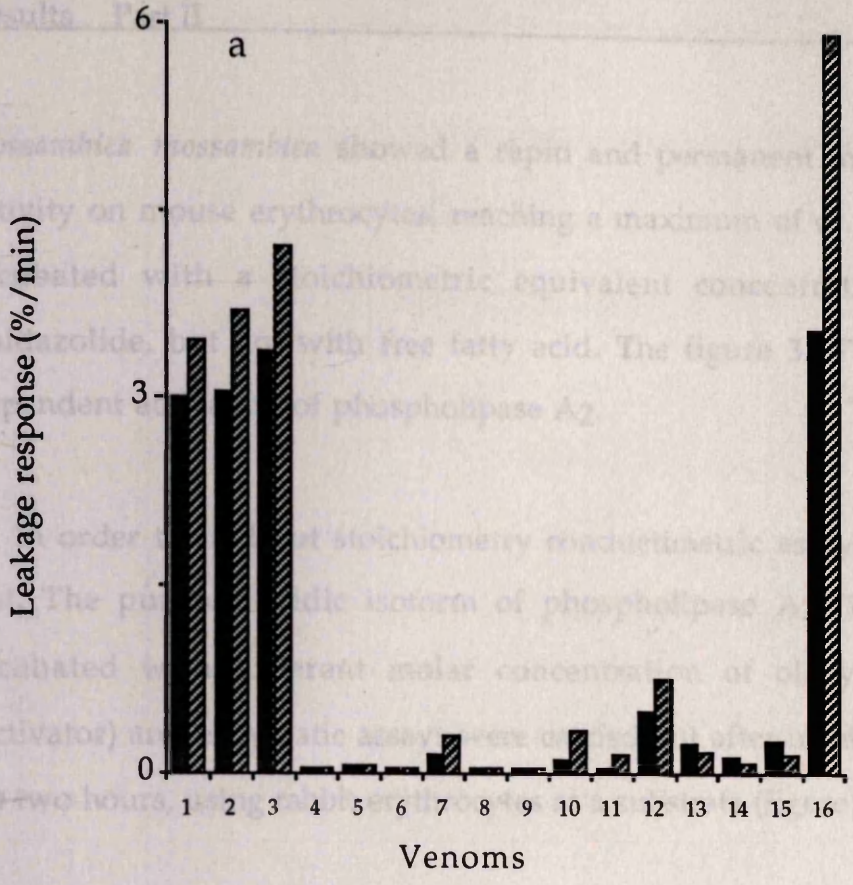
3.2.7.) Activation of the acidic isoform of Phospholipase A_2 from the venom of *Naja mossambica mossambica*.

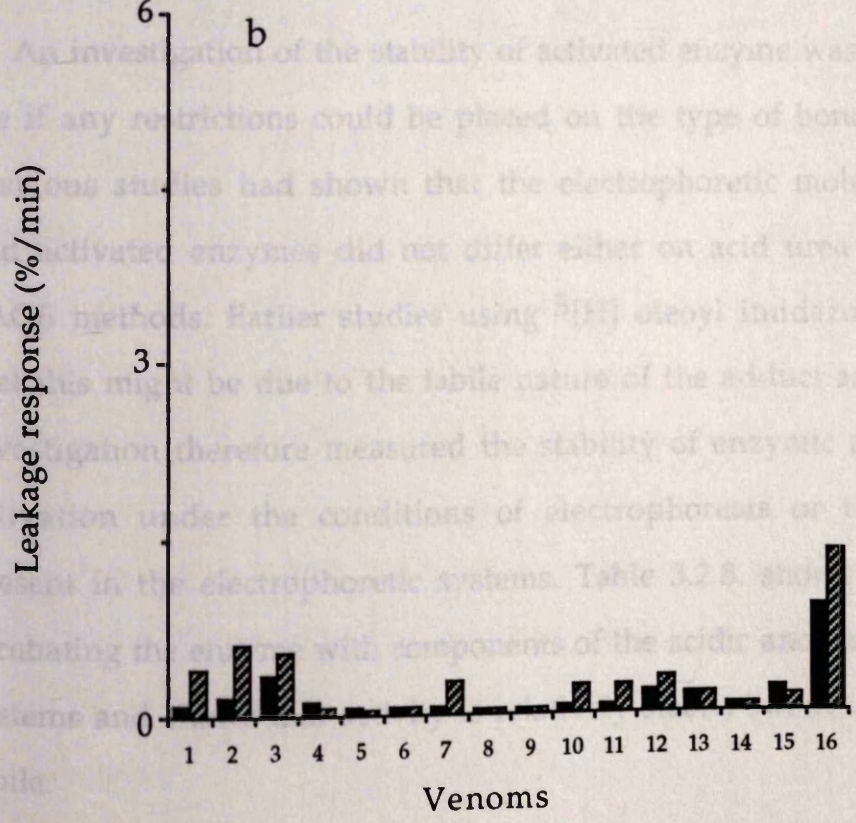
The acidic isoform of phospholipase A_2 from the venom of *Naja*

Figure 3.2.6. The activation of different snake venoms on mouse and rabbit erythrocytes.

Crude snake venoms were activated by incubation of 100 µl aliquots of solutions of whole venoms (1 mg/ml in 10 mM triethanolamine buffer, pH 8.0) with 2 µl of 4 mM solution of oleoyl imidazolide (solution in acetone) for 2 hours at 37 °C. Two µl sample was tested for the ability to promote leakage either on rabbit (a) or mouse (b) erythrocytes suspended in isotonic sucrose medium buffered at pH 7.4 with 10 mM MOPS/Na^+ and in the presence of 10 mM albumin.

1: *Naja nigricollis nigricollis*. 2: *Naja nigricollis crawshawii*. 3: *Naja nigricollis pallida*. 4: *Naja hannah* (King cobra). 5: *Naja nivea*. 6: *Naja haje*. 7: *Naja naja*. 8: *Crotalus atrox*. 9: *Crotalus adamanteus*. 10: *Naja melanoleuca*. 11: *Enhydrina schistosa* (sea snake). 12: *Russell's viper*. 13: *Agkistrodon piscivorus piscivorus*. 14: *Naja naja atra*. 15: *Bungarus fasciatus*. 16: *Naja mossambica mossambica*. Native venoms (■), treated venoms (▧).


a
Leakage response (%/min)
6
3
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Venoms


b
Leakage response (%/min)
6
3
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Venoms

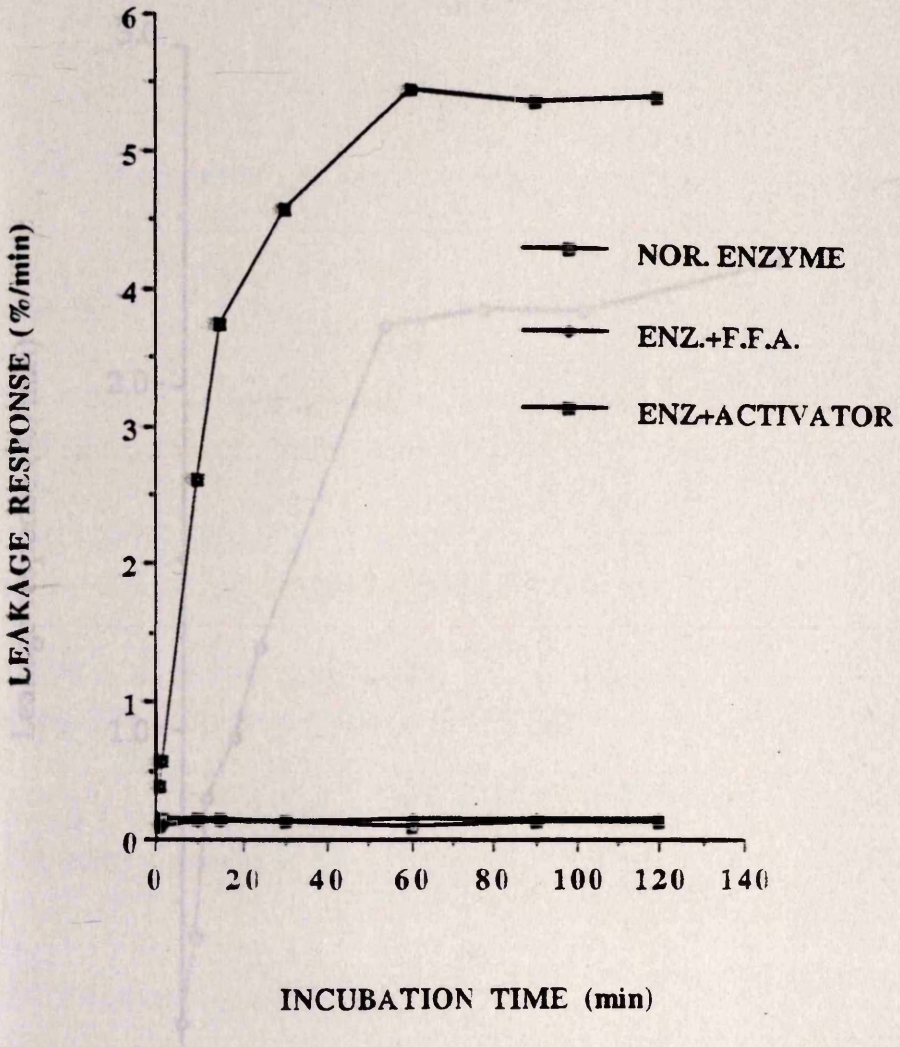
mossambica mossambica showed a rapid and permanent increase in lytic activity on mouse erythrocytes, reaching a maximum of ca. 60-fold, when incubated with a stoichiometric equivalent concentration of oleoyl imidazolide, but not with free fatty acid. The figure 3.2.7.I. shows time dependent activation of phospholipase A_2.

In order to find out stoichiometry conductimetric assays were carried out. The purified acidic isoform of phospholipase A_2 (1 mg/ml) was incubated with different molar concentration of oleoyl imidazolide (activator) and enzymatic assays were carried out after incubation at 37 °C for two hours, using rabbit erythrocytes as a substrate (figure 3.2.7.II.).

3.2.8.) The stability of the activated state of PLA_2 enzyme from the venom of *Naja mossambica mossambica.*

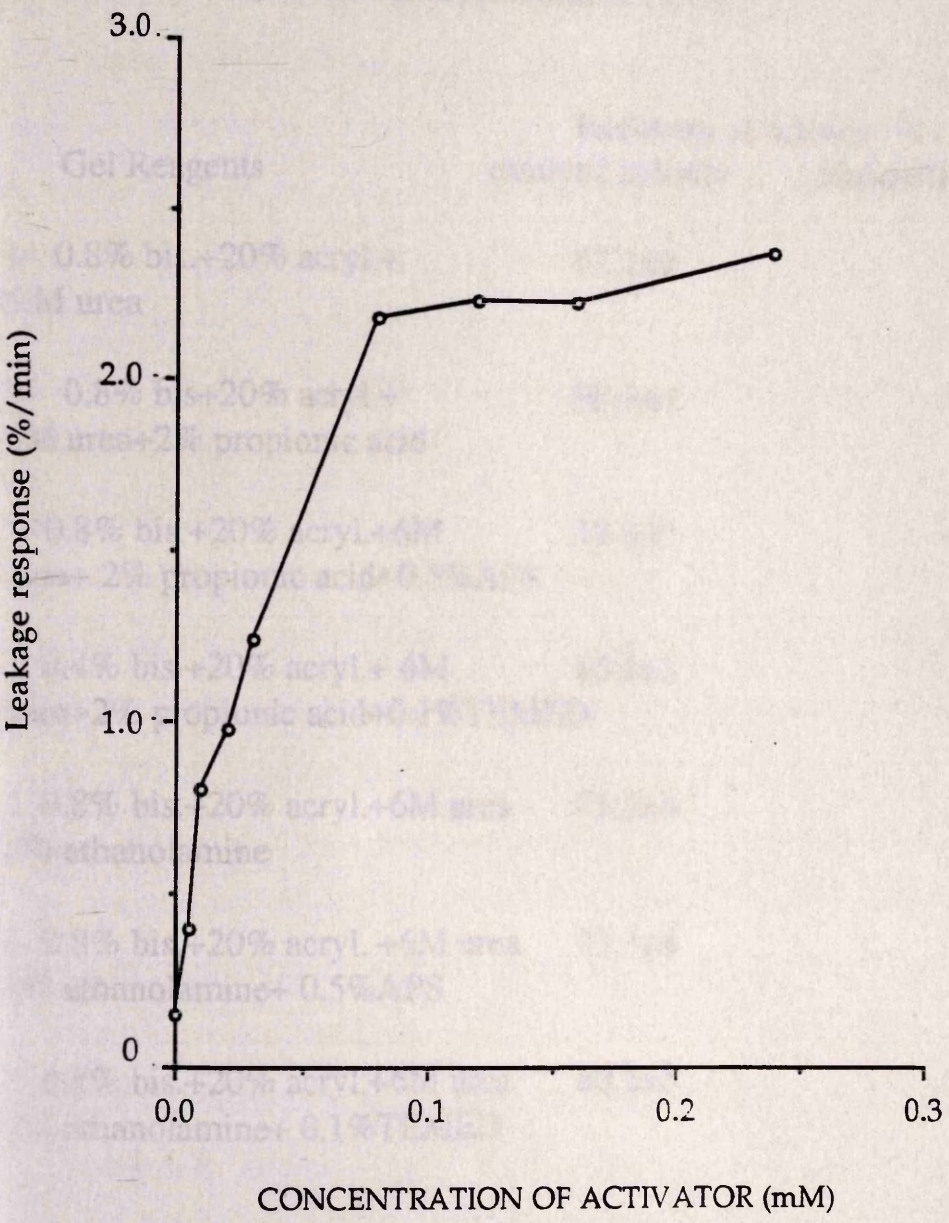
An investigation of the stability of activated enzyme was carried out to see if any restrictions could be placed on the type of bonding involved. Previous studies had shown that the electrophoretic mobility of native and activated enzymes did not differ either on acid urea or SDS based PAGE methods. Earlier studies using ^{3}[H] oleoyl imidazolide indicated that this might be due to the labile nature of the adduct and the present investigation therefore measured the stability of enzymic activity and of activation under the conditions of electrophoresis or to the reagent present in the electrophoretic systems. Table 3.2.8. shows the results of incubating the enzyme with components of the acidic and basic urea PAGE systems and shows that activity is relatively stable, but activation is quite labile.

Figure 3.2.7.I. Time dependent activation of phospholipase A_2.



Purified acidic isoform of phospholipase A_2 (1 mg/ml in 10 mM triethanolamine buffer of pH 8.0) from the venom of *Naja mossambica mossambica* was incubated with 2 µl each of either 4 mM oleoyl imidazolide (activator) or 4 mM free oleic acid (solution in acetone) at room temperature. Two µl samples were withdrawn from three ependorf tubes (one control and two treated) at different time intervals and activation was assayed by conductimetric analysis on mouse erythrocytes in the presence of 10 µM bovine serum albumin as described in materials and methods.

Figure 3.2.7.II. Stoichiometry of Activation of Phospholipase A_2.



The acidic isoform of phospholipase A_2 (1mg/ml in 10 mM triethanolamine buffer of pH 8.0) from the venom of *Naja mossambica mossambica* was activated with different molar concentrations of oleoyl imidazolide for two hours at 37°C and activation was checked on rabbit erythrocytes by conductimetric assay as described in figure 3.2.7.I.

TABLE 3.2.8. The effect of different gel electrophoresis solutions on the activated acidic isoform of PLA_2

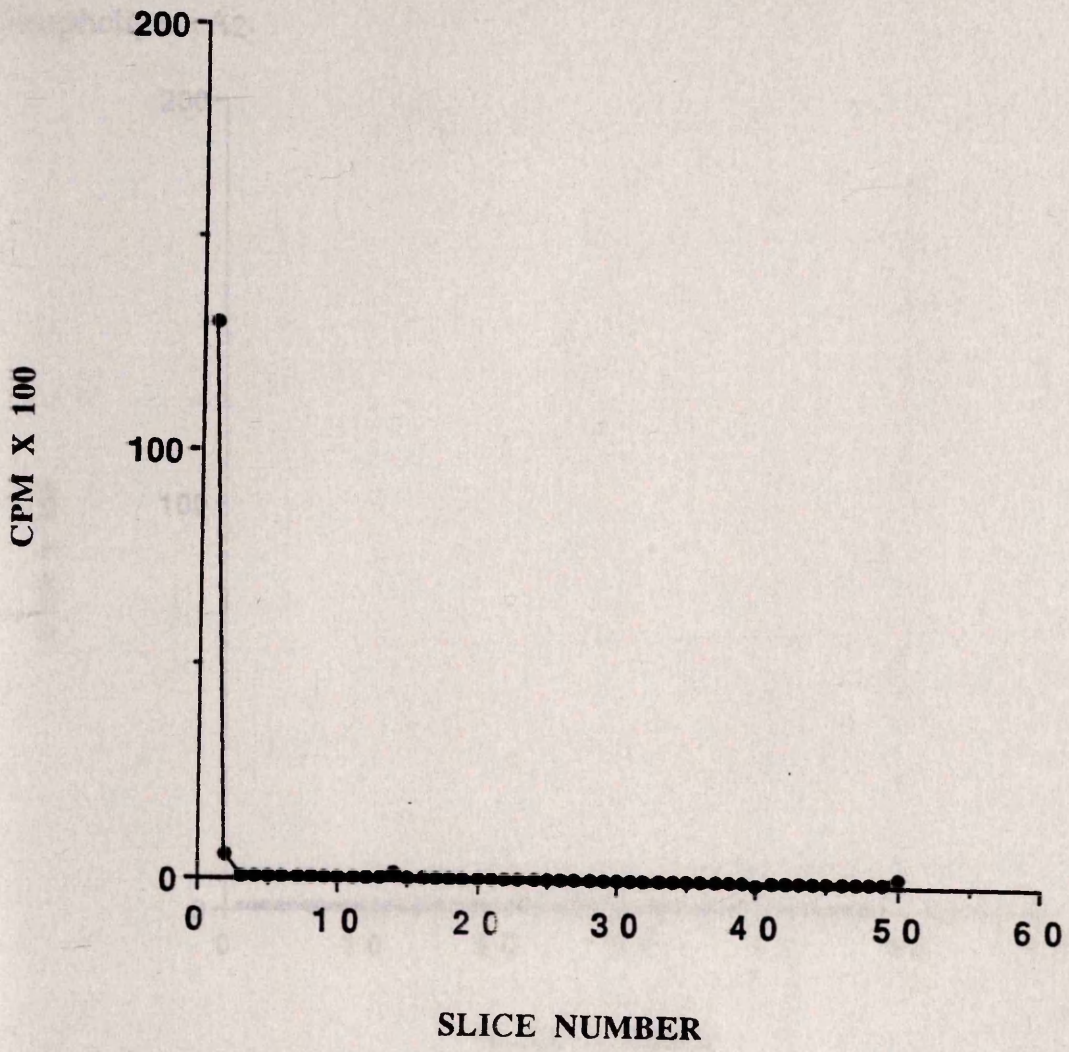
Gel Reagents	Recovery of activity (%) catalytic activity	Haemolytic activity
1- 0.8% bis.+20% acryl.+ 6 M urea	67.1±2	12.1±2
2- 0.8% bis+20% acryl.+ 6M urea+2% propionic acid	93.3±4	55.5±3
3- 0.8% bis.+20% acryl.+6M urea+ 2% propionic acid+0.5%APS	19.4±3	10.2±2
4. 0.4% bis.+20% acryl.+ 6M urea+2% propionic acid+0.1%TEMED	85.8±5	36.7±4
5. 0.8% bis.+20% acryl.+6M urea 2% ethanolamine	71.5±4	12.4±3
6. 0.8% bis.+20% acryl. +6M urea 2% ethanolamine+ 0.5%APS	93.3±4	11.7±2
7. 0.8% bis.+20% acryl.+6M urea 2% ethanolamine+ 0.1%TEMED	80.2±5	10.6±3

The purified acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* was incubated with different gel reagent solutions as indicated above and to see the recovery of activated PLA_2, the enzymatic assay were carried out both on substrate (dioctanoylphosphatidylcholine and on rabbit erythrocytes. The recovery of activity % was calculated as compared to activated enzyme (without treatment).

This was investigated further by recovering the enzymes after PAGE and examining both activity and activation. Thus these results support and extend earlier studies which indicated that the bound acyl group might be attached by a very labile bond which eliminates any possibility of an amide linkage to lysine residues or the N-terminal residue. The results also raise the possibility that no covalent bond might be involved, but all of the evidence from the time course of the reaction to the resistance of the activated state to albumin or organic solvents suggest that some chemical linkage is formed. In order to see the stability of the activated state of enzyme, all three types gel electrophoresis analysis were carried out using ^{3}[H]-labelled activator. Figure 3.2.8.I. shows the stability of activated acidic isoform of PLA_2 in SDS gel electrophoresis. It is clear that almost all radioactivity is lost from the enzyme during electrophoresis. Figure 3.2.8.II. shows that in the acidic urea gel the major band of radioactivity was found near the origin and a negligible amount co-ran with the protein band. Figure 3.2.8.III. shows that in basic/urea gel electrophoresis conditions, the major peak of radioactivity was found at the electrophoresis front, well separated from the enzymic activity. It is clear that the bond is very unstable. The fact that the peak of radioactivity was extremely sharp in both SDS and basic urea gel electrophoresis indicates that the residue was released from the protein very early in the procedure.

