REGULATION MECHANISMS FOR PHOSPHOLIPASE ENZYMES.

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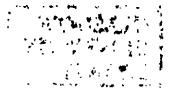

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Dedication

I dedicate this work to my mother and to those who have gone from my touch but never my heart, gone from my sight but never my memories. my late father and brother.

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Chapter four. DISCUSSION.

List of abbreviations.

APS:	Ammonium persulphate.
Bicine:	N,N-bis(2-hydroxyethyl)glycine.
ρ-BPB:	para-Bromophenacyl bromide.
BSA:	Bovine serum Albumin.
CMC:	Critical micelle concentration.
Csp:	Curve shape parameter.
DiC_6PC:	Dihexanoylphosphatidylcholine
DiC_8PC:	Dioctanoylphosphatidylcholine.
DiC_9PC:	Dinonanoylphosphatidylcholine.
$DiC_{10}PC$:	Didecanoylphosphatidylcholine.
$DiC_{11}PC$:	Diundecneoylphosphatidylcholine.
$DiC_{12}PC$:	Dilaurylphosphatidylcholine.
$1\text{-}C_{16},2\text{-}C_7PC$:	1-Palmatoyl,2-Heptanoylphosphatidylcholine.
$1\text{-}C_{16},2\text{-}C_8PC$;	1-Palmatoyl,2 Octanoylphosphatidylcholine.
$1\text{-}C_{16},2\text{-}C_9PC$:	1-Palmatoyl,2Nonanoylphosphatidylcholine.
DCC:	Dicyclohexylcarbodiimide.
DMF:	Dimethylformamide.
EDTA:	Ethylenediaminetetraaceticacid.
EGTA:	Ethylenebis(oxyethylenenitrilo)tetraaceticacid.
FA:	Fatty acid.
GPC:	Glycerophosphorylcholine.
IRS;	Interface recognition site.
LPC:	Lysophosphatidylcholine.
MOPS:	Morpholinopropane sulphonic acid.
Nmm:	*Naja mossambica mossambica.*
NTA:	Nitrilotriaceticacid.
PA:	Phosphatidic acid.
PAGE:	Polyacrylamide gel electrophoresis.
PC:	Phosphatidylcholine.
PE:	Phosphatidylethanolamine.
PEtOH:	Phosphatidylethanol.
PMeOH:	Phosphatidylmethanol.
PLA_2:	Phospholipase A_2.

PLB:	Phospholipase B.
PLC:	Phospholipase C.
PLD:	Phospholipase D.
PS:	Phosphatidylserine.
SDS:	Sodium dodecyl sulphate.
TEMED:	N,N,N,N,-Tetramethylethylenediamine.
TLC:	Thin layer chromatography.

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Aims of the project.

Many proteins are subject to modification by long-chain fatty acylation, but there is no known case in which this acylation affects the catalytic activity of the enzyme *in vivo*. *In vitro* studies showed that only in the case of venom phospholipases A_2 is there any evidence that the acylation can change the catalytic activity of these enzymes. Two models for activation have been suggested:-

1) That the acyl-chain acts as a hydrophobic anchor to the lipid surface and

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 aim of the project was to find out whether or not these enzymes were acylated by a mechanism that was capable of acting *in vivo*. This work continued studies of the bee venom phospholipase A_2 enzyme (Drainas 1978) and also of various snake venom enzymes (Chettibi 1990). The two major aspects of the investigation were : -

1) Studies of the effect of acylation on the reaction kinetics and in particularly on the relationship of acyl-group activation to metal ion activation.

2) Studies of the dependence of activation on the chemical and physical nature of the substrate; primarily investigating substrates capable of undergoing changes from micellar to monomeric and of bilamellar to micellar morphologies.

Chettibi, (1990) investigated the activation of PLA_2 enzymes from different sources and showed that sensitivity to the acylating agents varied widely. He also obtained results that strongly supported the suggestion of Drainas (1978) that more than one Ca^{2+} ion binding site might exist, one of which might have a role in acyl group activation.

Two phospholipase A_2 enzymes; those from bee venom and the basic non-toxic isoform from *Naja mossambica mossambica* which are evolutionary very distinct from each other, were selected for the present work in order to study the possible effects of fatty acyl residues and to elucidate other features of the action of these enzymes that had remained unexplained, particularly certain unusual effects of metal ion activation/inhibition.

Studies of the substrate structure were also extended by investigating the effect of head group substitution using the transferase characteristics of certain phospholipase D enzymes.

Summary.

The effect of hydrophobicity on susceptibility of phosphatidylcholine derivatives to PLA_2 attack was studied by synthesising two series of compounds, the saturated symmetrically distributed derivatives and compounds with one long acyl-chain in the first position and a selected short-chain in the second position. The first series were used to investigate the effect of substrate morphology over the range from free monomer substrates through micelle to bilayer forms, the second series were used to examine the region of the micelle/bilayer transition at higher resolution.

The symmetric substrates showed the expected properties with susceptibility increasing with hydrophobicity in the free monomer series and a sharp transition seen at the CMC for the shorter chain substrates. No such change was seen for longer chain substrates with any of these enzymes and the concept of a hydrophobic anchor increasing the rate of attack on condensed rather than monomeric substrates was questioned. These compounds were used to study the action of enzyme activated by treatment with oleoyl-imidazolide. The biggest activation factors were seen for asymmetric bilayer-forming substrates in propanolic solution, but the physico-chemical form of the substrates under these conditions was not established. Fatty acyl activation was found to alter the response to calcium activation and suggested a two-calcium site model.

Studies of calcium activation showed very strong evidence for two kinetically important sites. Metal ion inhibition showed that barium and large cations were competitive inhibitors for calcium, but zinc and cadmium were not and appeared to inhibit a component of activation only found at high calcium activation. This lead to the proposal that zinc and barium bind to the enzyme at different calcium binding sites.

Hydrolysis curves were shown to vary in shape depending on calcium concentration and the anomalous shape was associated with the presence of calcium at a single binding site. Addition of zinc removed the anomalous shape and without giving further enzyme activation whilst

addition of calcium changes the curve shape and was activating. Some evidence was presented to suggest that the presence of a metal ion in the second (zinc) site was important for modulating the activation of the enzyme with surfaces.

The use of the conductimetric assay was extended to the purification and characterisation of PLD enzymes acting as both transferases and hydrolases The transferase activity was used to generate phosphatidyl alcohols from DiC_8PC and the methanol derivative was shown to be better substrate than the PC equivalent but to posess chartacteristics of the monomeric form. Polar phosphatidic acid compounds which should have high CMC values sould enable the relationship between structure, morphology and susceptibility to PLA_2 to be examined in detail.

Chapter One

Introduction

General introduction:

1-1) Phospholipase A_2 enzymes.

Phospholipase A_2 (PLA_2) (EC 3.1.1.4) enzymes are estereolytic hydrolases catalysing the specific hydrolysis of 3-*sn* phosphoglycerides at the second position to form lysophospholipid and fatty acid (de Haas *et al.*, 1968., Kini and Evans, 1989). They are a ubiquitous class of enzymes and both secretory and intracellular forms have been identified (Shen and Law, 1979., van den Bosch, 1980).

The extra-cellular phospholipases are abundant in pancreatic tissues as well as 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 extensively studied. These enzymes are a closely related family of small proteins with a molecular weight of about 15-18 kDa and serve a variety of functions. Mammalian pancreatic phospholipase A_2 enzymes clearly have a digestive function, whereas the venom phospholipases which share 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, hemolytic, anti-coagulant, convulsant and hypotensive effects in the experimental animals (Karlsson, 1979., Howard and Gundersen, 1980).

Much less is known about the intra-cellular phospholipases which are found in all the tissues, but at a very low concentrations (van den Bosch, 1980). Several recent studies reported that a number of different iso-enzymes exist and some of these enzymes are immunologically distinct from secretory forms (De Jong *et al.*, 1987). These enzymes are reported to be involved in the regulation of many processes and are especially associated with the arachidonic acid cascade which is the rate-limiting step in the formation of a variety of bioactive inflammatory mediators such as;

prostaglandins, thromboxanes and leucotrienes (Flower and Blackwell, 1976 ,1979., Blackwell *et al.*, 1980., Lewis and Austen, 1981., Gupta *et al.*, 1984). Other types of these enzymes are involved in the protection of the cell membrane from lipid peroxidation damage (van Kuijk *et al.*, 1987) and also in the regulation of membrane structure by the de-acylation/re-acylation and lipid turnover cycles, as well as in the modulation of cell adhesion (Curtis *et al.*, 1975) and play a considerable role in the protection of the host cell against bacterial infections by degrading bacterial membranes (Elsbach and Weiss, 1983., Forst 1986 a, b).

1-2) Regulation of Phospholipase A_2 activities.

The activities of all PLA_2 enzymes under physiological conditions are 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.*, 1972., Drainas *et al.*, 1978), but the effect of each of these factors on the other and the modulation of the enzymic activity is not well understood. The present work was carried out to answer some of the many questions about the effects of these factors on the enzymic activity.

1-2-1) The activation of PLA_2 enzymes by long-chain fatty acylation.

Very many proteins are subject to modification by acylation *in vivo*, but there is no other known example of an enzymic activity being affected by this modification. Two studies have been carried out in order to understand the biochemical side of this modification. Lawrence and co-workers have examined the selective modification of some of these proteins by weak acylating agents, and de Haas and co-workers have used specific blocking methods to achieve selective acylation. The results from these two approaches have been interpreted in terms either of activation

by changing the protein conformation (Drainas, 1978), or by changing the interfacial interaction due to increased hydrophobicity (de Haas *et al.*, 1971).

Some of the earlier results indicated that activation by acylation might change the metal ion dependence of the enzyme and also that it might change the response of the enzyme to the different physical states of the substrate (Drainas, 1978). Sensitivity to long chain fatty acylating agents such as oleoyl-imidazolide, was found to be widespread among these enzymes (Chettibi *et al.*, 1990).

The present work involved a study of the properties of two of these enzymes, that from honey bee (*Apis mellifera*) venom and one isoform from the spitting cobra *Naja mossambica mossambica* to see to what extent the three types of regulation mechanisms were interdependent.

Drainas (1978), and Lyall (1984), have studied the activation of bee venom phospholipase A_2 by long-chain fatty acylation and showed that the acylating agent binds to the protein in an equimolar ratio and increases its activity by about 65-fold. Interestingly this increase in activity was observed only with long-chain phosphatidylcholine derivatives as substrates, whereas the activation factor did not exceed 20 % if the substrate was a simple micellar form and hardly any activation was observed on monomeric substrates. No simple explanation was provided for this phenomena, however, it was concluded that the activator causes a conformatinal change in the protein which is quite specific for the interaction with long-chain aggregated substrates.

Later Chettibi (1990), studying the activation of different forms of phospholipase A_2 enzymes from snake venoms and the bee venom enzyme, used different chain length acylating agents and showed that the highest degree of activation was obtained with the myristic acid derivative. In addition, the sensitivity to the activator differed from one enzyme to another. These enzymes must be considered as widely separated on the evolutionary scale and have very different regulation properties. It was therefore of interest to determine whether or not they shared common

structural and functional features that might explain the sensitivity to long-chain acylating agents.

Comparative analysis of the primary structure of the enzymes susceptible to activation suggested that a six amino acid sequence at positions 20, 21, 22, 23, 24, and 25 with a histidine residue in position 22 might be the activator binding region. In particular, enzymes which lacked the histidine residue did not show any activation. Unfortunately the bee venom phospholipase A_2 does not have the sequence of the suggested binding region, but showed a high activation factor.

1-2-2) 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 the 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. Nevertheless, these distinctions are not always well defined, especially for the monomer/micelle transition. This is summarised in **Figure 4-1** (discussion).

1-2-2-1) 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 that 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 b). Wells (1972) working on the action of the dimeric phospholipase A_2 from *Crotalus adamanteus* on the monomeric substrate dibutyrylphosphatidylcholine gave the first detailed kinetic analysis and showed that the enzyme acts at an optimum pH of 8 - 8.5 with Ca^{2+} as the only cation able to support activity. He introduced a model for snake venom phospholipases in which the addition of Ca^{2+} to the enzyme

preceeds substrate binding. 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.

Very recently Fujii *et al.*, (1991) studying the role of Ca^{2+} binding on the ionization of amino acid residues in the active site and on the kinetics of the hydrolysis of the monodispersed dihexanoylphosphatidylcholine by bovine pancreatic phospholipase A_2 reported that substrate binding is Ca^{2+} and pH independent. This result was in good agreement with the previous results obtained by other workers in which it was found that the binding of substrates to group I PLA_2 is independent of the Ca^{2+} binding whereas the binding to group II PLA_2 is facilitated more than 10 times by the Ca^{2+} binding (Teshima *et al.*, 1989).

1-2-2-2) 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 (Pieterson *et al.*, 1974). These results suggested that the active form of the pancreatic PLA_2 in contrast to its zymogen, contain a hydrophobic region which was 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 due to enforced hydrophobic interaction 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 the non-hydrolisable substrate analogues, 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 the monomeric dibutyryl-phosphatidylcholine (DiC_4PC) was about 3000 times lower than that observed on the micellar form of dioctanoylphosphatidylcholine (DiC_8PC). Although these observations showed a clear specificity for the substrate form, they cannot be generalised for all phospholipase A_2 enzymes. In particular the bee venom enzyme does not show similar properties (Shipolini *et al* , 1974).

1-2-2-3) Micellar substrates.

The micelle is now acknowleged to be a complex state with many sub-forms. It is clearly the form 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 show 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 the enzyme hydrolysed dioctanoyl phosphatidylcholine with a specific activity of 6 mmoles/min/mg, whereas didecanoyllecithin was not hydrolysed at all (Verger *et al* 1972). In contrast, by using a monolayer technique it was found that the rates of hydrolysis of all lecithins with acyl chain varying C_8 to C_{12} were quite similar (Zografi *et al.*, 1971).

The term "substrate quality " was then used to explain the difference in the enzymic activity on these substrates. Other factors were also found to be important in the regulation of PLA_2 on this type of substrates such as the difference in the head group and the electrical charge of phospholipid molecules . Hill *et al.* (1983 a, b) examined the effect of charge on the binding of pancreatic PLA_2 to its substrate. They used different negatively charged detergents and showed that increasing the detergent concentration increased the enzymic activity, but gave no change in the activity of the pro-enzyme.

1-2-2-4) Bilayer structures.

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 (van Deenen *et al.*, 1963., de Haas *et al.*, 1968).

The action of porcine pancreatic PLA_2 on fully saturated long-chain phosphatidylcholine derivatives was studied by Op den Kamp and co-workers (1974 ,1975). At the thermotropic phase transition where these compounds 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 uni-lamellar or multi-lamellar vesicles was also found to increase their susceptibility to PLA_2 hydrolysis (Wilschut *et al.*, 1976, 1978). Because of the difficulties in solubilising long-chain phospholipids it was extremely hard to obtain accurate initial rates or to give any kinetic analysis.

To overcome the problem of 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 (1978), and Drainas *et al.*, (1978) to study the activation of bee venom phospholipase A_2 by long-chain fatty acylation. They included 20% n-propanol in the assay buffer and used dioleoyl phosphatidylcholine as a 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.

The dramatic increase in the activity of PLA_2 enzymes upon the aggregation of substrates in the micelle form was summarised in three different theories:-

1- The enzyme theory: in which it is believed that a conformational change in the enzyme controlled by the lipid-water interface could result in optimising the active site.

2- Substrate theory: in which the assumption was based on the idea that the substrate at the lipid-water interface is in a better configuration for attack.

3- Product theory: this assumption was based on that the release of the product is slow in water and faster in a hydrophobic environment at lipid-water interface.

I-2-2-5) Erythrocyte membranes as substrates for phospholipase A_2.

Many workers have reported the use of erythrocytes as substrates for PLA_2 enzymes in order to study the membrane phospholipid composition and other characteristics such as the fragility, permeability and also the degree of sensitivity in different types of erythrocytes.

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 lysophospholipid resulting from the hydrolysis by *Naja naja* PLA_2 could be extracted by albumin without causing haemolysis. These results supported those obtained by Vaysse *et al.* (1986, 1987) using bee venom phospholipase A_2 on rabbit and human red blood cells. The authors showed that the addition of the enzyme to intact rabbit erythrocytes caused about 65% cleavage of PC with no haemolysis, whilst PE, PS and SM were hardly attacked at all.

Lawrence, studying the synergism of PLA_2 by oleic acid showed that lysophospholipid 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. He invoked the concept that lysis was determined at least in part by the rate of phospholipid cleavage, rather than the extent of cleavage.

Drainas and Lawrence, (1978)., Drainas *et al.* (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 FA in the presence of albumin showed different effects on the leakage rates determined partly by addition order of lysophospholipid and fatty acid. The present work examines this phenomenon in more detail.

1-2-3) The regulation of PLA_2 activity by divalent metal ions.

Full kinetic analysis would require a detailed study of all substrate morphologies. In particular the effect of metal ion activation/inhibition should be examined both on the K_m and V_{max} kinetic parameters of the substrate. All phospholipase A_2 enzymes are reported to be regulated by divalent cations, being activated by Ca^{2+} and inhibited by Cd^{2+}, Zn^{2+}, Ba^{2+}, Cu^{2+} and Pb^{2+} (Wells, 1974) but, the mechanism by which these metals activate or inhibit PLA_2 is not well understood and very little relevant information available. The metal ion dependence of two venom phospholipase A_2 enzymes (bee venom and the non-toxic basic isoform from *Naja mossambica mossambica*) will be discussed in detail below.

1-3) Purification of venom enzymes.

Venoms are, in principle, ideal sources for purification of enzymes. The range of contaminating proteins is generally low and the activity of specific components is high. Thus PLA_2 ranges from 0-15% by weight of dry venom. Although the composition is relatively simple, few workers have produced gel data to illustrate the overall venom composition. The components of venoms 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. For this reason, conventional protein gel

electrophoresis has been little used and the normal criteria of purity are those used in HPLC techniques, such as curve shape and elution characteristics.

1-3-1) Bee Venom Phospholipase A_2.

The common European honey bee (*Apis mellifera*) was reported to be the richest source for phospholipase A_2 enzyme (Shipolini *et al.*, 1971; Banks and Shipolini 1986). It has been purified by a five-step procedure described by Shipolini *et al.* (1971) where it was shown to comprise about 12% of the dry weight of the venom (Shipolini *et al.*, 1974 b). Further studies on the chemical properties of this enzyme showed that it shares with many other phospholipases a high pI of about 10.5, but unlike the snake venom phospholipases it was reported to have a 14 carbohydrate moieties in (1:1:8:4) fructose: galactose: mannose: and glucosamine respectively. In addition, this enzyme interacted strongly with mellitin, the major peptide of the venom and has been used as a model for studying the activity of a venom PLA_2 in combination with a synergistic peptide.

1-3-1-1) Structure and function of bee venom phospholipase A_2.

Bee venom phospholipase A_2 was reported to be a single chain glycosylated protein of 128 amino acid with a molecular weight of 14,555 Da based on the amino acid composition. The carbohydrate residues which increase the molecular weight to 15,800 Da are attached to the amino acid number 13 (asparagine). Originally the protein was thought to have 4 SS bridges linking the amino acids in positions 9, 31, 37 and 59 to those at positions 30, 89, 107 and 99 respectively (Shipolini *et al.*, 1974).

Maraganore *et al.* (1986) analysed the primary structure of phospholipase A_2 enzymes in order to characterise a hypothetical ancestor sequence. The alignment of bee venom phospholipase A_2 with the proposed ancestor sequence suggested that the disulphide bridge pattern of Shipolini *et al.* (1974 b) might be incorrect. Thus they proposed that the

half cysteines at positions 9, 31, 37 and 59 should link those at positions 30, 107, 99 and 89 respectively (Maraganore *et al.*, 1986 b).

Recently, the cDNA analysis presented by Kuchler *et al.* (1989) confirmed that the protein consists of 134 amino acid residues, and also confirmed the presence of an extra disulphide bridge. The enzyme has recently been crystallised and comparison of structure analysis at 2.0 Å resolution, showed 5 SS bridges that link the residues at positions 9, 30, 37, 61 and 105 to those in positions 31, 70, 63, 95, and 113 respectively (Scott *et al.*, 1990).

Despite the extensive structural differences between the bee venom phospholipase A_2 and the other phospholipases A_2 species, this enzyme shows very similar response to standard activators and inhibitors such as calcium, EDTA and ρ-bromophenacylbromide (Shipolini *et al.*, 1971., Abe *et al.*, 1977). It has been also reported that the bee venom component mellitin stimulates the enzyme to its optimal activity (Dempsey and Watts, 1987).

1-3-2) *Naja mossambica mossambica* (The spitting cobra) phospholipase A_2

Snake venoms are a rich source of both structural and functional varieties of phospholipase A_2 enzymes (EC 3.1.1.4), for example the sub species of *Naja nigricollis* family, the South African cobra *Naja mossambica mossambica* contains three phospholipase A_2 enzymes which are slightly different in their biochemical structure but very different in their catalytic activity and in their pharmacological effects. The three forms of PLA_2 have been purified by gel filtration on Sephadex followed by ion-exchange chromatography on CM-cellulose (Joubert, 1977), hence they were called CM-I, CM-II and CM-III, they each contain 118 amino acid residues and are cross-linked by seven disulphide intra-chain bridges.

The primary structure of these three phospholipases has been elucidated and they closely resemble those from other snake venoms and also the pancreatic phospholipase A_2. This is especially true for the sequence of the invariant amino acid residues. The amino acid sequence of the 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., Maraganore *et al.*, 1986 ., Djikstra *et al.*, 1989). 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 positions 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 the basic form PLA_2 from *Naja nigricollis* showed a replacement of only one amino acid (Leu) in position 67 for Phe in the CM-III which is also a highly basic protein (Joubert, 1977., Dufton and Hider, 1983., 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' and co workers 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 and Menashe' *et al.*, 1980) and CM-III (Joubert, 1977). This isoform of the enzyme was purified and shown to be highly basic (pI 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).

Ortho-phenanthroline was found to activate some of these enzymes by binding endogenous inhibitory metal ions which are thought to be Zn^{2+} and Cu^{2+} (Tu, 1977., Chettibi *et al.*, 1990). Ba^{2+} was also found to competitively inactivate these enzymes (Lawrence *et al.*, unpublished results).

Chettibi (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 (PI 8.8) was highly activated by long-chain fatty acylation, whereas the toxic form bound the activator, as found by gel electrophoresis, but was not activated by it. Activation of CM-II form was ca. 10-fold faster than the bee venom enzyme. This result suggested a very high specificity of interaction of the reagent with the activating site.

1- 4) Crystallographic studies of phospholipase A_2 enzymes.

The crystal structures of some phospholipase A_2 enzymes have been deduced, but the difficulties lie in obtaining crystals of adequate quality with the enzymes from different sources, The problem of including the substrates in the active site has been partially solved by the use of non-hydrolisable substrate analogues.

Dijkstra *et al.* (1978, 1981) gave a detailed crystal structure of bovine pancreatic phospholipase A_2 at 2.4 Å and at 1.7Å resolution in which they showed a clear picture of the position of 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 dam Mieras *et al.*, 1975).

The idea was 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 the seven di-sulphide 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 den Bergh *et al.*, 1989). The occupation of the 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 the enzyme facilitates the substrate binding.

Recently, 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 as 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 the 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 uninhibited form. The second Ca^{2+} ion was suggested to serve a function as a weak additional nucleophile possibly aiding the catalytic activity provided by the occupation of the primary site.

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 a), but the recent 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). The enzyme has been crystallised recently 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.

The Ca^{2+} binding site was found to be hepta-coordinated, but in this case Asp 35 corresponds to the invariant Asp 49 in the other classes of phospholipases. The Asp 99 residue was reported to be replaced by Asp 64 (Phe in Maraganore*et al.*, 1986 a) which interacts with His 34 (48 in the other phospholipases). This interaction was thought to play a role in the neutralisation of the positive charge which was created during catalysis. The interaction of the conserved Tyrosine residues at positions 52 and 73 with Asp 99 in the class I/II enzymes was replaced by the interaction between Tyr 87 (Thr in Maraganore *et al.*, 1986 a) and Asp 64 in the bee venom enzyme.

Kuchler *et al.* (1989), reported that the bee venom PLA_2 is derived from a precursor that is cleaved by a signal peptidase most likely after Ser 11 or Gly 13. Also, Scott *et al.* (1990), reported that enzyme undergoes the same kind of transformation from a pro-enzyme to its activated form as the pancreatic PLA_2 by the creation of a new amino terminus, but this amino

terminus does not form an α helix and is not involved in the interaction with the active site. The Ca^{2+} binding loop was shown to have the same consensus sequence as all catalytically active PLA_2 enzymes vis (X-Cys-Gly-X-Gly)

The effect of the carbohydrate moieties attached to Asp 13 on the catalytic activity and the allergenecity of bee venom enzyme has not yet been clarified.

1-5) Metal ion dependence of phospholipase A_2 enzymes.

Phospholipase A_2 enzymes are calcium dependent being catalytically inactive in its absence (Pietreson *et al.*, 1974). So far, only Gd^{3+} has been shown to substitute Ca^{2+} with retention of some enzymic activity (Hershberg *et al.*, 1976).

Binding of Ca^{2+} to *Notechis scutatus scutatus* II-1 (Halpert and Eaker, 1976), *Taipoxin* (Fohlman *et al.*, 1979) and *Notexin* (Halpert *et al.*, 1976) induced almost identical ultraviolet difference spectra to those observed with pancreatic PLA_2 and its pro-enzyme (i.e. Pancreatic phospholipase A_2 and its zymogen induce ultraviolet difference spectra characterised by a large peak at 242 nm and two small peaks at 282 and 288 nm (Pieterson *et al.*, 1976., Slotboom *et al.*, 1978). These direct chemical studies showed that only one Ca^{2+} ion was bound per one mole of the protein except in the case of *Taipoxin* PLA_2 which was found to bind two Ca^{2+} ions.

Abe *et al* (1977) demonstrated that β *bungarotoxin* binds one Ca^{2+} molecule per one mole protein with a $K_{Ca^{2+}}$ of 150μM. Wells (1973) using equilibrium dialysis showed that the dimeric *Crotalus adamanteus* PLA_2 has two cation binding sites per dimer with a dissociation constant of about 50μM at pH 8.0.

The UV difference spectra revealed that Ca^{2+}, Ba^{2+} and Sr^{2+} bind to this enzyme in an apparently similar manner, But Zn^{2+} caused a different spectral shift with a lower dissociation constant (about 2μM). The Ca^{2+} binding to this enzyme was found to be pH dependent, showing that the proton functions as a non-competetive inhibitor of Ca^{2+}.

Purdon *et al.* (1976) working on a closely related enzyme from *Crotalus atrox* showed that there was hardly any perturbation of the absorption spectra even up to 20 mM Ca^{2+}. On the other hand a change in the circular dichroism (CD) spectrum suggested that 100μM Ca^{2+} induced a structural change, from which it was concluded that the dissociation constant for Ca^{2+} was about 1mM. Phospholipase A_2 from *Laticauda semifasciata* was shown to be activated by Ca^{2+} ions and to a lower degree by Mg^{2+}, but Zn^{2+} and Cd^{2+} were found to be strong inhibitors even at relatively high Ca^{2+} concentrations (Tu, *et al.*, 1977).

Viljoen *et al.* (1975), using the dimeric enzyme from *Bitis gabonica* reported the presence of two Ca^{2+} binding sites per dimer. The authors suggested that Ca^{2+} binding produced a conformational change which enhanced the substrate binding. The Ca^{2+} binding was also found to be pH dependent and regulated by a residue with a pK of 6.4 (Viljoen and Botes, 1979). van Eijk *et al.* (1984 b) using the same PLA_2 showed that Ca^{2+} perturbed the protonation of Asp 49. Fleer *et al* (1981) working on porcine pancreatic phospholipase A_2, showed that the change in the UV spectra caused by Ca^{2+} binding to both the active enzyme and the zymogen were pH dependent and proposed that the histidine residue at position 48 was involved in the catalytic activity together with a carboxylate group with an apparent pK of 5.2 thought to be Asp 49. The dissociation constants were estimated as 100 mM at pH 4.00 while at pH 10, the K_d was ca. 200μM.

The differences between the pancreatic PLA_2 or its precursor and the snake venom phospholipases were suggested to be due to the change of a charge in the region of Tryptophan residue. Andersson *et al.* (1981) using NMR measurements for pancreatic phospholipase A_2 investigated the binding of $^{43}Ca^{2+}$ and found that the association constant (2.5 mM) was close to that found by other methods (gel filtration and spectrophotometry) which was reported as 2-4 mM. Slotboom *et al.* (1978) have raised a possibility of the presence of a second Ca^{2+} binding site located in the N-terminal region with a higher K_d of about 20 mM.

Donne-Op den Kelder *et al.* (1983) also reported that an additional binding site is present at alkaline pH, and the occupation of this site was shown to increase the affinity of the enzyme for lipid-water interface at high pH. The essential residue for the binding of the second Ca^{2+} was determined as Glutamine 71. This result left a possibility that all phospholipase A_2 enzymes which contain Gln 71 might have a second Ca^{2+} binding site.

The binding of Ca^{2+} to bee venom phospholipase A_2 was studied early in 1971 by Shipolini *et al.* who showed that the enzyme requires Ca^{2+} for its activity, and is inhibited by heavy metal ions such as Zn^{2+}, Cu^{2+} and Ba^{2+}. Tsai *et al.* (1985) using ultraviolet difference spectroscopy in the presence and absence of Ca^{2+} found a large peak at 249 nm and two small peaks at 287.6 and 302 nm and reported a Ca^{2+} dissociation constant which was similar to that reported by Shipolini *et al.* (1971) of about 2.5 mM. They also found that Cd^{2+} binds to the same site as Ca^{2+} but gave different spectral shifts indicating that it caused a different conformational change, thereby explaining why one ion could activate whilst the other inhibits.

1-6) Mechanism of catalytic action of phospholipase A_2 enzymes.

The action of phospholipase A_2 on different forms of the substrate and its preference for aggregates have been previously reviewed, but the chemistry of binding and catalysis are still under extensive study.

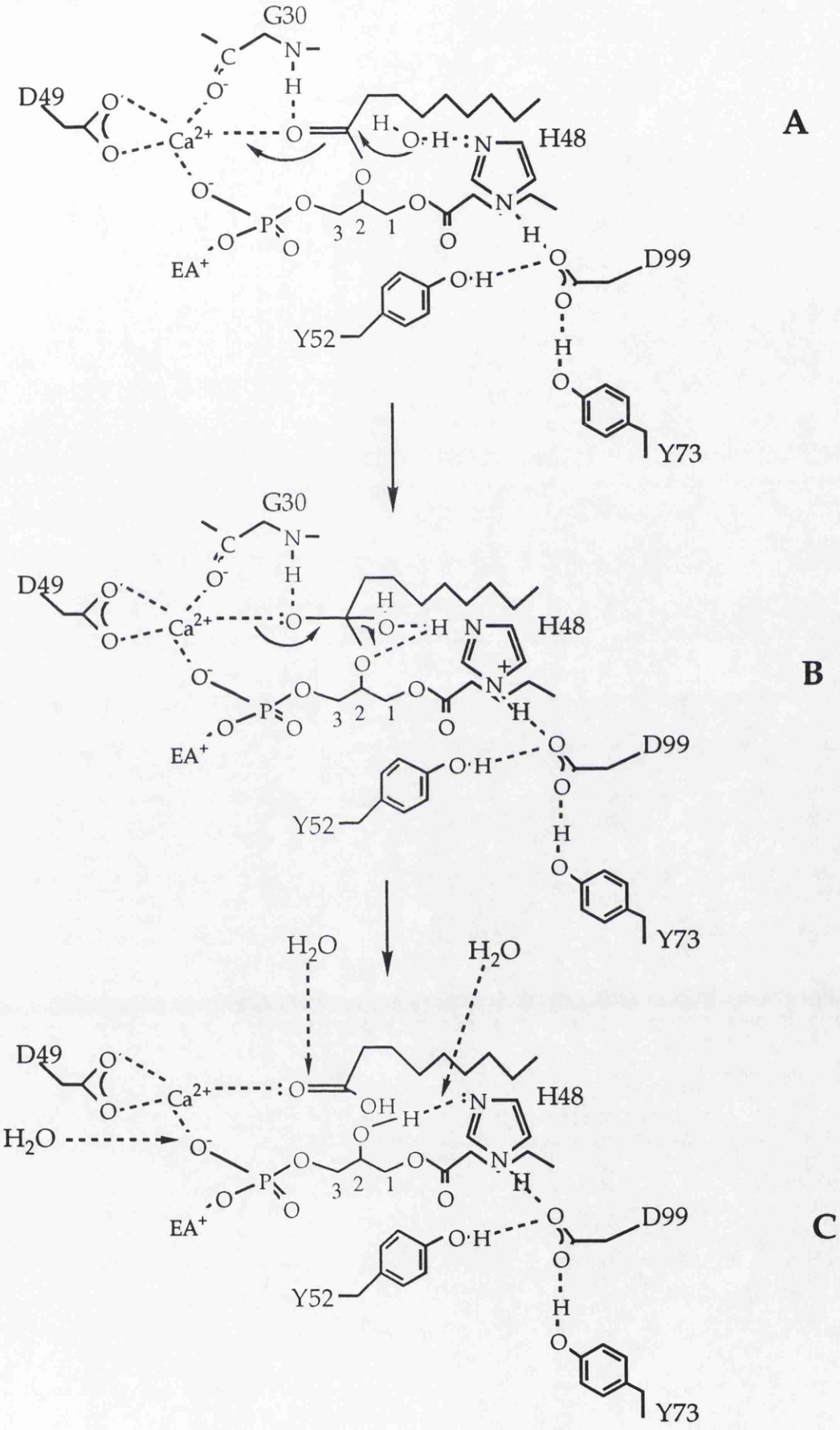
Scott *et al.* (1990) have introduced a model for the mechanism of action of PLA_2 based on the reported crystal structures of two phospholipases (Bee venom PLA_2 and *Naja naja atra* PLA_2) with the phosphonate transition analogue, and the full stereo-chemical picture was completed by the structure of the uninhibited form of the *Naja naja atra* enzyme. The authors outlined some very important concepts concerning the chemical adjustment in both the protein and the ligand caused by the binding and hydrolysis of the substrate and showed that:

1) there was no conformational adjustment for optimal binding of the substrate or catalysis.

2) Some additional positive charges might be required for the stabilisation of the transition state analogue.

3) The stabilisation of the transition state involves the conservation of the geometric co-ordination of the primary Ca^{2+} ion with two water molecules which are displaced by the oxyanion of the substrate tetrahedral intermediate and the non-bridging oxygen of the *sn* -3 phosphate.

The catalytic mechanism was elucidated based on the assumption that the phosphonate analogue emulates the tetrahedral intermediate of esterolysis and the interactions of the phosphonate with the enzyme catalytic site were outlined in which the Nδ1 of the active site His 48 (34 in bee venom PLA_2) is hydrogen-bonded to the non-bridging phosphonate oxygen. This was found to represent the attacking hydroxyl formed between the histidine residue and a water molecule in the uninhibited enzyme from *Naja naja atra* . The water molecule was thought to be the only source for the nucleophile to attack the carbonyl function (Verheij *et al.,* 1980., Dijkstra *et al.,* 1981). The position of the bridging oxygen *sn*-2 of the phosphonate was thought to be ideal for protonation by the histidine abstracted proton. The presence of Ca^{2+} ion is essential for both binding of the substrate and for catalysis because its binding facilitates the nucleophile attack. In this structure Ca^{2+} is bound by 2 oxygens of Asp 49 (35) in addition to the non bridging oxygen of the phosphonate and the oxygen of the C=O bond of Glycine 30 (10). (See the model proposed by Scott *et al.* 1990).



Mechanism of the catalytic action of phospholipase A_2 enzymes
A model proposed by Scott *et al* (1990).

1-7) The relationship between the structure and function of phospholipase A_2 enzymes.

A very large number of phospholipase A_2 enzymes have been sequenced and their structure and pharmacological effects have been characterised. Pancreatic and snake venom phospholipase A_2 enzymes have been reported to share some general aspects such as the similarities in the overall structure and the resistance and stability against denaturing agents.

Heinrikson *et al.* (1977) have classified these enzymes into two groups according to their structure and positions of the disulphide bridges. Group I contains the enzymes of pancreas and of *Elapidae* and *Hydrophidea* while group II contains *Viperidae* and *Crotalidae* phospholipases. This classification has been expanded by Dufton and Hider, (1983) to include the amino acid sequences surrounding the active site.

Snake venom phospholipases induce different pharmacological effects including pre-synaptic neurotoxicity (Fohelman *et al.*, 1976., Habermann *et al.*, 1978), Carditoxicity (Lee *et al.*, 1979), myotoxicity (Habermann *et al.*, 1978), hypotensive (Fletcher *et al* 1980) and blood anti-coagulant effect (Boffa *et al.*, 1976., Verheij *et al.*, 1980). Because of the high degree of homology and the similar catalytic and lytic activities of these enzymes, their classification was found to be very difficult, but several attempts have been made to classify them according to such a characteristic.

The snake venom phospholipase A_2 enzymes have been classified according to their anti-coagulant properties into three classes, strong anti-coagulant, weak and non anti-coagulant enzymes. In this case the amino acid sequence of the region 54-77 was called the anti-coagulant region and it was found to be positively charged in strong anti-coagulant class but, negatively charged in both weak and non anti-coagulant classes (Kini and Evans, 1987).

1-8) Assay of phospholipase A_2 enzymes.

A wide variety of assays for phospholipase A_2 enzymes have been developed, but the earliest fully quantitative and probably the most important assay is the automated titration method (pH stat). de Haas *et al.* (1971) and Wells, (1972) have used this method 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 a colorimetric assay for phospholipase A_2 based on the fact that the proton release due to the ester hydrolysis changed the spectrum of some sensitive dyes. One disadvantage of this method is the inhibition of phospholipase A_2 by some of the dyes used. Radio-labelling and thin layer chromatography were also used to determine the activity of phospholipase A_2 enzymes. van den Bosch and Aarsman (1979), Grossman *et al.* (1974)., Shakir (1981)., Dey (1982) and Katsumata *et al.* (1986) have reported the use of this method for the detection of intra-cellular phospholipase A_2 by using either a labelled fatty acyl chain or labelled lysophospholipid.

Spectrophotometric assays were introduced for the measurement of phospholipase A_2 activity by Aarsman (1976), and was based on the use of thio-ester substrates which can be detected specrtophotometrically 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 ca. 100-fold greater than the titrimetric method.

Moores and Lawrence, (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 PLA_2, PLC and PLD activities in addition to protease activities (Drainas and Drainas, 1985). 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 assay system used multiple open stirred conductivity cells which were temperature controlled to within ± 0.01 °C. The multi-channel feature allowed the use of one or more reaction blanks to be employed.

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 substracted to leave only the conductance change due to the reaction. Applying this method it was possible to detect the activity of 1ng protein with a high degree of accuracy.

1-9) Gel electrophoresis.

Gel electrophoresis is one of the major tools for characterising proteins, but it has not been fully exploited in the case of PLA_2 enzymes. These proteins are small and do not give very sharp bands on SDS-PAGE. More seriously, the different isoforms normally present in any venom tend to have similar MWs and are not resolved by this method. In this respect acid/urea PAGE is more satisfactory because, it relies on charge differences for resolution. In the case of *Naja mossambica mossambica* PLA_2, the different isoforms can be resolved by this method. More significantly, alkaline/urea PAGE leads to even greater resolution of the isoforms (Lawrence *et al.*, unpublished work). These methods allow the PLA_2 composition of complex venoms to be characterised according to:

1- Their molecular weight by electrophoresis on SDS-PAGE in which the SDS binds proteins in a ratio of about 1:2 SDS: amino acid and gives the protein complex a net negative charge. This method was mainly applied for the detection of high molecular weight proteins (i,e MW>10 KDa).

2- In the absence of SDS the separation of proteins depends on the ratio of charge to molecular weight of the protein species, the quality of separation can be altered by controlling the degree of acidity or basicity .

Acid/urea PAGE has been widely used for the study and analysis of the charge modification of basic poly-peptides such as histones (Panyim and Chalkly, 1969., Riggs *et al.*, 1977., Mardian and Isenberg, 1978). SDS-PAGE and acetic acid/urea PAGE were both applied to study the components of the bee venom (King *et al.*, 1976). Acid/urea PAGE gave better resolution and revealed the presence of high mobility components that were not visible in the SDS-PAGE.

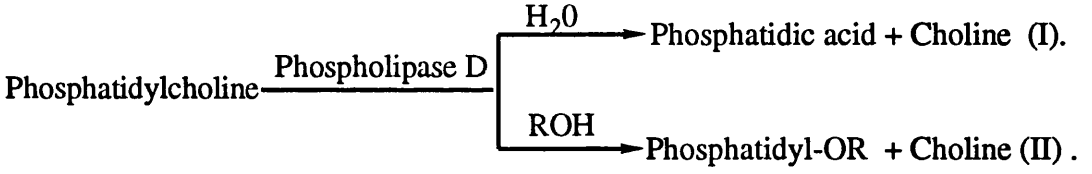
The use of the acetic acid/urea gels for the study of bee venom phospholipase A_2 and other components (Lyall, 1984 and Camero-Diaz *et*

al., 1985) gave irreproducible results because the gels did not set uniformly. Raising the pH of the mixture very slightly gave a considerable improvement in both resolution and reproducibility. However, Chettibi and Lawrence, (1989) investigated the effect of TEMED on the gel setting and reported the best separation of peptides was obtained at low TEMED concentrations (0.08%). An investigation of other aliphatic acids showed that the higher acids (e.g propionic acid) gave considerabely better resolution for small peptides, probably because this give the gels a slightly higher setting pH.

1-10) Phospholipase D.

Whilst phosphatidyl choline derivatives with different side chains can be synthesised relatively easily, changing the molecular properties by changing the head group was more difficult. However one simple approach is to use the enzyme phospholipase D which is reported to catalyse not only the hydrolytic removal of the base , but also to catalyse its replacement with other simple alcohols.

Phospholipase D (EC.3.1.4.4) is a lipolytic enzyme which catalyses the hydrolysis of the phospho-ester bond in phospholipids to yield the free polar head group and phosphatidic acid **(I)**, (Tzur and Shapiro, 1972., Heller 1978., Allgyer and Wells, 1979., Eibl and Covatchev, 1981., Ben-Av and Liscovich, 1981). It was also reported that the same enzyme mediates the transphosphatidylation reaction by which the phosphatidic acid moiety is transferred to an acceptor alcohol **(II)**, (Yang *et al.*, 1967., Dawson, 1967., Heller *et al*, 1975).



The enzyme was first detected in carrot extracts (Hanahan and Chaikoff, 1948) and later was reported to exist in other plants, mainly in the genus *Brassica* (Davidson and Lang, 1958., Quarles and Dawson, 1969), also in micro-organisms and mammals (Dils and Hubscher, 1961., Heller, 1978). Much attention was given to the plant phospholipase D because, its capacity to transphosphatidylate could be used as a biochemical tool in lipid synthesis .

1-10-1) Purification of phospholipase D.

The complete purification of phospholipase D from peanut seeds was reported by Tzur and Shapiro (1970) and Heller *et al.* (1974). They showed an increase of 1200-fold in the enzymic activity in comparison with the starting homogenate. The authors used preparative disk gel electrophoresis and found that the minimal molecular weight of the protein was about 20 kDa, with multiforms of about 50 kDa and 200 kDa. They reported that the purified enzyme was inactivated at low pH (around 4.5). In 1979, Allgyer and Wells reported a 600-fold purification of phospholipase D from Savoy cabbage. They used a combination of ammonium sulphate fractionation, gel filtration on Sephedex G200 and agarose gel-based hydrophobic affinity chromatography. The enzyme stability was increased by including 50% phenylglycol in all buffers. The molecular weight of the protein was determined by (SDS-PAGE) as 112 kDa and by (sedimentation equilibrium centrifugation) as 116 kDa. Both methods also showed the presence of higher multiforms. This enzyme was also found to be pH sensitive, but in a Ca^{2+}-dependent manner, for example at 0.5 mM Ca^{2+}, the pH optimum was 7.25 whereas it was 6.0 at 50 mM Ca^{2+}.

Very recently, Lambrecht and Ulbrich-Hofman (1992), reported a simple procedure for the purification of cabbage phospholipase D using affinity chromatography based on specific calcium-dependent hydrophobicity (as used for calmodulin). The enzyme was extracted from savoy cabbage leaves according to the method of Dawson and Lang, (1967) and then applied to an octyl-sepharose column in the presence of calcium, and eluted by EDTA-containing buffer. The molecular weight and the Iso-electric point were determined electophoretically and gave values of 87 kDa and 4.7 respectively. The authors reported kinetic analyses of the action of the purified enzyme on mixed micelles and on pure short-chain phosphatidylcholine derivatives below and above their critical micelle concentrations. The results showed that the enzymic activity rises

markedly above the CMC. Reaction progress curves were sigmoidal below the CMC, and hyperbolic above the CMC.

The term "substrate quality " was also used here to indicate that the behaviour of the enzyme depended on the physico-chemical form of the substrate. The results also confirmed the assumption that PLD like other lipolytic enzymes contains amino-acid residues which are involved in the recognition of the substrate .

1-10-2) Transphosphatidylase catalysed reactions.

Transphosphatidylation which is also termed base exchange reaction is a common characteristic for plant phospholipase D for which a variety of alcohols were found to act as phosphatidate acceptors. It was also reported that the free hydroxyl group of lysophospholipids serve as acceptors for the phosphatidate leading to the formation of the cyclic compound "1-Acyl-*sn*-2,3-phosphoglycerol" (Kates 1956., Lang *et al.*, 1967). van Deenen, (1966) reported that phosphatidylmethanol was formed after methanol extraction of spinach leaves and concluded that this was due to phospholipase D action.

Yang *et al.* (1967), have shown that the hydrolase and transferase catalysed reactions by PLD were quite similar, differing only because some alcohols serve as better acceptors for the phosphatidate than water. Their results suggested that concentrations of 0.7, 0.3, 1.1 and more than 10%, Ethanol, Ethanolamine, Glycerol and Serine respectively gave equal rates of hydrolysis and transphosphatidylation. Dawson *et al.* (1967) examined the transferase reactions catalysed by cabbage phospholipase D using about 20 acceptors, and showed that all primary alcohols including the poly-functional ones acted as acceptors for the phosphatidate residue, whereas secondary alcohols and acids that have a hydoxyl group (such as citric acid) were very poor substrates for the transphosphatidylation system. Stanacev *et al.* (1973) reported that phospholipids which have a hydroxyl group in the base moiety could be used as acceptors. Hence cardiolipin could be

synthesised by the cabbage enzyme from two phosphatidylglycerol molecules.

It is important to point out that some reactions in which other phospholipids were used have failed because of the lack of the primary hydroxyl group or else, because of the rapid reaction observed in the presence of Ca^{2+} which was found to enhance the hydrolysis reactions to greater extent the transferase activity.

Bacterial phospholipase D enzymes were also shown to catalyse transphosphatidylation reactions and were used to synthesise novel types of phospholipids. Some of these compounds have important physiological and therapeutic activities such as anti-leukemic activities (phosphatidyl nucleosides) (Shuto *et al.*, 1988) and cytostatic activity (O-alkyl glycerophospho-L-serine) (Brachwitz *et al.*, 1990).

Very recently, Nagao *et al.* (1991) investigating cellular defence against oxygen toxicity and membrane lipid peroxidation, used phospholipase D from *Streptomyces* species to synthesise compounds that can serve as anti-oxidants on the surface of the membrane. They found that L-Ascorbic acid, which contains a primary hydroxyl group necessary for the transphosphatidylation, was a very good acceptor for the phosphatidate.

1-10-3) The metal ion requirement and other regulatory factors of Phospholipase D activity.

The regulation of phospholipase D activity has been studied by many investigators who have raised many questions concerning the metal ion dependence and the physico-chemical form of the substrate.
General remarks based on the results obtained by many workers suggested that:
1) The preferred form of the substrate was the micellar or aggregated substrates rather than the monomeric substrates.

2) The enzyme worked optimally at the lipid-water interface in which the aggregated substrates can not be packed too tightly.
3) Negatively charged substrates were preferred .
4) Metal ions, especially Ca^{2+} were essential for the activity.

Metal ion requirement of phospholipase D was studied by Heller *et al.* (1976, 1978). It was suggested that the role of Ca^{2+} is quite complicated. The possibility that more than one calcium ion could be required was based on the high inhibitory concentrations of EDTA (8-10 mM). Einest and Clark, (1958) have examined the effect of different divalent cations on carrot phospholipase D and found that the degree of stimulation was in the order : $Ca^{2+} > Ni^{2+} > Co^{2+} > Mg^{2+} = Mn^{2+} > Zn^{2+}$ and in all these cases it was found that high concentrations of the metal ion is required for full activation, suggesting that these metal ions have more than a catalytic function. Metal ion binding was reported to cause a shift in the pH optimum (Allgyer and Wells, 1979) suggesting that low affinity binding of divalent metal ion caused a conformational change of the protein. In addition, the action of the enzyme against dihexanoylphosphatidylcholine was found to increase rapidly at a concentration of ca. half the CMC (4.3) whilst not at the true CMC. This observation raised the possibility that a lipid or a lipid-enzyme aggregate is formed at that concentration.

Dawson and Hemington, (1967) studying the effect of surface charge on the activity of a number of PLD enzymes reported that all but the cabbage enzyme showed a direct relation between surface charge and hydrolysis rate.

I-10-4) Phospholipase D assays.

Most of the assays for phospholipase D either measure proton release or the release of the free head group, which is usually choline .

Allgyer and Wells, (1979) in a kinetic study of cabbage phospholipase D described three types of assay in which they used direct pH titration, titration by continuous spectrophotometric determination of indicator protonation and a single sample method using an indicator dye. Because

the transferase reaction is not protogenic, assays of the transferase activity require the direct estimation of choline release or of the phosphatidate products. Some workers have used sensitive methods based on the separation and determination of the radio-labelled head groups.

In comparison with the reaction catalysed by PLA_2 enzymes, that catalysed by PLD is even more suitable for the conductimetric approach. The conductance changes in this case would have two components, that due to the buffer protonation, operative above pH 6.5 and that due to separation of the residual positive charge (due to the choline release) and the negative charge of the phosphatidate. These changes should be of similar size, but the second would be independent of pH and of buffer type. The interesting aspect of the conductimetric assay is that, in principle, it can be used as a direct assay for transferase activity.

Chapter Two

Materials and Methods

2-1) The conductimetric apparatus.

The conductimetric apparatus was developed by Lawrence *et al.,* (1971) and based on a published design (Lawrence *at al.,* 1975). The reaction cell was a glass tube of 1 cm diameter with 2 platenum electrodes of ca 2mm diameter fused into the wall of the cell and sealed from the outside by silicone adhesive. The contents were stirred continousely by small magnetic pellet (**Fig 2-1 a, b**).

The cells were mounted in a water bath, temperature controlled by a high precision thermostor-operated relay working as a high-gain on/off switch. Eight independent bridges were sampled by 13 bit analogue-to-digital convertor with microcomputer control.

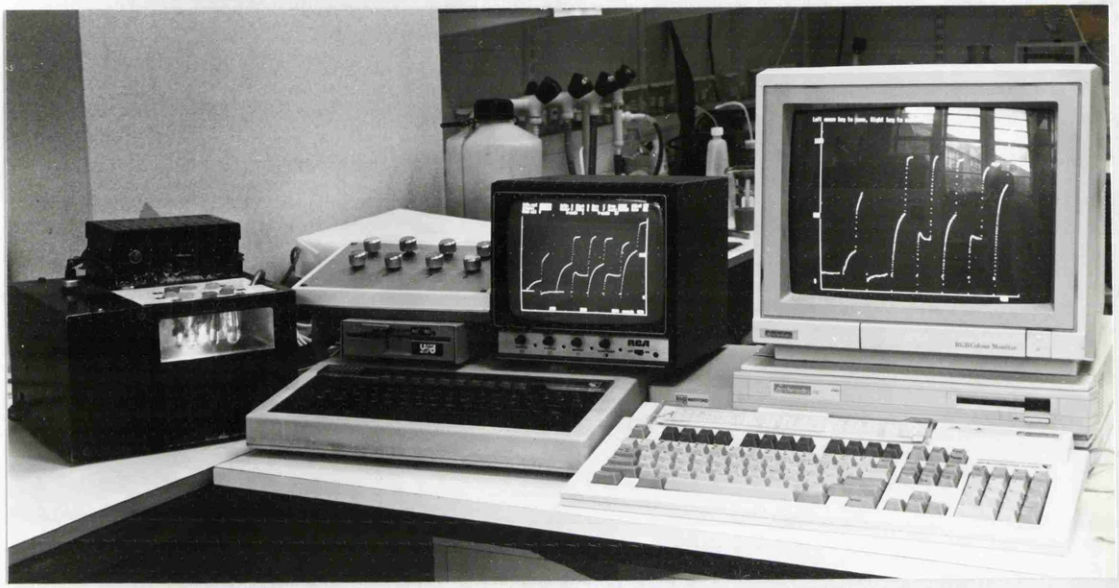
Results were presented in either numerical or graphical form as conductance values or differences between successive readings. Automatic substraction facilities were provided for any cell selected as a blank (control).