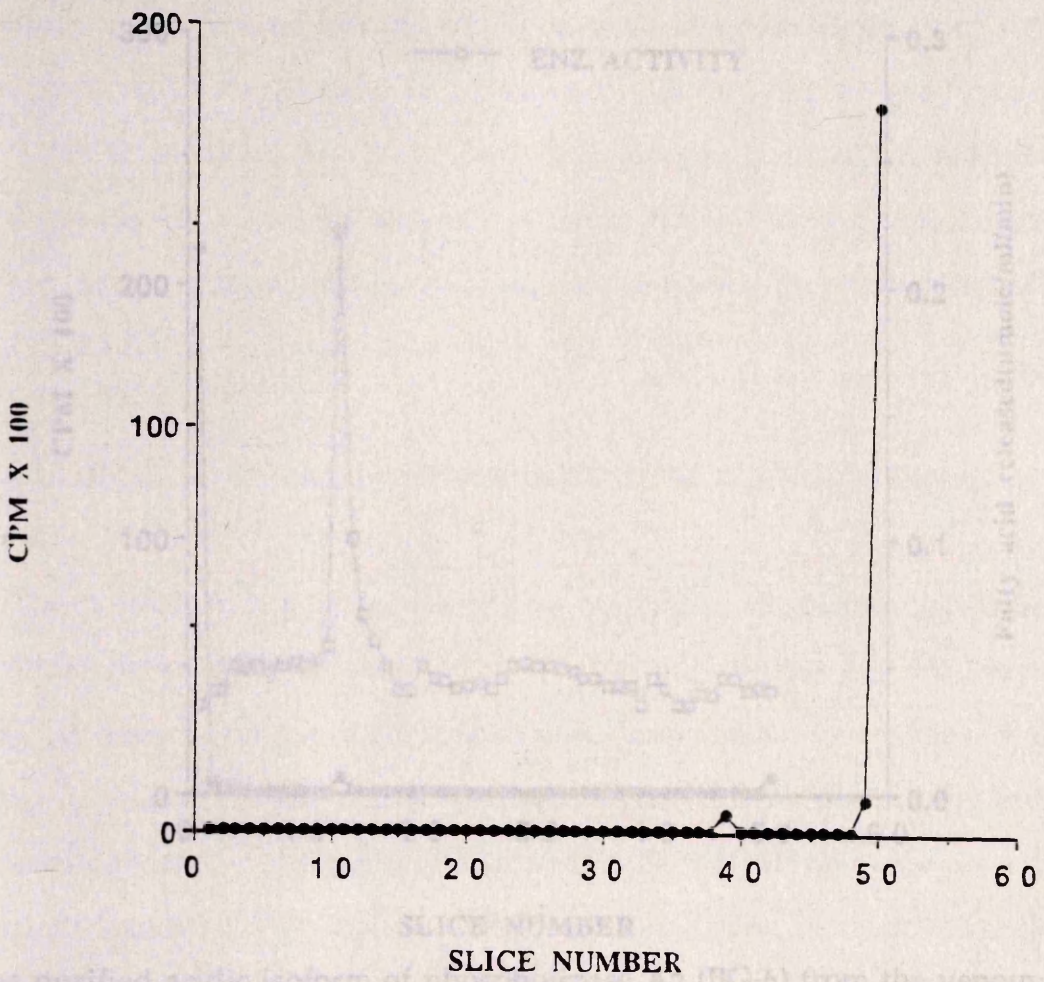
Activation had the progressive nature of a chemical reaction and the modified protein was stable to dilute organic solvents and also to albumin, which was necessary for the expression of lytic activity in the erythrocyte leakage assay. Radiolabelling experiments using ^{3}[H]-oleic acid showed

Figure 3.2.8.I. SDS gel electrophoresis of activated phospholipase A_2



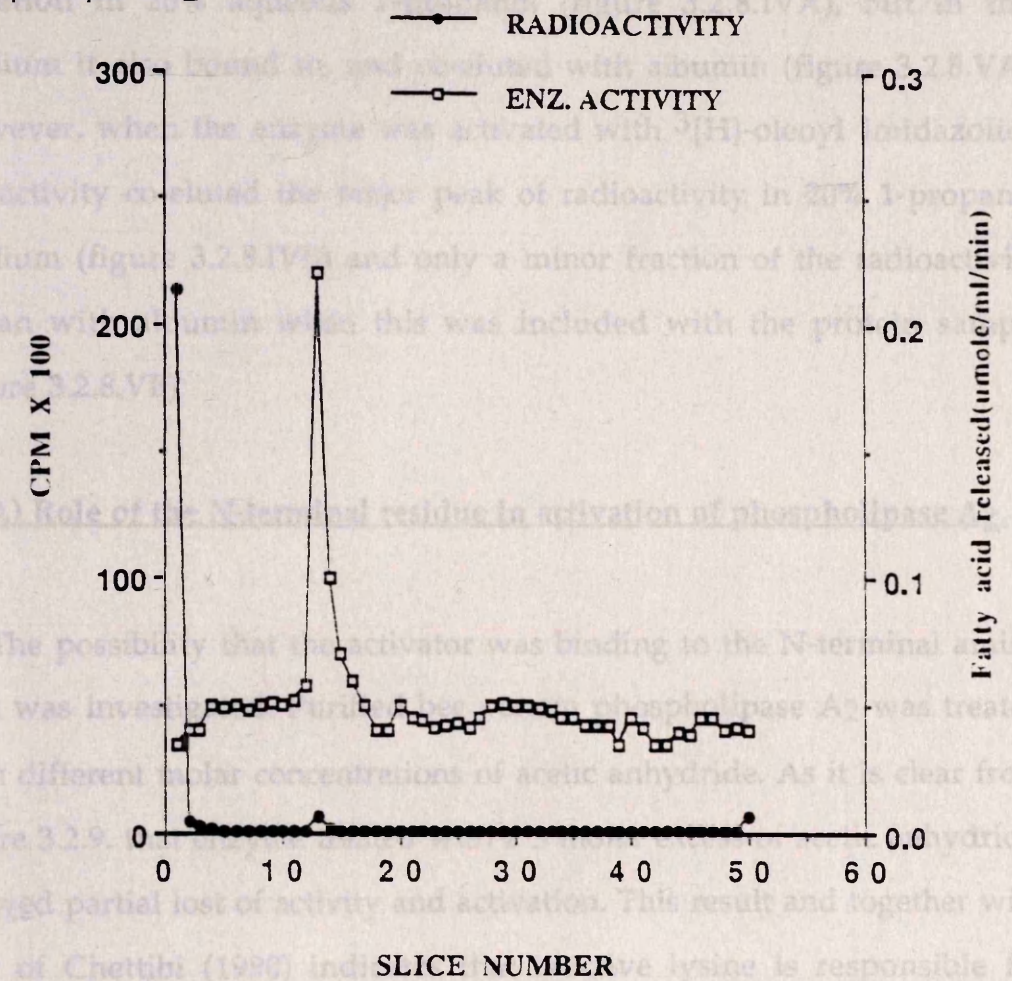
The purified acidic isoform of phospholipase A_2 (BG-6) from the venom of *Naja mossambica mossambica* was activated with ^{3}H-oleoyl imidazolide and was passed through gel filtration BioGel P-30 column in order to remove free radioactivity and then ran on SDS gel electrophoresis in two lanes, experimental and guide lane. The guide lane was stained with coomassie blue and destained and aligned with the experimental lane and experimental was cut into 1 to 1.5 mm slices starting from dye front and enzyme and radioactivity were extracted as described in materials and methods. Radioactivity was determined. Slice number 14 indicates enzyme position.

Figure 3.2.8.II. The acidic/urea gel electrophoresis of the activated phospholipase A_2.



The purified acidic isoform of phospholipase A_2 (BG-6) from the venom of *Naja mossambica mossambica* was activated with ^{3}[H]-oleoyl imidazolide and was passed through gel filtration BioGel P-30 column in order to remove free radioactivity and then ran on the acidic urea gel electrophoresis in two lanes, experimental and guide lane. The guide lane was stained with Coomassie blue and destained and aligned with the experimental lane and experimental lane was cut into 1 to 1.5 mm slices starting from dye front and enzyme and radioactivity were extracted as described in materials and methods. Radioactivity was determined. Slice number 39 indicates enzyme position.

Figure 3.2.8.III. The basic/urea gel electrophoresis of the activated phospholipase A2.



The purified acidic isoform of phospholipase A2 (BG-6) from the venom of *Naja mossambica mossambica* was activated with ^{3}[H]-oleoyl imidazolide and was passed through gel filtration BioGel P-30 column in order to remove free radioactivity and then ran on the basic urea (unmodified) gel electrophoresis in two lanes, experimental and guide lane. The guide lane was stained with Coomassie blue and destained and aligned with the experimental lane and experimental lane was cut into 1 to 1.5 mm slices starting from dye front and enzyme was extracted as described in materials and methods. Radioactivity and enzymatic activity was determined. Slice number 13 indicates enzyme position.

that free oleic acid could be separated from the native enzyme by gel filtration in 20% aqueous 1-propanol (figure 3.2.8.IVA), but in this medium it also bound to, and co-eluted with albumin (figure 3.2.8.VA). However, when the enzyme was activated with ^{3}[H]-oleoyl imidazolide the activity co-eluted the major peak of radioactivity in 20% 1-propanol medium (figure 3.2.8.IVB) and only a minor fraction of the radioactivity co-ran with albumin when this was included with the protein sample (figure 3.2.8.VB).

3.2.9.) Role of the N-terminal residue in activation of phospholipase A_2.

The possibility that the activator was binding to the N-terminal amino acid was investigated. Purified bee venom phospholipase A_2 was treated with different molar concentrations of acetic anhydride. As it is clear from figure 3.2.9. that enzyme treated with a 3 molar excess of acetic anhydride, showed partial lost of activity and activation. This result and together with that of Chettibi (1990) indicates that reactive lysine is responsible for activity and thus mask any possible effect on activation but the results indicate that the most reactive NH residue has little effect on any aspect of catalytic activity. From this experiment and previous experiments the possibility of the N-terminal amino acid in binding the activator can be ruled out.

3.2.10.) Fourier Transform Infrared Spectroscopy.

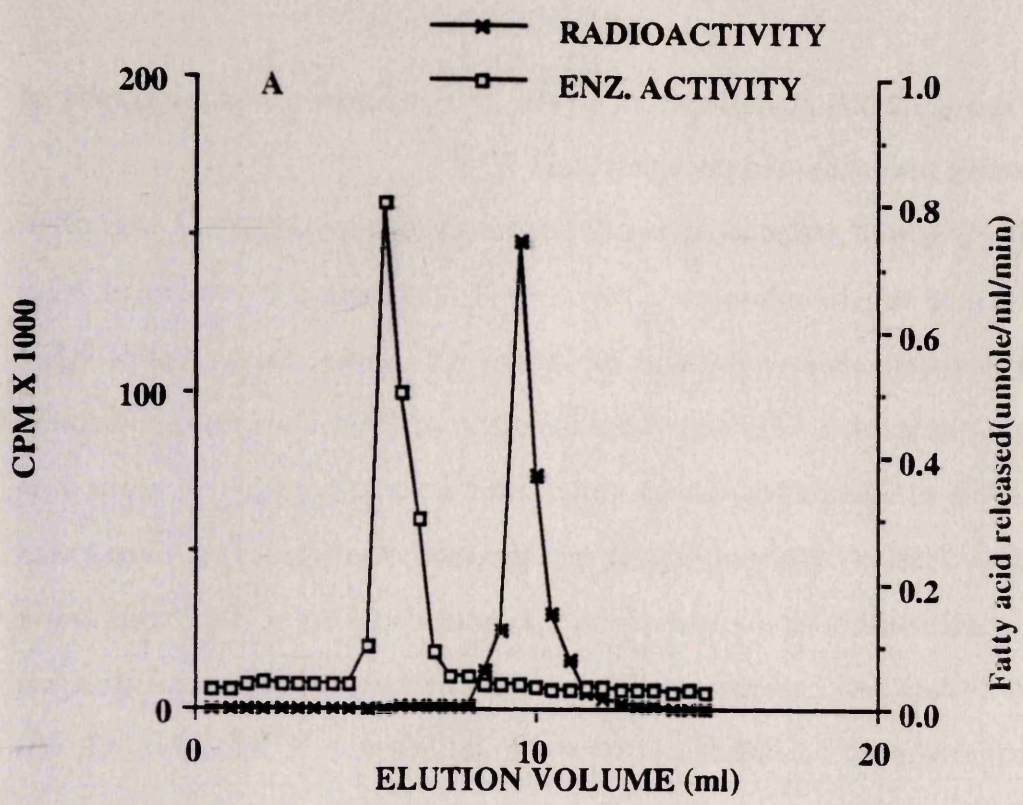
In order to investigate the nature of the chemical linkage a Fourier Transform Infrared Spectroscopic (FTIR) investigation was planned which

Figure 3.2.8.IV. Gel filtration Bio-Gel P-30 column chromatography of native and activated phospholipase A_2.

The purified acidic isoform of phospholipase A_2 (100 µl of 1 mg/ml in 10 mM triethanolamine buffer of pH 8.0) from the venom of *Naja mossambica mossambica* was incubated with molar equivalent of ^{3}[H]-oleic acid and ^{3}[H]-oleoyl imidazolide at 37°C for 2 hours. Bio-Gel P-30 column (1 x 16 cm) was equilibrated with 10 mM triethanolamine buffer of pH 8.0 containing 20 % aqueous 1-propanol. The sample was then applied to gel filtration column and 0.5 ml fractions were collected in 5 minutes. All the fractions were then analysed for enzymic activity and radioactivity.

A) Enzyme incubated with ^{3}[H]-oleic acid

B) Enzyme incubated with ^{3}[H]-oleoyl imidazolide.


RADIOACTIVITY
ENZ. ACTIVITY
A
200
100
0
CPM X 1000
1.0
0.8
0.6
0.4
0.2
0.0
Fatty acid released(umole/ml/min)
0
10
20
ELUTION VOLUME (ml)

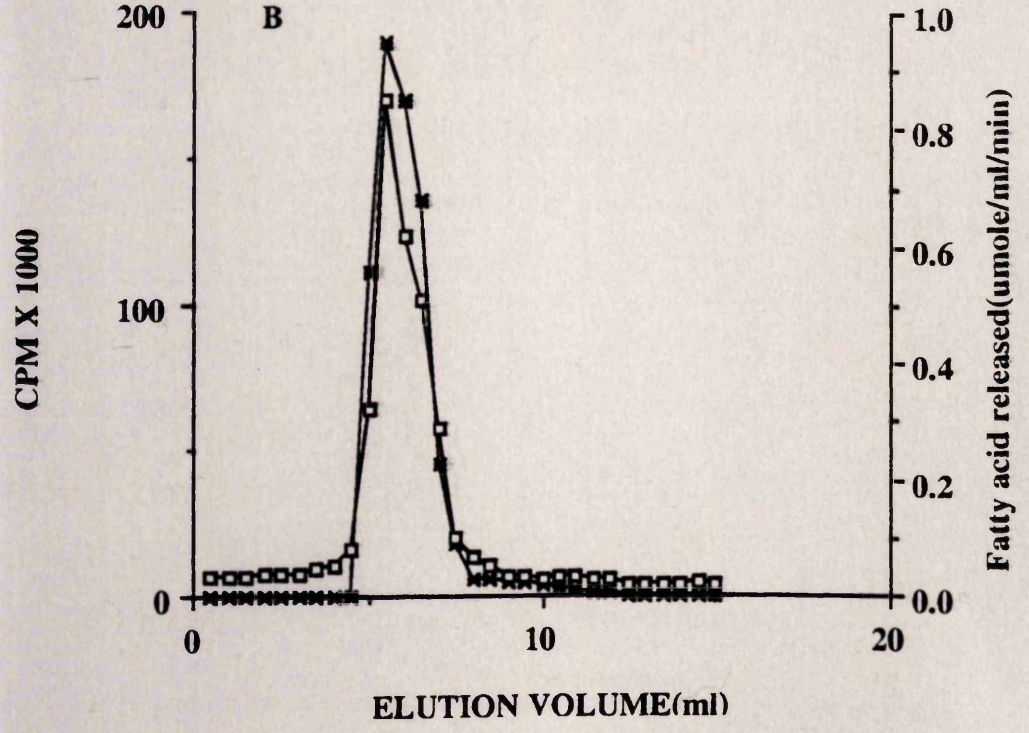
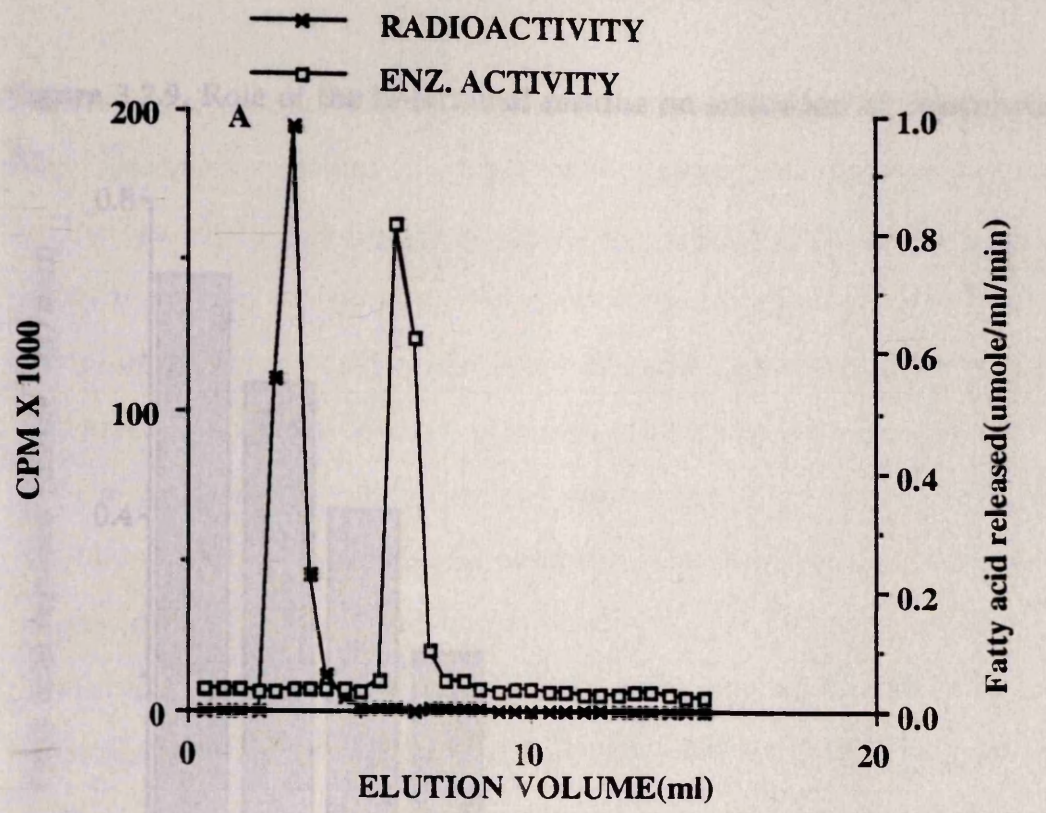

B
200
100
0
CPM X 1000
1.0
0.8
0.6
0.4
0.2
0.0
Fatty acid released(umole/ml/min)
0
10
20
ELUTION VOLUME(ml)

Figure 3.2.8.V. Gel filtration BioGel P-30 column chromatography of native and activated phospholipase A_2

The purified acidic isoform of phospholipase A_2 (100 µl of 1 mg/ml in 10 mM triethanolamine buffer of pH 8.0) from the venom of *Naja mossambica mossambica* was incubated with molar equivalent of ^{3}[H]-oleic acid and ^{3}[H]-oleoyl imidazolide at 37°C for 2 hours. After 2 hours of incubation 8-fold molar excess bovine serum albumin was added before applying to column. BioGel P-30 column (1 x 16 cm) was equilibrated with 10 mM triethanolamine buffer of pH 8.0 containing 20 % aqueous 1-propanol. The sample was then applied to gel filtration column and 0.5 ml fractions were collected in 5 minutes. All the fractions were then analysed for enzymic activity and radioactivity.

A) Enzyme incubated with ^{3}[H]-oleic acid

B) Enzyme incubated with ^{3}[H]-oleoyl imidazolide.


RADIOACTIVITY
ENZ. ACTIVITY
A
200
100
0
CPM X 1000
1.0
0.8
0.6
0.4
0.2
0.0
Fatty acid released(umole/ml/min)
0
10
20
ELUTION VOLUME(ml)

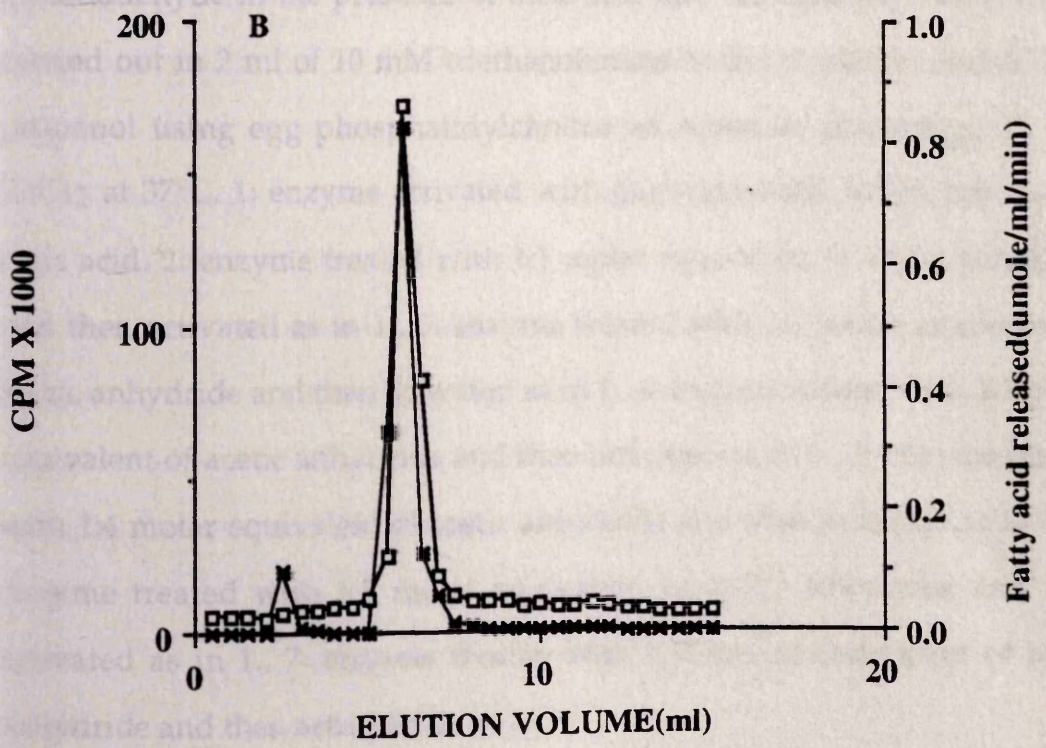
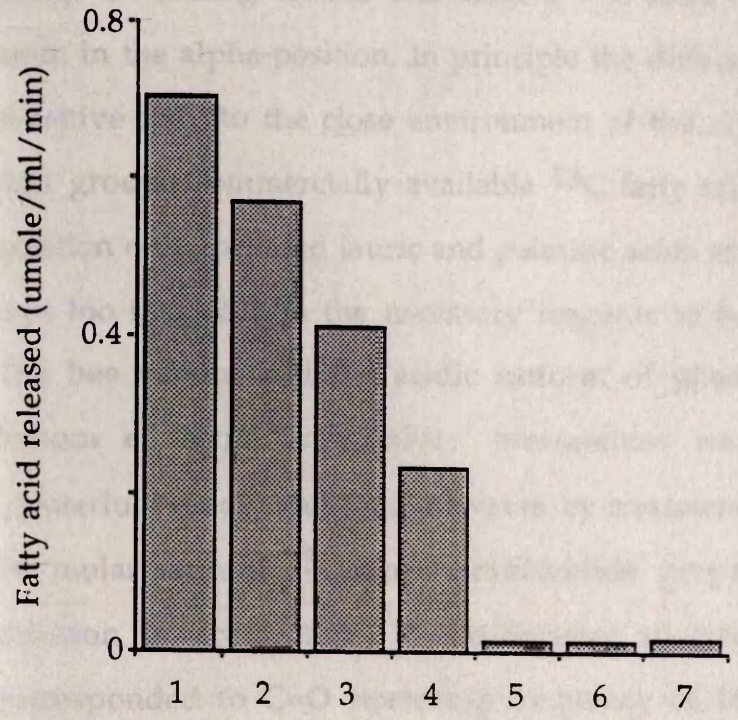

B
200
100
0
CPM X 1000
1.0
0.8
0.6
0.4
0.2
0.0
Fatty acid released(umole/ml/min)
0
10
20
ELUTION VOLUME(ml)

Figure 3.2.9. Role of the N-terminal residue on activation of phospholipase A_2.