2-1-1) The circuit.

The measuring circuit is an AC bridge with reference (balance) 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 themsleves.

Fig 2-1 a, The conductimetric apparatus.



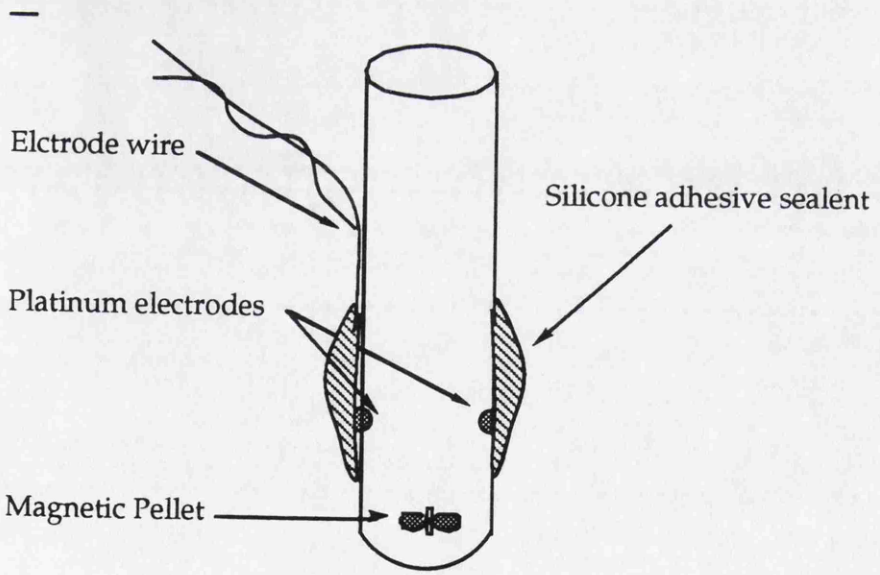


Fig 2-1 b, The conductivity cell.

2-1-2) Calibration.

The distance **L** between the two electrodes and the surface area of the electrode **A** are constants for each cell, and the ratio **L/A** is called the cell constant **K**. The variation of this constant between the cells (which was found to be about ± 10 %), required a correction factor for each cell. This correction was originally applied as 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 (cell constant), the following procedure was adopted. Cells were filled with buffer and the thermostat switched on to allow temperature to rise. Cell conductance readings were plotted continuously to give a set of straight lines, and the cell constants were determined from the slopes of these lines. The values obtained were then normalised by taking the middle value of the set as 100. Typical values of the correction factors were found to lie within a range of 90-110 %. The cell constants were then stored in a computer disk and loaded for use in data processing.

2-2) Preparation of Buffers.

For conductimetric assays, the sensitivity falls as the ion concentration increases. Buffers were therefore chosen to include the lowest possible concentration of ions, consistant with good buffering power. To this end, the compounds chosen were Imidazole, Tris, and Triethanolamine as cationic buffers (i,e. $B + H^+ \longrightarrow BH^+$) and Acetate, Bicine, MOPS, and Borate as anionic buffers (i,e. $B^- + H^+ \longrightarrow BH^0$). All these buffers were used at 10mM concentration based on the ionised forms. Thus cationic buffers were prepared by adding the buffer base to 10m moles of HCl in a total volume of one litre, and anionic buffers by the addition of free acid to 10m moles of NaOH in a total volume of one litre.

By for the greatest part of this work was carried out with triethanolamine buffer, a stock solution of 200mM was prepared by

titrating 200 mmoles of HCl with triethanolamine free base (Sigma Chemical, Co, Ltd) and the final volume was made up to 1000ml at pH 8.0. This was diluted 1/50 for use.

2-2-1) Metal ion chelating Buffers.

These buffers were used in the study of metal ion dependence of PLA_2 enzymes. Amongest the widely used chelators were NTA, EDTA and O-phenanthroline, they were prepared as follows:-

- 1mM solution of NTA buffer was prepared by dissolving 257mg of NTA (Sigma Chemical, Co, Ltd) in one litre of 10mM triethanolamine/HCl buffer at pH 8.0.

- A stock solution of 100mM EDTA was prepared by dissolving 292mg of EDTA (BDH, Co, Ltd) in 10ml of distilled water and diluted for use.

- O-phenanthroline buffer was prepared either as a 100mM solution in acetone by dissolving 192mg in 10ml of acetone and diluted to 50μM in 10mM triethanolamine/HCl buffer at pH 8.0, or else by dissolving 10mg of the reagent in a litre of 10mM triethanolamine/HCl buffer at pH 8.0.

2-2-2) Erythrocyte assay buffer (isotonic succrose buffer).

The erythrocyte leakage assay required a low conductance isotonic buffer which was prepared by dissolving 98 g of sucrose in 800 ml distilled water in the presence of 10 ml of 1 M NaOH. The pH was adjusted to 7.4 with MOPS free acid (Sigma Chemical, Co, Ltd), and the final volume was made up to one litre with distilled water.

2-2-3) Isotonic Saline.

This solution was used in the preparation of mammalian erythrocytes, It was prepared by dissolving 9g of sodium chloride (NaCl) in 900ml

distilled water in the presence of 10ml of 1M NaOH, adjusting the pH to 7.4 with MOPS, and the final volume was made up to one litre with distilled water.

2-3) Erythrocyte preparation.

Approximately 20ml of blood from New Zealand white rabbits (from the animal house, Physiology Dept, University of Glasgow) were collected over heparin, (Sigma, London; 100 units/ml, 250 units for 10ml of blood). The blood was centrifuged at 3000 rpm for 15 minutes, the serum and white blood cells were removed by aspiration with a vacuum pump and the red cells were resuspended in isotonic saline at 3000 rpm for 10 min. The procedure was repeated three times and finally the erythrocytes were made up to 33% v:v with saline, stored at 4 ^{o}C and used within one week.

2-4) Preparation of phospholipid substrates.

2-4-1) Analysis and detection of phospholipids.

Phospholipids were detected and analysed on silica gel coated plastic TLC sheets (Mark AG Darmstaf, Germany) and normally, detection was with molybdenum blue reagent.

To detect phospholipids, a drop of the solution was spotted on the TLC sheet, dried and dipped in a beaker containing molybdenum blue and rinsed with tap water. Acyl phospholipids give a strong blue colour, whereas GPC gives a greenish blue colour which fades quite quickly. For analysis, the chromatogram was developed by a solution of chloroform/methanol/acetic acid/water, 25/15/4/2 respectively, dried, dipped in the staining solution and rinsed with tap water. To detect the amino phospholipids PE and PS, 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.

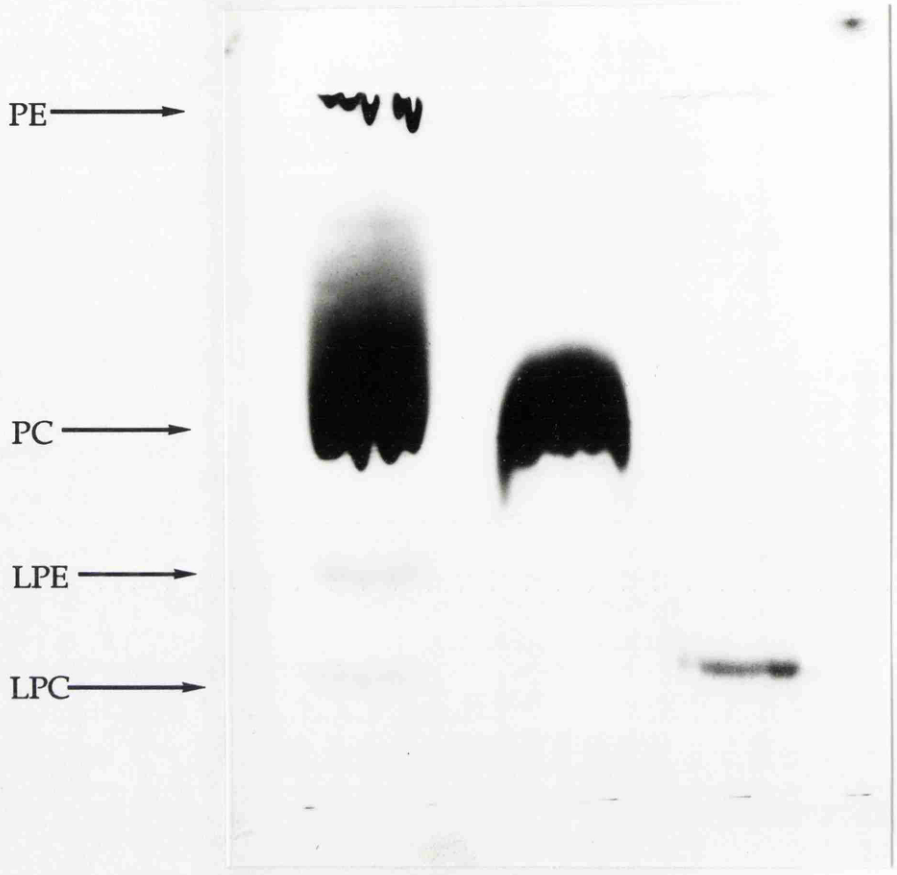


Fig 2-4-3, Thin layer chromatography of purified lecithin derivatives.

The chromatogram was developed with a solution containing 50, 30, 8, 4 v:v of chloroform, methanol, acetic acid, water respectively.

Phospholipids were stained with ninhydrin and molybdenum blue reagents.

PE = Phosphatidylethanolamine, PC = Phosphatidylcholine

LPE = Lysophoshatidylethanolamine, LPC = Lysophosphatidylcholine

2-4-2) Preparation of molybdenum blue.

The molybdenum blue reagent was prepared according to the method of J. C. Dittmer and R. L. Lester (1964). Two solutions were prepared separately in which, the first was prepared by the addition of 40.20g of MoO_3 to one litre of 25M H_2SO_4, stirred and boiled until all the MoO_3 is completely dissolved. The second solution was prepared by the addition of 4.0g of molybdenum powder to the first solution. The mixture was then stirred and boiled for about 25 min, it was then cooled down and filtred to get rid of any undissolved residues.

The reagent was finally prepared by the addition of 2 litres of distilled water to the mixture which became greenish yellow coloured

2-4-3) 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, homogenized and extracted three times in acetone in order to remove the yellow pigment and oils. After the third stage of extraction, the white residue was dissolved in ethanol, stirred for half an hour and filtered. The precipitate was discarded and the filtrate was evaporated to dryness. The product (about 60g) was checked by thin layer chromatography (TLC). and showed two major bands corresponding to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (**Fig 2-4-3**).

2-4-4) Preparation of GPC (Glycerophosphorylcholine).

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

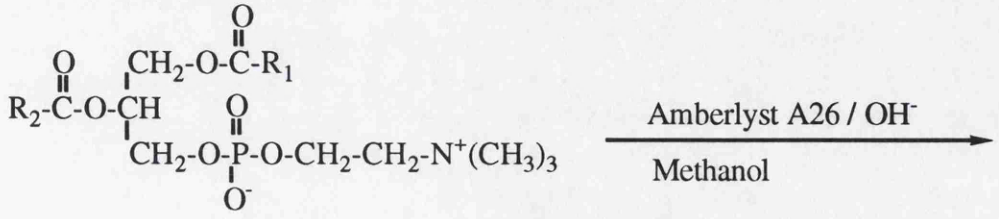
methanol. The mixture of lecithin-resin was stirred at 56 °C.

Estimation of reaction progress was done using the fact that PC and lysoPC give a strong blue colour with molybdenum blue reagent, but GPC give a greenish blue colour that fades quickly. Thus the assessment of the reaction progress was made by spotting 5-10µl of the mixture on silica gel coated sheets and checking for the loss of the blue colour. By this indicator, the reaction was found to reach completion within three hours.

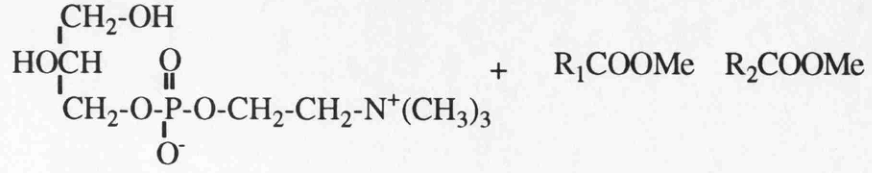
The resin was removed by filtration and the solvent evaporated to dryness leaving an oily residue believed to be GPC contaminated with fatty acid esters and glycerol. The residue was washed with DMF (dimethyl formamide) followed by three washes with ethyl acetate to remove exess DMF. The product was then dissolved in methanol and deionised by mixing with a mixed bed resin Dowex MR-3 (Sigma Chemical, Co, Ltd), stirring continuously and checking the conductance every 2 minutes. When the conductance reading of the mixture was equal to that of methanol, the resin was removed by filtration and the solvent evaporated completely. The GPC was finally extracted by precipitation with chloroform/methanol mixture.

The purification made use of the fact that GPC is insoluble in all organic solvents that are not alcohols or acids. One special feature of the purification was the use of DMF which dissolves glycerol but not GPC. Without this step the removal of free glycerol is extremly difficult.

The chemical reaction proceeds as follows:



Lecithin (PC)

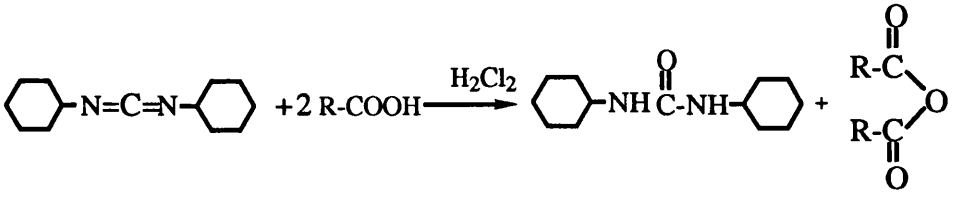


Glycerophosphoryl Choline Methyl Esters

2-4-5) Preparartion of fatty acid anhydrides.

Fatty acid anhydrides were prepared by the method of Selinger and Lapidot (1966), but using Dichloromethane (CH_2Cl_2) instead of petrolium ether.

Two molar equivalents of fatty acid were mixed with one molar equivalent of the cross linking reagent D.C.C (Dicyclohexylcarbodiimide) (Sigma Chemical, Co, Ltd), in the presence of Dichloromethane as outlined below:



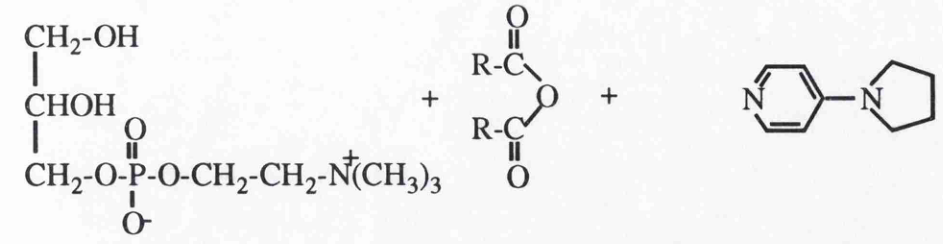
DicyclohexylCarbodiimide Fatty acid Dicyclohexylurea Anhydride

The white precipitate (Dicyclohexylurea) was removed by filtration and the solvent was rotary evaporated to yield the anhydride. Heptanoic anhydride was purchased from sigma.

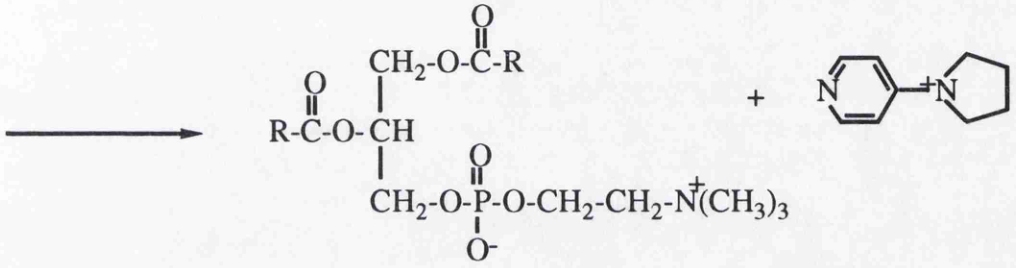
2-4-6) Synthesis of Di-acyl phosphatidylcholine derivatives.

Di-acyl phosphatidylcholine derivatives were prepared by a modification of the method of Patel *et al.*, (1979) using GPC free base instead of the $CdCl_2$ adduct.

The derivatives were then prepared by adding two moles exess of fatty acid anhydride to one mole of GPC in the presence of 1m mole of the catalyst 4-Pyrrolidinopyridine (Sigma Chemical Co, Ltd) as outlined below:



Glycerophosphoryl Choline F.A.Anhydride 4-Pyrrolidinopyridine



Diacylphosphatidyl Choline

The mixture was stirred overnight at 55 °C and the progress of the reaction checked by thin layer chromatography. When the reaction is over (i,e all the GPC was dissolved and a single, molybdate positive band on TLC sheet), the mixture was dissolved in chloroform and applied to an Alumina column (Neutral, Activated, Aldrich chemical Co, Ltd).

The column was washed with chloroform and the derivative eluted with 8:1 (v:v) chloroform /methanol. The elueate which contains most of the diacyl compund was evaporated to dryness and again analysed by thin layer chromatography.

The product was dissolved in methanol and deionized with a mixed bed resin Dowex MR-3 and the conductance checked continuously. After deionisation, the resin was removed by filtration and the solvent was evaporated leaving a clear residue (pure substrate) which was weighed and made up to a desired concentration in methanol and stored at 4 °C.

2-4-7) Preparation of 2-acyl-lecithin derivatives.

2-4-7-1) Preparation of pure egg phosphatidylcholine.

This was prepared by applying a solution of crude lecithin to an alumina column, washing the column with chloroform, and eluting with a mixture of 8:1 chloroform/methanol. The TLC analysis of the eluted compound showed a single ninhydrin negative, molybdate positive band which corresponded to phosphatidylcholine.

2-4-7-2) Purification of egg Lysophosphatidylcholine.

Lysophosphatidylcholine was prepared by dissolving pure phosphatidylcholine in 10mM triethanolamine buffer which contained 20 % n-propanol and 1mM $CaCl_2$ at pH 8.0. De-acylation was carried out enzymatically by the addition of a concentrated bee phospholipase A_2 enzyme, and the reaction pH was maintained at 8.0 by continuous addtion of 1M NaOH.

The conversion of phosphatidylcholine to lysophosphatidylcholine was assesed by TLC. When the reaction is over, the product was separated by phase extraction with butanol, the solvent phase (which contained the lysophosphatidyl choline) was evaporated to dryness leaving the solid which was dissolved in chloroform and applied to an alumina column. The elution of lysophospholipid was carried out by a mixture of 1:2 v:v methanol/chloroform respectively. The eluted samples were mixed, rotary evaporated to dryness, weighed and stored at $4^{o}C$.

2-4-7-3) The synthesis of 2-acyl phosphatidylcholine derivatives.

Lysophosphatidylcholine was re-acylated at the second position chemically by addition of an equivalent amount of fatty acid anhydride in the presence of the catalyst 4-Pyrrolidinopyridine and stirred at 56 ^{o}C.

Reaction progress was assessed by spotting the solution on silica gel coated sheet and assessing the colour yielded with molybdenum blue reagent. This reaction was found to be very quick especially when short chain fatty acid anhydrides were used because, in contrast to GPC, LPC is highly soluble in the mixure. Generally the reaction is completed within one hour. After completion the mixture was dissolved in chloroform, applied to an alumina column and eluted as described above, as diacyl phosphatidylcholine derivatives.

2-5) Phospholipase A_2 assays.

Two different conductimetric assay methods were used in order to study the kinetics of phospholipase A_2 enzymes. The first assay was used to study the catalytic activity of these enzymes directly by following the hydrolysis of various phospholipid substrates, whereas the second was applied on erythrocytes by following the progress of leakage of electrolytes from intact cells, this was mainly used for activation studies of phospholipases. In each case the buffers were degassed at the start of the work period.

2-5-1) Phospholipid hydrolysis.

In this assay 2ml of 10mM triethanolamine/HCl buffer pH 8.0 was added to each cell and 20µl of phospholipid substrate added.

The standard activity assay used a pure aqueous buffer with 20µl of 40mg/ml dioctanoyl phosphatidylcholine in methanol to give a final concentration of approximately 0.35mM. The total hydrolysis of this substrate was found to give ca 2% of the total conductance change, and the rates of hydrolysis were measured using a computer line-drawing program.

Fig 2-5-1, shows the standard hydrolysis curves of 0.4 mM of dioctanoyl phosphatidyl choline (DiC_8PC) by native bee venom (*Apis mellifera*) phospholipase A_2 and the basic non-toxic isoform of phospholipase A_2 from *Naja mossambica mossambica*plotted both from the conductance values in the absence and presence of calcium.

2-5-1-1) Calibration of the enzyme assay.

Calibration of phospholipase A_2 assay was carried out basing on the fact that the release of free fatty acid anion and a proton would cause an increase in the conductance change if cationic buffers such as triethanolamine were used as follows:

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

Therefore, the linearity of the assay as a function of fatty acid release was checked by the addition of 2µl aliquots of 0.5 M of different fatty acids to cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 either in the presence or absence of the substrate, and in both cases the conductance changes were found to give linear plots with a standard variation less than 1% (**Fig 2-5-1-1**).

2-5-2) Erythrocyte leakage assay.

The application of conductimetric assay method on erythrocyte leakage was carried out by incubating 20µl of 33% (v:v in isotonic saline) washed red blood cells in 2ml of isotonic succrose buffer pH 7.4 at 37 °C and added to it 10µM bovine serum albumin (Sigma Chemical, Co, Ltd). After balancing the conductivity cells, 2µl of either native or activated phospholipase A_2 were injected into the mixture and the results were recorded and analysed.

Fig 2-5-1, The hydrolysis of dioctanoyl phosphatidyl choline by bee venom (1) and *Naja mossambica mossambica* (2) phospholipase A_2 enzymes in the absence (○) and presence (▲) of Ca^{2+}.

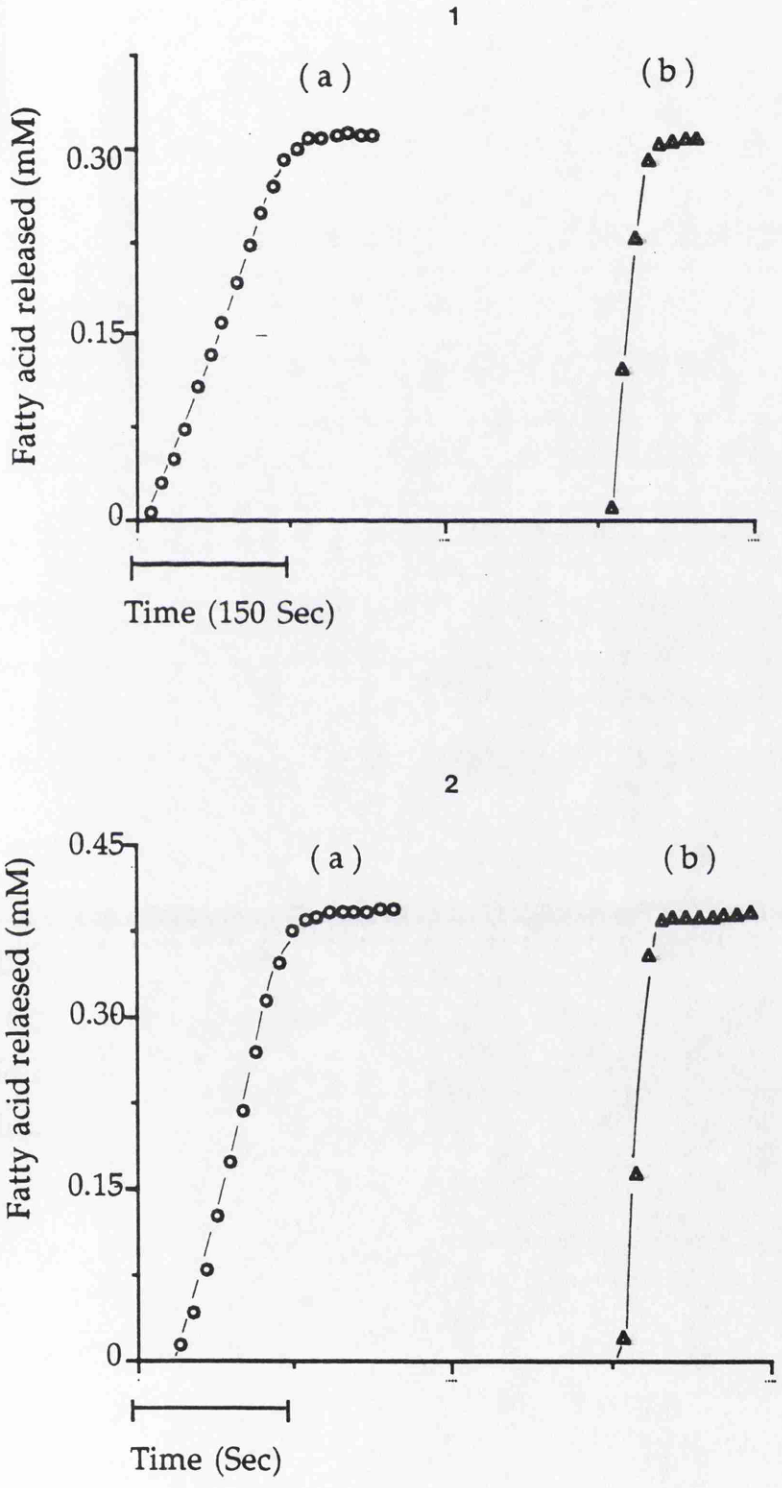
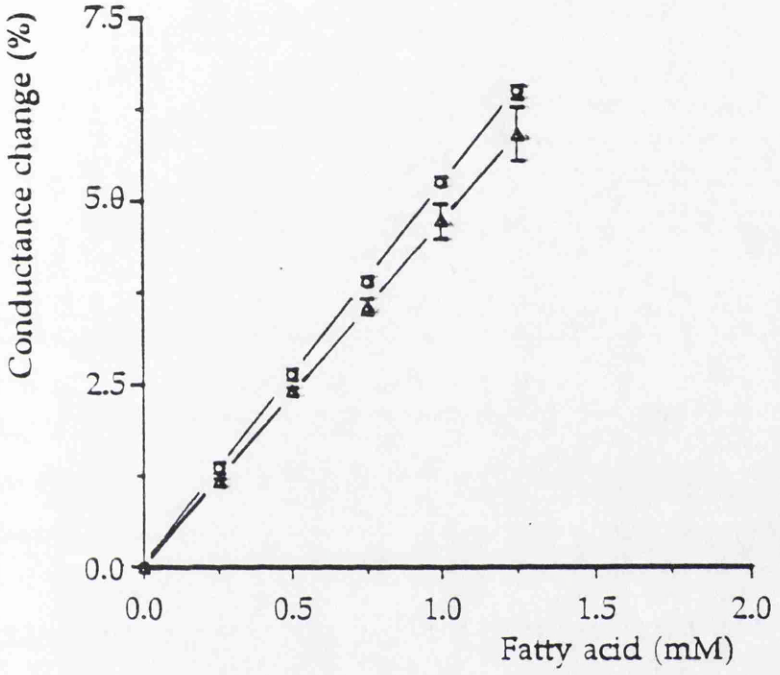
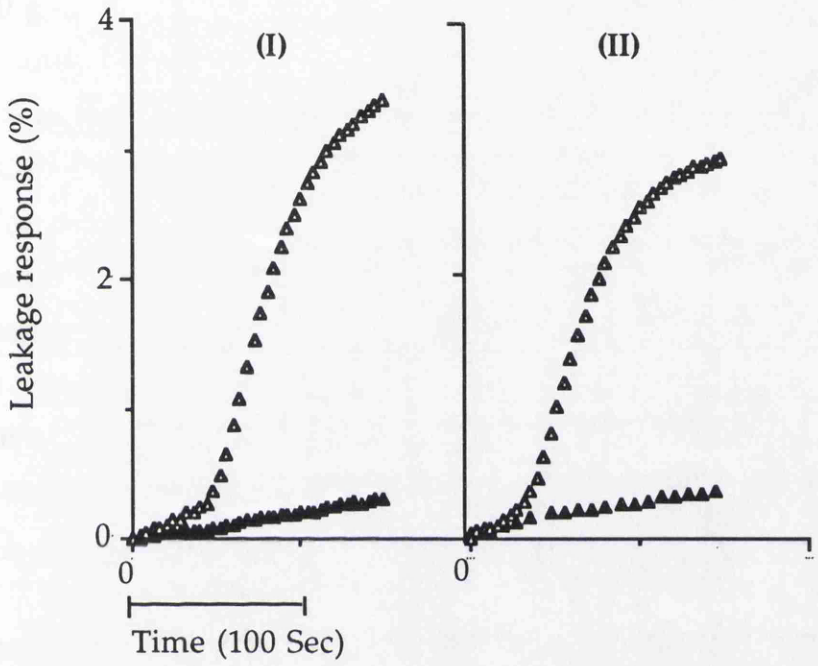


Fig 2-5-1-1, . Calibration of the conductimetric assay with fatty acids.



The calibration was carried out by the addition of 2µl aliquotes of 0.5 M Octanoic (○) or Lauric (△) acids to conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 at 37 °C.
The conductance values were measured as described in the text.

Fig 2-5-2, shows a typical leakage response curves of rabbit erythrocytes induced by native and activated phospholipase A_2 enzymes from bee venom and *Naja mossambica mossambica* venom.



The reactions were carried out by injecting 20µl of rabbit erythrocytes into conductivity cells containing 2ml of isotonic succrose buffer pH 7.4 at 37 °C in the presence of 10µM bovine serum albumin, followed by the addition of:-

(I) 2µl of 1mg/ml native (▲) and activated (△) bee venom PLA_2.

(II) 2µl of 0.1mg/ml native (▲) and activated (△) basic non-toxic PLA_2 from *Naja mossambica mossambica* venom.

2-6) Purification of phospholipase D.

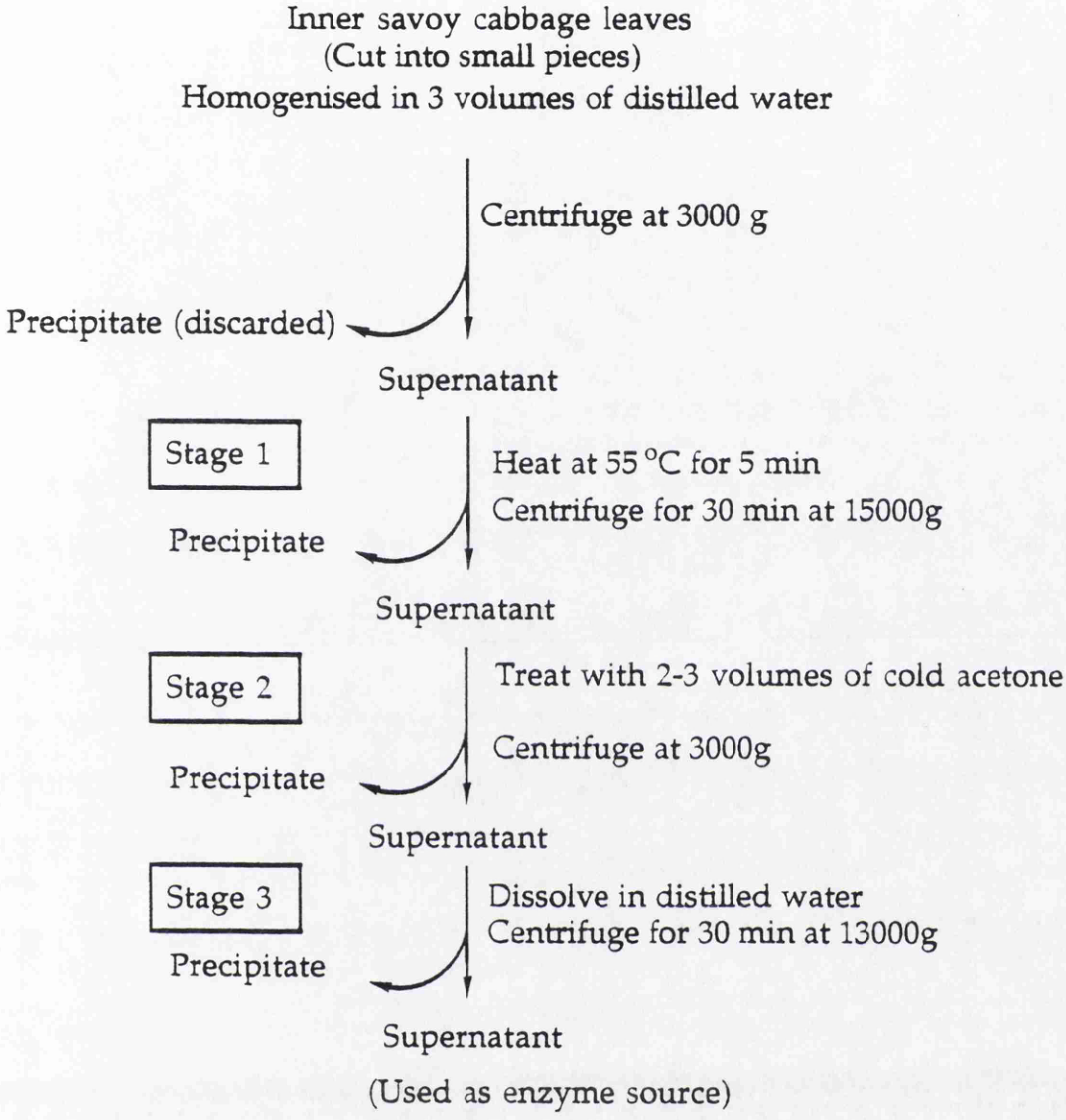
Phospholipase D was purified from the inner leaves of savoy cabbage according to the method of Davidson and Lang (1958), with slight modifications in the procudure.

About 500 g of inner yellowish green cabbage leaves were cut into very small pieces and homogenized in three volumes of distilled water, the homogenate was filtred and spun down for 15 minutes at 3000g to get rid of the debris, then the precipitate was discarded and the supernatant was centrifuged for 30 minutes at 15000g, the supernatant was heated at 55 ^{o}C for 5 minutes followed by centrifugation as above. The supernatant at this stage was treated with 3 volumes of cold acetone and centrifuged for an 15 minutes, the white precipitate obtained from this step was dissolved in small volume of distilled water and centrifuged for 30 min at 15000g. The supernatant was used as the enzyme source with no further purification (**scheme 2-6**). All the centrifugation steps were run at -15

2-6-1) Phospholipase D assay.

The assays for phospholipase D activity were carried out using the conductimetric assay method which is based on 8 cells cleaned with weak NaOH, methanol, and twice with distilled water followed by a wash with the assay buffer then filled with 2 ml of fresh, degassed buffer and left for about 2 minutes to achieve thermal equilibrium,

An appropriate amount of the substrate was put into the cells which were balanced afterwards to ±100 using the balance control, the experiment was then started by activating the computer to run on a preliminary programme first to check the degree of stability of the cells, and then when the readings are stable the computer was run onto the experimental mode in which the enzyme was injected into the cells containing the substrate, the data was fully recorded starting from zero second. When the reaction is over, the data was then transferred to an Archimedes computer for



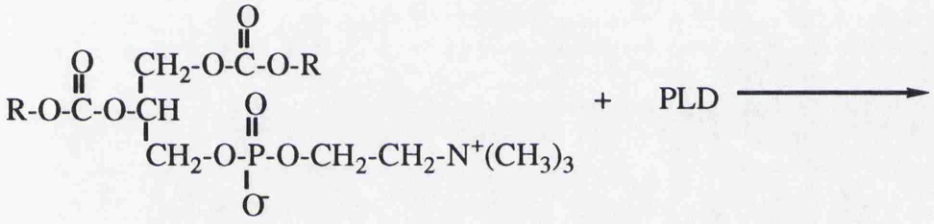
Scheme 2-6, Partial purification of savoy cabbage phospholipase D.

kinetic analysis.

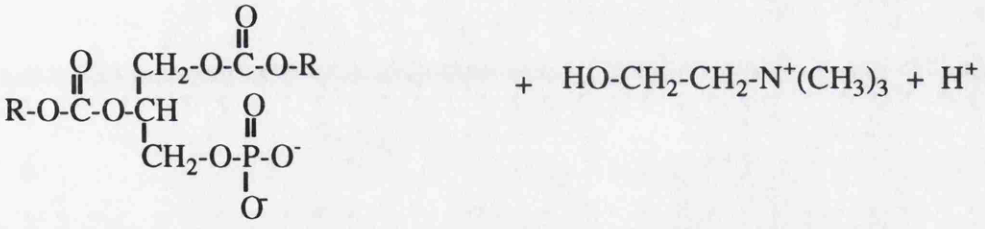
Fig 2-6-1, shows the hydrolysis curves of DiC_8PC by the purified cabbage PLD and a bacterial phospholipase D (purchased from Sigma Chemical, Co, Ltd) plotted from the conductance values. The substrate conversion is expressed in the percentage of the total conductance change.

2-6-2) Calibration of phospholipase D assay.

The calibration of phospholipase D assay was analysed according to the release of the product both in the hydrolysis and transphosphatidylation reactions. The hydrolysis of phosphatidylcholine by phospholipase D yields phosphatidic acid and choline moiety as follows:



Phosphatidyl Choline



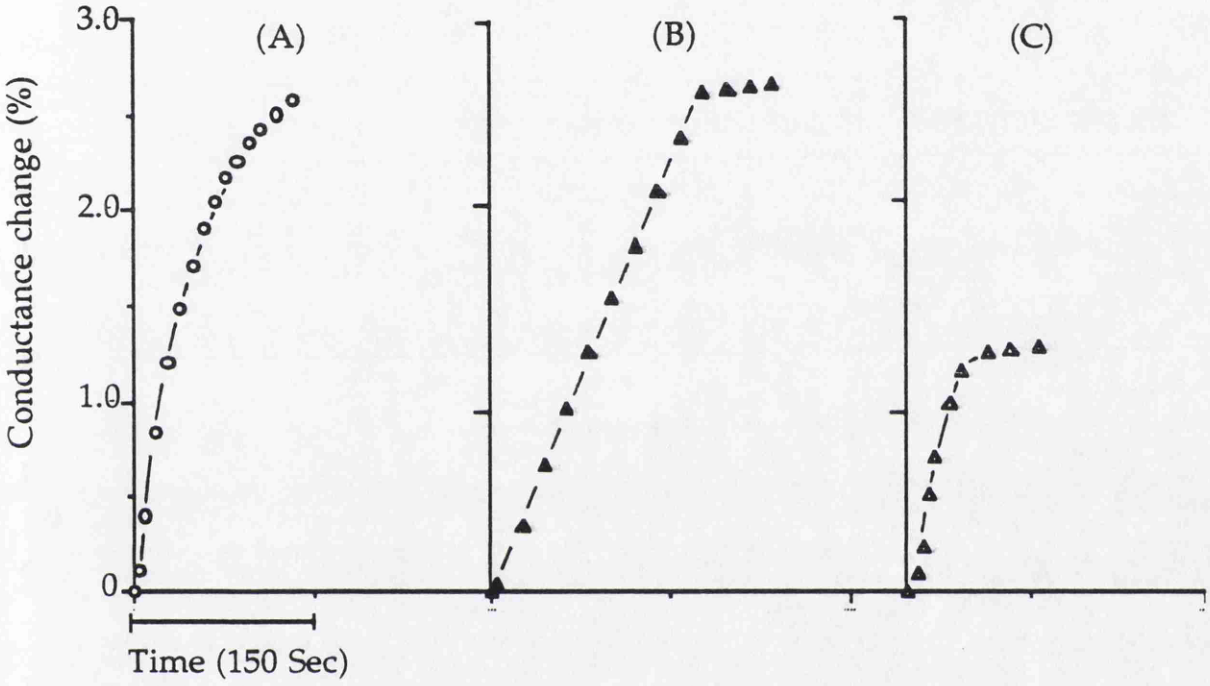
Phosphatidic acid

Choline

Therefore, two positive charges were gained which cause an estimated increase in the conductance change double to that obtained with phospholipase A_2.

The results of phospholipase D catalysed reactions were expressed in the percentage of the conductance change which was found to be in the the range of 1-4%, this was calculated by measuring the voltage between the potentiometers which corresponds to both the conductance change given by the buffer alone and that given by the total hydrolysis of a standard

Fig 2-6-1. The hydrolysis of DiC_8PC by savoy cabbage and bacterial phospholipase D enzymes.



The reactions were carried out by injecting 10µl of the purified cabbage phospholipase D (A), 2µl of 1mg/ml of phospholipase D from Streptomyces chromofuscus (B) and 2µl of 1mg /ml bee venom phospholipase A_2 (C) into cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20µl of 30mg/ml dioctanoyl phosphatidyl choline substrate.

The result was expressed in percentage of conductance change caused by the release of free fatty acid and choline.

amount of the substrate, the values were then substracted from each other to give standard values of the change in conductance.

2-7) Gel electrophoresis.

2-7-1) Propionic acid/urea/polyacrylamide gels (acidic gels).

This type of gel was developed by Chettibi and Lawrence (1989) for the study of bee venom peptides and was based on the method of Panyim and Chalkly (1969). The gel solution was made up by dissolving 22.5 g of acrylamide monomer (CH_2-CH-CO-NH_2) (Koch-Light Ltd; Haverhill Suffolk England), with 1g of N,N'-methylenebis-acrylamide (Koch-Light Ltd; Haverhill Soffolk England), and 36 g urea (BDH Co, Ltd) in 50 ml of distilled water. The mixture was stirred at 45 ^{o}C and when all components had dissolved, the solution was cooled and the final volume made up to 100 ml with distilled water. To 30 ml of this solution was added 0.6ml of propionic acid (Riedel-de Haen Ag Seelze-Hannover; Germany), 200µl of 10 % w/v freshly prepared ammonium persulphate solution (Sigma Chemical, Co, Ltd) and 25µl of TEMED (Sigma Chemical, Co, Ltd).

The solution was mixed thoroughly and poured into the gel plates with the comb positioned. and then incubated at 37 ^{o}C (usually for about 10 min) in order to obtain uniform setting.

2-7-1-1) Gel running.

Protein samples were prepared by mixing at 1:1 v/v with neutral red dye solution (0.1% neutral red and 50% glycerol) and loaded in the gel using a microsyringe. Gels were run with 2 % acetic acid in both anode and cathode compartments at 20 mA (ca 400 V). When the marker dye reached the bottom of the gel (which takes about 2 hours) the electophoresis was stopped and the gel was removed from the plates and put into a dish containing the staining solution (0.1% Coomassie brilliant blue G (Sigma Chemical, Co, Ltd) prepared with methanol/water/acetic acid, 50/50/7 respectively), for about 15 min (or for overnight in a weak stain in case of very low concentrations of protein samples) then finally

destained with a mixture of methanol/acetic acid/water, 50/70/880 respectively.

2-7-2) Ammonia and Ethanolamine/urea/polyacrylamide gels (Basic gels).

These were prepared by the same procedure described above, but substuting ethanolamine or ammonia for propionic acid as gel electrolyte (Lawrence, A. J. unpublished work). the dye used in this case is 0.1% bromophenol blue, and the gels were run with 2% ethanolamine or ammonia solutions respectively in both anode and cathode compartments.

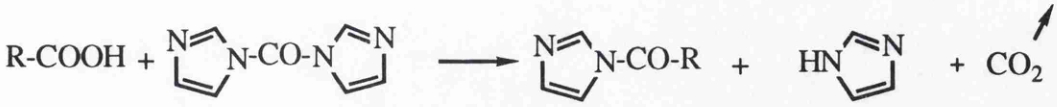
2-8) Preparation of the acivator (oleoyl-imidazolide).

Oleoyl-imidazolide was prepared as a stock solution of 2% w/v, and this was done by mixing 0.1 M free fatty acid (Sigma Chemical, Co, Ltd) with 0.2M of N,N'-Carbonyldiimidazole (Aldrich Chemical, Co) in 1 ml dry acetone and used without further purification.

Lawrence *et al.,* (unpublished work) demonstrated a simple method for the preparation of pure acyl-imidazolides according to the following procedure:

One molar equivalent of free fatty acid in acetone was treated with two molar equivalents of N, N'-carbonyldiimidazole as outlined in the scheme below.

The acetone was evaporated after 10 min and the residue was extracted twice with dry petroleum ether to give Imidazole as a precipitate. This was filtred and the filtrate was evaporated to yield pure fatty acyl-imidazolide which was weighed and stored at 4 ^{o}C.



Fatty acid N,N'-Carbonyldiimidazole Acyl-imidazolide

2-9) Activation of phospholipase A_2 by oleoyl-imidazolide.

The acylation process was carried out by mixing 100µl of 1mg/ml phospholipase A_2 with 2µl of 0.2% Oleoyl-imidazolide at pH 8.0, This was calculated to give equimolar ammounts of both protein and acylating agent (Camero-Diaz *et al.*,1985)

The mixture was then incubated at 37 ^{o}C for two hours and assayed for activation using erythrocyte leakage assay as described above.

Chapter Three

Results

Part One

3-1-1) The action of phospholipase A_2 at interfaces:

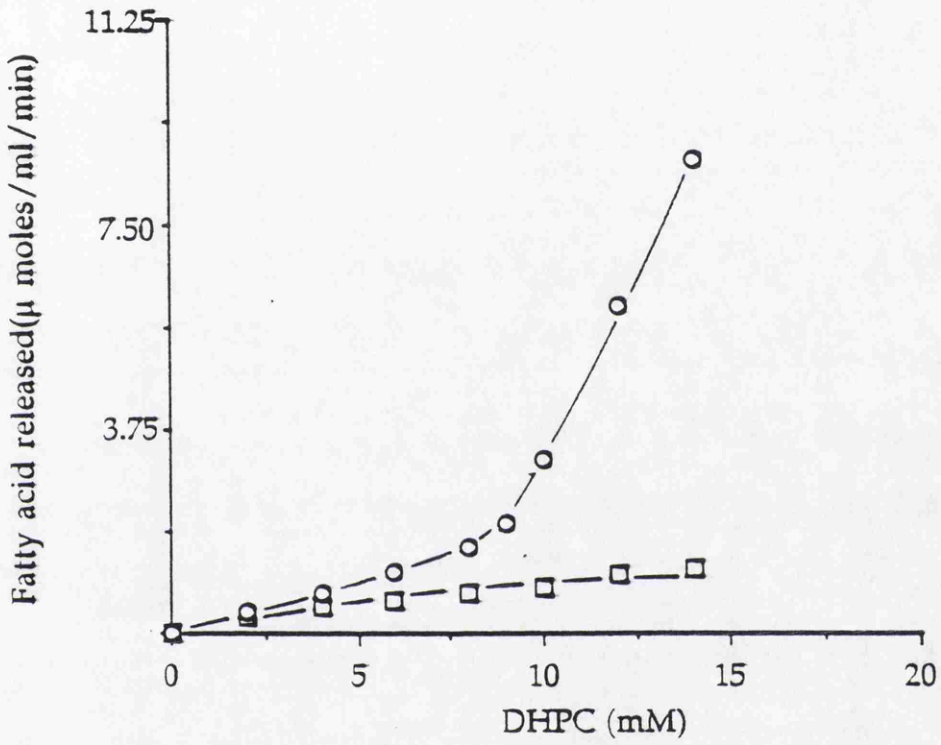
3-1-1-1) Monomeric substrates.

Wells (1974) and de Haas and co-workers, investigated the effect of substrate structure on susceptibility of phosphatidyl choline derivatives to phospholipase A_2 enzymes and characterised the specific changes that occur when the substrate changes from monomeric form to micellar morphology at the critical micelle concentration (CMC). The CMC itself falls very rapidly with increasing acyl chain length, ie for dibutyryl, dihexanoyl, diheptanoyl and dioctanoyl phosphatidylcholine the values are 40, 10, 1.3 and 0.3 mM respectively. Most of these studies were carried out with dibutyryl and dihexanoly phosphatidylcholine derivatives, and for most phospholipase A_2 species tested there was a very dramatic acceleration as the substrate concentration passes through the CMC. The results have been interpreted in terms of an interfacial binding site that greatly increases the catalytic activity against the condensed substrate form. Shipolini *et al* (1974), in contrast, failed to show any such effect for the bee venom enzyme and proposed that the enzyme might be specific for substrates in the monomeric state.

Part of the interest in the current study is to understand why phenomena that could be attributed to the monomer/micelle transition are not observed with dioctanoyl phosphatidylcholine derivative. Many assays require concentrations of this substrate > 0.3mM and in such cases the hydrolysis should take the concentration through the CMC. The present work confirms that the bee venom phospholipase A_2 enzyme does not appear to sense the CMC whilst the enzymes from snake venom *Naja mossambica mossambica* shows a very characteristic response.

Fig 3-1-1-1, shows the hydrolysis of the monomeric substrate dihexanoyl phosphatidylcholine by both bee and snake venom phospholipase A_2 enzymes. This result clearly shows the sudden increase in the catalytic activity of snake venom PLA_2's which is dependent upon the change in the physico-chemical form of the substrate. The critical

Fig 3-1-1-1, The action of bee venom and*Naja mossambica mossambica* phospholipase A_2 enzymes on dihexanoyl phosphatidylcholine substrate.



The reactions were carried out by injecting 2 μg of *Naja mossambica mossambica* PLA_2 (○) and 4 μg of bee venom PLA_2 (□) into conductivity cells containing 2ml of triethanolamine/HCl buffer pH 8.0 and different concentrations of 100mM DiC_6PC (DHPC) in the presence of 1mM $CaCl_2$.

micelle concentration (CMC) was found to be very similar to the value reported by other workers. interestingly, the initial rates for the bee venom enzyme catalysed reactions increased without interruption through the CMC.

3-1-1-2) Micelle forming substrates.

Various diacyl short-chain phosphatidylcholine substrates which adopt micellar morphology, were synthesised as described in the material and methods section, and used to study their susceptibility to bee venom phospholipase A_2. The effect of detergents and organic solvents on the enzymic activity were also investigated. The results obtained confirm that in pure aqueous solution (**Fig 3-1-1-2**), peak activity was found with the dioctanoyl phosphatidylcholine derivative and there was an abrupt fall in the enzymic activity at $DiC_{12}PC$ derivative consistent with the model that this substrate was the first compound of the series to be present in the from monomer/bilamellar rather than monomer/micelle, and that the bilamellar state is very much less susceptible to the enzyme than the corresponding micellar transition.

The neutral detergent triton X-100 was found to act as a weak inhibitor for the monomer and micelle-forming compounds, but a powerful activator in the case of $DiC_{12}PC$ derivative (**Fig 3-1-1-2**) consistent with the proposal it induced a bilamellar/micelle transition for this compound.

N-propanol was expected to alter the CMC for the monomer/micelle transition and perhaps to raise the degree of side chain hydrophobicity required to stabilise the bilamellar rather than micellar morphology. It was found to inhibit the activity of the enzyme on both monomeric and micellar substrates, but also to change the position of peak susceptibility from the DiC_8PC to the DiC_9PC derivatives (**Fig 3-1-1-2**), suggesting that it might raise the CMC, so that the DiC_8PC derivative was not in the micellar state under the conditions used. In addition, n-propanol activated the enzyme quite substantially against the $DiC_{12}PC$ derivative suggesting that it might stabilise the micellar rather than bilamellar morphology.