The purified bee venom phospholipase A_2 was treated with different molar concentrations of acetic anhydride. The PLA_2 was activated with glutaraldehyde in the presence of oleic acid and the activation assays were carried out in 2 ml of 10 mM triethanolamine buffer at pH 8.0 and 20 % 1-propanol using egg phosphatidylcholine as substrate containing 0.1 mM $CaCl_2$ at 37°C. 1: enzyme activated with glutaraldehyde in the presence of oleic acid. 2: enzyme treated with 1:1 molar equivalent of acetic anhydride and then activated as in 1., 3: enzyme treated with 1:2 molar equivalent of acetic anhydride and then activated as in 1., 4: enzyme treated with 1:3 molar equivalent of acetic anhydride and then activated as in 1., 5: enzyme treated with 1:4 molar equivalent of acetic anhydride and then activated as in 1., 6: enzyme treated with 1:5 molar equivalent of acetic anhydride and then activated as in 1., 7: enzyme treated with 1:10 molar equivalent of acetic anhydride and then activated as in 1.

involved the comparison of enzymes activated with a long-chain acyl group containing on the one hand a ^{12}C atom and on the other a ^{13}C atom in the alpha-position. In principle the difference spectrum should be sensitive only to the close environment of the alpha carbon atom of the acyl group. Commercially available ^{13}C fatty acids labelled in the alpha position only included lauric and palmitic acids and of these, palmitic acid was too insoluble in the necessary reagents to be used with confidence. The bee venom and the acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* were dissolved in D_2O (deuterium oxide) and then activated by treatment with an approximately 1:1 molar ratio of ^{13}C-lauryl imidazolide prepared as a concentrated solution in acetonitrile. The difference spectrum obtained by FT-IR corresponded to C=O stretching frequency of 1696 nm which does not correspond to the values for any of the model compounds tested, (table 3.2.10.) Such a spectrum could be attributed to an ester hydrogen bonded to a positively charged residue. The data indicate that an acyl imidazolide might also produce a similar spectrum, but larger perturbations would be required. These results are completely incompatible with the presence of an amide linkage. Figure 3.2.10.I. shows the spectrum of acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* after subtraction of enzyme treated with ^{12}C-lauryl imidazolide from enzyme treated with ^{13}C-lauryl imidazolide. Similarly the figure 3.2.10.II. shows spectrum of bee venom PLA_2 after subtraction of enzyme treated with ^{12}C-lauryl imidazolide from enzyme treated with ^{13}C-lauryl imidazolide.

Table 3.2.10.

Model Studies of a Variety of Esters in a Number of Solvents

Frequency of ester acyl carbonyl stretching vibration in wavenumber (cm^{-1})

Solvent	Dielectric Constant	ethyl hydrocinnamate	phenyl acetate	*N*-hydrocinnamoyl imidazole	*N*-methyl acetamide	hydrocinnamic acid
hexane	2	1743	1781, 1772	1745	x	1715
diisopropyl ether	4				1687, 1667	
methanol	33	1717	1769, 1741	1749	1661, 1638	1733, 1711
acetonitrile	40	1731	1765, 1746	1711	1675	1740, 1715
^2H_2O	78	1715	1734	1738	1623	1700

x = immiscible, use diisopropyl ether instead

ethyl hydrocinnamate | Ø-CH_2-CH_2-(C=O)-O-CH_2-CH_3 |, aliphatic oxy-ester, modelling seryl ester.
phenyl acetate | CH_3-(C=O)-O-Ø |, oxy-ester, modelling tyrosyl ester.
N-hydrocinnamoyl imidazole | Ø-CH_2-CH_2-(C=O)-imidazole |, imidazole ester, modelling histidyl ester.
N-methyl acetamide | CH_3-(C=O)-NH-CH_3 |, amide, modelling lysyl ester.
hydrocinnamic acid | Ø-CH_2-CH_2-COOH |, free acid, to model non covalently bound free acid.

|^{12}C=^{16}O| acyl carbonyl stretch for both enzymes was at 1696cm^{-1}

Work was done by C. Wharton

Figure 3.2.10.I. FT-IR spectrum of the binding of fatty acid to the acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica*.

The purified acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* was dissolved in deuterium oxide (99.9%D) to final concentration of 1 mM and kept in deuterium oxide for two days before activation. The enzyme was divided into two equal portions and activated with molar equivalent of either ^{13}C-lauryl imidazolide or ^{12}C-lauryl imidazolide (solution in acetonitrile and prepared as described in materials and methods) for two hours at 37°C in incubator. FT-IR spectrum was recorded using a Nicolet 60SX spectrometer equipped with a Nicolet 1280 computer and with a mercury-cadmium telluride type A detector. The instrument was purged continuously with dry air to maintain a very low water-vapour pressure (-70 °C dew point); the sample cell (CaF2, 50 μm pathlength) was filled and emptied from outside the instrument in order to maintain the purge. For each spectrum 1000 scans were averaged (500 ms/scan) at a resolution of 2 cm^{-1}. The spectrum shown is substration of the spectrum of enzyme activated with ^{13}C-lauryl imidazolide from that of enzyme activated with ^{12}C-lauryl imidazolide.

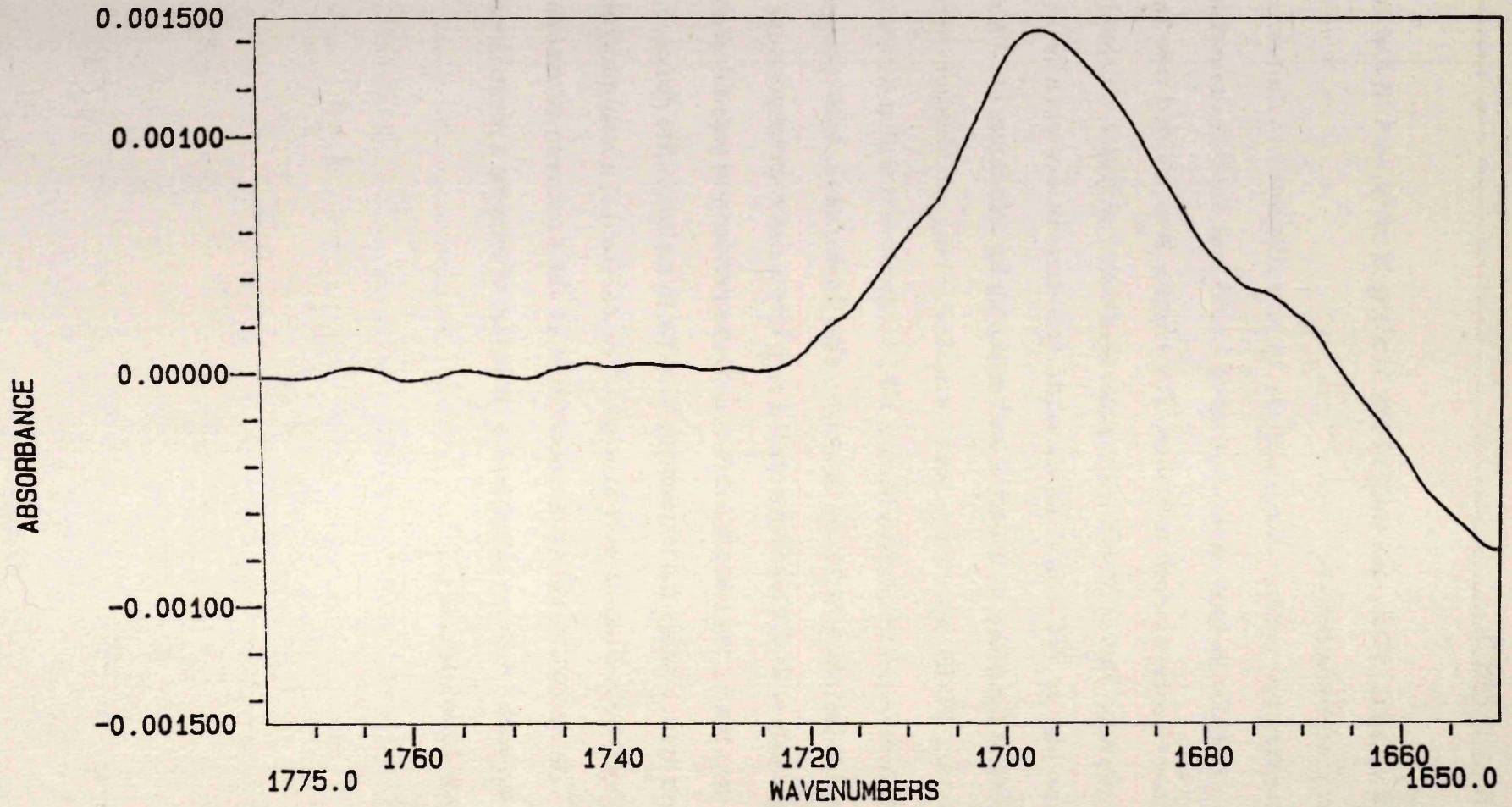
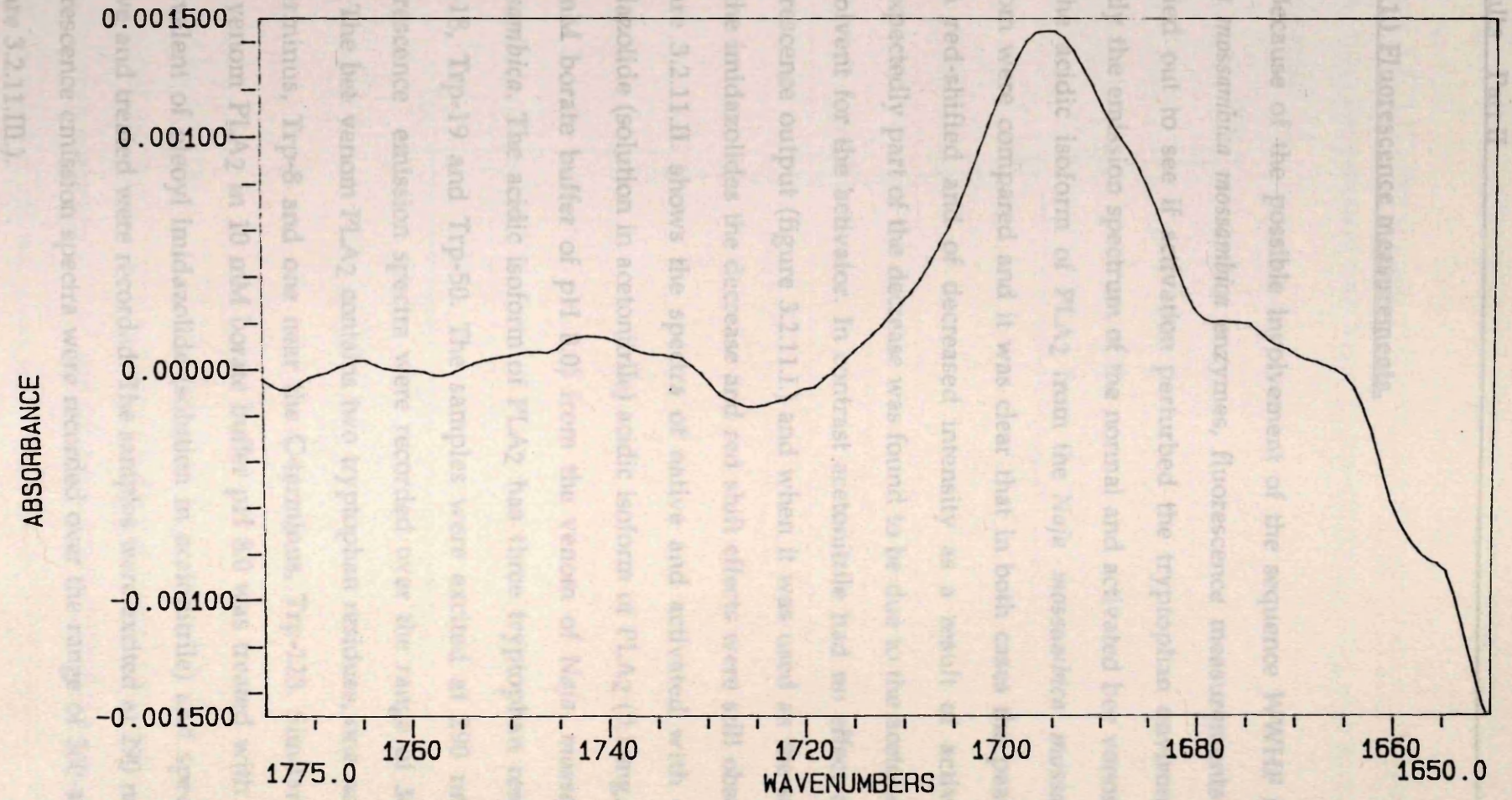

0.001500
0.00100
0.00000
-0.00100
-0.001500
ABSORBANCE
1775.0
1760
1740
1720
1700
1680
1660
1650.0
WAVENUMBERS

Figure 3.2.10.II. FT-IR spectrum of the binding of fatty acid to the bee venom phospholipase A_2.

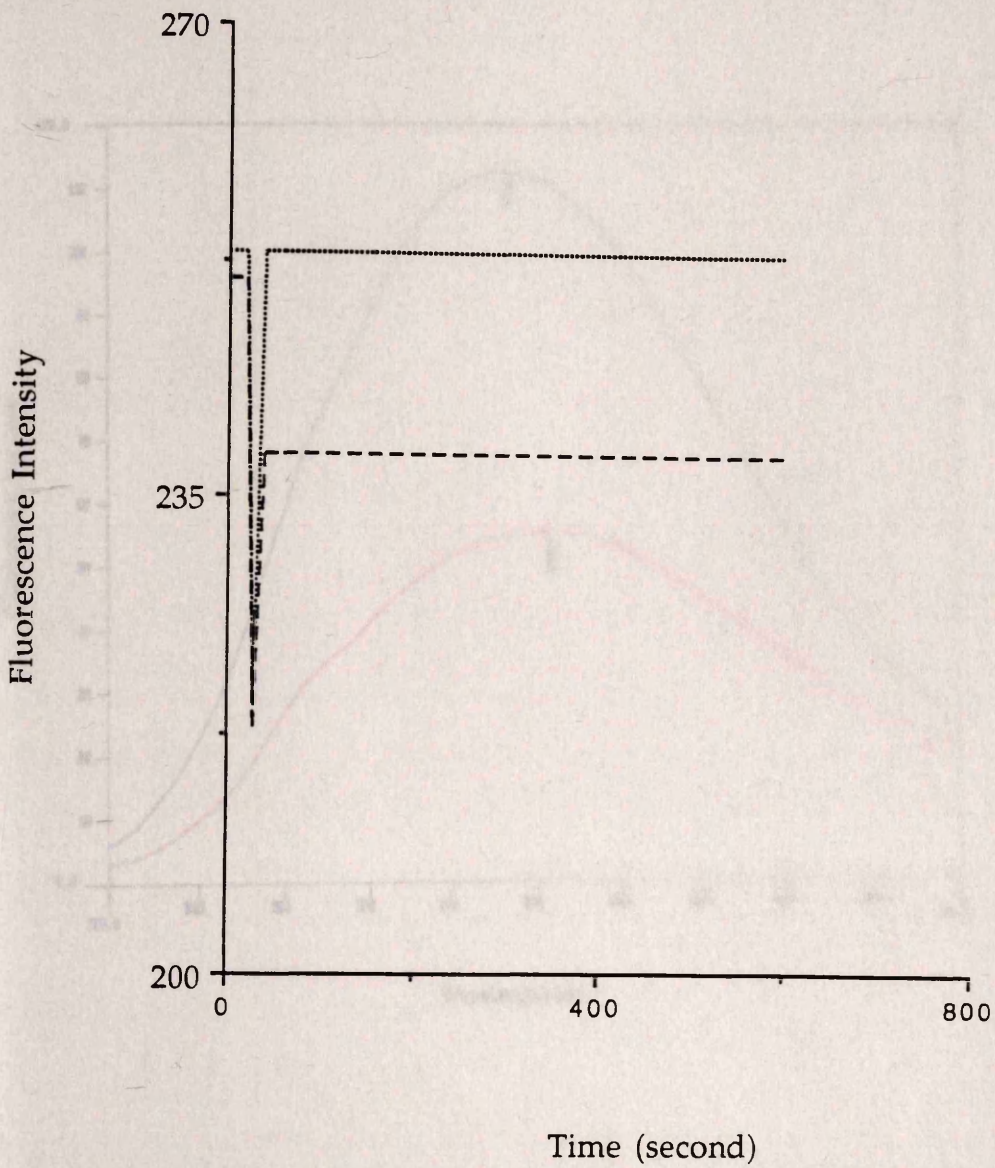
The purified bee venom phospholipase A_2 was dissolved in deuterium oxide (99.9%D) to final concentration of 1 mM and kept in deuterium oxide for two days before activation. The enzyme was divided into two equal portions and activated with molar equivalent of either ^{13}C-lauryl imidazolide or ^{12}C-lauryl imidazolide (solution in acetonitrile and prepared as described in materials and methods) for two hours at 37°C in incubator. FT-IR spectrum was recorded using a Nicolet 60SX spectrometer equipped with a Nicolet 1280 computer and with a mercury-cadmium telluride type A detector. The instrument was purged continuously with dry air to maintain a very low water-vapour pressure (-70 °C dew point); the sample cell (CaF2, 50 μm pathlength) was filled and emptied from outside the instrument in order to maintain the purge. For each spectrum 1000 scans were averaged (500 ms/scan) at a resolution of 2 cm^{-1}. The spectrum shown is substration of the spectrum of enzyme activated with ^{13}C-lauryl imidazolide from that of enzyme activated with ^{12}C-lauryl imidazolide.


0.001500
0.00100
0.00000
-0.00100
-0.001500
ABSORBANCE
1775.0
1760
1740
1720
1700
1680
1660
1650.0
WAVENUMBERS

3.2.11.) Fluorescence measurements.