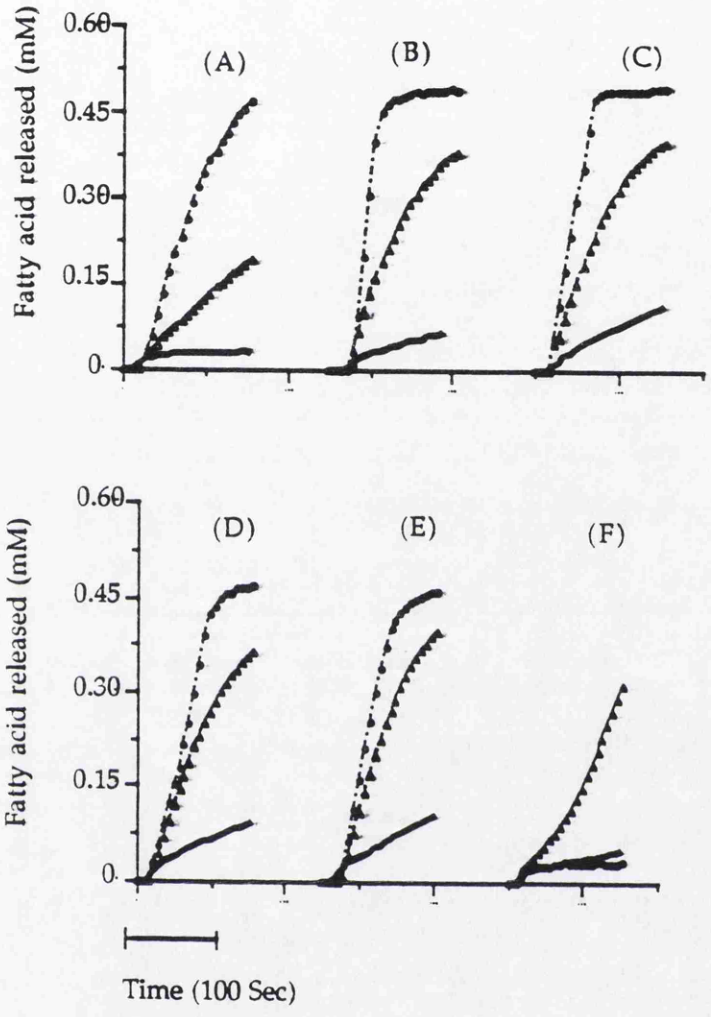
Fig 3-1-1-2. The hydrolysis of diacyl phosphatidylcholine substrates by bee venom PLA_2.

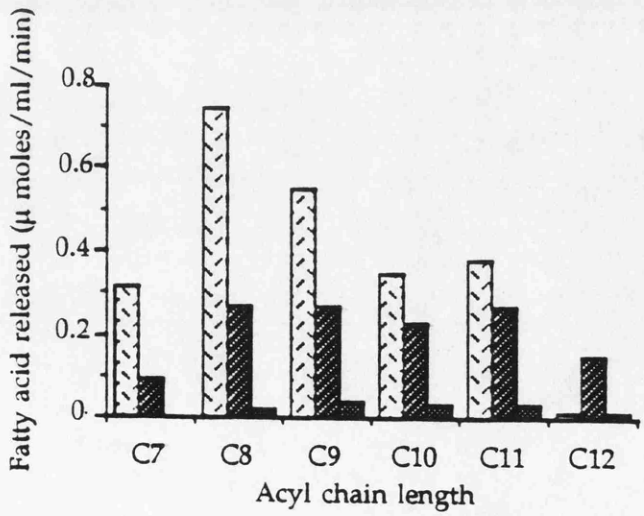
The hydrolysis reactions were carried out by injecting 2µl of 1mg/ml bee venom PLA_2 into conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 at 37 °C, and 20 µl of the substrate in the absence (•). and in the presence of 0.025 % triton X-100 (▲) and 20% n-propanol (■).

Substrates concentrations were adjusted to give equal, total conductance changes.

A: DiC_7PC
B: DiC_8PC
C: DiC_9PC
E: $DiC_{10}PC$
F: $DiC_{11}PC$
G: $DiC_{12}PC$

Initial rates of the hydrolysis of these substrates repectively were measured in each case (ie, aqueous buffer (▽), in the presence of triton X-100 (▨) and in the presence of 20% n-propanol (▩)). by conductimetric analysis of the results based on the calibration of the assay method by free fatty acid.


Fatty acid released (mM)
0.60
0.45
0.30
0.15
0
(A)
(B)
(C)
(D)
(E)
(F)
Time (100 Sec)


Fatty acid released (μ moles/ml/min)
0.8
0.6
0.4
0.2
0
C7
C8
C9
C10
C11
C12
Acyl chain length

3-1-2) The sensitivity of the conductimetric assay method.

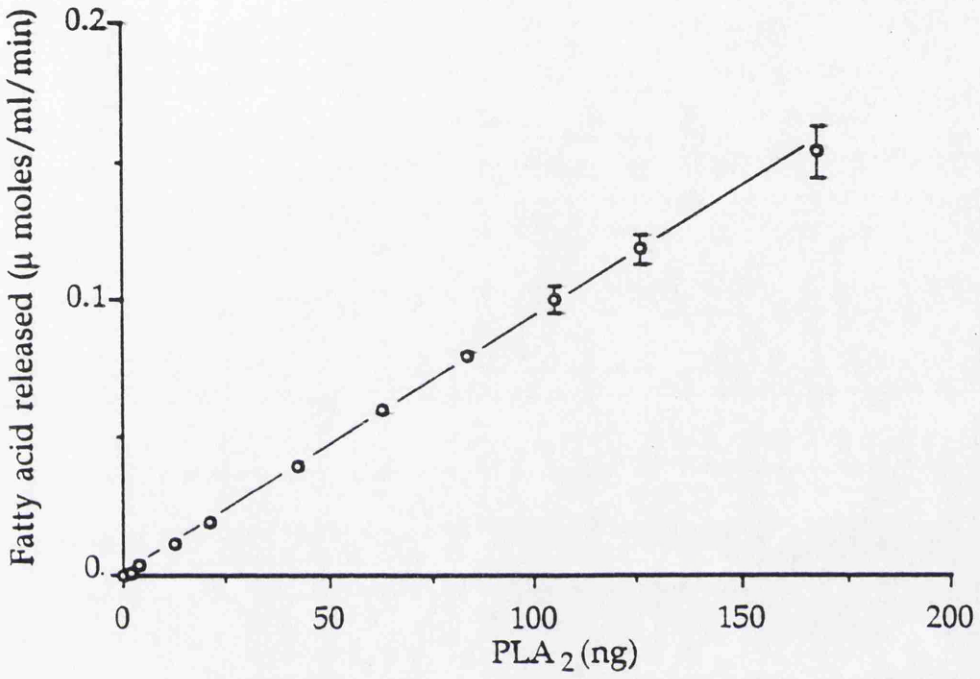
The results described above confirmed that the DiC_8PC derivative is the most sensitive substrate under normal assay conditions. measurements were therefore carried out to determine the limiting sensitivity for the conductimetric assay. This required the use of saturating calcium concentration and also the presence of transition metal ion chelators (eg, o-phenanthroline) to minimise transition metal ion inhibition. Conductimetric analysis of the results showed that the activity of 1 ng protein could be easily and reliably measured (**Fig 3-1-2**). This result also confirmed that the method was both highly sensitive and technically a simple one to use for studying kinetics of different classes of enzymes and lytic agents.

3-1-3) The hydrolysis of DiC_8PC by native and acylated bee venom PLA_2.

Treatment of bee venom phospholipase A_2 with oleoyl-imidazolide has been shown to produce a stable albumin-resistant activation against erythrocytes and on long-chain substrates in the presence of n-propanol. The action against micellar substrates in pure aqueous solution was never shown to be increased by more than 50% by this treatment (Drainas, 1978., Drainas and Lawrence, 1978., Chettibi, 1990., Chettibi *et al* ,1990).

The present work was undertaken to see if this activation could be increased by changing the reaction conditions, for example by working at non-saturating calcium levels or at non-saturating substrate concentrations. The results obtained in this study (**Fig 3-1-3**) indicated that a maximum enhancement of the catalytic activity of the enzyme against DiC_8PC of ca 4-fold occurred at intermediate levels of calcium activation. Because of the high sensitivity of this substrate to these enzymes, an assay based on this effect would be the most sensitive available for measuring activation.

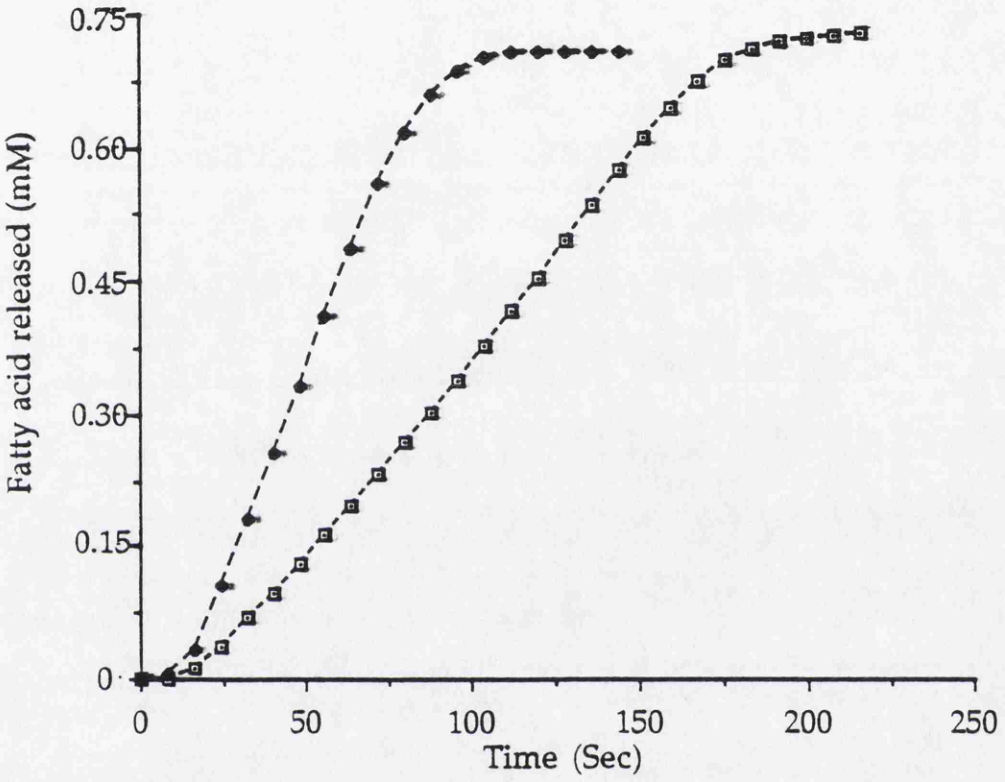
Fig 3-1-2, The hydrolysis of dioctanoyl phosphatidylcholine by bee venom PLA_2



The reactions were carried out by the addition of different concentrations of bee venom phospholipase A_2 to cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20μl of 0.3mM DiC_8PC in the presence of 50μM O-phenanthroline and 1mM $CaCl_2$.

The initial rates were measured as described elsewhere and the values are the means of three experiments

Fig 3-1-3 , The hydrolysis of dioctanoyl phosphatidylcholine by native and activated bee venom PLA_2.



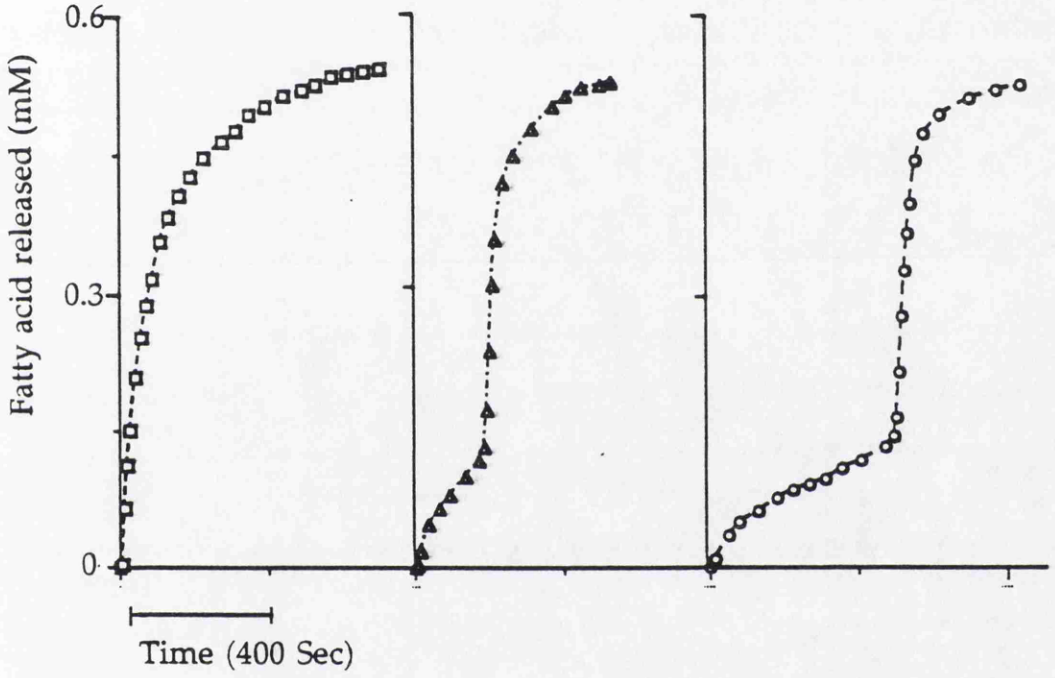
The reactions were carried out by injecting 2µl of 1mg/ml native (▫) and activated (▪) bee venom PLA_2 into cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20 µl of 50mg/ml DiC_8PC.

3-1-4) The action of native and activated bee venom phospholipase A_2 on 2-acyl-chain lecithins.

The selective acylation of the egg lysophosphatidylcholine, prepared as described in material and methods, gave a series of PC derivatives with a defined short acyl-chain at the second position and mixed substitution in the first position, corresponding to palmitoyl, stearoyl and oleoyl residues in the ratios 6/3/1 respectively. In addition, pure palmatoyl lysoPC was synthesised and reacylated to give defined 1-long, 2-short, acyl-chain substituents. The characteristics of these compounds as substrates for bee venom phospholipase A_2 are shown in **Fig 3-1-4 a**. The 1-palmatoyl, 2-hexanoyl PC gave a monophasic hydrolysis curve, but the 2-octanoyl and higher derivatives gave sharply biphasic responses which suggest that there is a sudden transition in the substrate morphology during hydrolysis. This result indicated that these substrates were ideal to study the bilamellar/micellar transition, largely because for the early members of the series, the rates of hydrolysis in each phase were not too different.

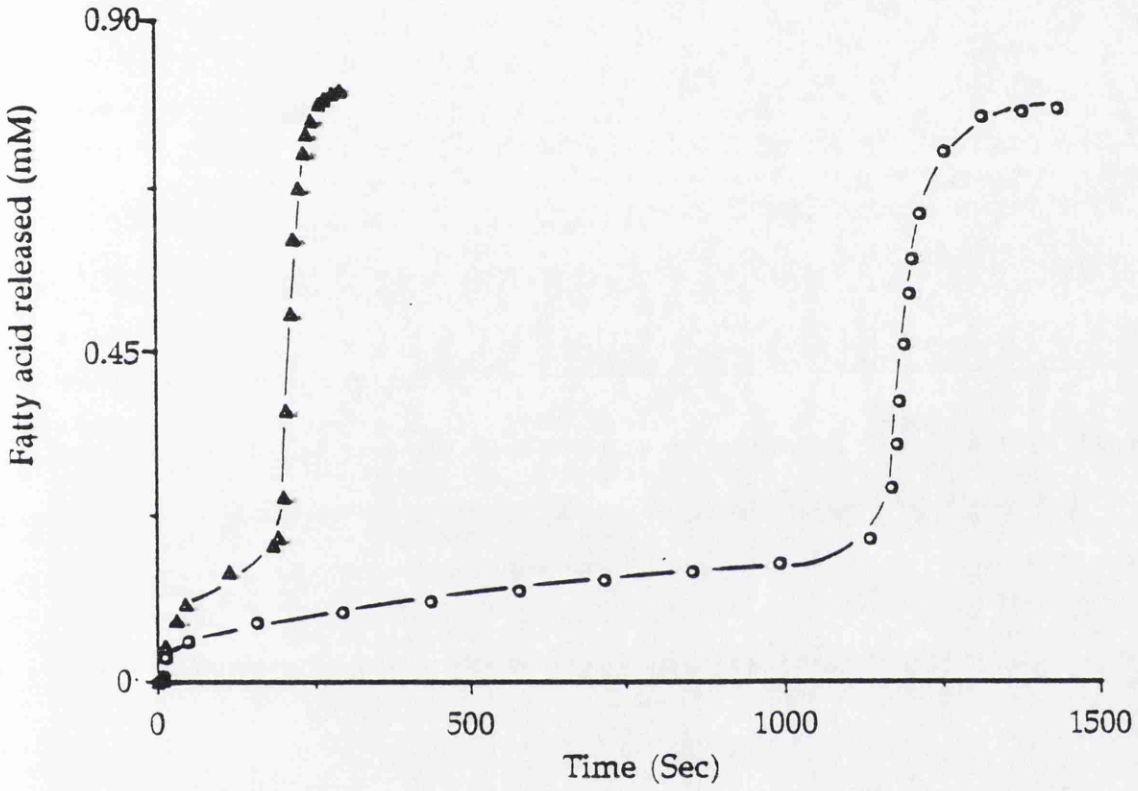
Much of the present work was carried out with the less well defined derivatives made from egg lysophosphatidylcholine, but the overall characteristics were very similar. The early work of Drainas, (1978) indicated that the activation of bee venom phospholipase A_2 enzyme by long-chain fatty acylation might be highest against the bilamellar substrates. These compounds therefore, allowed this hypothesis to be tested directly. The results (**Fig, 3-1-4 b**), confirmed the model showing that the activated form of the enzyme is more active and more strongly activated in the first phase of the hydrolysis reaction than in the second. It is quite clear that the rate transition occurs at a constant amount of the substrate conversion and can therefore be attributed to a bilamellar/micelle transition promoted by the release of detergent-like reaction products.

Fig 3-1-4 a , The action of bee venom phospholipase A_2 on different 2-acyl chain phosphatidylcholine substrates.



The reactions were carried out by injecting 5μg of bee venom PLA_2 into cells containing 2 ml of triethanolamine/HCl buffer pH 8.0 and 20μl of 1-C_{16},2-C_6PC (□), 1-C_{16},2-C_8PC (▲) and 1-C_{16},2-C_9PC (○).

Fig 3-1-4 b , The hydrolysis of 2-nonanoyl phosphatidylcholine by native and activated bee venom phospholipase A_2.



The reactions were carried out by injecting 4µl of 1mg/ml native and activated (by mixing 100 µl of 1mg/ml PLA_2 with 2µl of 0.2% Oleoyl imidazolide and incubated for 120 min at 37°C) bee venom PLA_2 into cells containing 2ml of triethanolamine/HCl buffer pH 8.0 and 20µl of 50mg/ml of $2C_9PC$

(o) The reaction progress curve of the hydrolysis by native PLA_2.

(▲) The hydrolysis by activated enzyme.

3-1-4-1) The effect of calcium on the action of bee venom phospholipase A_2 on 2-C_9PC substrate.

Calcium activated the enzyme in both phases of the hydrolysis reaction, but also increased the difference between the activity of the activated and the normal enzymes in the first stage of the reaction (**Fig 3-1-4-1**). This result does not confirm the earlier suggestion that calcium activation and acyl-group activation are complementary to each other.

3-1-4-2) The effect of the reaction products on the hydrolysis of 2-C_9PC by native bee venom PLA_2.

Experiments to determine the underlying basis of the sharp rate transition were of two types. The first was remove the reaction products by the addition of albumin. It was quite clear the albumin could delay the transition, but also affect the rates of reaction in both phases (**Fig 3-1-4-2 a**). Importantly, low concentrations of albumin were found to cause a large increase in the catalytic activity of both forms of the enzyme and its effect was rather greater on the first phase of the reaction (**Fig 3-1-4-2 b**).

Conversely the effect of exogenous reaction products (ie lysophospatidylcholine and oleic acid) were examined and found to decrease the time of onset of transition without affecting the reaction rates (**Fig 3-1-4-2 c**). This suggests that the transition is indeed promoted by the presence of the reaction products consistent with a detergent-induced transition from bilamellar form to micellar morphology. The complex effects of albumin will be discussed elsewhere.

Fig 3-1-4-1, The effect of calcium on the hydrolysis of 2-C_9PC by native and activated bee venom PLA_2.

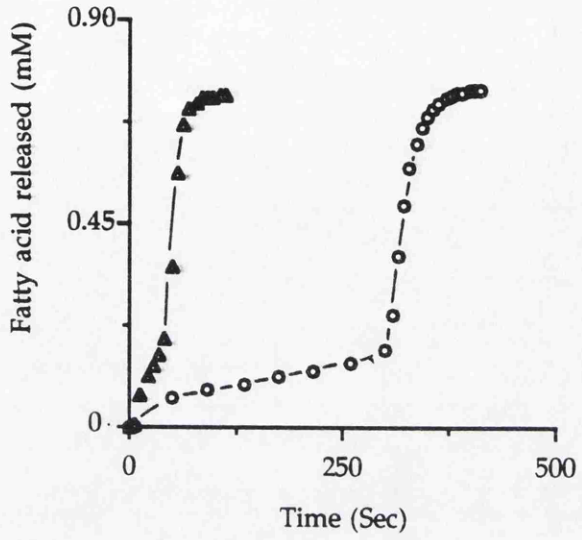
The reactions were carried out as described earlier but in the presence of 1mM $CaCl_2$ with native (○) and activated (▲) PLA_2

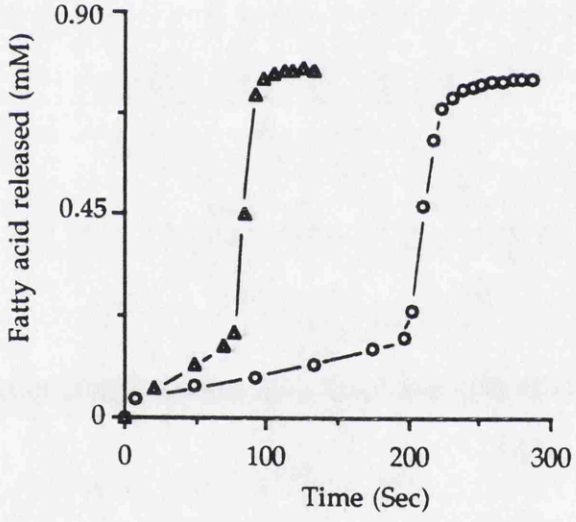
Fig 3-1-4-2 a, The effect of albumin of the hydrolysis of 2-C_9PC by native and activated bee venom PLA2.

The reactions were carried out by injecting 4µl of 1mg/ml native (▲) and activated (○) bee venom phospholipase A_2 into cells containing 2 ml of 10mM triethanolamine/HCl buffer pH8.0 and 20µl of 50mg/ml 2-C_9PC substrate in the presence of 5µM bovine serum albumin.

Fig 3-1-4-2 b, The effect of albumin on the hydrolysis of 2-C_9PC by native bee venom PLA_2.

The hydrolysis reaction were carried out as above but with the enzyme added in the absence (○) and presence of 10µM albumin (•) and 20µM albumin.(▲).


0.90
0.45
0
Fatty acid released (mM)
0
250
500
Time (Sec)


0.90
0.45
0
Fatty acid released (mM)
0
100
200
300
Time (Sec)

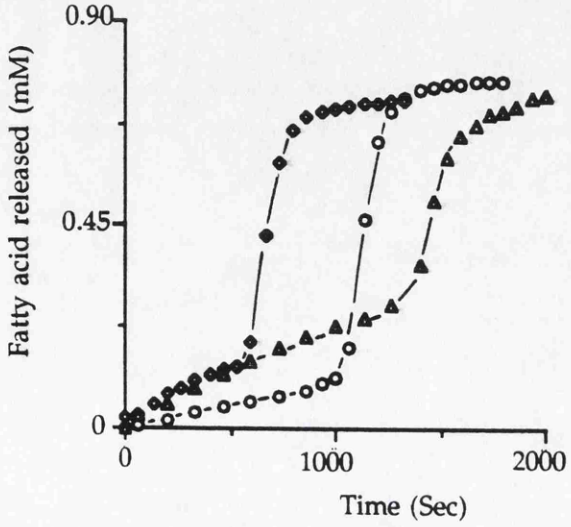
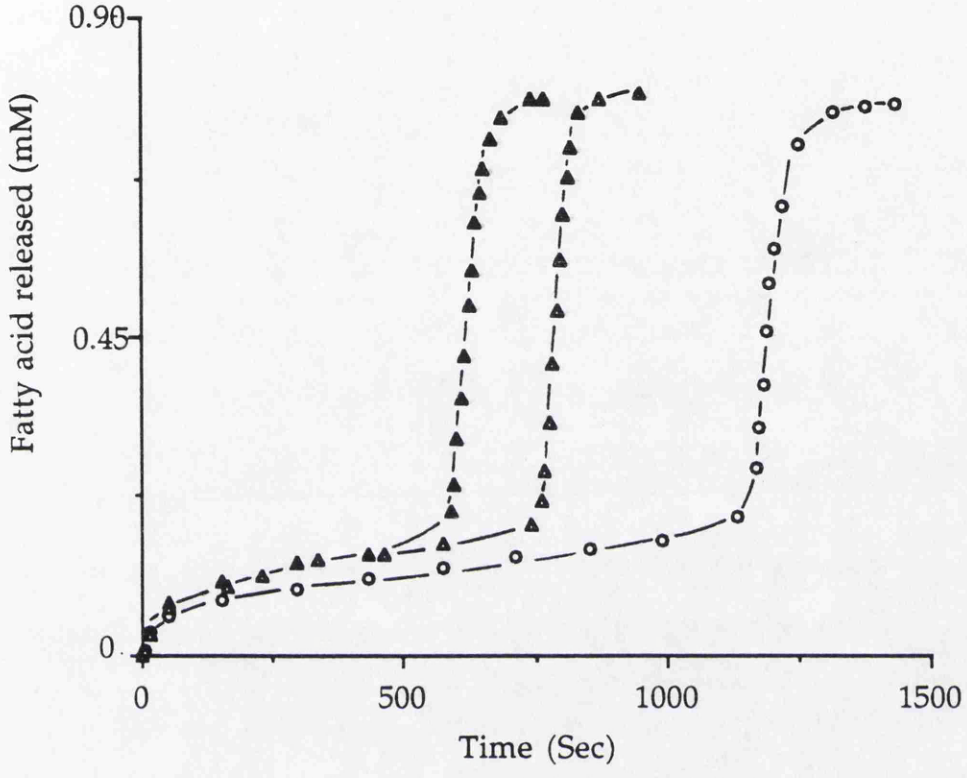

0.90
0.45
0
Fatty acid released (mM)
0
1000
2000
Time (Sec)

Fig 3-1-4-2 c, The effect of lysophosphatidylcholine and Oleic acid on the hydrolysis of 2-C_9PC by native bee venom phospholipase A_2.



The reactions were carried out injecting 4µl of 1mg/ml native bee venom PLA_2 into cells containing 2ml of standard buffer and 20µl of 50mg/ml of 2-C9PC substrate in the absence (○) and presence of 2µg lysophosphatidyl choline (▲) and 4µg of oleic acid (△).

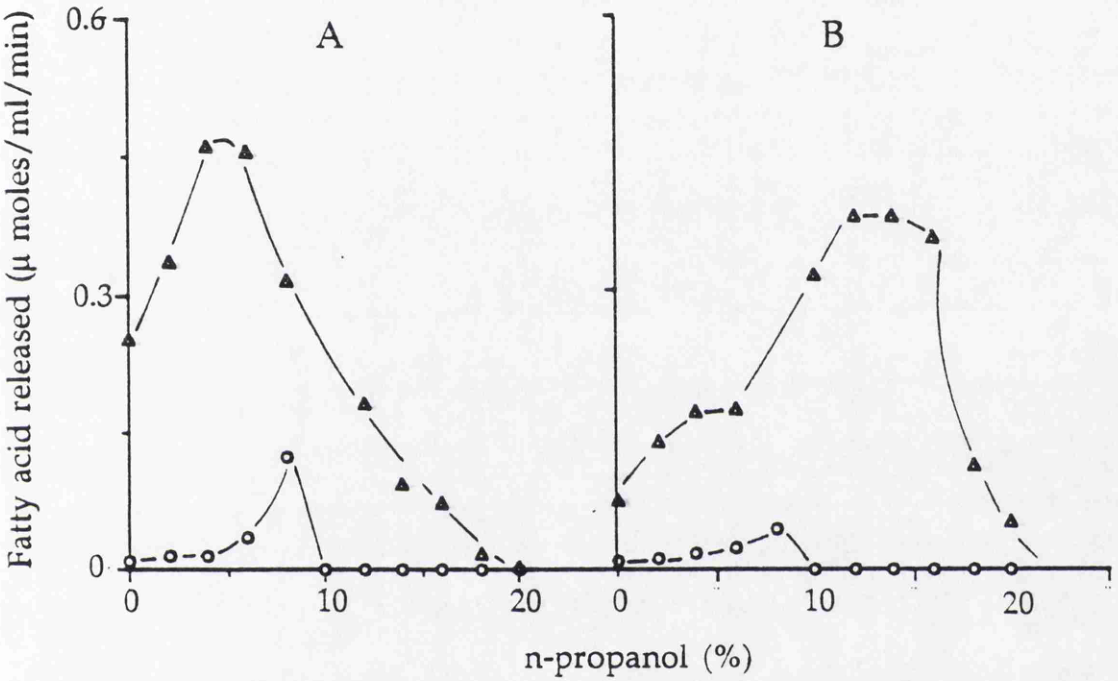
3-1-4-3) The effect of organic solvents on the hydrolysis of 2-C_9PC by bee venom PLA_2:

This was examined by using 1-palmatoyl, 2-nonanoyl phosphatidylcholine as a substrate for bee venom phospholipase A_2. The results obtained showed that the addition of n-propanol progressively abolished the initial slow phase of the hydrolysis reactions, but low concentrations were found to increase the enzymic activity in the second phase of the reaction. Most significantly, there was a sharp contrast between the native and activated forms of the enzyme (**Fig 3-1-4-3**). Peak activity for the activated enzyme occurred at about 15% n-propanol where the curve was entirely monophasic, whilst under the same conditions, the normal enzyme showed a very low activity. These results show that the activation by acylation makes the enzyme more resistant to the organic solvent. Therefore this type of reaction in 16% n-propanol could represent an ideal assay for the activation reaction.

3-1-5) The hydrolysis of phospholipase D-synthesised substrates by bee venom phospholipase A_2.

To extend the study of the acyl-chain reactions, attempts were made to synthesise compounds with different polar head-groups. Purified phospholipase D enzyme from savoy cabbage is known to catalyse the head group exchange reactions, thus this was used to synthesise other head-group containing phospholipids. The syntheses were carried out using dioctanoyl phosphatidylcholine as a substrate for this enzyme in presence of different phosphatidate acceptors (see material and methods section). The results confirmed the expectation that the total conductance change in the presence of the acceptors (transferase) would be halved compared to that in their absence (hydrolase) (This analysis will be discussed elsewhere).

Fig 3-1-4-3, The effect of n-propanol on the hydrolysis of 1-C_{16} 2-C_9PC by native and activated bee venom phospholipase A_2.



The reactions were carried out by injecting 2μl of 1mg/ml native (A) and activated (B) bee venom phospholipase A_2 into cells containing 2ml of triethanolamine /HCl buffer pH 8.0 and 20 μl of 50mg/ml of 1-C_{16}2-C_9PC in the presence of different concentrations of n-propanol.

Initial rates (○) and maximum rates (△) were measured as described elsewhere.

The product of the transesterfication reactions were used as substrates for PLA_2 in order to see whether or not the new type of phospholipids are susceptible to hydrolysis by PLA_2 enzymes.

Fig 3-1-5 a, b and **c** show the hydrolysis of dioctanoyl derivatives with different head-group substituents by native bee venom PLA_2, these results clearly show that phosphatidylmethanol (PMeOH) derivative is more susceptible to PLA_2 attack than the parent choline derivative, whilst phosphatidylserine (PS) is a very poor substrate. It is also found that the acceptors which lack a primary hydroxyl group were not exchanged for choline, but did affect slightly the hydrolsis of the original substrate (DiC_8PC). It should be noted that the hydrolysis of these new substrates by PLA_2 suggest that the Km for the methanol adduct is lower than the choline derivative.

Fig 3-1-5 a, b, c, and **d,** The hydrolysis of dioctanoyl phosphatidyl alcohol derivatives (synthesised by phospholipase D) by bee venom phospholipase A_2.

The reactions were carried out by injecting of 10µl of the partially purified savoy cabbage phospholipase D into conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20µl of 30mg/ml dioctanoylphosphatidylcholine substrate in the absence and presence of 2% concentrations of different phosphatidate acceptors.
When the reactions were over, 2µl of 1mg/ml bee venom phospholipase A_2 were added.

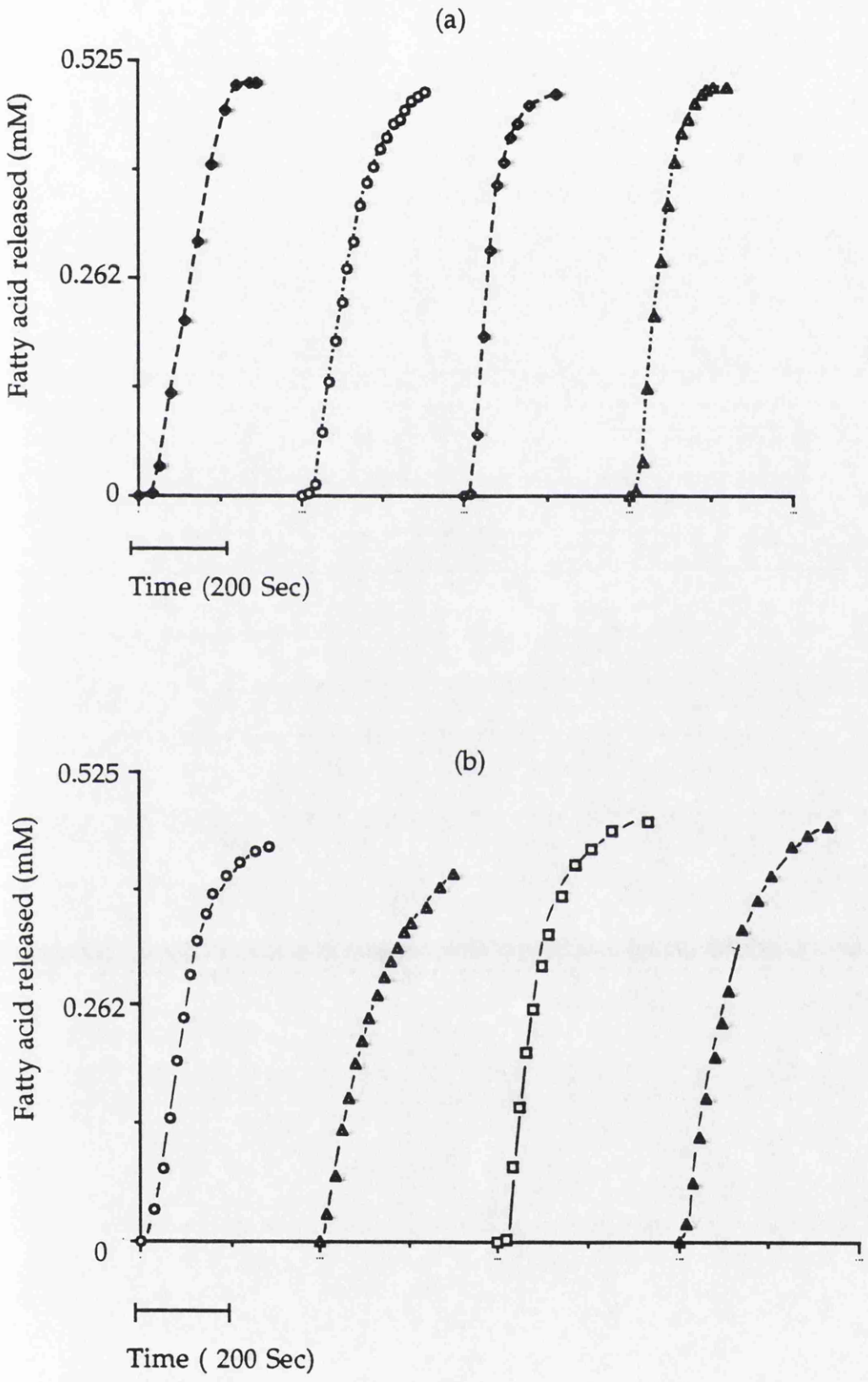
The figures represent the action of PLA_2 on:-

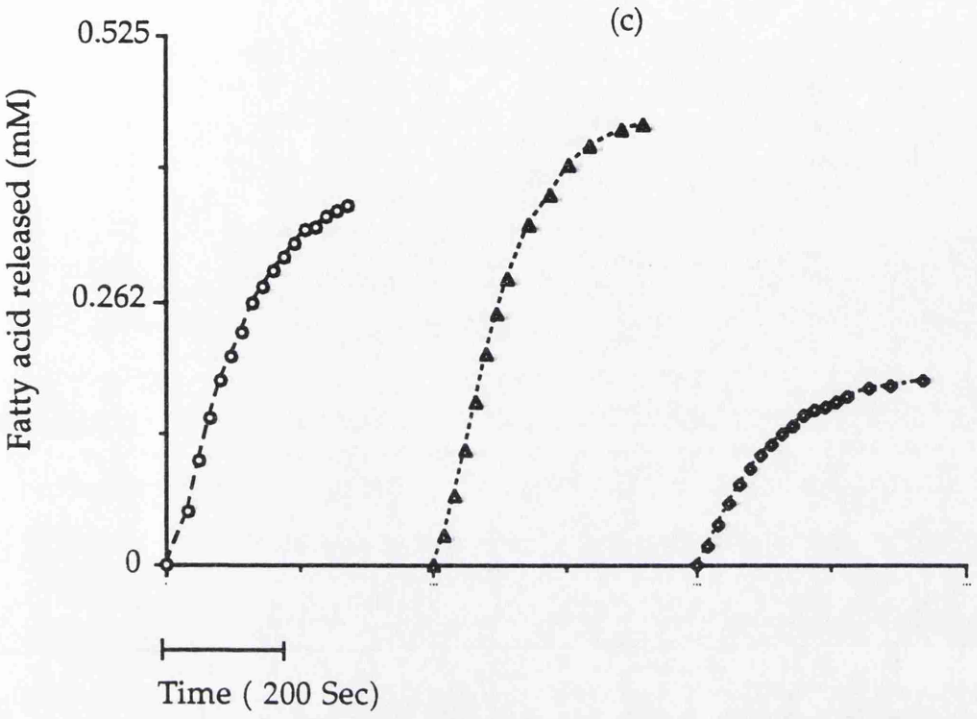
a) - Control DiC_8PC (■) , DiC_8PA(○), DiC_8P-MeOH (□) and DiC_8P-EtOH (△).

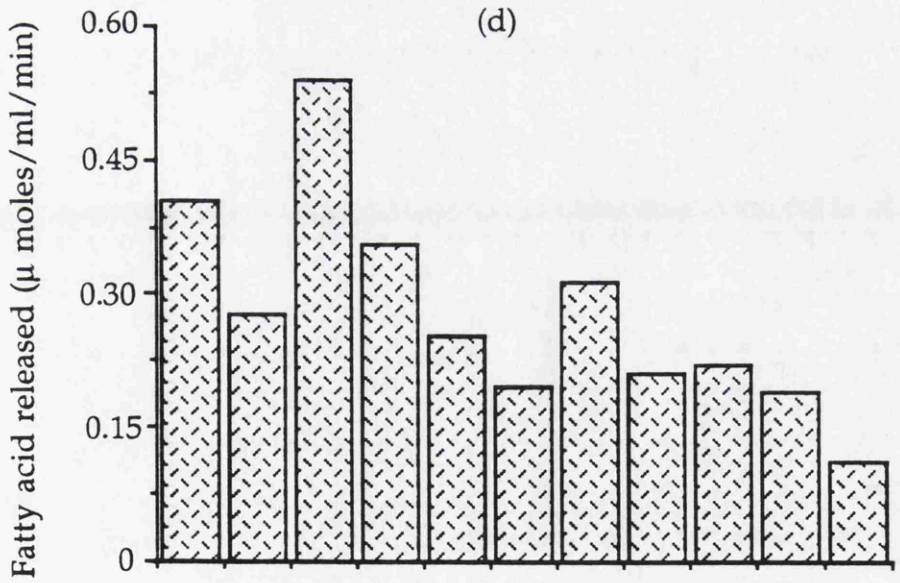
b) - DiC_8P-(n-Propanol)(○) , DiC_8P-(2-propanol) (△), DiC_8P-butanol (□) and DiC_8P-(1,6 propanedi-ol) (▲).

c) DiC_8P-(1,6 diethylhexane di-ol) (○), DiC_8P-(Thiodiglycol) (▲) and DiC_8PS (□).

d) The Initial rates of the hydrolysis of these substrates by bee venom PLA_2 were measured by conductimetric analysis and the values were recorded and drawn in the order as described in parts a, b, and c (above).


(a)
0.525
0.262
0
Fatty acid released (mM)
Time (200 Sec)
(b)
0.525
0.262
0
Fatty acid released (mM)
Time (200 Sec)


(c)
0.525
0.262
0
Fatty acid released (mM)
Time (200 Sec)


(d)
0.60
0.45
0.30
0.15
0
Fatty acid released (μ moles/ml/min)

3-1-6) The use of erythrocytes as substrates for bee venom PLA_2.

Erythrocyte membranes were reported to be poor substrates for some PLA_2 enzymes. Drainas and Lawrence (1981) showed that the acylation of bee venom PLA_2 by oleoyl-imidazolide increased the lytic activity by ca 65-fold in the presence of albumin. Chettibi and Lawrence (1990) have shown that other PLA_2 enzymes could be activated by acylation and both the catalytic and lytic activities were totally inhibited by EDTA which indicated that the lytic activity requires the catalytic action. Some comparative experiments were carried out here to study the activation of two phospholipase A_2 enzymes that were widely separated on the evolutionary scale and had quite divergent primary sequences, ie that from bee venom and the basic non-toxic isoform from *Naja mossambica mossambica*. Both enzymes were acylated by treatment with a 1:1 equimolar ratios of oleoyl-imidazolide, incubated for 120 min and assayed for activation against rabbit and rat erythrocytes. The results showed that both enzymes were strongly activated in the presence of albumin, the haemolytic activity of the *Naja mossambica mossambica* PLA_2 was much greater than that of bee venom enzyme and rat erythrocytes were ca 5 times more sensitive to both native and activated enzymes (**Fig 3-1-6 a, b**). This advantage was slightly offset by the fact that the rat cells had a higher basal leakage rate than the rabbit erythrocytes. In the absence of albumin neither enzyme showed much lytic activity either in native or activated state

Albumin has been proposed to act by binding the fatty acid and lyso phospholipid, the reaction products of phospholipase A_2 catalysed reaction. Lawrence (1975) has shown that lysophospholipids inhibit the lytic action of phospholipase A_2 enzymes on rabbit erythrocytes, whilst fatty acids potentiate them

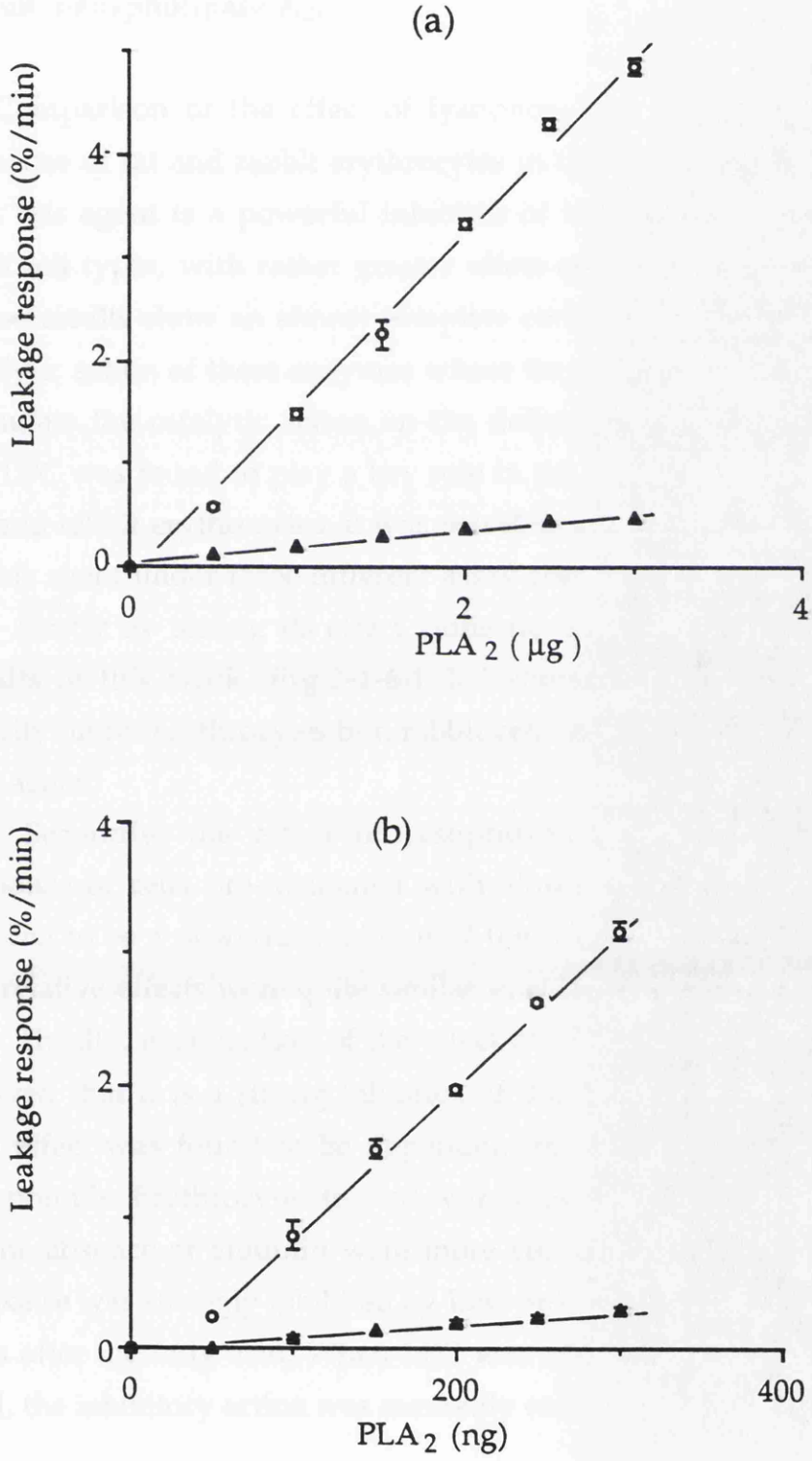
Fig 3-1-6, The leakage response of rat and rabbit erythrocytes to activated venom phospholipase A_2 enzymes.

a) The reactions were carried out by injecting 2µl of 1mg/ml activated bee venom PLA_2 into conductivity cells containing 2ml of isotonic succrose/MOPS buffer pH 7.4 and 20µl of washed rat (○) and rabbit erythrocytes (▲) in the presence of 10µM bovine serum albumin.

Initial rates were measured from the conductance change values which are expressed as a percentage of the total leakage of electrolytes from washed erythrocytes. the values represent the mean of three different assays.

b) As previous but using 2µl of 0.1mg/ml of the basic non-toxic PLA_2 from *Naja mossambica mossambica.*

Fig 3-1-6, The action of phospholipaseA_2 enzymes on rat and rabbit erythrocytes.



3-1-6-1) The effects of exogenous lysophosphatidylcholine and fatty acid on the leakage response of rat and rabbit erythrocytes induced by bee venom phospholipase A_2.

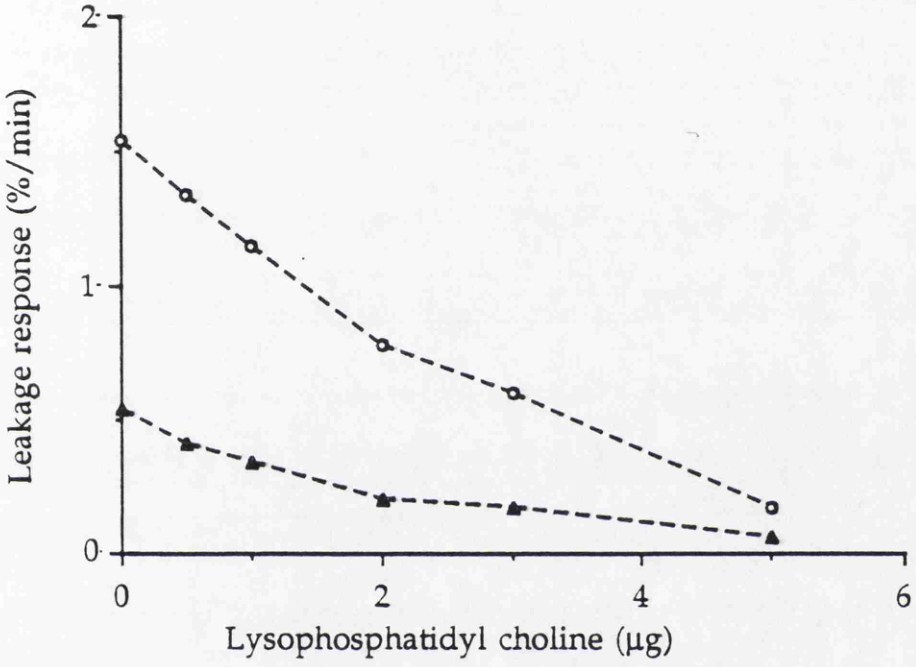
Comparison of the effect of lysophosphatidylcholine on the leakage response of rat and rabbit erythrocytes in the presence of albumin showed that this agent is a powerful inhibitor of the activated enzyme acting on both cell types, with rather greater effect on the rat cells (**Fig 3-1-6-1 a**). These results show an almost complete contrast with similar studies of the catalytic action of these enzymes where the lyso-compounds were found to stimulate the catalytic action on the defined bilayer structures. Moreover the LPC was found to play a key role in the PLA_2-induced leakage of both rat and rabbit erythrocytes, it was therefore of interest to examine the effect of this agent under three different assay conditions:

- Firstly by testing its effect alone (ie. in the absence of albumin), the results of this work (**Fig 3-1-6-1 b**) showed that it stimulates the lytic activity on rat erythrocytes but, rabbit cells were very much less sensitive to this agent.

- Secondly, the effect of lysophosphatidylcholine on the leakage response of cells pre-incubated with albumin. Under these conditions, it showed to be a powerful inhibitor of the lytic action on both cell types, but the relative effects were quite similar in either cases.

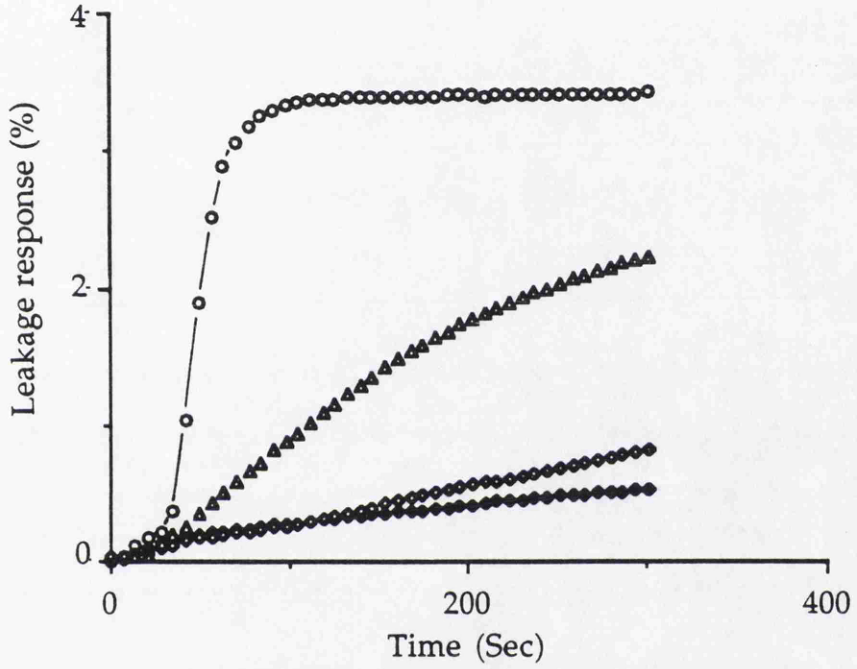
- Thirdly, examination of the effect of LPC in the presence of fatty acid showed that it is a strong inhibitor of the lytic action of the enzyme, but this effect was found to be dependent on the addition order of the two compounds. Erythrocytes treated with sub-lytic concentrations of fatty acid in the absence of albumin were more susceptible to PLA_2 attack, but this response was strongly inhibited by LPC provided that LPC was added to the cells after the fatty acid. When LPC was added to the cells before the fatty acid, the inhibitory action was markedly reduced (**Fig 3-1-6-1 c**).