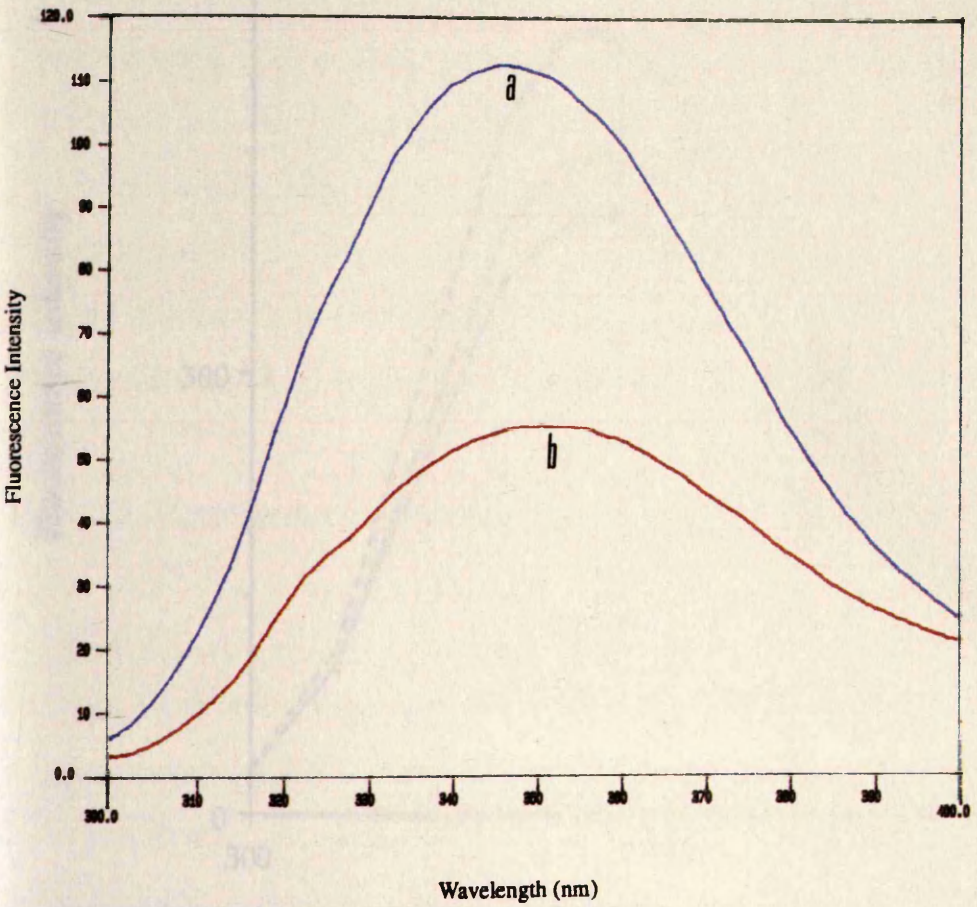
Because of the possible involvement of the sequence WWHF in the *Naja mossambica mossambica* enzymes, fluorescence measurements were carried out to see if activation perturbed the tryptophan environment. Firstly the emission spectrum of the normal and activated bee venom and of the acidic isoform of PLA_2 from the *Naja mossambica mossambica* venom were compared and it was clear that in both cases the peak was both red-shifted and of decreased intensity as a result of activation. Unexpectedly part of the decrease was found to be due to the acetone used as solvent for the activator. In contrast acetonitrile had no effect on the fluorescence output (figure 3.2.11.I.) and when it was used as the solvent for the imidazolides the decrease and red shift effects were still observed. Figure 3.2.11.II. shows the spectra of native and activated with oleoyl imidazolide (solution in acetonitrile) acidic isoform of PLA_2 (0.1 mg/ml in 10 mM borate buffer of pH 8.0) from the venom of *Naja mossambica mossambica*. The acidic isoform of PLA_2 has three tryptophan residues, Trp-18, Trp-19 and Trp-50. The samples were excited at 290 nm and fluorescence emission spectra were recorded over the range of 300-400 nm. The bee venom PLA_2 contains two tryptophan residues, one near the N-terminus, Trp-8 and one near the C-terminus, Trp-128. Similarly the bee venom PLA_2 in 10 mM borate buffer pH 8.0 was treated with molar equivalent of oleoyl imidazolide (solution in acetonitrile) and spectra of native and treated were recorded. The samples were excited at 290 nm and fluorescence emission spectra were recorded over the range of 300-400 nm (figure 3.2.11.III.).

Figure 3.2.11.I. Time courses of fluorescence changes of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* treated with acetone and acetonitrile.



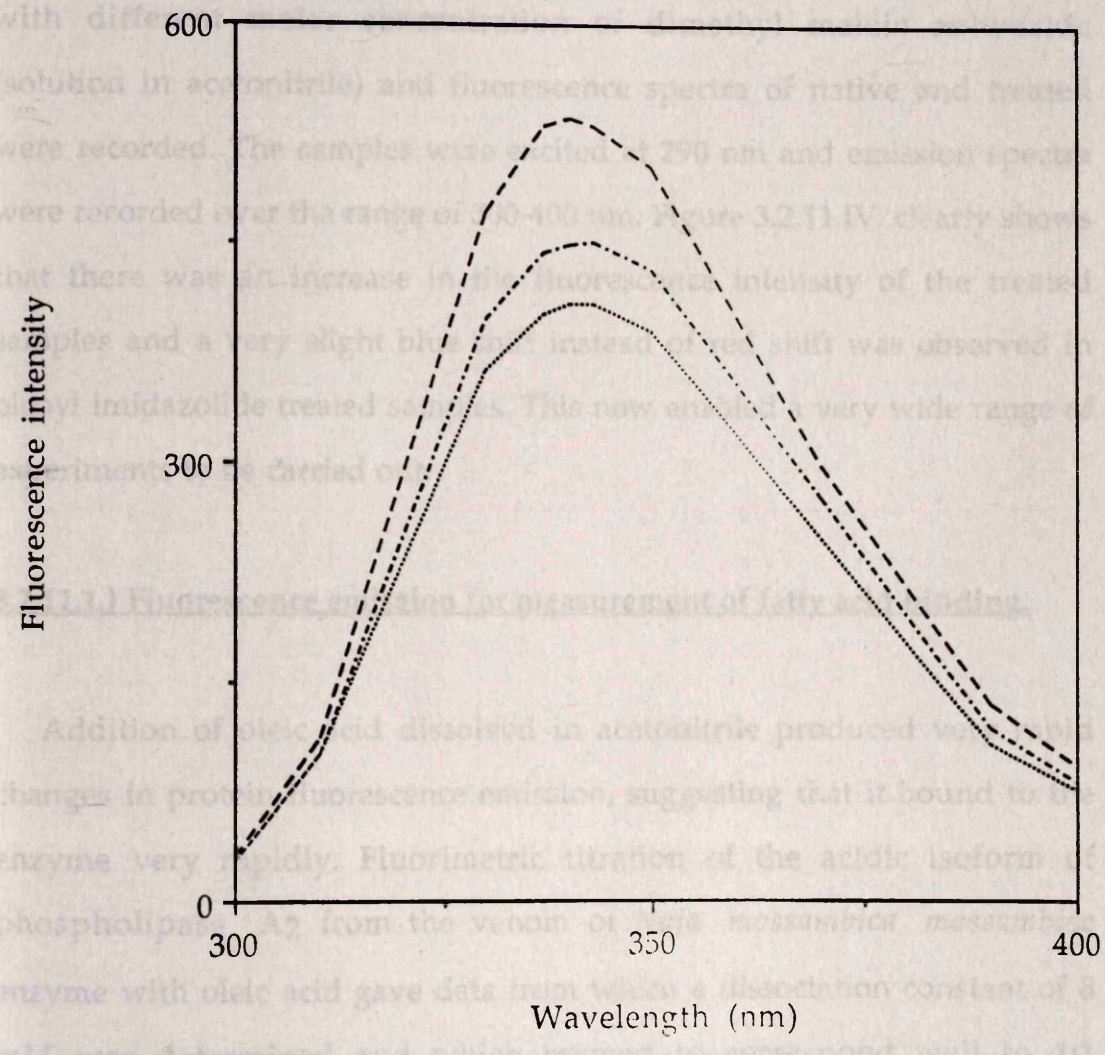
The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated either with acetone (- -) or with acetonitrile (.....) and time courses of tryptophan fluorescence were recorded at 340 nm using a Perkin-Elmer LS 50 spectrofluorimeter at 25°C. Samples were excited at 290 nm.

Figure 3.2.11.II. Tryptophan fluorescence emission spectra of native and treated acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* with oleoyl imidazolide.



The acidic isoform of PLA_2 (0.1 mg/ml) from the venom of *Naja mossambica mossambica* was treated with molar equivalent (8μM) of oleoyl imidazolide and fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and fluorescence emission spectra were recorded over the range of 300-400 nm. Native enzyme (a) and treated enzyme (b).

Figure. 3.2.11.III. Tryptophan fluorescence spectra of native and oleoyl imidazolide treated bee venom phospholipase A2.



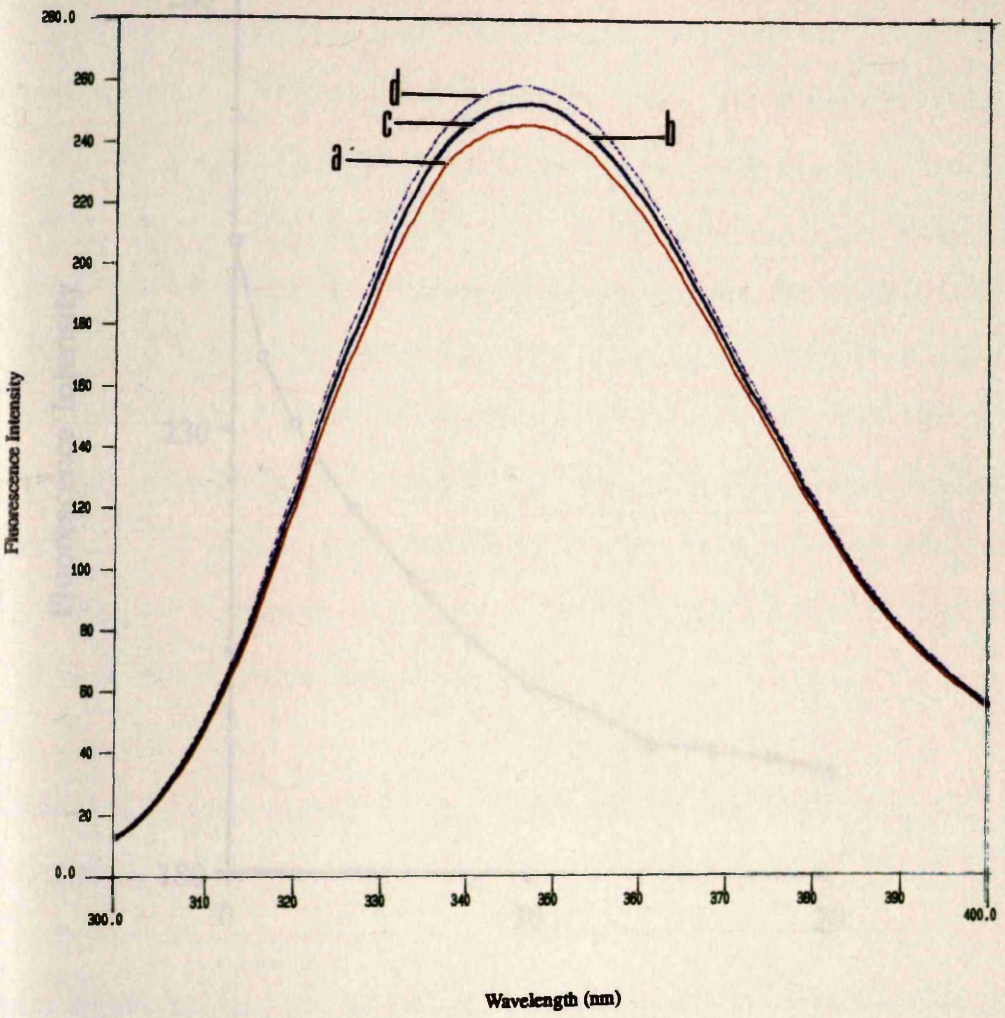
Fluorescence measurements were made at 25°C using Perkin-Elmer LS 50 spectrofluorimeter. Enzyme solutions were prepared at 0.1 mg per ml (ca. 7 μM and the samples were excited at 290 nm and emission spectra over the range 300-400 nm were recorded for native enzyme (--), enzyme after 10 minutes of treatment with 8 μM oleoyl imidazolide (-·-·-), and after 60 minutes of treatment with 8 μM oleoyl imidazolide (·····).

In order to see the specificity of the activator (oleoyl imidazolide), the acidic isoform of phospholipase A_2 (0.1 mg/ml in 10 mM borate buffer of pH 8.0) from the venom of *Naja mossambica mossambica* was treated with different molar concentration of dimethyl maleic anhydride (solution in acetonitrile) and fluorescence spectra of native and treated were recorded. The samples were excited at 290 nm and emission spectra were recorded over the range of 300-400 nm. Figure 3.2.11.IV. clearly shows that there was an increase in the fluorescence intensity of the treated samples and a very slight blue shift instead of red shift was observed in oleoyl imidazolide treated samples. This now enabled a very wide range of experiments to be carried out.

3.2.11.1.) Fluorescence emission for measurement of fatty acid binding.

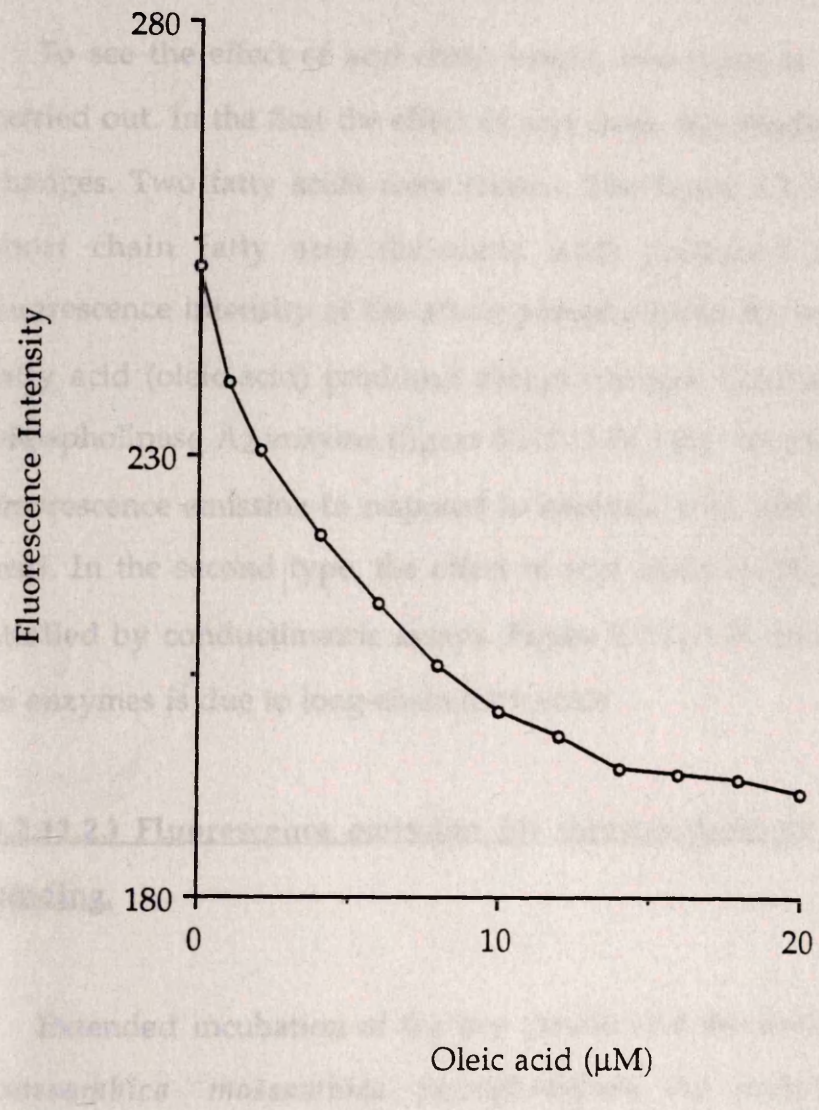
Addition of oleic acid dissolved in acetonitrile produced very rapid changes in protein fluorescence emission, suggesting that it bound to the enzyme very rapidly. Fluorimetric titration of the acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* enzyme with oleic acid gave data from which a dissociation constant of 8 mM was determined and which seemed to correspond well to 1:1 stoichiometry. Figure 3.2.11.1.I. shows the titration of acidic isoform with different molar concentration of free oleic acid (solution in acetonitrile), after addition of 30 seconds of oleic acid, fluorescence intensities were recorded at 340 nm. In the second type of experiments, the phospholipase A_2 enzyme was dissolved in 10 mM borate buffer pH 8.0 at the concentration of 0.1 mg/ml and incubated with different molar concentration of oleoyl imidazolide. After one hour of treatment, the

Figure 3.2.11.IV. Tryptophan fluorescence emission spectra of native and treated acidic isoform of PLA_2 from the venom of *Naja mossambica mossambioca* with different molar concentration of dimethyl maleic anhydride.



The acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* was prepared at concentration of 0.1 mg/ml solution in 10 mM borate buffer of pH 8.0 and divided into four equal fraction and three of them were treated with different molar concentration of dimethyl maleic anhydride. After about two hours of treatment the fluorescence spectra of native 1:0 (a), treated with 1:1 (b), 1:2 (c) and 1:3 (d) were recorded at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter over the range of 300-400 nm. The enzyme samples were excited at 290 nm.

Figure 3.2.11.1.I. Fluorimetric titration of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* with free oleic acid.



The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated with different molar concentration of free oleic acid (in acetonitrile) and fluorescence intensities were recorded after each addition of free oleic acid at 340 nm using a Perkin-Elmer LS 50 spectrofluorimeter at 25°C. Samples were excited at 290 nm.

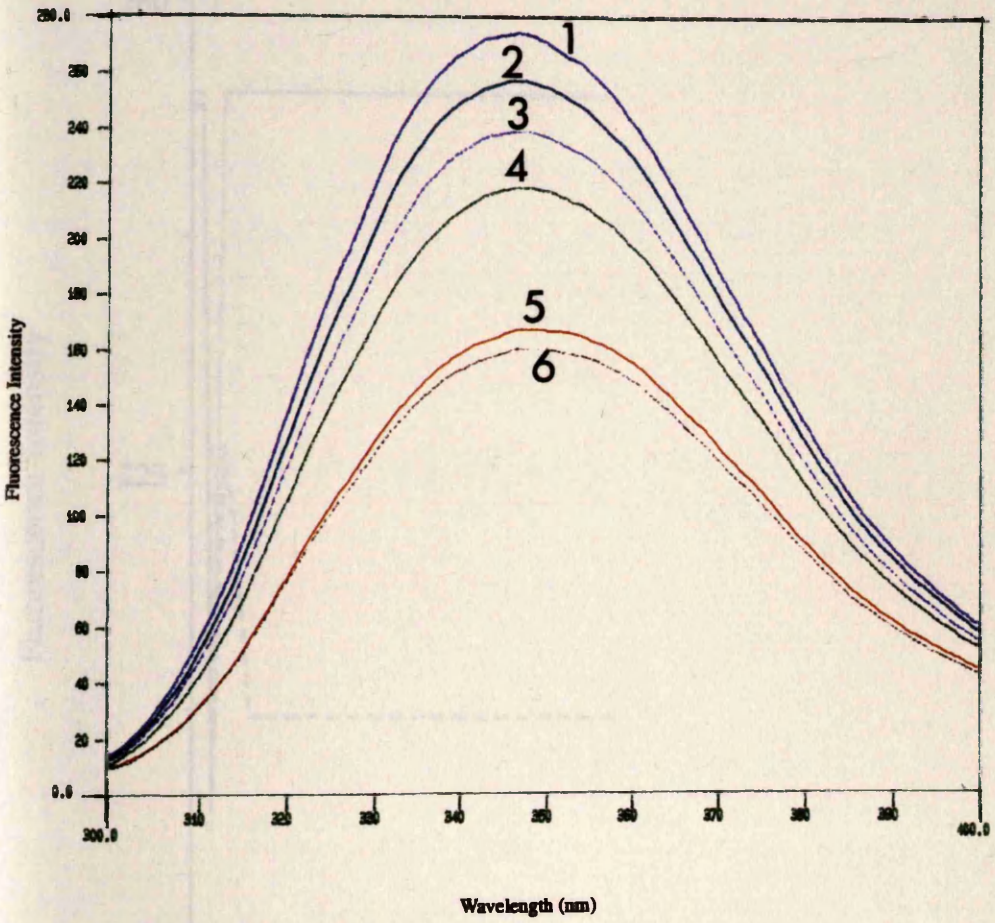
tryptophan fluorescence spectra of native and treated samples were recorded at 25 °C (3.2.11.1.II.).

To see the effect of acyl chain length, two types of experiments were carried out. In the first the effect of acyl chain was studied by fluorescence changes. Two fatty acids were chosen. The figure 3.2.11.1.III. shows that short chain fatty acid (hexanoic acid) produced no effect on the fluorescence intensity of the acidic phospholipase A_2, whereas long-chain fatty acid (oleic acid) produced abrupt changes. Similarly the bee venom phospholipase A_2 enzyme (figure 3.2.11.1.IV.) did not show any changes in fluorescence emission in response to hexanoic acid, but responded to oleic acid. In the second type, the effect of acyl chain on PLA_2 activation was studied by conductimetric assays. Figure 3.2.11.1.V. shows that activation of enzymes is due to long-chain fatty acids.

3.2.11.2.) Fluorescence emission for measurement of acyl imidazolide binding.

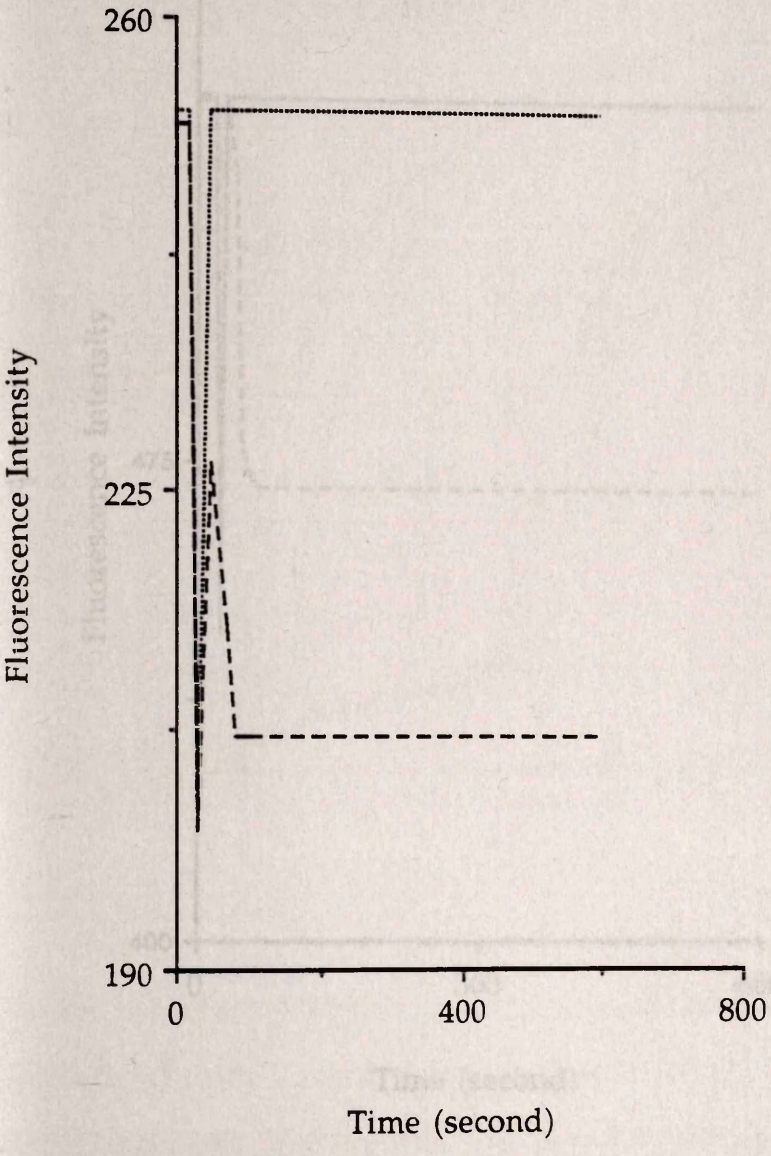
Extended incubation of the bee venom and the acidic isoform of *Naja mossambica mossambica* phospholipase A_2 enzymes with oleoyl imidazolide resulted in very considerably reduced fluorescence emission and a distinct red shift for the maximum absorption. When this was studied in the kinetic mode two distinct processes were revealed. The first was a very rapid fall in emission and this was followed by a long slow fall which eventually ended at a low stable level. The interpretation of these events was that the first part could represent binding of the reagent to the protein and the second represent the subsequent chemical reaction leading

Figure 3.2.11.1.II. Tryptophan fluorescence emission spectra of the acidic isoform of phospholipase A2 from the venom of *Naja mossambica mossambica* treated with different molar concentrations of oleoyl imidazolide.



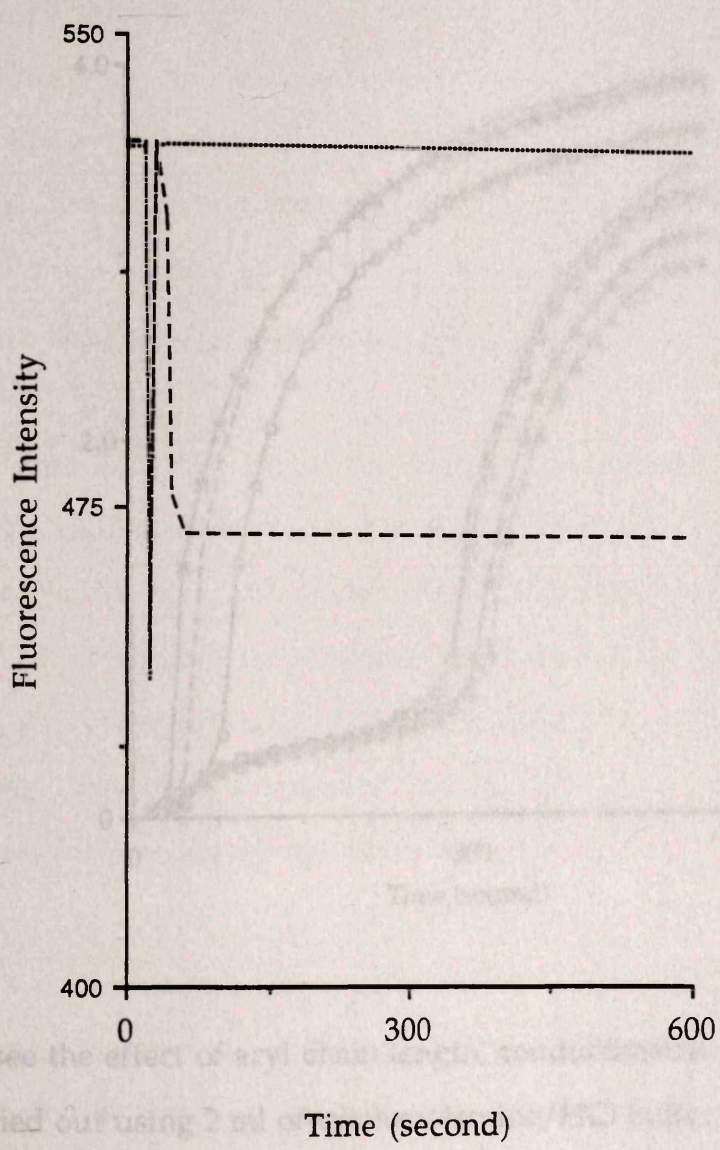
The acidic isoform of phospholipase A2 from the venom of *Naja mossambica mossambica* was prepared at concentration of 0.1 mg/ml solution in 10 mM borate buffer of pH 8.0 and divided into six equal fraction and five of them were treated with different molar concentration of oleoyl imidazolide. After one hour of treatment the fluorescence spectra of native 1:0 (1), treated with 1:0.125 (2), 1:0.25 (3), 1:0.5 (4), 1:1 (5) and 1:2 (6) were recorded at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter over the range of 300-400 nm. The enzyme samples were excited at 290 nm.

Figure 3.2.11.1.III. Time courses of fluorescence emission changes of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* treated with hexanoic acid and oleic acid.



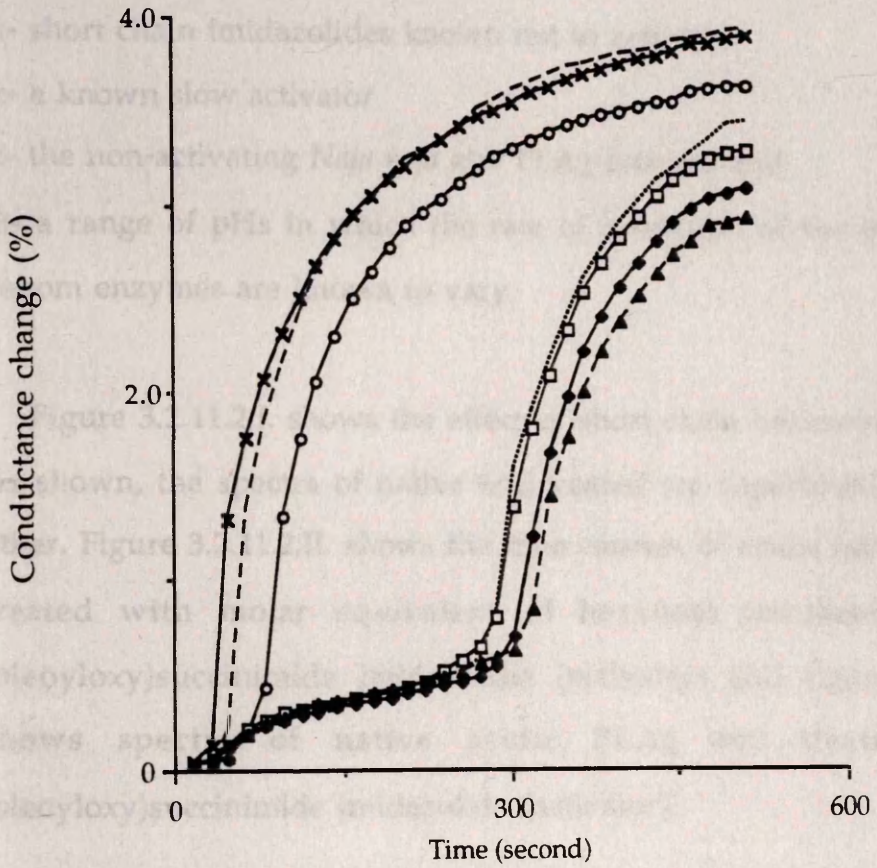
The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated either with the hexanoic acid (short chain fatty acid) (·····) or with the oleic acid (long chain fatty acid) (--) and time courses of tryptophan fluorescence were recorded at 340 nm using a Perkin-Elmer LS 50 spectrofluorimeter at 25°C. Samples were excited at 290 nm.

Figure 3.2.11.1.IV. Time courses of fluorescence emission changes of the bee venom phospholipase A_2 treated with hexanoic acid or oleic acid.



The bee venom phospholipase A_2 (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated either with hexanoic acid (short chain fatty acid) (┄┄) or with oleic acid (long chain fatty acid) (--) and time courses of tryptophan fluorescence changes were recorded at 340 nm using a Perkin-Elmer LS 50 spectrofluorimeter at 25°C. Samples were excited at 290 nm.

Figure 3.2.11.1.V. The effect of acyl chain length on the activation of phospholipase A_2



To see the effect of acyl chain length, conductimetric enzyme assays were carried out using 2 ml of triethanolamine/HCl buffer of pH 8.0 containing 20 % 1-propanol. The substrate used was purified egg phosphatidyl choline in the presence of 0.1 mM $CaCl_2$. Free fatty acid (0.2 mM) was included in the assay buffer. Two µl of bee venom phospholipase A_2 (1mg/ml) was used. (·····) Native enzyme, (—×—) oleic acid+enzyme, (- - -) palmitic acid+enzyme, (—o—) myristic acid+enzyme, (-□—) octanoic acid+enzyme, (-◆—) heptanoic acid+enzyme, (- -▲-)hexanoic acid+enzyme.

to stable modification. In order to determine whether these processes correspond to activation, a number of experiments were carried out. These included the use of;

a:- short chain imidazolides known not to activate,

b:- a known slow activator

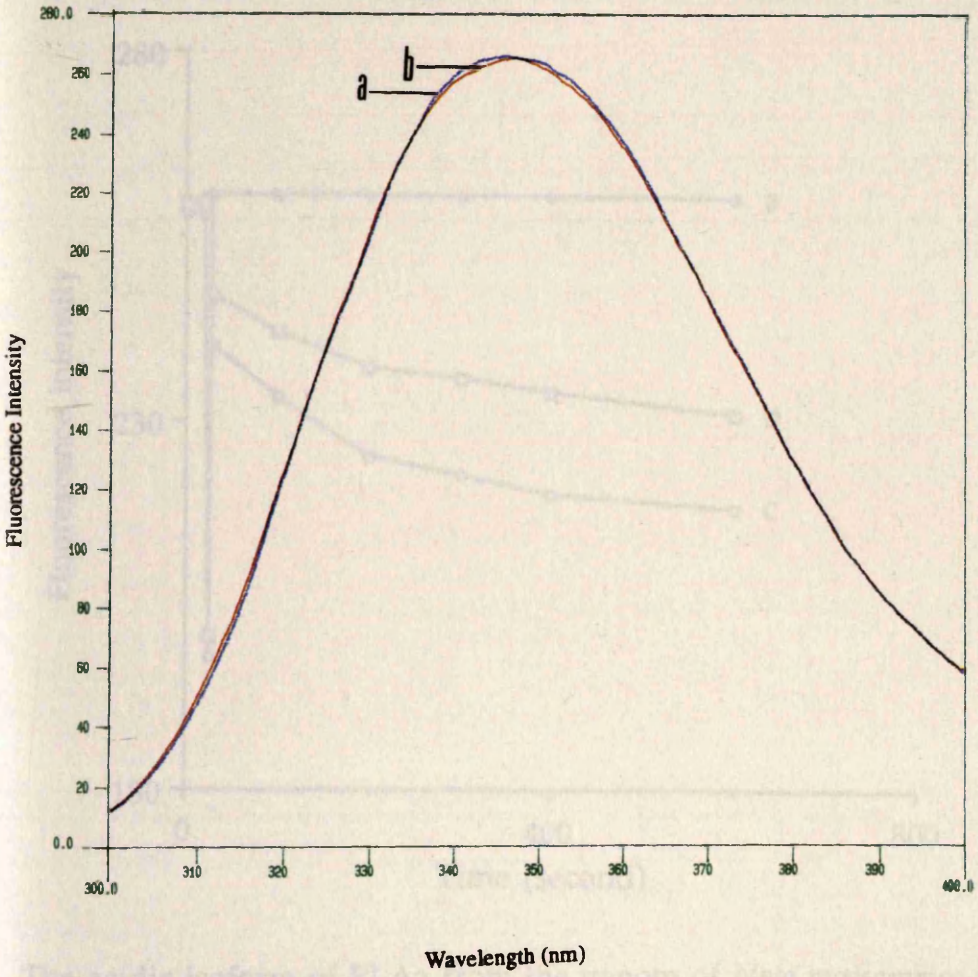
c:- the non-activating *Naja naja atra* PLA_2 enzyme and

d:- a range of pHs in which the rate of activation of the bee and snake venom enzymes are known to vary.

Figure 3.2.11.2.I. shows the effect of short chain hexanoyl imidazolide. As shown, the spectra of native and treated are superimposable on each other. Figure 3.2.11.2.II. shows the time courses of acidic isoform of PLA_2 treated with molar equivalent of hexanoyl imidazolide and N-(oleoyloxy)succinimide imidazolide (activator) and figure 3.2.11.2.III. shows spectra of native acidic PLA_2 and treated with N-(oleoyloxy)succinimide imidazolide (activator).

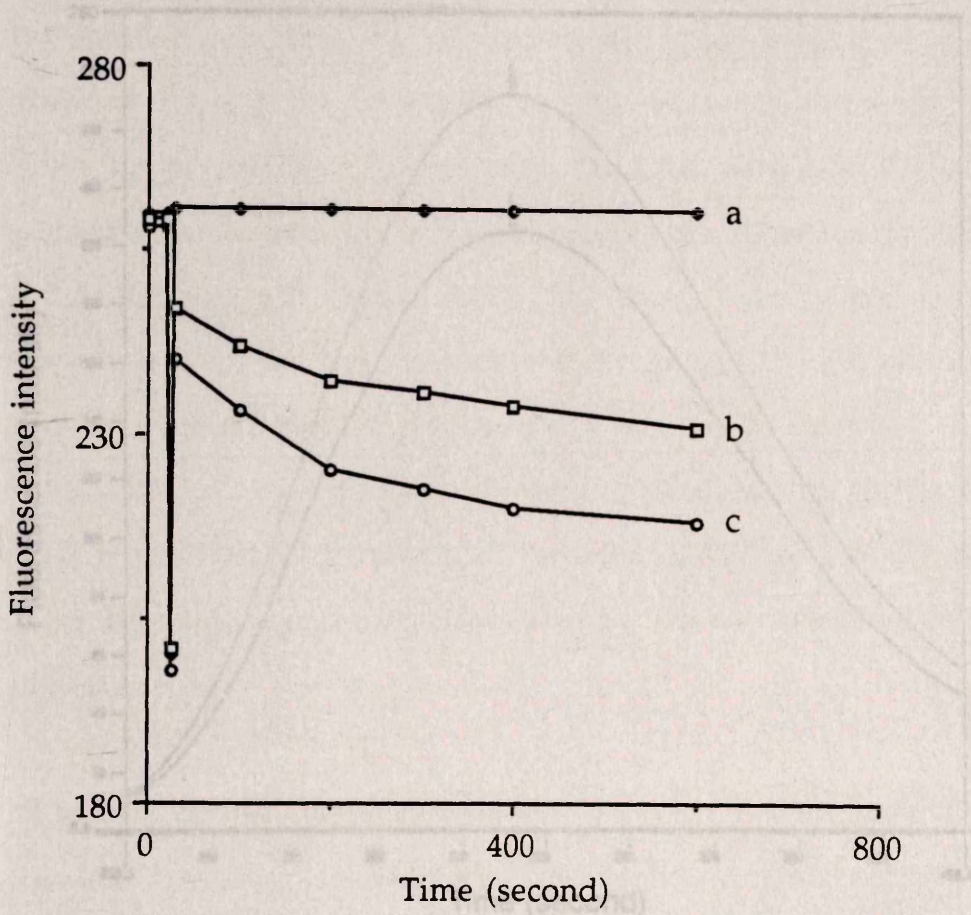
In order to characterise the nature of the group which binds the activator, an extensive pH dependent activation kinetic studies of three different isoforms of phospholipase A_2 enzymes from three different venoms were carried out. The first approach was to study pH dependent activation kinetics by using an erythrocyte assay, as this is the most convenient method of detecting activation. The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (1 mg/ml) was incubated in 10 mM buffers of either bis tris pH 7.0 or borate pH 8.0. and 9.0 and incubated with the molar equivalent of oleoyl imidazolide at room

Figure 3.2.11.2.I. Tryptophan fluorescence emission spectra of native and treated acidic PLA2 from the venom of *Naja mossambica mossambica* with hexanoyl imidazolide.



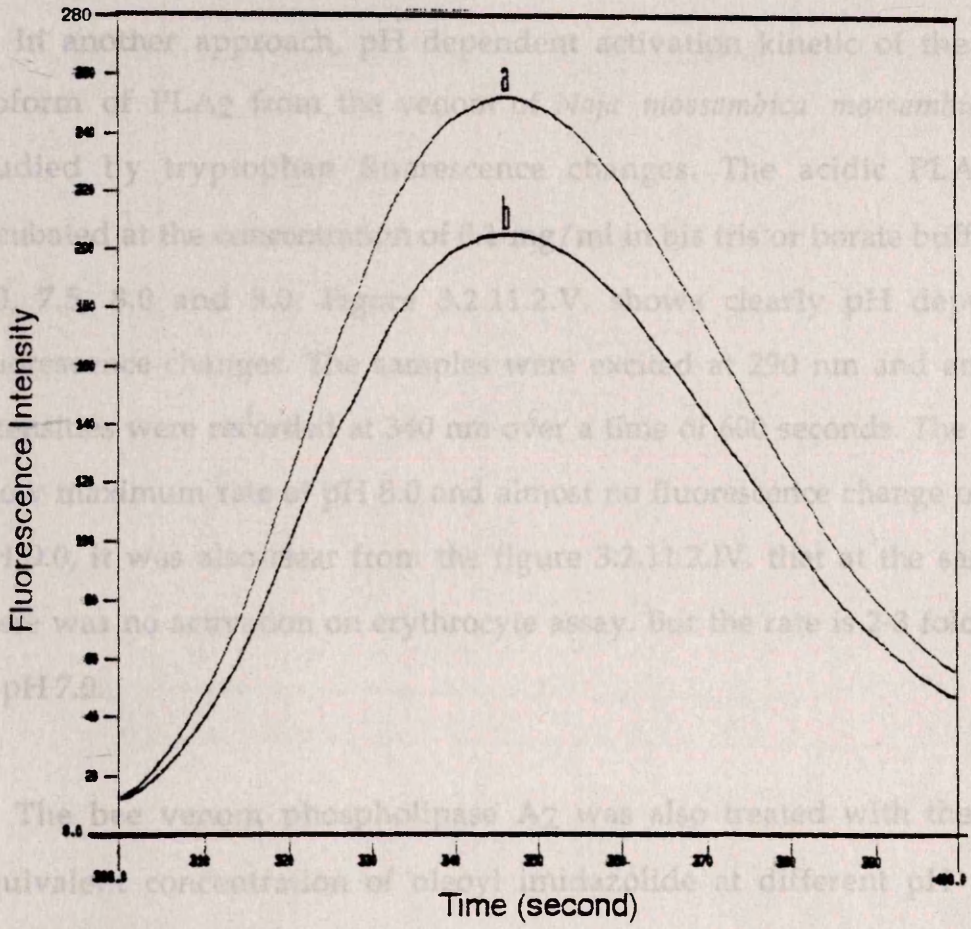
The acidic isoform of PLA2 (0.1 mg/ml) from the venom of *Naja mossambica mossambica* was treated with molar equivalent (8μM) of hexanoyl imidazolide and fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and fluorescence emission spectra were recorded over the range of 300-400 nm. Native enzyme (a) and treated enzyme (b).

Figure 3.2.11.2.II. Time courses of tryptophan fluorescence emission of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambioca.* treated with hexanoyl imidazolide, N-(oleoyloxy)succinimide imidazolide and oleoyl imidazolide.



The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated with molar equivalent of hexanoyl imidazolide, N-(oleoyloxy)succinimide imidazolide (activator) and oleoyl imidazolide (activator) and fluorescence measurements were made of hexanoyl imidazolide (a), N-(oleoyloxy)succinimide imidazolide (b) and oleoyl imidazolide (c) PLA_2 treated samples at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and emission intensity recorded at 340 nm.

Figure 3.2.11.2.III. Tryptophan fluorescence spectra of native and treated acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* with N-(oleoyloxy)succinimide imidazolide (activator).



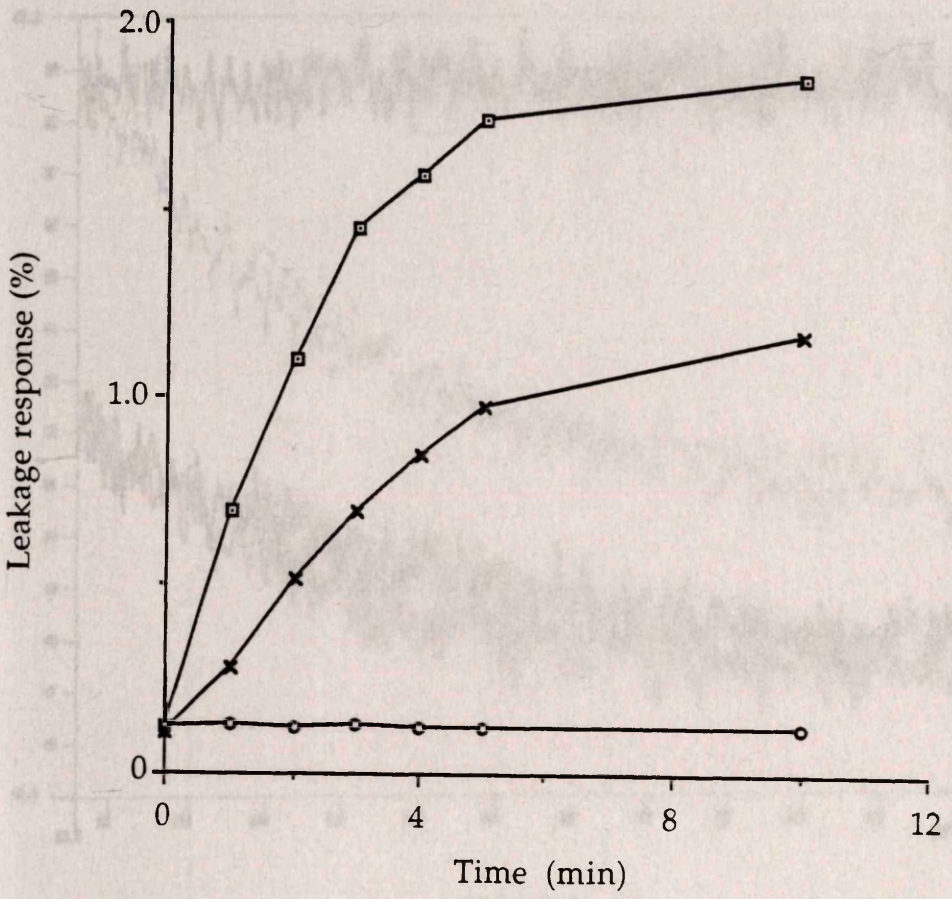
The acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* (0.1 mg/ml) in 10 mM borate buffer of pH 8.0 was treated with molar equivalent of N-(oleoyloxy)succinimide imidazolide (activator).and fluorescence measurements were made of native (0.1 mg/ml in 10 mM borate buffer of pH 8.0) and treated PLA_2 samples at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and emission spectra over the range 300-400 nm were recorded for native (a) and treated PLA_2 samples(b).

temperature. At different time intervals 2 µl samples were withdrawn and conductimetric assays were carried out on mouse erythrocytes (figure 3.2.11.2.IV.).