Fig 3-1-6-1-a, The effect of lysophosphatidylcholine on the action of activated bee venom phospholipase A_2 on rat and rabbit erythrocytes.



The reactions were carried out by injecting 2µl of 1mg/ml activated bee venom phospholipase A_2 into cells containing 10 mM isotonic succrose buffer pH 7.4 and 20 µl of 33% v/v washed rat (○) and rabbit (▲) erythrocytes in the absence and presence of different concentrations of lysophosphatidyl choline and 10µM bovine serum albumin.

Fig 3-1-6-1 b , The effect of lysophosphatidylcholine on the action of activated bee venom phospholipase A_2 on rat erythrocytes in the presence and absence of albumin.



The reactions were carried out as described above, but with activated PLA_2 added to rat erythrocytes in the presence of 10μM albumin (○), 10μM albumin + 4μg LysoPC (◇), In the absence of albumin (◆), and with 4μg Lyso PC in the absence of albumin (△).

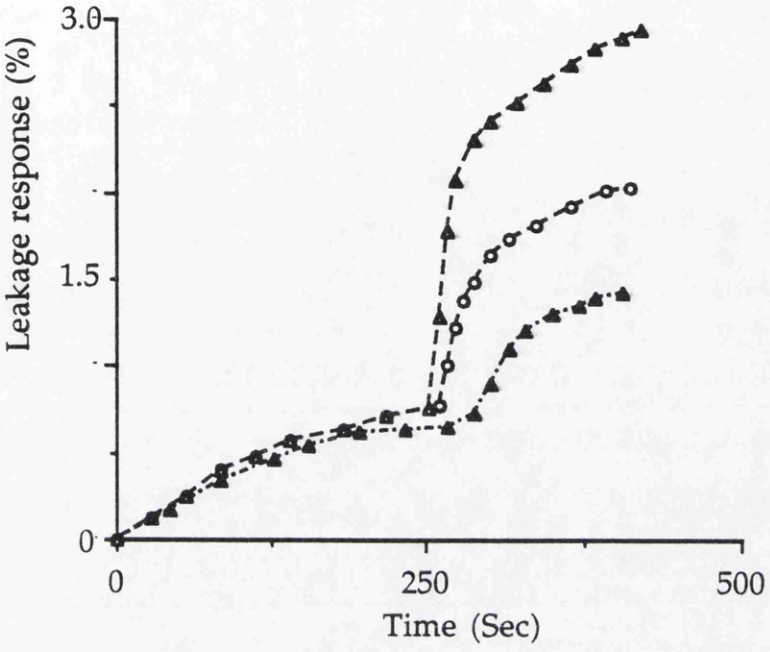
When these experiments were repeated with albumin added to the system after LPC/FA additions, a new phenomenon was observed. Removal of FA by albumin increased the leakage response quite dramatically, the simultaneous presence of traces of LPC with the fatty acid inhibited this response, but the degree of inhibition was greater if LPC was present before rather than after the fatty acid (**Fig 3-1-6-1 d**). This effect. which appears to be the opposite of that obtained in the absence of albumin (as mentioned above), was found with both rat and rabbit erythrocytes.

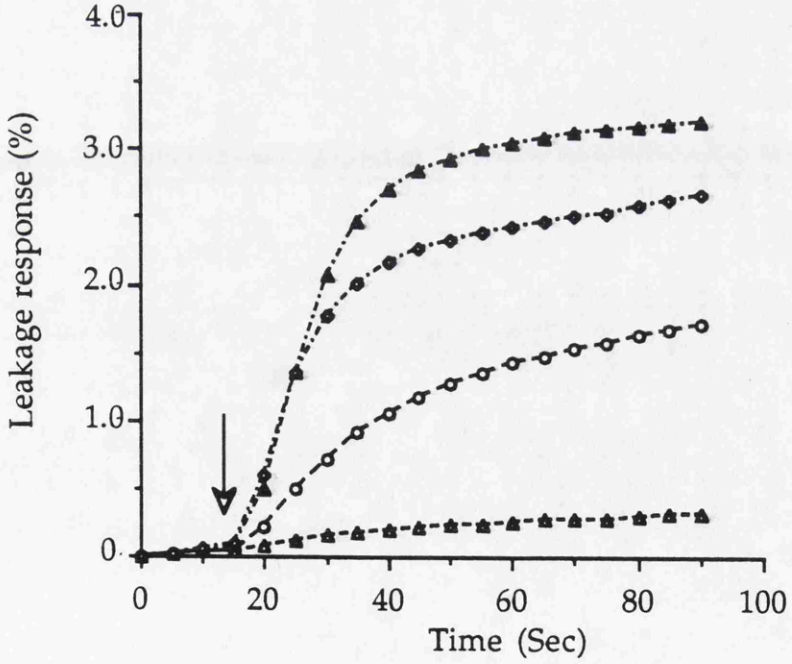
Fig 3-1-6-1-d, The effect of the addition order of fatty acid and lysophospholipid on the susceptibility of rabbit erythrocyes to PLA_2 attack.

I) The leakage response of washed rabbit erythrocyte was obtained by the treatment of RBC with 2µl of 1mg/ml oleic acid followed by 2µg PLA_2 (Δ), oleic acid + 2µl of 1mg/ml lysophosphatidylcholine +PLA_2 (▲) and lysophosphatidylcholine + oleic acid +PLA_2 (○).

II) The effect of the additon order of LPC nad FA and their extraction by albumin ofn the leakage of rabbit erythrocytes.

The assays were carried out as described above but with FA + albumin (▲), FA + LPC + albumin (◇) and LPC +FA + albumin (○).


3.0
1.5
0
Leakage response (%)
0
250
500
Time (Sec)


4.0
3.0
2.0
1.0
0
Leakage response (%)
0
20
40
60
80
100
Time (Sec)

Summary

A complete analysis of the behaviour of phospholipase A_2 enzymes requires the use of substrates that form the free monomer, micelle and bilamellar structures. This work has approached this problem by developing a range of phosphatidyl choline derivatives that span all three morphologies while remaining accessible to the simple conductimetric assay method.

Studies of the monomer/micelle transition have revealed that some enzymes are very sensitive to this change of state when it occurs with short-chain substrates, but there is no trace of such behaviour with longer-chain derivatives. Thus no PLA_2 enzyme responds to the monomer/micelle transition in dioctanoylcholine and the reasons for this are obscure.

Studies of the micelle/bilayer transition have been facilitated by the development of a series of 1-long-chain 2-short-chain phosphatidylcholine derivatives in which the short chain alone is varied. These compounds can be used to illustrate the behaviour of the enzyme at the onset of the transition and to see how it varies as the relative stability of the bilayer form increases.

These substrates have proved to be particularly useful in the studies of activation of PLA_2 by long-chain fatty acylation. None of these enzymes are activated significantly towards the monomeric forms or to simple micellar forms, but activation increases as the conditions for stability of the bilayer form are reached. For a given compound the bilayer form is always considerably more sensitive to the effects of acylation than the monomeric form. The work has also established that attack on the bilayer form of such a substrate is complex and an initial phase seems to have the highest sensitivity to activation.

The most significant study of activation by acylation shows that interaction with a bilammellar substrate stabilises the acylated enzyme, but not the native enzyme against inactivation by n-propanol, whilst interaction with a micellar substrate does not.

To a first approximation the metal ion dependence of the enzyme appears to be independent of the substrate morphology. The activity of the enzyme against a micellar substrate that does not sense activation by acylation when tested at basal calcium concentration or at maximum calcium stimulation, is greatly enhanced at intermediate calcium concentrations, confirming that acylation is involved with calcium binding and providing evidence for two calcium functions.

Part Two

3-2-1) Calibration of the conductimetric assay and calculation of free Ca^{2+} in distilled water:

Studies of the metal ion dependence of PLA_2 enzymes show that the vast majority have an absolute requirement for calcium, but there is considerable confusion with respect to the binding affinities. Values vary from ca 1μM to 2.5mM for enzymes from different sources and in some cases there is a very large variation for enzymes from the same source (eg. bee venom). The two approaches to measuring exact binding affinities are either to use a classic metal ion buffer system to control calcium levels, or else to use pure solutions and add specified metal ions. This latter approach is not possible in most cases because the range of calcium concentration present in apparently pure water varies from ca. 2--20 μM and this is the range in which the majority of activation is seen for very many of these enzymes. In addition distilled water contains significant quantities of inhibitory divalent cations and quite considerable increase in catalytic activity has been demonstrated when low levels of chelators are added.

Among the commonly used chelators for calcium that can be used as 'buffers' are EDTA/EGTA, NTA and citrate in decreasing order of binding affinity. EDTA and EGTA have very high affinity for calcium and can be used to prepare calcium buffers that operates in the range 0.01-1μM. Preliminary experiments with NTA showed that used at 1mM concentration, variation of calcium from 0-2mM gave almost the full range of rate variation with the enzymes used here. Citrate has a lower affinity than NTA and did not seem likely to form the basis of a useful calcium buffer system.

Because the metal ion affinity of these compounds varies considerably both with pH and ionic strength, the literature values were not considered to form a useful basis for calculating free calcium concentrations. Conductimetric titration of NTA was therefore carried out to determine the calcium binding characteristics. **Figure 3-2-1 b**, shows the results of a

difference titration in which calcium was added either to assay buffer or assay buffer containing NTA (**Fig 3-2-1 a**). The conductance deficit between the control and the NTA solution was due to show the complex (Ca^{2+}/NTA) formation and it was clear that at low calcium concentrations this deficit was almost a linear measure of added calcium. At higher calcium concentrations there was a significant departure from linearity. This departure was used as shown to calculate free calcium, bound calcium, free NTA and bound NTA. These values were then put into the equation; $K_d = [Ca^{2+}].[NTA]/[Ca^{2+}\text{-NTA}]$ and a range of K_d values calculated. For NTA, the result was K_d = 8μM.

This result does not take into account the amount of free calcium present on the initial solution, but this was not considered to introduce serious errors because the concentration of NTA was at least 50 times greater than free calcium.

The second problem with studies of metal ion dependence concerned inhibitory divalent cations. Chettibi (1990), has shown that transition metal ion chelators with low affinity for divalent ions of the alkaline earth series (Mg^{2+}-Ba^{2+}) activated PLA_2 quite significantly whilst titration with EDTA enhance activity before inhibiting it. Thus a significant part of the behaviour of these enzymes was determined by inhibition by endogenous cations.

Use of metal ion buffers enabled the role of calcium to be studied in the absence of these ions, but the reverse was not true, All chelators fro alkaline earth elements have much higher affinities for transition metal ions. Thus although it is possible, though difficult to calculate free Sr^{2+} and Ca^{2+} in the presence of NTA because the affinities of the ligand for these ions are quite similar, it is not possible to determine Zn^{2+} in the presence of calcium under the same conditions. This has the consequence that inhibitory transition metal ions can only be studied in the presence of the endogenous divalent cations which include calcium and whatever other ions are present. measurement of the endogenous calcium concentration was done in two ways. firstly conductimetric titration with EDTA was done to find the total divalent ion concentration. (ca. 5μM).

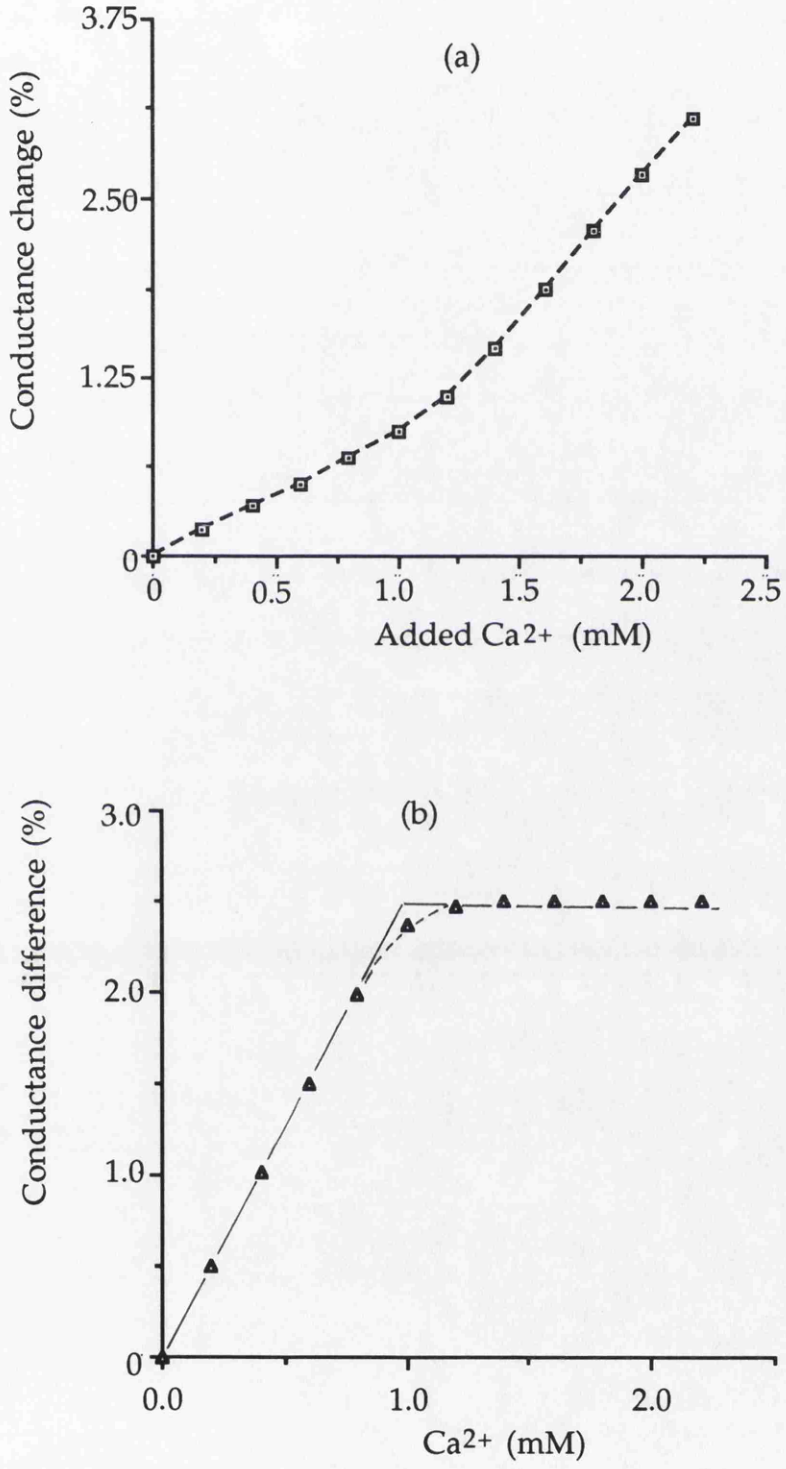
Fig 3-2-1 a, The calibration of the conductimetric assay method by $CaCl_2$.

The calibration assay was carried out by addition of 2µl aliqouts of 100mM $CaCl_2$ to conductivity cells containing 2ml of 10 mM triethanolamine/HCl buffer pH 8.0 in the presence of 1mM Nitrilotriacetic acid (NTA).

Fig 3-2-1 b, The calibration of the conductimetric assay and calculation of free Ca^{2+} concentration present in distilled water.

The curve represents the difference in the conductance change resulted from the subtraction of the values obtained from the figure above, from those obtained from a similar titration assay in which $CaCl_2$ was added to 10mM triethanolamine/HCl buffer pH 8.0 in the absence of NTA.

Fig 3-2-1, Calibration of the conductimetric assay method by calcium.



Secondly the enzyme activity was determined in the absence of added calcium but in the presence of o-phenanthroline. It was then determined in a range of Ca^{2+}/NTA buffers based on 1mM NTA. the concentration of 'free' calcium that gave the same reaction rate as the non-buffered solution was then considered to represent the endogenous calcium concentration. This was clearly a first approximation and would be improved by using the result to correct the concentrations used to measure the Kd, but the correction was considered to lie within the range of experimental error. The results indicated hat calcium was the major endogenous divalent cation (ca. 2μM).

3-2-2) Metal ion requirement of phospholipase A_2-catalaysed reactions.

3-2-2-1) Progress curve shapes at low calcium concentrations.

Chettibi (1990) reported that the hydrolysis curve of the micellar substrate DiC_8PC by bee venom phospholipase A_2 in aqueous solution in the presence of transition metal ion chelator o-phenanthroline had a sigmoidal shape rather than the hyperbolic shape which was observed in the absence of the metal ion chelator. This effect was examined further using other chelators. Phospholipase A_2 enzymes are strongly inhibited by EDTA consistent with the absolute requirement for Ca^{2+}. However over a range of low concentrations in a standard assay conditions, EDTA was found to increase the enzymic activity (**Fig 3-2-2-1 a**). This activation was accompanied with a very clear shape change in the hydrolysis curve from the hyperbolic to the sigmoidal form. Removal of the inhibitory ion either by EDTA or o-phenanthroline was the major factor in enhancing the sigmoidal curve shape (**Fig 3-2-2-1 b**). At high calcium concentrations the curve remained hyperbolic even in the presence of transition metal ion chelators. These results suggest that the enzyme activated at a low calcium concentration gives sigmoidal time courses, but they do not indicate what the origin of this unusual curve shape might be. They also indicate that the shape becomes

Fig 3-2-2-1 a, The effect of EDTA on the hydrolysis of $DiC_{10}PC$ by the basic non-toxic PLA_2 from*Naja mossambica mossambica*.

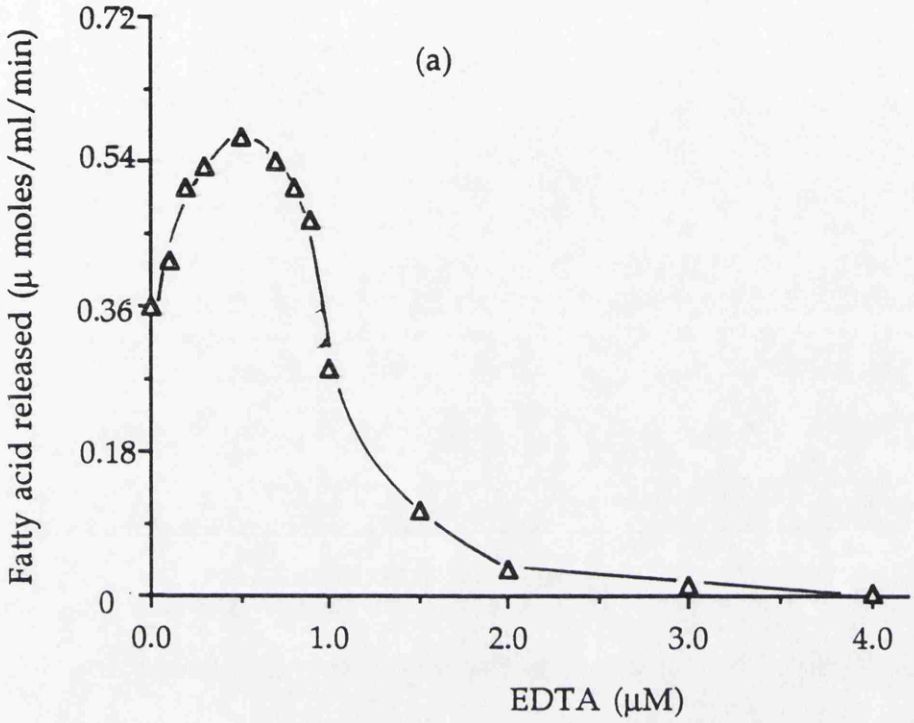
The hydrolysis reactions were carried out by injecting 2µl of PLA_2 into cells contaning 2ml of triethanolamine/HCl buffer pH 8.0 and 20µl of $DiC_{10}PC$ substrate in the presence of different concentrations of EDTA.

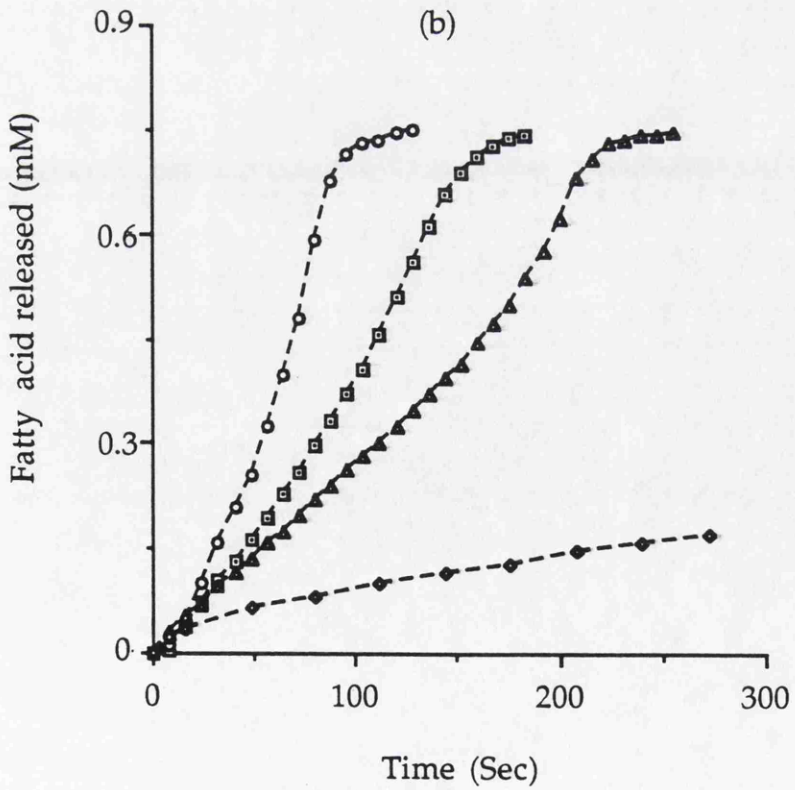
The reaction rates were measured as described elsewhere.

Fig 3-2-2-1 b, The effect of different concentrations of EDTA on the curve shape of the hydrolysis of didecanoylphosphatidylcholine substrate by the basic non-toxic PLA_2 from *Naja mossambica mossambica* venom

The reactions were carried out out as described above but in the absence (△) and presence of 0.6 µM (○), 1µM (⊡) and 2µM (□) EDTA.

Fig 3-2-2-1, The effect of EDTA on the hydrolysis of $DiC_{10}PC$ by the basic non-toxic PLA_2 from *Naja mossambica mossambica*..





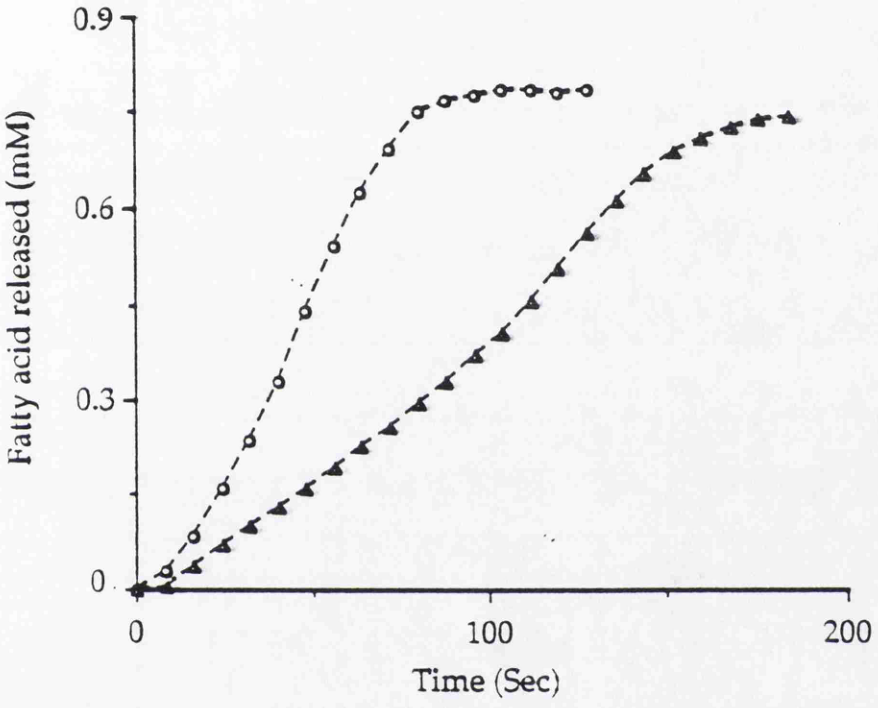
more conventional when a second metal ion either Ca^{2+} or a transition metal ion binds to the enzyme

These results indicated that the conventional hyperbolic curve shape requires either saturating levels of calcium, or else low levels of calcium together with an unknown divalent metal cation. This result also indicated that calcium may have two quite distinct effects, one of which is involved with the curve shape determination and is associated with activation, but this function can be replaced by another non-activating metal ion.