In another approach, pH dependent activation kinetic of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* was studied by tryptophan fluorescence changes. The acidic PLA_2 was incubated at the concentration of 0.1 mg/ml in bis tris or borate buffer pHs 7.0, 7.5, 8.0 and 9.0. Figure 3.2.11.2.V. shows clearly pH dependent fluorescence changes. The samples were excited at 290 nm and emission intensities were recorded at 340 nm over a time of 600 seconds. The results show maximum rate at pH 8.0 and almost no fluorescence change occur at pH 9.0, it was also clear from the figure 3.2.11.2.IV. that at the same pH there was no activation on erythrocyte assay. But the rate is 2-3 fold lower at pH 7.0.

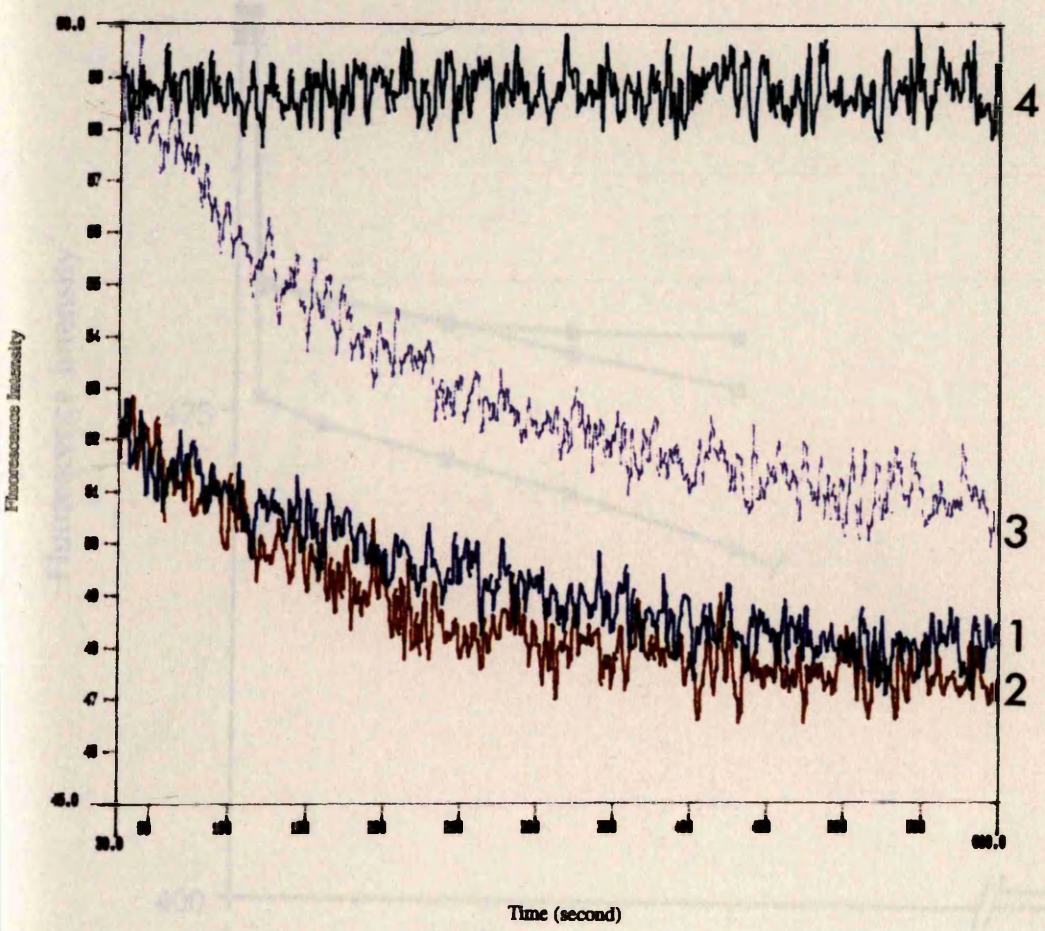
The bee venom phospholipase A_2 was also treated with the molar equivalent concentration of oleoyl imidazolide at different pH values. Figure 3.2.11.2.VI. shows the time course of fluorescence changes of bee venom PLA_2 treated at pH 7.0, 8.0 and 9.0 using 10 mM buffer of bis tris or borate. Similarly as in the case of the acidic isoform, bee venom PLA_2 shows a maximum rate at pH 8.0 and the rate at pH 7.0 is two to three fold lower, but in this case the rate at pH 9.0 is slightly lower than rate at pH 8.0. Figure 3.2.11.2.VII. shows spectra of bee venom PLA_2 at different pH values. The samples were excited at 290 nm and spectra were recorded over the range 300-400 nm. It is also clear from this figure that maximum fluorescence changes were produced at pH 8.0.

Figure 3.2.11.2.IV. pH dependence activation of the acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica*.



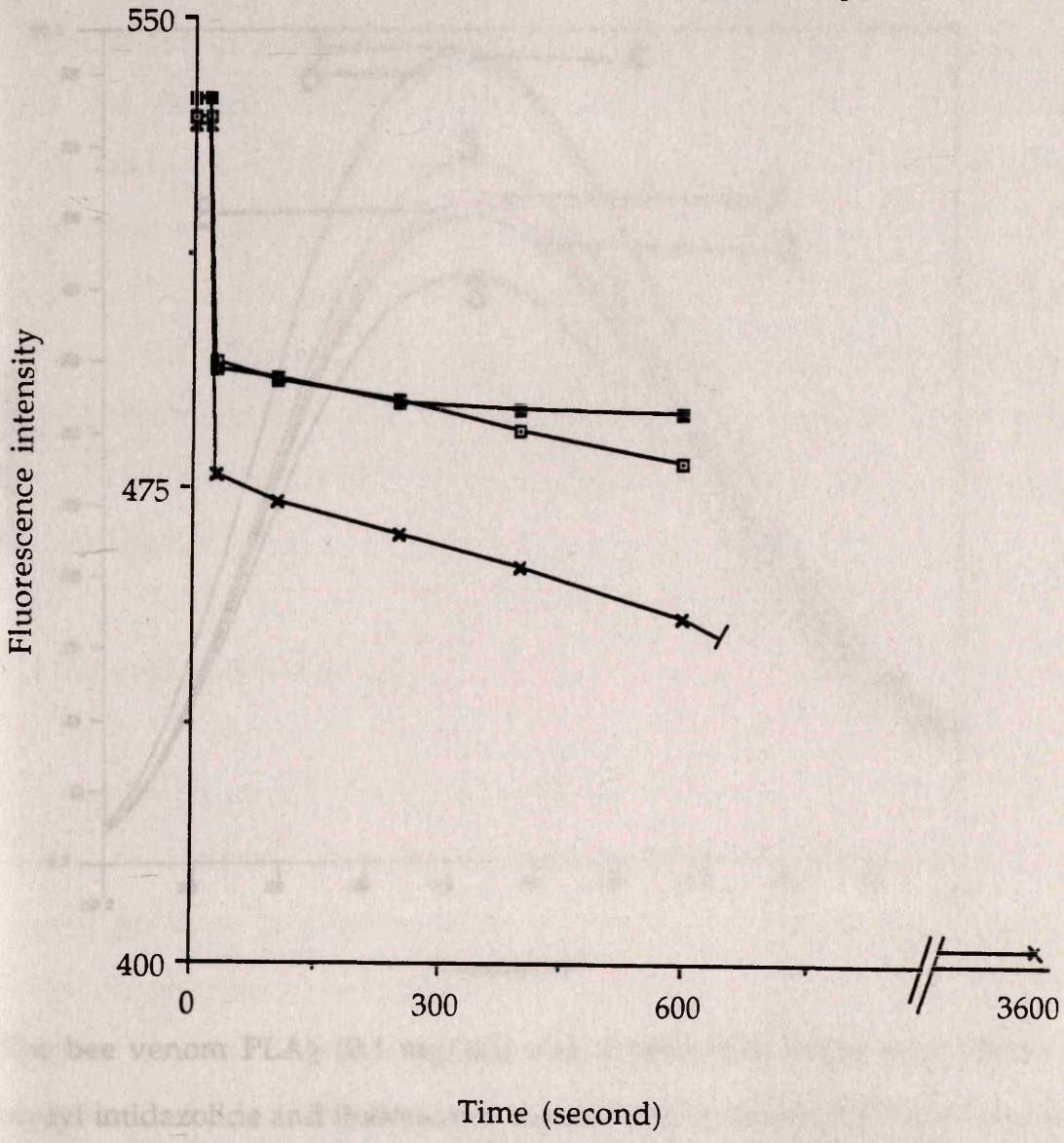
The acidic isoform of PLA_2 (1 mg/ml) from the venom of *Naja mossambica mossambica* was treated with molar equivalent (80 µM) of oleoyl imidazolide at room temperature and 2 µl sample was withdrawn at different time intervals and activation assays were carried out as described in materials and methods but using mouse erythrocytes as substrate. pH 7 (—✕—), pH 8 (—□—), and pH 9 (—○—)

Figure 3.2.11.2.V. Time courses of tryptophan fluorescence emission of the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica* treated with oleoyl imidazolide at different pHs.



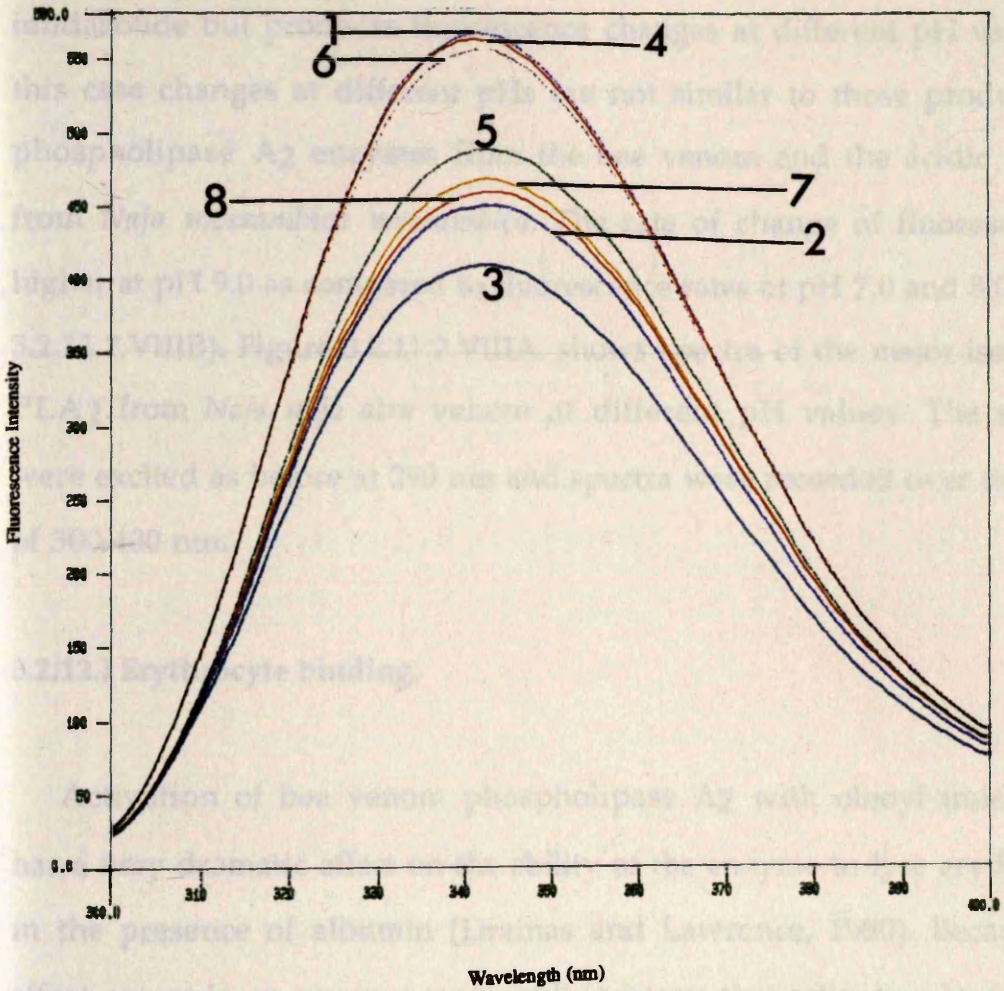
The acidic isoform of PLA_2 (0.1 mg/ml) from the venom of *Naja mossambica mossambica* was treated with molar equivalent (8μM) of oleoyl imidazolide and fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and emission intensity recorded at 340 nm. Time courses of PLA_2 treated with oleoyl imidazolide were recorded at pH 7.0 (enzyme in 10 mM Bis tris buffer) (1), at pH 7.5 (enzyme in 10 mM Bis tris buffer) (2) at pH 8.0 (enzyme in 10 mM borate buffer) (3) and at pH 9.0 (enzyme in 10 mM borate buffer) (4).

Figure 3.2.11.2.VI. Time courses of tryptophan fluorescence emission of the bee venom PLA_2 treated with oleoyl imidazolide at different pHs.



The bee venom PLA_2 (0.1 mg/ml) was treated with molar equivalent of oleoyl imidazolide and fluorescence measurements were made of at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and emission intensity recorded at 340 nm. Time courses of bee venom PLA_2 treated with oleoyl imidazolide were recorded at pH 7.0 (enzyme in 10 mM Bis tris buffer), at pH 8.0 (enzyme in 10 mM borate buffer) and at pH 9.0 (enzyme in 10 mM borate buffer). pH 7.0 (—■—), pH 8.0 (—×—) and pH 9.0 (—□—)

Figure 3.2.11.2.VII. Tryptophan fluorescence spectra of bee venom PLA_2 at different pH.



The bee venom PLA_2 (0.1 mg/ml) was treated with molar equivalent of oleoyl imidazolide and fluorescence measurements were made at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Samples were excited at 290 nm and emission spectra over the range 300-400 nm were recorded at pH 7.0 (enzyme in 10 mM Bis tris buffer) native (4) and after 10 minutes of treatment (5), at pH 8.0 (enzyme in 10 mM borate buffer) native (1), after 10 minutes of treatment (2) and after 60 minutes of treatment (3) and at pH 9.0 (enzyme in 10 mM borate buffer) native (6), after 10 minutes of treatment (7) and after 60 minutes of treatment (8) ,

The major isoform of phospholipase A_2 with WWDF sequence from the venom of *Naja naja atra* does not activate in response to oleoyl imidazolide but produces fluorescence changes at different pH values. In this case changes at different pHs are not similar to those produced by phospholipase A_2 enzymes from the bee venom and the acidic isoform from *Naja mossambica mossambica*. The rate of change of fluorescence is higher at pH 9.0 as compared to fluorescence rates at pH 7.0 and 8.0 (figure 3.2.11.2.VIIIB). Figure 3.2.11.2.VIIIA. shows spectra of the major isoform of PLA_2 from *Naja naja atra* venom at different pH values. The samples were excited as before at 290 nm and spectra were recorded over the range of 300-400 nm.

3.2.12.) Erythrocyte binding.

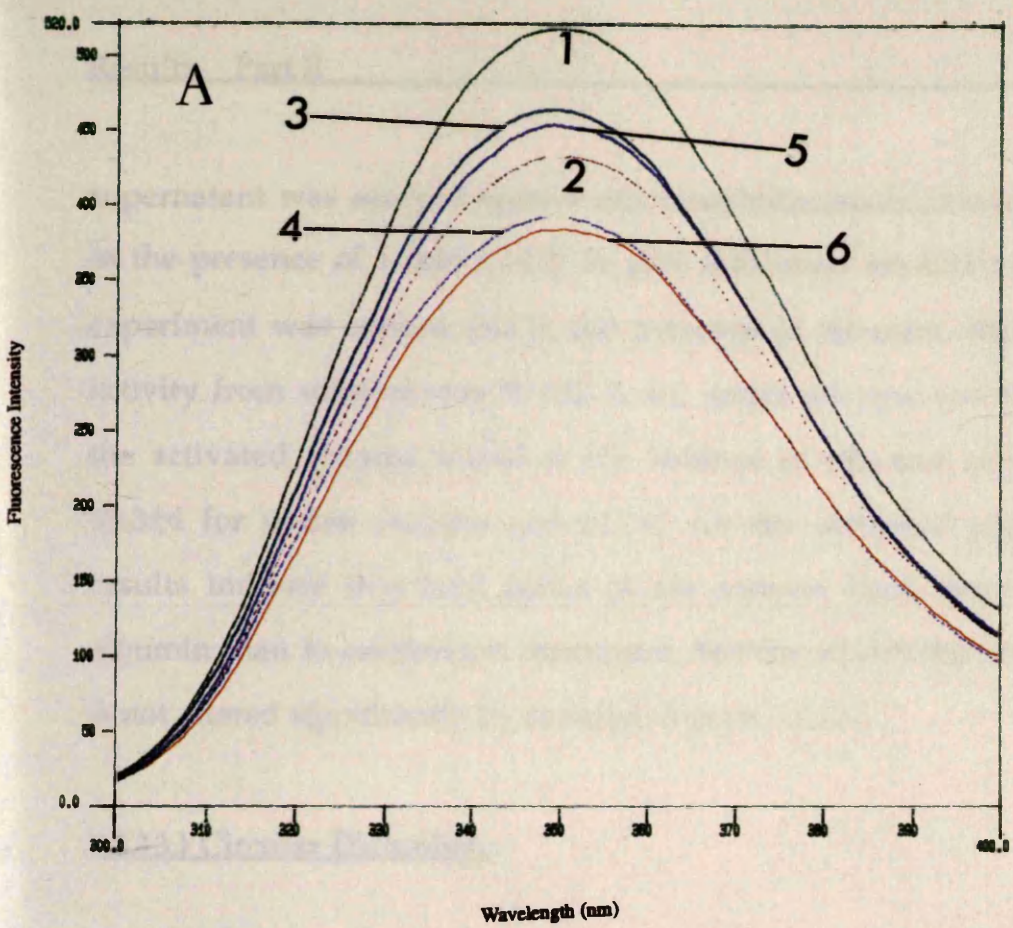
Activation of bee venom phospholipase A_2 with oleoyl imidazolide has a very dramatic effect on the ability of the enzyme to lyse erythrocytes in the presence of albumin (Drainas and Lawrence, 1980). Because this effect occurs in an aqueous medium it indicates that activation by free fatty acids or acylation might have a physiological role. Although neither the native nor the activated enzyme are lytic in the absence of albumin, the enhanced activity of the activated enzyme in the absence of albumin has been clearly demonstrated. Enhancement of the lytic action of the enzyme could be attributed either to increased binding of the enzyme to the erythrocyte membrane or else to increases in the lytic properties of the enzyme itself. To investigate the former possibility, erythrocytes were incubated with low quantities of normal and activated enzyme and after a brief incubation the cells pelleted and the residual PLA_2 present in the

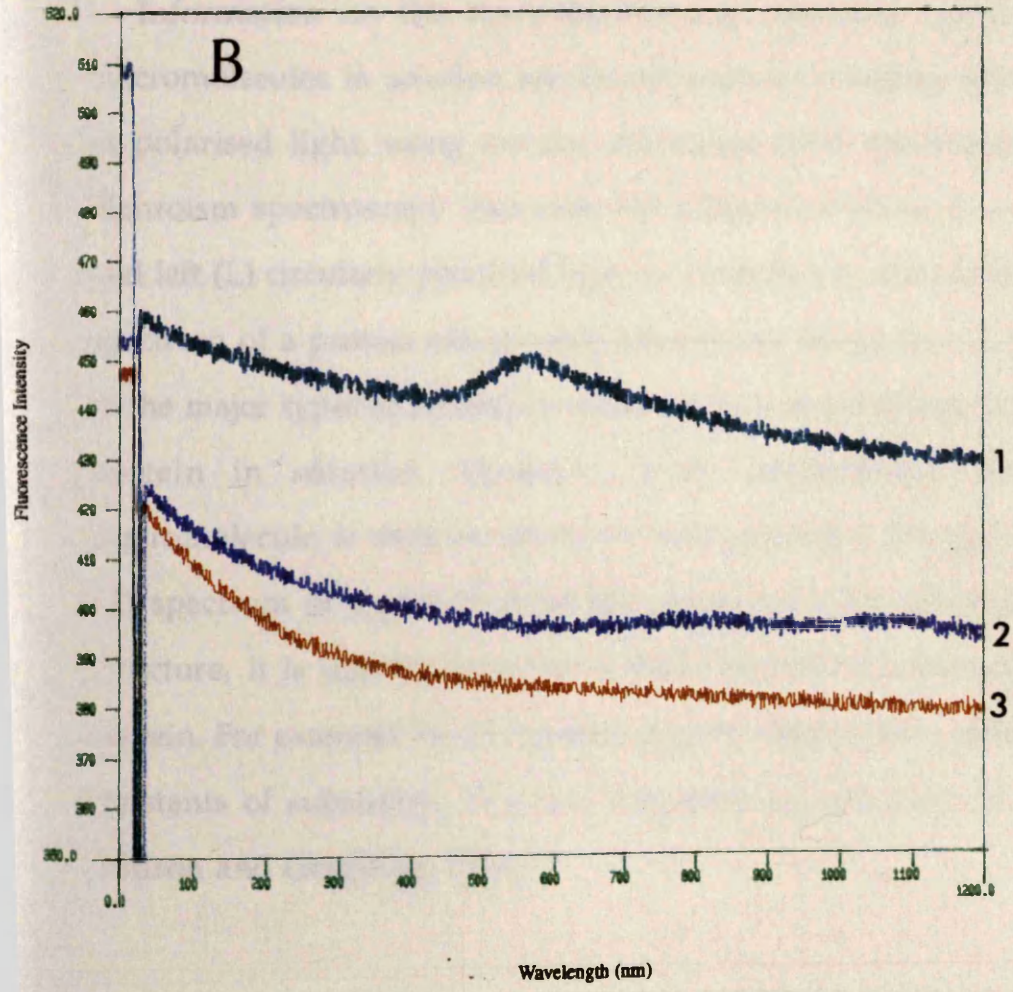
Figure 3.2.11.2.VIII. Perturbation of the tryptophan fluorescence of the major PLA_2 isoform from the venom of *Naja naja atra* by oleoyl imidazolide at different pHs.

The major isoform of PLA_2 (0.1 mg/ml) from the venom of *Naja naja atra* was treated with molar equivalent (8μM) of oleoyl imidazolide and fluorescence measurements were recorded at 25°C using a Perkin-Elmer LS 50 spectrofluorimeter. Enzyme solutions were prepared at 0.1 mg/ml (ca 7.5 μM) and emission spectra for excitation at 290 nm obtained

(A) Emission spectra over the range 300-400 nm were recorded at pH 7.0 (enzyme in 10 mM Bis tris buffer) native (1) and after 10 minutes of treatment (2) and at pH 8.0 (enzyme in 10 mM borate buffer) native (3) and after 10 minutes of treatment (4) and at pH 9.0 (enzyme in 10 mM borate buffer) native (5) and after 10 minutes of treatment (6)

(B) Emission intensity recorded at 340 nm. Time courses of fluorescence emission changes of PLA_2 treated with oleoyl imidazolide were recorded at pH 7.0 (enzyme in 10 mM Bis tris buffer) (1), at pH 8.0 (enzyme in 10 mM borate buffer) (2) and at pH 9.0 (enzyme in 10 mM borate buffer) (3).


A
1
2
3
4
5
6
Fluorescence Intensity
Wavelength (nm)


B
1
2
3
Fluorescence Intensity
Wavelength (nm)

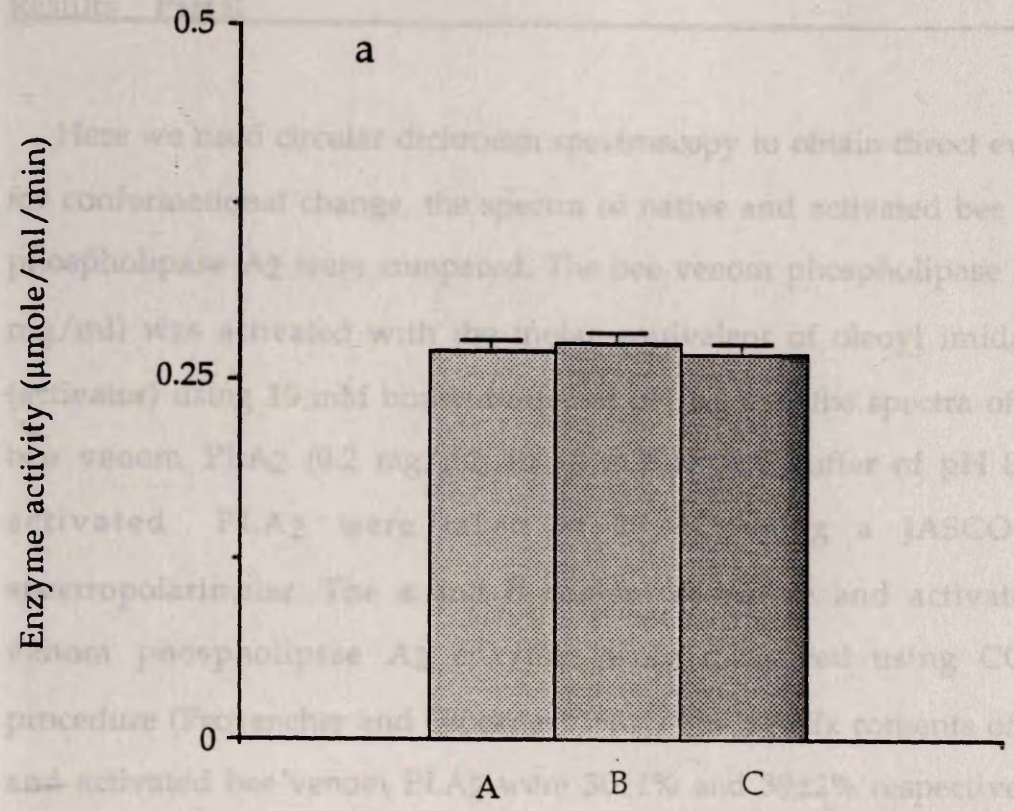
supernatant was assayed against dioctanoylphosphatidylcholine substrate in the presence of 1 mM $CaCl_2$ to give maximum sensitivity. When this experiment was carried out in the presence of albumin, the recovery of activity from solution was 97.6±2 % for native enzyme and 96.1±.3 % for the activated enzyme whilst in the absence of albumin recovery fell to 63.3±4 for native enzyme and 61.5±3 for the activated enzyme. These results indicate that both forms of the enzyme bind more strongly to albumin than to erythrocyte membrane, but the affinity for the membrane is not altered significantly by acylation (figure 3.2.12.).

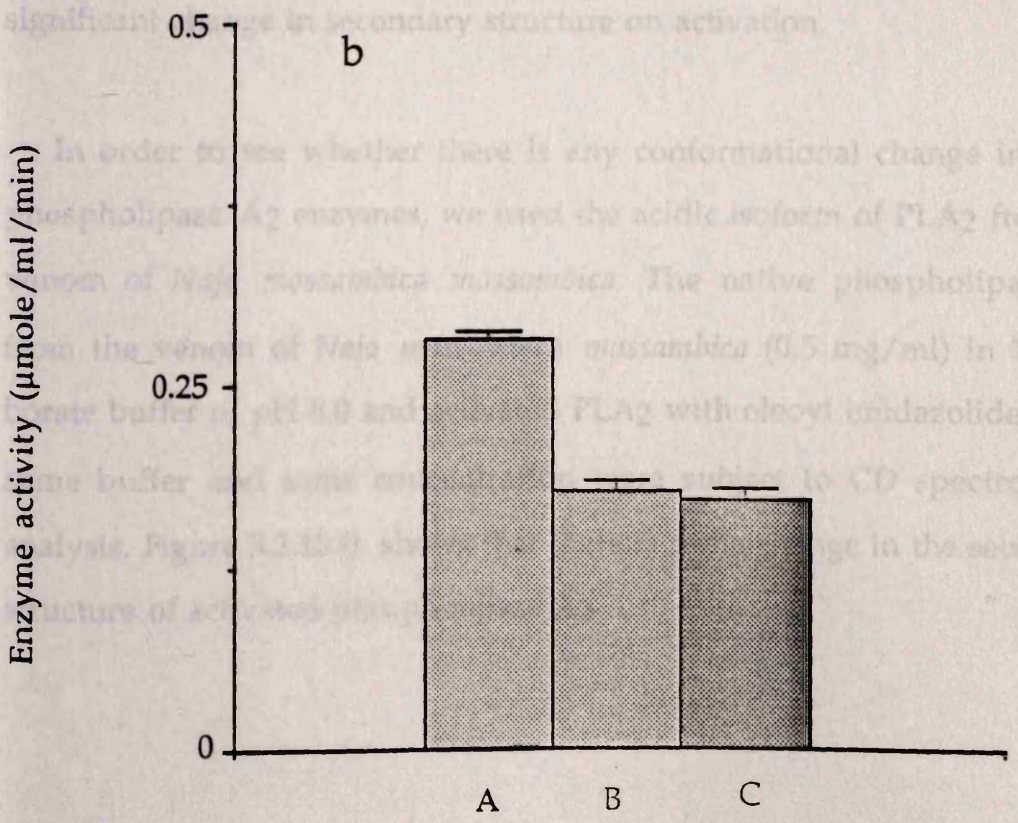
3.2.13.) Circular Dichroism.

Information on the three-dimensional structure (conformation) of macromolecules in solution can be obtained by studying their absorption of polarised light, using circular dichroism (CD) spectroscopy. Circular dichroism spectroscopy measures the differential absorption of right (R) and left (L) circularly polarised light as a function of a wavelength. The CD spectrum of a protein can provide information about the relative amounts of the major types of secondary structure (a, b and random coil) within the protein in solution. However, more importantly, the CD of a macromolecule is very sensitive to conformational changes. Even if the CD spectrum of a protein is far too complex for the determination of its structure, it is usually possible to study almost any interaction with the protein. For example the CD spectra may be used to determine the binding constants of substrates, cofactors, inhibitors or activators of any enzyme (Wilson and Goulding, 1986).

Figure. 3.2.12. Erythrocyte binding of Phospholipase A_2.

Native and activated with oleoyl imidazolide the bee venom phospholipase A_2 (2 μg) was incubated with mouse erythrocytes in 2 ml of 10 mM isotonic sucrose buffer of pH 7.4, either a) in the absence of albumin or b) in the presence of albumin and then centrifuged and supernatants were tested for phospholipase A_2 activity using dioctanoylphosphatidylcholine as a substrate and 1 mM $CaCl_2$ to give maximum sensitivity in 2 ml of 10 mM triethanolamine/HCl buffer of pH 8.0 A) Bee venom PLA_2 (2 μg) diluted in 2 ml of isotonic sucrose buffer of pH 7.4. B) Native bee venom PLA_2 C) Activated bee venom PLA_2 with oleoyl imidazolide.


a
0.5
0.25
0
Enzyme activity (µmole/ml/min)
A
B
C


b
0.5
0.25
0
Enzyme activity (µmole/ml/min)
A
B
C

Here we used circular dichroism spectroscopy to obtain direct evidence for conformational change, the spectra of native and activated bee venom phospholipase A_2 were compared. The bee venom phospholipase A_2 (0.2 mg/ml) was activated with the molar equivalent of oleoyl imidazolide (activator) using 10 mM borate buffer of pH 8.0 and the spectra of native bee venom PLA_2 (0.2 mg/ml) in 10 mM borate buffer of pH 8.0 and activated PLA_2 were taken at 25 °C using a JASCO J-600 spectropolarimeter. The α and β content of native and activated bee venom phospholipase A_2 enzymes were measured using CONTIN procedure (Provencher and Glöckner, 1981). The α-helix contents of native and activated bee venom PLA_2 were 30±1% and 39±2% respectively and that of β-sheet were 38±1% and 45±2% respectively and the remainder of secondary structure for native and activated were 32±1% and 16±3% respectively. The result figure 3.2.13.I. shows clearly that there is a significant change in secondary structure on activation.

In order to see whether there is any conformational change in other phospholipase A_2 enzymes, we used the acidic isoform of PLA_2 from the venom of *Naja mossambica mossambica*. The native phospholipase A_2 from the venom of *Naja mossambica mossambica* (0.5 mg/ml) in 10 mM borate buffer of pH 8.0 and activated PLA_2 with oleoyl imidazolide in the same buffer and same concentration were subject to CD spectroscopic analysis. Figure 3.2.13.II. shows that there is some change in the secondary structure of activated phospholipase A_2.

Figure. 3.2.13.I. Comparison of the circular dichroism (CD) of native and oleoyl imidazolide activated bee venom PLA_2.

The bee venom phospholipase A_2 (0.2 mg/ml) in 10 mM borate buffer of pH 8.0 was activated with molar equivalent of oleoyl imidazolide (solution in acetone). CD spectra of native (a) and the modified (b) enzymes were recorded at 25°C using a JASCO J-600 spectropolarimeter.

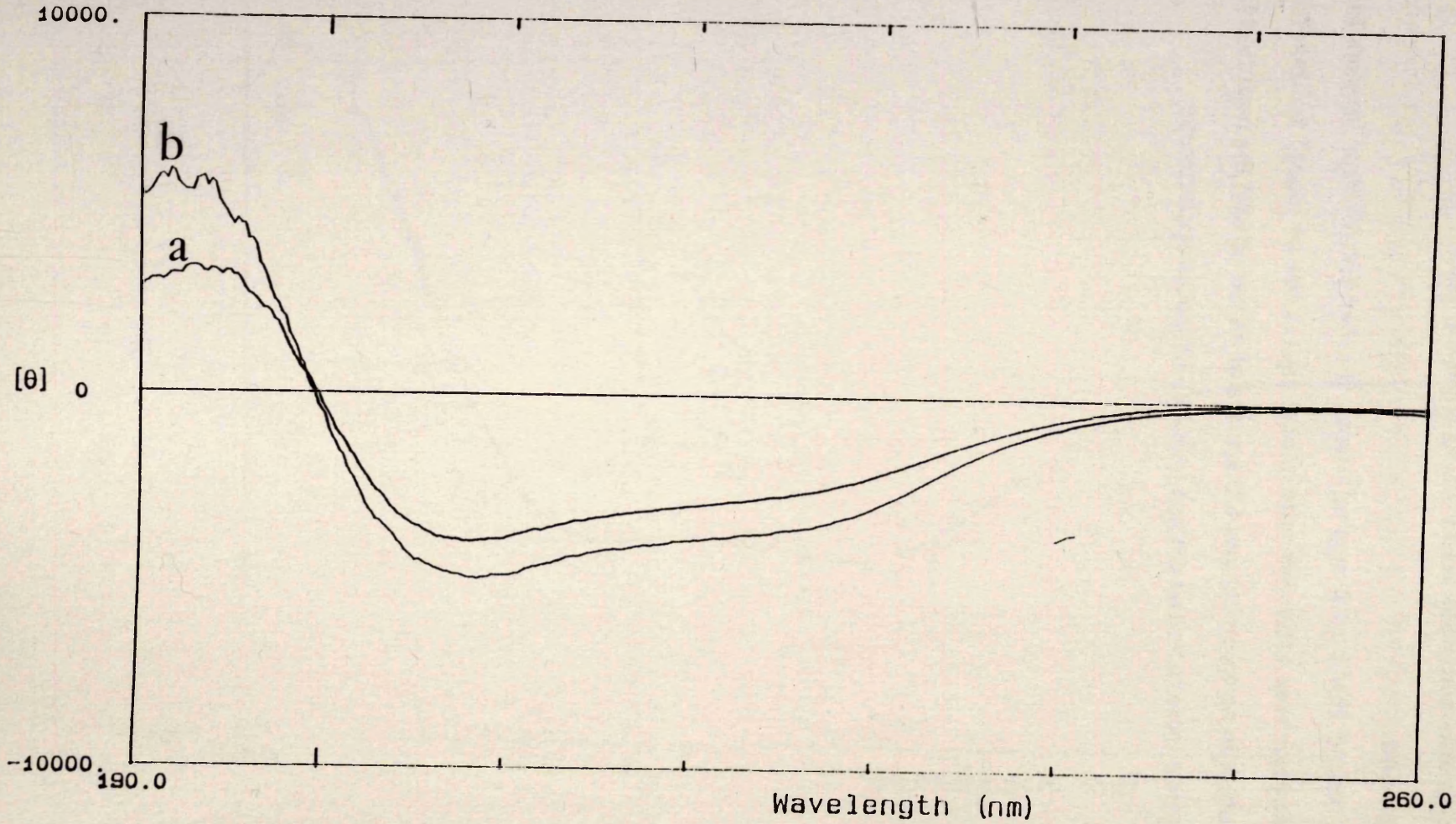
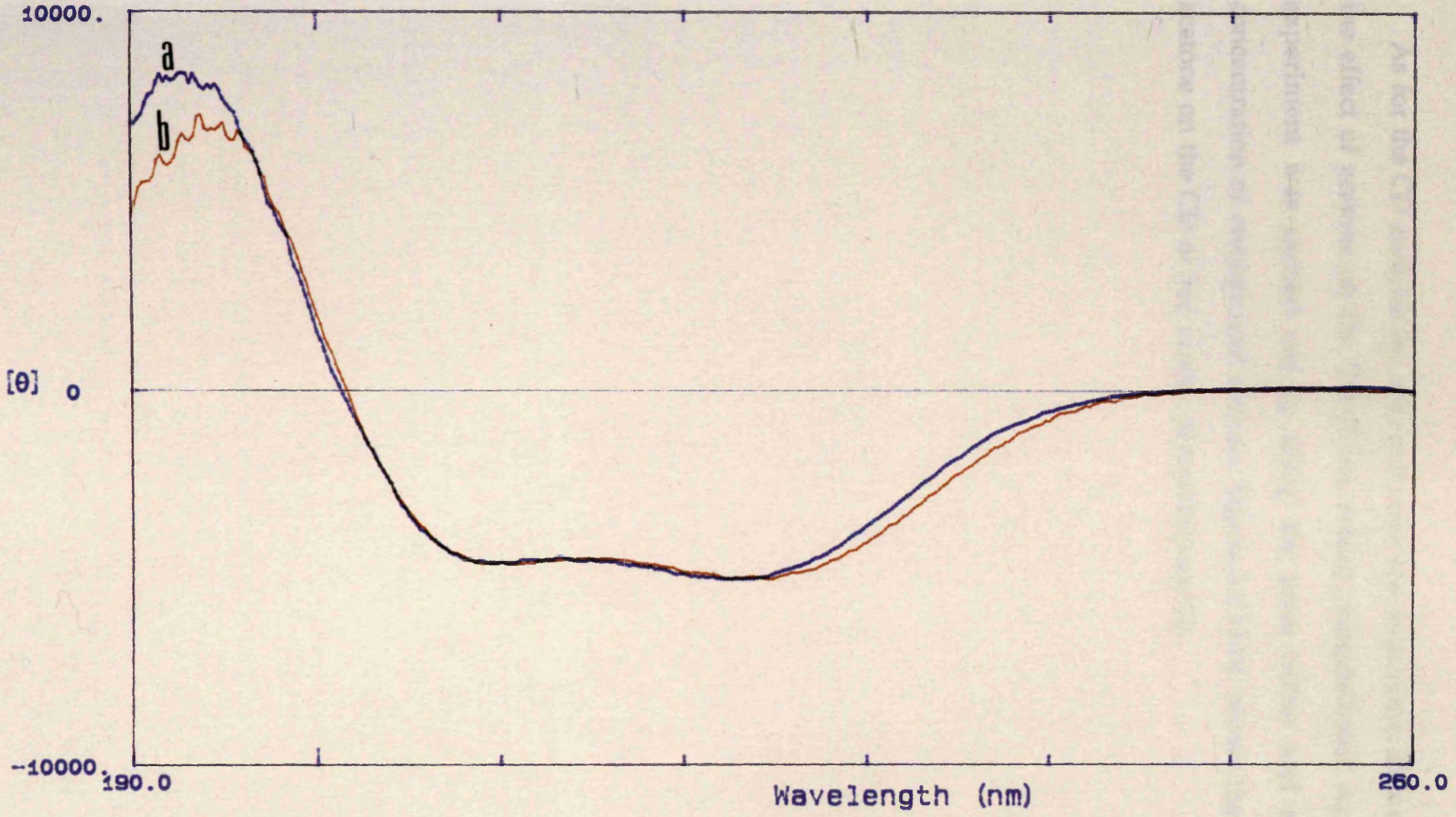

10000.
b
a
[θ]
0
-10000.
190.0
Wavelength (nm)
260.0

Figure 3.2.13.II. Comparison of the circular dichroism of native and oleoyl imidazolide activated acidic PLA_2 from the venom of *Naja mossambica mossambica*.

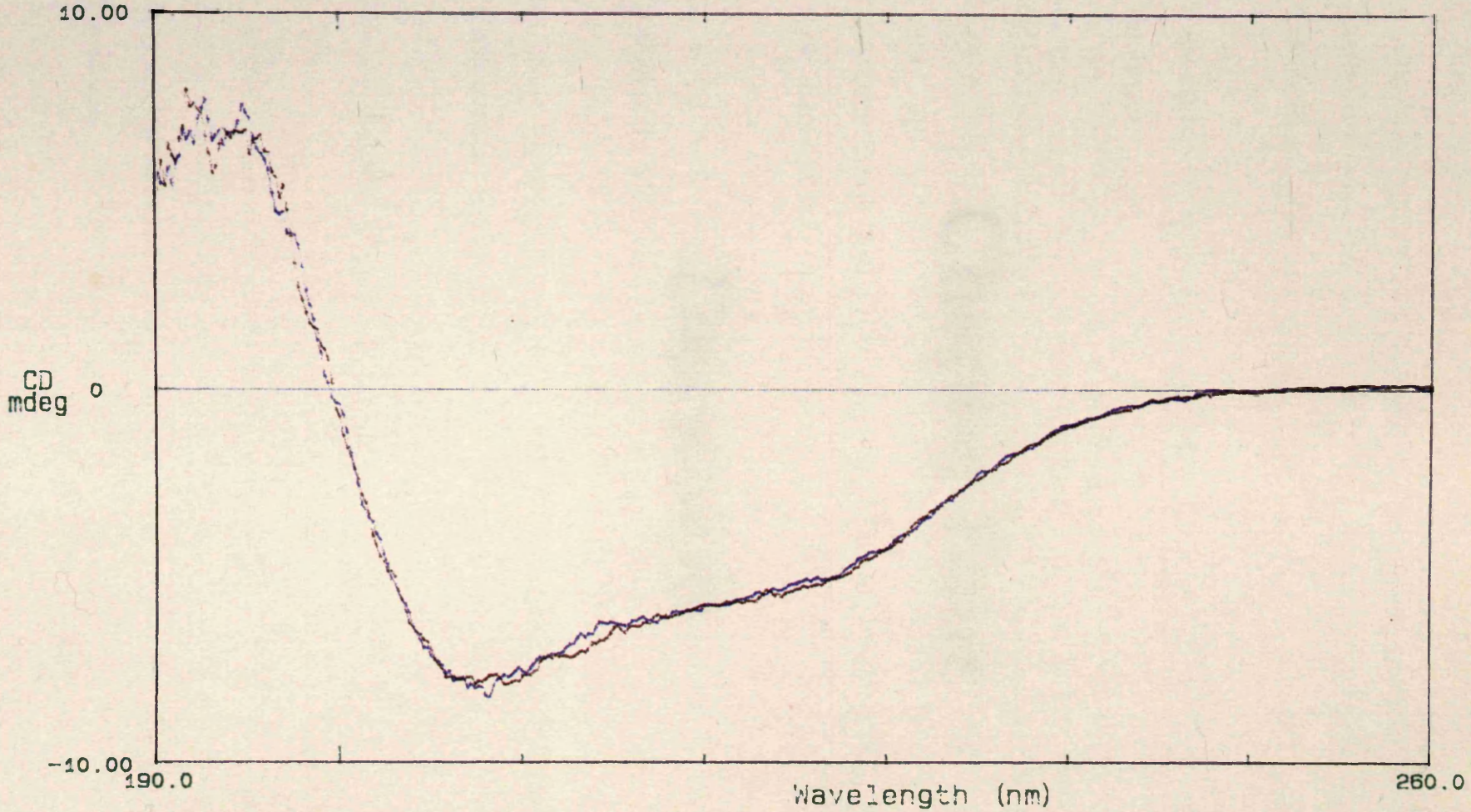
The acidic PLA2 (0.2 mg/ml) from the venom of *Naja mossambica mossambica* was activated with molar equivalent of oleoyl imidazolide (solution in acetonitrile) and CD spectra of native (a) and the modified (b) enzymes were recorded using a JASCO J-600 spectropolarimeter.


10000.
a
b
[θ] o
-10000.
190.0
260.0
Wavelength (nm)

As for the CD analysis the activator used was in acetone, in order to see the effect of acetone on the CD of bee venom phospholipase A_2, a blank experiment was carried out by using the same buffer and the same concentration of enzyme and acetone. Figure 3.2.13.III. shows the effect of acetone on the CD of bee venom phospholipase A_2.

Figure. 3.2.13.III. Effect of acetone on circular dichroism (CD) of native bee venom PLA_2.

the bee venom phospholipase A_2 (0.2 mg/ml in 10 mM borate buffer of pH 8.0) was recorded at 25°C using a JASCO J-600 spectropolarimeter.


10.00
CD
mdeg
0
-10.00
190.0
Wavelength (nm)
260.0

Chapter Four

Discussion

The investigations were carried out in this workhelp to resolve many questions concerning the activation of venom phospholipase A_2 enzymes and these are most conveniently listed.

1) Is there a fatty acid operated activation site in the majority of these enzymes?

2) Can the action of free fatty acids at this site have any other effect than to stabilise the enzyme against denaturation by organic solvents?

3) Is this the same site that reacts with long-chain acyl imidazolides in those enzymes activated by these reagents?

4) What is the nature of the bond formed when these enzymes are activated by acyl imidazolides?

5) Does activation involve the hydrophobic anchor model or the conformation change model?

4.1.) Purification of venom phospholipase A_2 isoforms.

The work started with purification of PLA_2 isoforms with the specific aim of obtaining enzymes of known sequence but with different response to potential activators. The results obtained were generally useful, but did not succeed in discovering useful new activating enzymes.

The preliminary aims of purification were two-fold. The first being to have a ready supply of a highly purified non-activating PLA_2 species and the second was to compare the characterised isoforms of PLA_2 from the venom of *Naja mossambica mossambica*. Here the development of high resolution basic urea PAGE coupled with the activity stain method proved crucial. Firstly it was clear that of all readily available venoms only that of the honey bee showed a simple PLA_2 composition with a single isoform and its glycosylated major variant. Among the snake venoms tested only that of *Naja naja atra* contained a single major isoform. Here there was good evidence for the existence of a minor isoform that had been reported previously and several additional novel PLA_2 components.

Studies of the venom of *Naja mossambica mossambica* gave rather different results from those of Chettibi *et al.*, (1990). Those workers used enzymes purchased from Sigma chemical Co ltd, but did not possess the analytical methods to distinguish the forms. In the present case 7 forms were identified of which four were major components and which appeared to correspond to the known acidic, basic non-toxic and basic toxic forms, but in this case the basic non-toxic form, hitherto considered to be the most abundant form could be resolved into two components. Activation studies did not fully support the results of Chettibi *et al.*, (1990) because only the acidic form responded to activator in the way they had described for the non-toxic basic form. A major technical problem encountered was that the whole venom supplied by Sigma appeared to be specifically depleted of the acidic isoform. Nevertheless the acidic isoform supplied by Sigma satisfied the criteria established for venom in which it was abundant.

Following the success of the activity staining method, a large number of venom from related species were studied both for PLA_2 isoform pattern and response to activator. The results appeared to be encouraging and indicated that the venoms from the spitting cobra species *Naja nigricollis nigricollis* and *Naja melanoleuca* (which all contain the WWHF motif) could be activating.

This work therefore succeeded in identifying three PLA_2 isoforms for the most intensive study, the major isoform from *Naja naja atra*, and the acidic isoform from *Naja mossambica mossambica* and bee venom PLA_2. The majority of the work however remained a comparison of the honey bee enzyme and the acidic isoform from *Naja mossambica mossambica*. and the major use of gel electrophoresis to examine the stability of the activated enzyme. The results showed quite conclusively that the oleoyl residue implanted by oleoyl imidazolide was more strongly bound than free oleic acid, but did not survive any electrophoretic technique. In this respect the bee venom enzyme adduct was significantly more stable than the snake venom enzyme adduct. Thus all of these results support the original conclusion that the linkage of the acyl residue to the protein does not involve an amide bond.

4.2.) Activation of bee venom phospholipase A_2 by glutaraldehyde in the presence of oleic acid.

The second part of the investigation centred on the activation of the bee venom phospholipase A_2 by glutaraldehyde in the presence of long-chain fatty acid (oleic acid). Here it was confirmed that glutaraldehyde did

stabilise the activated form generated by oleic acid and that this effect was eventually cancelled by the general inactivation produced by the cross linking agent. It proved to be very easy to stabilise the enzyme against inactivation by treatment with sodium borohydride at the point when maximum activation was reached. At this point the enzyme was about 8-fold activated and could be activated another 8-fold by the presence of free fatty acid in the assay solution. This result alone indicated that cross-linking did not cause the activating fatty acid to be retained in the enzyme and therefore that a significant part of the activation was due to a change but glutaraldehyde could only produce if the fatty acid were present, most probably a conformation change.

The stabilisation enabled the enzyme to be treated in order to remove oleic acid and the results of this treatment showed that the behaviour was hardly change by removing >98% of the oleic acid. Therefore it is certain that a major part of the activation is caused by a conformation change. This indicates that the fatty acid is in a buried site and therefore is not well positioned to act as a hydrophobic anchor. It is therefore possible that the full activation effect is also due to conformation change and that the change which can be stabilised by glutaraldehyde is not sufficient for maximum activation. The present results question one aspect of earlier work where it was reported that glutaraldehyde treatment alone stabilised the non-activating form of the enzyme without general inactivation. The present data indicate that there is partial inactivation which delays the onset of fatty acid inactivation and gives the appearance of non activation kinetics. If the hydrolysis reaction is continued long enough the biphasic curve is seen.

Activation by cross-linking depends on fortunate placing of primary amino groups as it is impossible for this function to relate to any natural phenomenon. It was not therefore expected to be found in diverse enzymes and the present work failed to find a significant effect in any snake venom enzyme.

This study of fatty acid activation revealed one peculiar feature. Careful inspection of the biphasic reaction progress curves showed that the first part was extremely linear. The model of activation by a single fatty acid molecule is not consistent with linearity and suggests either that more than one fatty acid molecule is involved or else that the fatty acids released from the substrate (largely oleic and linoleic acids) have different activating power and the least effective is liberated first. Comparison of the results with those of Drainas (1978), who used synthetic dioleoyl PC as substrate show that the progress curves had less initial linearity and supports the differential hydrolysis rate model.

4.2.1.) The activation Mechanism of venom phospholipase A_2 enzymes by fatty acyl derivatives.

The bee venom phospholipase A_2 has high activity towards short-chain phospholipid substrates which form micelles and very low activity toward long-chain phospholipid 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 mechanisms. One favoured by Verger and 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 Vmax 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.

Another model for activation of phospholipase A_2 enzymes, is called dimer model. According to this model, the activation of soluble phospholipase A_2 involves formation of enzyme dimers on the vesicles surface. This model was deduced by quantitative analysis of kinetic data obtained with porcine pancreatic phospholipase A_2 (Bell and Biltonen, 1989). Direct evidence for the formation of stable enzyme dimers following activation has been reported for the pancreatic enzyme, the phospholipase

A_2 from the venom of *Agkistrodon contortrix* and the monomeric (D-49) enzyme from the *Agkistrodon piscivorus piscivorus* (Cho, 1988).

The present work showed that for bee venom and for the acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica*, the activation is not due to dimerisation of enzymes as there was no difference on acidic/urea and SDS gel electrophoresis and as well as on gel filtration Bio-gel P-30 column chromatography.

The function of long-chain acyl residues in proteins is a matter of debate. They may stabilise protein oligomers or facilitate both stable or transient protein-membrane interactions (Grand, 1989; Resh, 1994) where the acyl chain may extrude from the protein and penetrates the lipid layer acting as a hydrophobic anchor. Such a model would appear to be a logical feature for a phospholipase and enable the protein to 'scoot' (Jain and Berg, 1990) on the substrate surface. Nevertheless the results obtained here provide good reason to believe that it does not apply in the present case.

Studies of activation in 1-propanolic medium carried out by Mezna (1993) in this laboratory showed very clearly that the major effect is to stabilise the enzyme against denaturation by the solvent. Thus there appears to be a site on the protein that can bind long-chain fatty acids or fatty acyl residues and reverse the inactivating effect of 1-propanol. However activation is substrate-dependent and not observed for a short-chain compound such as dioctanoyl phosphatidylcholine, but is present with the 1-palmitoyl,2-nonanoyl PC derivative, hence the presence of both activator and long-chain substrate is required to reverse the denaturing effect of 1-propanol.

The present work has extended by obtaining proof that a conformation change occurs during activation. This has been obtained by analysis of glutaraldehyde fixation in the presence of oleic acid where the present results show that the major component of activation by this modification persist even when the fatty acid has been removed.

To extrapolate from stabilisation against organic solvent to a role for the activating modification in an aqueous medium it is necessary to show that the enzyme is capable of undergoing a conformation change under these conditions and that this can be correlated with the activating modification. Although the three dimensional structure of the enzyme is stabilised by five disulphide bridges, the protein nevertheless undergoes a significant conformational change on activation as shown by the changes in circular dichroism.

This conclusion was strengthened by the changes in tryptophan fluorescence which indicate that occupation of the fatty acid binding site causes a conformation change. On the basis of the red shift in the emission maximum, the conformational change increases the exposure of at least one tryptophan residue to the solvent. As anticipated, the effect is instantaneous when induced by the free fatty acid, but when produced by oleoyl imidazolide has an appropriate rate and pH dependence to correlate with activation. Of the two tryptophan residues present in this protein, Trp-8 is in a relatively internal hydrophobic environment near the active site, whilst Trp-128 is in a less compact region of the protein with a lower density of hydrophobic residues, hence Trp-8 is the more likely source of the observed changes.

The most cogent argument against the hydrophobic tail model is that direct determination of the binding of the enzyme to erythrocyte membranes is not significantly altered by activation. Thus on balance the most plausible model is that acylation involves a buried acyl residue that forces a conformation change which makes the enzyme more effective against long-chain substrates whilst having no effect against short-chain substrates. This includes the possibility that contact with the substrate surface itself actually forces the enzyme into a low activity conformation and the effect of the activator is to overcome this induced change.

This part of study has been confined to the bee venom enzyme for the principal reason that it is the only such enzyme that can be activated by the combination of glutaraldehyde and free fatty acid and therefore provides unique evidence for conformation change.

4.3.) Stability of acyl linkage.

The acidic isoform of phospholipase A_2 from the venom of *Naja mossambica mossambica* shows a rapid and permanent increase in lytic activity, reaching a maximum of ca. 60-fold, when incubated with a stoichiometric equivalent concentration of oleoyl imidazolide. The hydrolytic activity against dioctanoyl phosphatidylcholine was very slightly affected by this treatment. Activation had the progressive nature of a chemical reaction and the modified protein was stable to dilute organic solvents and also to albumin, which was necessary for the expression of lytic activity in the erythrocyte leakage assay. The results showed that the activation of this enzyme by oleoyl imidazolide involves

the formation of a covalent linkage that appears to be extremely stable, but is readily broken in the presence of denaturing agents. And that the activity of native enzyme was stable to incubation with both types of urea gel reagents in the absence of ammonium persulphate, but that activation was rapidly lost. The experiments those were undertaken to examine the linkage of the oleoyl group directly, confirmed the earlier results by showing that it (oleoyl residue) is lost extremely rapidly during electrophoresis. In both urea gel systems the enzyme was applied to the gel in neutral solution without a denaturant and therefore the deacylation reaction should only occur after electrophoresis commenced. This was confirmed in the case of the acidic system because free oleic acid (pKa ca 5.0), could not enter the gel and therefore must have been released after the start of electrophoresis. The urea gels run at mildly acidic (pH 3.2) or mildly basic (pH 11.6) conditions. Typical esters which are sensitive to base hydrolysis have quite short half-lives above pH 11, they are more stable to the equivalent acidic conditions but might be close to their limit of stability in basic system. However, the instability of the linkage under the conditions of SDS-PAGE (pH 8.9) suggests that the presence of the denaturant may be more important than the actual pH. Taken together these results suggest that the linkage of the acyl group to the protein is inherently unstable but resists hydrolysis indefinitely when the enzyme is stored in aqueous solution in the absence of denaturing agents.

4.4.) Long-chain fatty acid binding site.

The present work shows that fatty acid binding in aqueous media can be measured directly by fluorescence emission and the initial results

showed that both bee venom and the acidic isoform from *Naja mossambica mossambica* bind oleic acid strongly and it is possible to determine a dissociation constant for the interaction. In contrast binding of the short chain fatty acids nonanoic and hexanoic acid was not detected. This therefore gives independent proof of the existence of a long chain fatty acid binding site on these proteins which can be inferred to be the activation site.

Studies of the acylation phenomenon were also greatly facilitated by the florescence emission method. Firstly the fact that fatty acids and their derivatives produce similar, but not identical responses, indicate that they act at the same site in slightly different ways. There is no direct proof that the fluorescence response measures the same event that causes activation, but the correlation is extremely good. Short chain derivatives that do not activate do not produce the fluorescence change. Oleoyl hydroxyethyl succinimde which is a slow activator in comparison with oleoyl imidazolide produces the changes but very slowly. The most convincing correlation however came from studies of the pH dependence. Earlier kinetic studies had shown that the bee venom enzyme activated very slowly below pH 7 and rates increased slowly above pH 8. These results are consistent with control by a single ionising group with pKa ca. 6.5. Fluorescence studies of the acidic isoform of the enzyme from *Naja mossambica mossambica* now showed a rate decrease above pH 8.5 and when the appropriate kinetic test of activation was carried out there was found to be a very clear pH optimum at ca 8.0. However no similar optimum was found by fluorescence studies of the bee venom enzyme.

Taken together with the fact that the snake venom enzyme activates significantly faster than the bee venom enzyme these results support the following conclusion:-

Long-chain fatty acid derivatives bind very rapidly to a hydrophobic cleft and perturb the conformation in the neighbourhood of a trp residue in the same way that fatty acids do. After binding there is a slower reaction which results in transfer of the acyl group from the imidazole ring to an acceptor residue in the protein. The transfer reaction is facilitated by a neighbouring residue.

The acylation of any residue will be controlled by the pKa of that residue because it must react in its non-protonated form. Where there is a pH optimum then two groups must be involved, one active in its non-protonated form and one in its protonated form. Lawrence and Moores (1975) had proposed such a model on the basis of the observation that oleic acid potentates the rate of activation by oleic anhydride, indicating that both reagents can bind to the site at the same time because it seems unlikely that the positively charged group that binds the carboxylate group of the fatty acid could also be the target for acylation. The pH rate data for the snake venom enzyme now confirms this model. It could be argued that this only accounts for the fact that activation of the snake venom enzyme is about ten times faster than that of the bee venom enzyme. Alternatively the group in the bee venom enzyme that binds the carboxylate group of the free fatty acid could be arginine and therefore would not be able to change its ionisation state.

The nature of the acceptor group for the acyl residue remains uncertain. It is clear however that the binding is highly labile to denaturation of the enzyme. The groups in this protein that are most reactive to acylating agents are the primary amino groups. Bell and Biltonen have demonstrated that in certain of these enzymes the catalytic reaction itself results in acylation of lysines which enhances aspects of the reactivity. This cannot be on the normal reaction pathway, because if it were there would be no delay and activation could not be detectable. It would therefore be a low probability event. If such a property were present then the features that promote acylation would also promote the reverse reaction. Acyl groups bound to such lysines would therefore be labile provided that the enzyme retained its native state, but stable when it was denatured. In the present system the opposite effect is seen.

Chettibi *et al.*, (1990) argued that the acceptor group was probably an H residue (H22 in the typical snake venom), based on two types of observation. Firstly the pH/activity studies of Lyall (1984) showed a pKa for activation of 6.5, close to the typical pKa of histidine. Secondly, snake venom PLA_2 enzymes were tested by him and those which activated had the tetrapeptide sequence XYHZ where X and Z were normally aromatic and Y was often aromatic. Sequences for activating non-activating enzymes are shown in Table 4.4.

4.4.1.) Target residue

In Terms of reactivity with acylating agents the most reactive target groups in phospholipase A_2 enzymes are:-

TABLE 4.4

Comparison of PLA_2 enzyme sequences

1) NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTAVDDLDR

2) NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTAVDDLDR

3) NLYQFKNMIHCTVPS-RPWWHFADYGCYCGRGGKGTPVDDLDR

4) NLYQFKNMIQCTVPS-RSWWDFADTGCYCGRGGSGTPVDDLDR

5) IIYPGTLWCGHGNKSSGPNELGRFKHTDACCRTHDMCPDVMSAG

1) CCQVHDNCYGEAEKL-GCWPYLTLYKYE-CSQGKLT-CSGGNNKCEA

2) CCQVHDNCYGEAEKL-GCWPYLTLYKYE-CSQGKLT-CSGGNNKCAA

3) CCQVHDNCYEKAGKM-GCWPYFTLYKYK-CSQGKLT-CSGGNSKCGA

4) CCQVHDNCYNEAEKISGCWPYFKTYSYE-CSQGTLT-CKGGNNACAA

5) ESKHGLTNTASHTRLSCDCDDKFYDCLKNSADTISSYFVGKMYFNL

1) AVCNCDLVAANCFAGAPYIDANYNVNLKERCQ

2) AVCNCDLVAANCFAGARYIDNYNINLKERCQ

3) AVCNCDLVAANCFAGARYIDANYNINFKKRCQ

4) AVCDCDRLAAICFAGAPYNNNNYNIDLKARCQ

5) IDTKCYKLEHPVTGCGERTEGRCLHYTVDKSKPKVYQWFDLRKY

1)CM-I from the venom of Naja mossambica mossambica, 2=CM-II Naja mossambica mossambica, 3=CM-III Naja mossambica mossambica, 4=Naja naja atra, 5=BEE VENOM,

The N-terminal amino group, ϵ-amino-groups, tyrosine, threonine and serine hydroxyl groups and the histidine imidazole group. Various pieces of evidence have been obtained which allow comments to be made about each of these as possible targets.

Amino groups:-

The best evidence that lysine is not the target residue for acylation comes from the extreme lability of the activated enzyme to any form of gel electrophoresis and especially from the fact that the enzyme can be apparently deactivated by removal of the residue without loss of basal activity. The possibility that a very reactive amino group existed in the native enzyme that was both acylated and deacylated extremely rapidly due to a facilitated reactivity has been suggested, but this reactivity should be lost upon denaturation but it is clear that the residue loses the bound group even more rapidly under denaturing conditions.

The present evidence confirms results of Chettibi (1990), who showed that primary amino groups were required for activation although were not necessarily targets for acylation.

Studies with dimethyl maleic anhydride produced interesting new information. This reagent inhibits the enzyme at high concentration, but it does not produce the fluorescence shift given by activators.

In terms of structural comparisons, the non activating enzymes of *Naja mossambica mossambica* have additional K residues to the activating forms.

Hydroxyl groups.

There is no evidence to support OH groups as the target residues except for FTIR data. On the surface this is compelling because the chemical shifts are in the bounds of possibility. However the difficulty arises in modelling the environment of the OH group and at present there is no pertinent information.

Otherwise threonine, serine and tyrosine esters are known to be stable to the conditions of SDS-PAGE. Furthermore the ST and Y composition of the various activating and non-activating isoforms show remarkable conservation. This is especially noticeable for tyrosines which are remarkably conserved in the type 1 and type 11 PLA_2 enzymes.

The imidazole group.

These enzymes have very few histidine groups. Stable acylation of the active histidine can be ruled out because p-bromophenacyl bromide is an irreversible inhibitor. Otherwise the enzymes tend to have two H residues near the N-terminus and in general these are either both present or both absent. Chettibi *et al.*, (1990) has shown evidence in favour of the WWHF sequence as the activator target and indeed it was with this in mind that the fluorescence experiments were undertaken. The fact that activation strongly perturbs the Tryptophan environment supports this model but cannot directly prove that the H residue is the acceptor. The non activating enzyme from *Naja naja atra* has the sequence WWDF and lacks H at position 20 and does not give similar fluorescence changes.

Additional evidence favouring H acylation is the fact that the *Naja mossambica mossambica* enzyme shows a pH optimum for activation. This suggests that two groups are involved either opposite ionisation states.

One, the presumed acceptor reacts in is basic unionised state, whilst the other, the facilitator acts in its ionised acidic state. Clearly a lysine amino group would be the preferred candidate for the facilitator residue and it should be possible to identify this by progressive blocking. However all acceptor groups should react increasingly rapidly with increased pH. In the bee venom enzyme, where no optimum is observed, the pKa controlling activation was ca. 6.5 and thus clearly indicated an H residue.

Chapter Five

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