This effect of a sudden rate increase towards the end of the reaction suggested that a CMC phenomenon might be involved. This kind of results would be predicted if the monomer morphology was more susceptible to PLA_2 attack than the micellar form. To examine this, experiments were carried out with longer chain substrates where the CMC should be 10-100-fold lower. The results (**Fig 3-2-2-1 c**) showed that with didecanoyl substrate the rate change phenomenon was more sharply defined, but occurred at roughly the same concentration level. Therefore a CMC-determined phenomenon can be eliminated. Similar results were obtained with both the bee venom enzyme and the non-toxic basic isoform from *Naja mossambica mossambica* and also a number of other enzymes, but not the toxic isoform from *Naja mossambica mossambica* or the 11' isoform from *Notechis scutatus scutatus.*

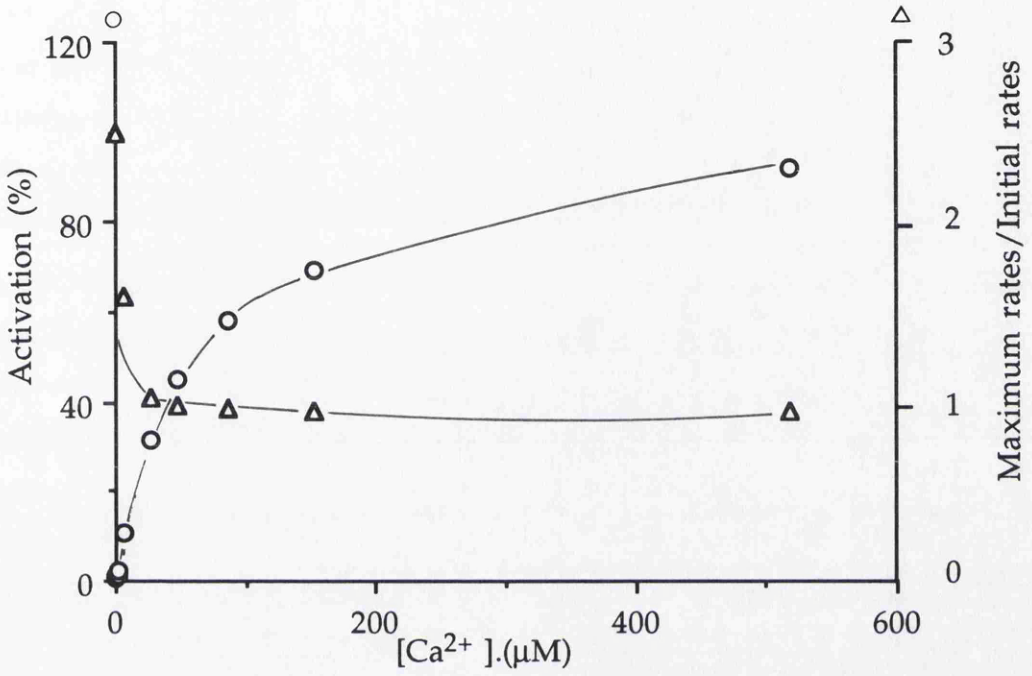
In summary, these results show that the sigmoidal curve shape is not found at high calcium concentrations and is greatly intensified at low concentrations provided that a transition metal ion chelator is present. The simplest explanation of these results is that there are two metal ion binding sites in the enzyme and that one of them must be unoccupied to produce the sigmoidal curve shape. to test the hypothesis that the site involved in the curve shape determination was also a calcium site, the effect of Ca^{2+} on the curve shape was examined in detail using an NTA-calcium buffer system. Under these conditions it was found that by increasing Ca^{2+} concentrations towards saturation, the degree of curvature decreases and the hydrolysis curve becomes more linear (**Fig 3-2-2-1 d**).

Fig 3-2-2-1 c, The hydrolysis of dioctanoyl and didecanoyl phosphatidylcholine derivatives by the basic non-toxic phospholipase A_2 from*Naja mossambica mossambica* .



The hydrolysis reactions were carried out by injecting 2µl of 0.1mg/ml of the basic non-toxic PLA_2 from *Naja mossambica mossambica* into conductivity cells containing 2ml of 10mM triethanolamine /HCl buffer pH 8.0 at 37 °C and 20 µl of 50mg/ml of DiC_8PC (○) and $DiC_{10}PC$ (△).

Fig3-2-2-1d, The effect of Ca^{2+} on the hydrolysis curve shape.



The reactions were carried out by injecting 2μl of 1mg/ml of the basic non-toxic PLA_2 from *Naja mossambica mossambica* into conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 in the presence of 1mM nitrilotriacetic acid (NTA) and 20μl of 40mg/ml $DiC_{10}PC$.

The reaction rates were measured as described elsewhere and plotted as a ratio of the active (late phase) rates to the initial rates against different calcium concentrations.

3-2-2-2) The effect of other divalent cations.

In order to find out whether or not divalent cations were involved in these phenomena the actions of Barium and Zinc were investigated. These divalent cations, which have been reported to be the most powerful inhibitors of many PLA_2 enzymes, were tested for their effect on the hydrolysis curve shape. Attempts to study barium inhibition in the presence of calcium/NTA showed a serious artefact, in that only a limiting degree of inhibition could be obtained. It was clear that barium , at high concentrations was pushing Ca^{2+} out of the NTA complex, therefore the interaction of different metal ions in a single chelating system was considered to be too complex to be analysed here. In the case of zinc, the affinity for NTA is so high that it is not possible to have significant zinc concentration in the presence of Ca^{2+}/NTA system. Therefore all measurements were done in the absence of chelators. This, on the other hand, had the problem that the initial concentration of Ca^{2+} and of inhibitory metal ion species could not be precisely known and compensated for.

The results of these studies were quite clear and showed that Ba^{2+} inhibited the enzyme without changing the curve shape, in the same manner as lowering Ca^{2+} concentration. In contrast, Zn^{2+} had a completely different effect and showed a typical hyperbolic shape similar to that observed in the absence of transition metal ion chelators (**Fig 3-2-2-2 a**).

One unexpected consequence of these results was the observation that zinc inhibited the enzyme much more strongly at high than at low calcium concentration. This finding profoundly alters any previously held views on the mechanism of action of the zinc ion. In contrast, inhibition by barium was completely in accord with conventional competitive inhibition (**Fig 3-2-2-2 b**). Comparison of the effects of the two metal ions is very clearly shown in **Fig 3-2-2-2 c**. The conclusion from this work is that an active form of the enzyme (ie. a form with a bound calcium ion) must also bind a zinc ion if its kinetic character is to be altered. This is very clear

Fig 3-2-2-2 a, The effect of Ba^{2+} and Zn^{2+} on the shape of the hydrolysis curve of $DiC_{10}PC$ catalysed by the basic non-toxic PLA_2 from*Naja mossambica mossambica* .

The hydrolysis reactions were carried out by injecting 2µl of 1mg/ml PLA_2 into a conductivity cell containing 2ml of 10mM triethanolamine buffer pH 8.0 and 20µl of 50mg/ml substrate (A), and 4µl of 1mg/ml PLA_2 to cells containing 0.5mM Ba^{2+} (B) and 25µM Zn^{2+} (C) both in the presesnce of 0.1mM $CaCl_2$.

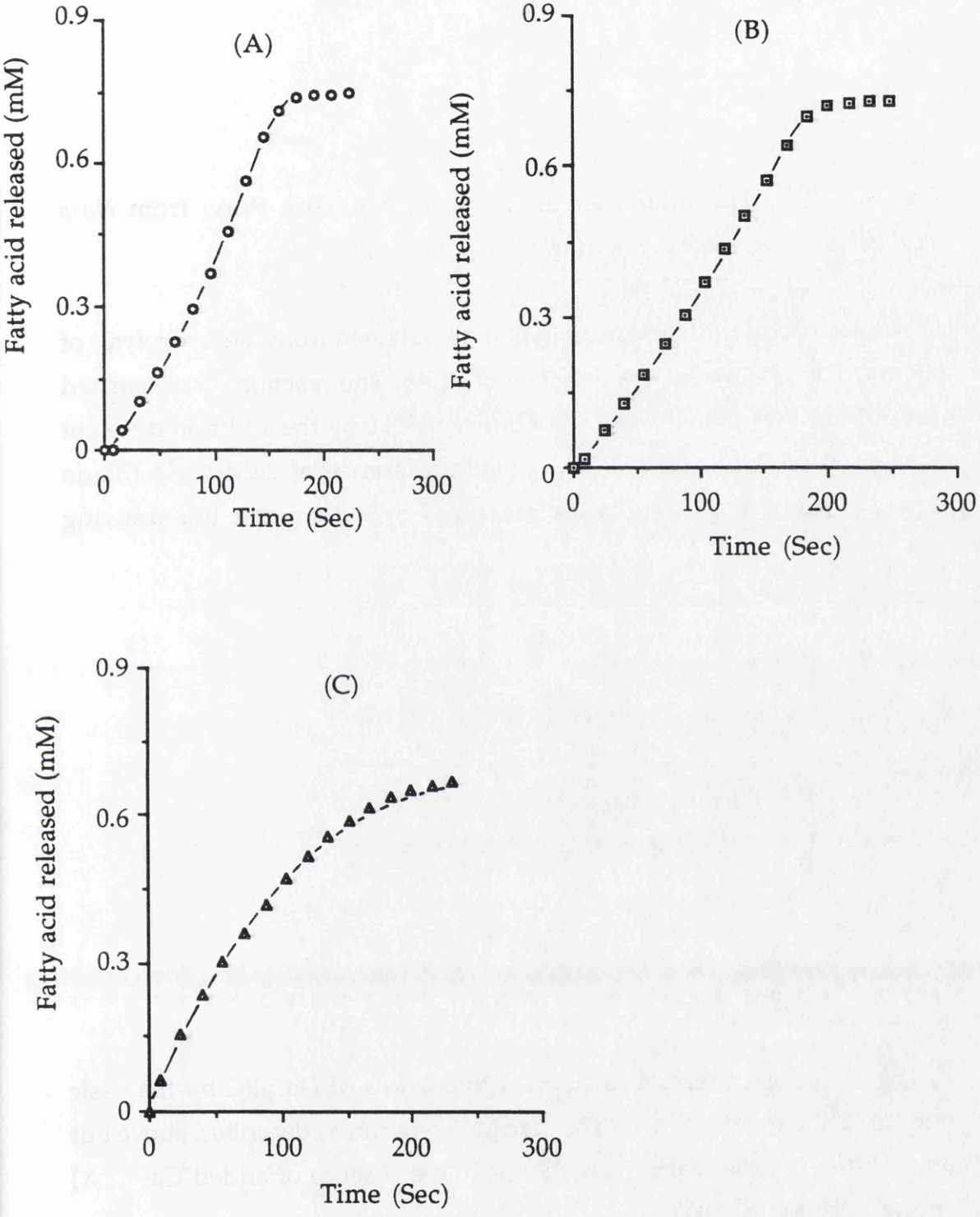

(A)
(B)
(C)
Fatty acid released (mM)
Time (Sec)
0.9
0.6
0.3
0
100
200
300

Fig 3-2-2-2 b, The inhibition of the basic non-toxic PLA_2 from *Naja mossambica mossambica* by barium (1) and zinc (2).

1) The reciprocal plot of barium inhibition obtained from the hydrolysis of 0.6 mM DiC_8PC by 2µl of 0.1mg/ml of PLA_2. The reactions were carried out in 10mM triethanolamine/HCl buffer pH 8.0 by the addition different concentrations of of 0.5mM $BaCl_2$ in the presence of 2µM Ca^{2+} (ie no added $CaCl_2$). Initial rates were measured by a computer line-drawing program.

2) The inhibitory effect of zinc on the hydrolysis of DiC_8PC by the basic non- toxic PLA_2 from *N m m*. The reaction were run as described above but by carrying out the reactions with zinc in the absence of added Ca^{2+} (Δ) and with 1mM Ca^{2+} (○).

Fig 3-2-2-2 b, The inhibition of the basic non-toxic PLA_2 from *Naja mossambica mossambica* by barium and zinc.

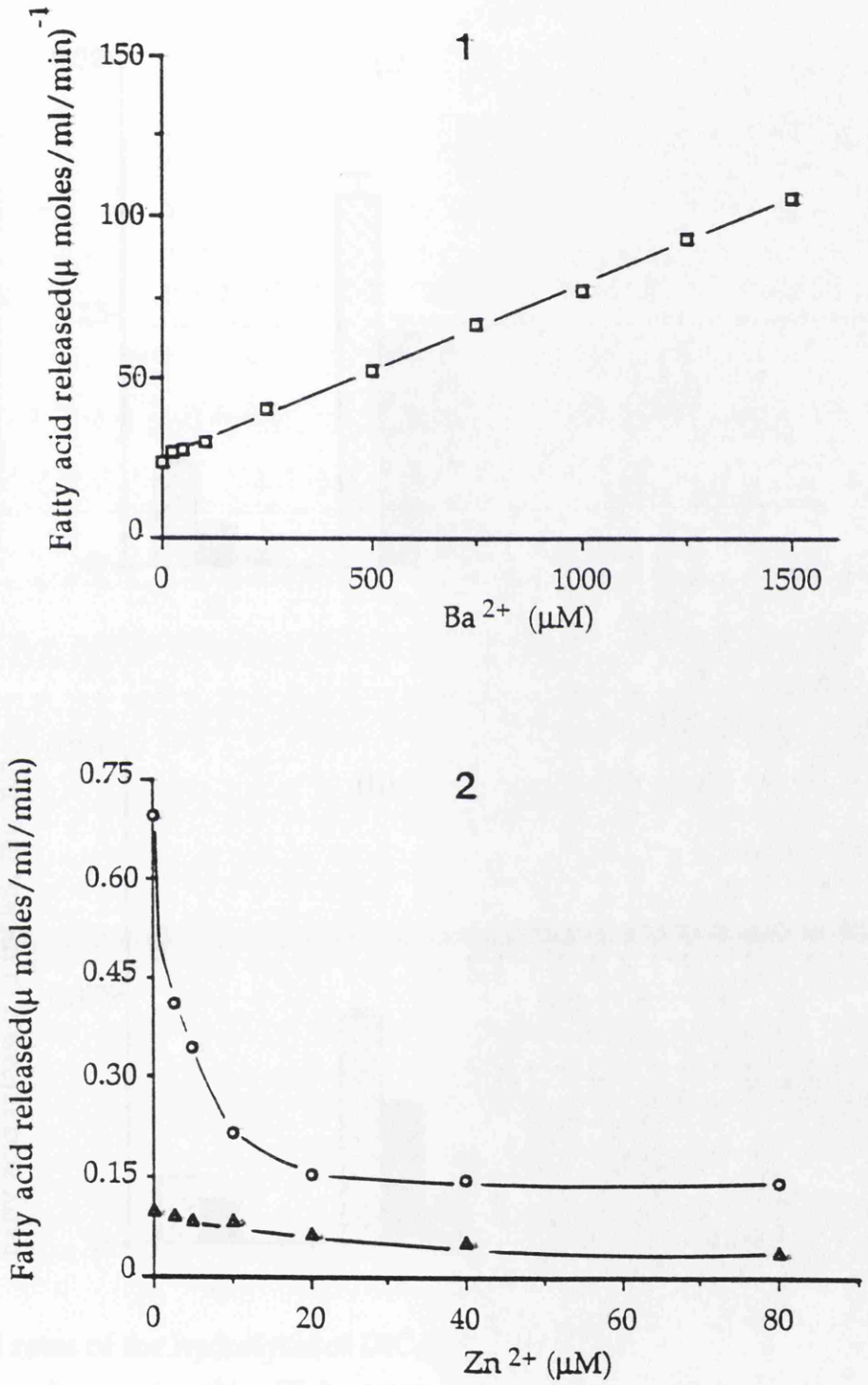
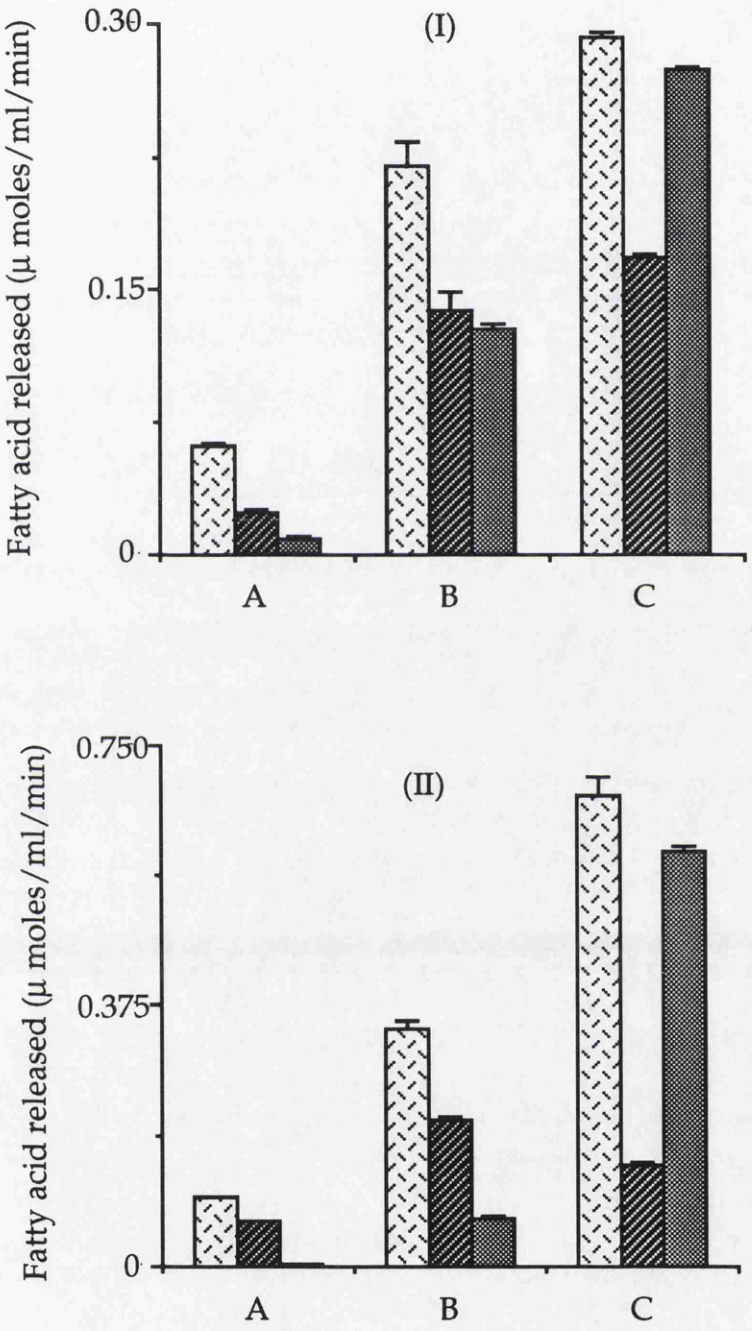


Fig 3-2-2-2C, The inhibition of bee venom and the basic non-toxic PLA_2 from *Naja mossambica mossambica* by barium and zinc.



Initial rates of the hydrolysis of DiC_8PC by bee venom (I) and CM-II from *Naja mossambica mossambica* PLA_2 were measured as described elsewhere, from reaction carried out by injecting the enzyme into cells containing 2ml of 10mM triethanolamine/HCl buffer at pH 8.0 (A) in the absence of added calcium (▨) with 20μM zinc (▩) and 0.5mM barium (■)
(B) in the presence of 0.1mM $CaCl_2$ and (C) at 1mM $CaCl_2$.

and direct evidence that the enzyme has two metal ion binding sites. The results also raise the possibility that the second metal ion binding site must also bind calcium.

3-2-3) Calcium requirement for phospholipase A_2 enzymes.

A full kinetic analysis was carried out in order to investigate the Ca^{2+} requirement of some phospholipase A_2 enzymes to see if there was any direct evidence for the existence of two kinetically significant calcium binding sites. The concept of a second site had already been raised by Drainas (1978) and Chettibi (1990), studying the bee venom phospholipase A_2, but the data were not adequate to prove this conclusively.

3-2-3-1) Ca^{2+} dependence of bee venom phospholipase A_2.

The calcium dependence of bee venom PLA_2 was then studied in the presence of 1mM nitrilotriacetic acid using a value of 2μM for the residual calcium concentration (see above). Plots of the reaction rates derived from the hydrolysis of dioctanoyl phosphatidyl choline substrate at different calcium levels either by the double reciprocal method or by the Eadie Hofstee method, were non-linear and could be analysed in terms of two, or possible more calcium binding sites and they also enabled the dissociation constants for these sites to be estimated. (**Fig 3-2-3-1 a, b**). Very similar results were also obtained for the non-toxic basic isoform PLA_2 from *Naja mossambica mossambica* although the quantitative features of the curves were slightly different (**Fig 3-2-3-1 c, d**). This data indicated that two quite distantly related enzymes both had more than one calcium ion present at, or near, the active site.

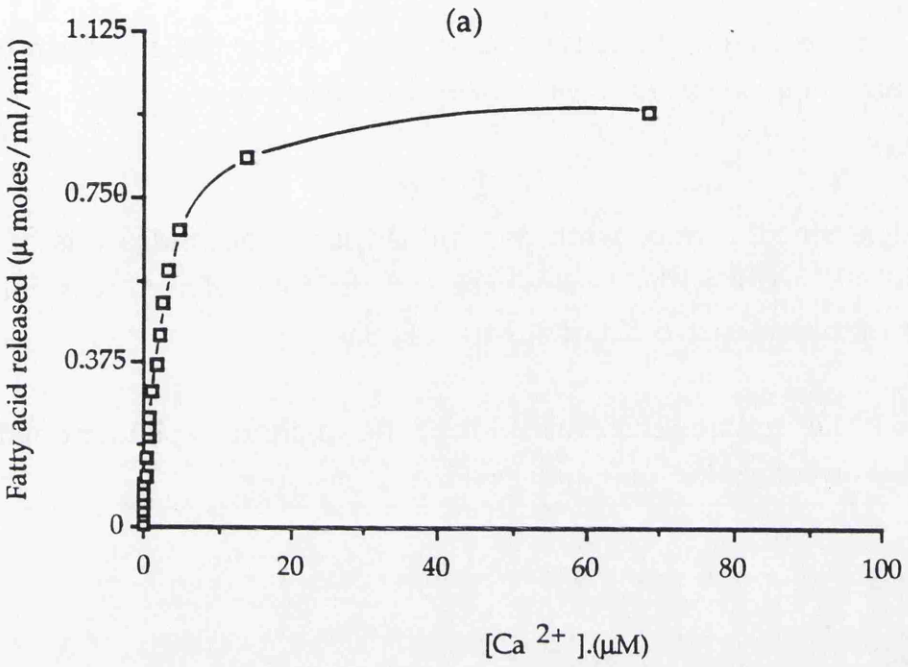
Measurements of this kind were found to be very hard to make. The accuracy required to show convincingly that the double reciprocal plots were not linear, was not present at low substrate concentrations and the strongest conclusion that could be drawn from this kind of data is that the results do not support the model of a single essential calcium ion.

Fig 3-2-3-1 a,b:

a) Activation of bee venom phospholipase A_2 by calcium

Reaction rates were determined by conductimetric measurments of the hydrolysis of 0.6mM DiC_8PC in 10mM triethanolamine/HCl buffer pH 8.0 at 37 °C, containing 1mM nitrilotriacetic acid by 2µg PLA_2. Calcium concentrations were varied by adding $CaCl_2$ and free calcium concentrations were calculated from the measured Kd as described in the text.

b) The double reciprocal plot of the calcium dependent activity of bee venom phospholipase A_2, plotted from Fig (a) above.


(a)
1.125
0.750
0.375
0
Fatty acid released (μ moles/ml/min)
0
20
40
60
80
100
[Ca 2+].(μM)

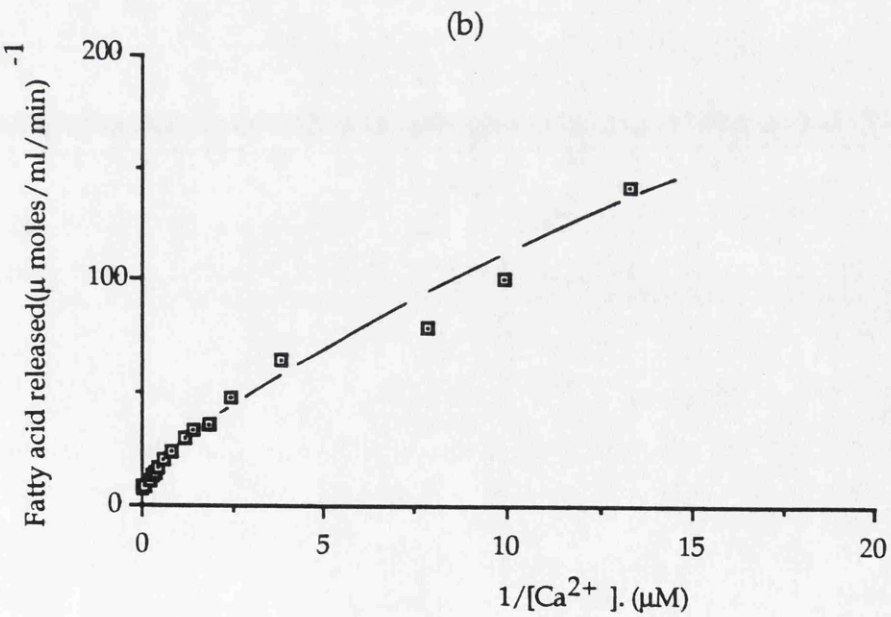
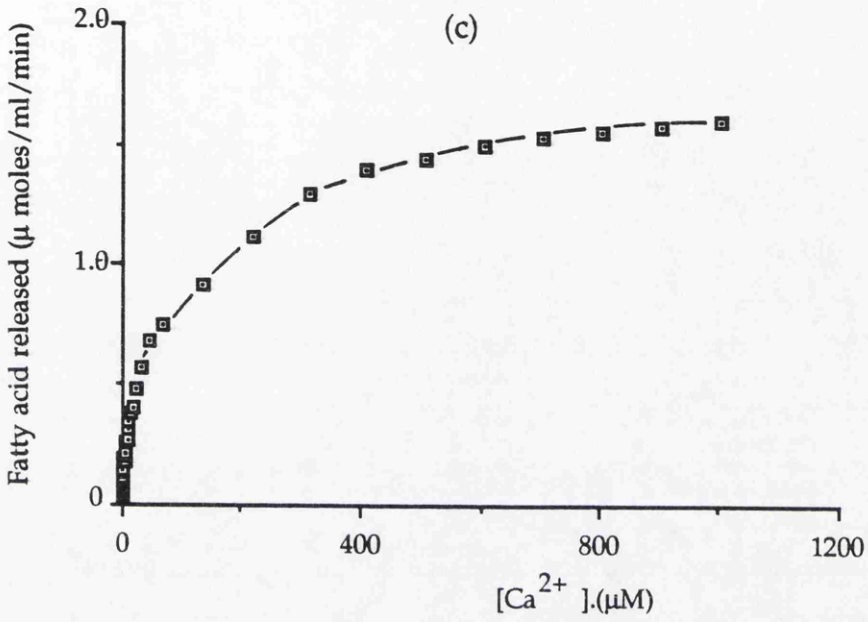
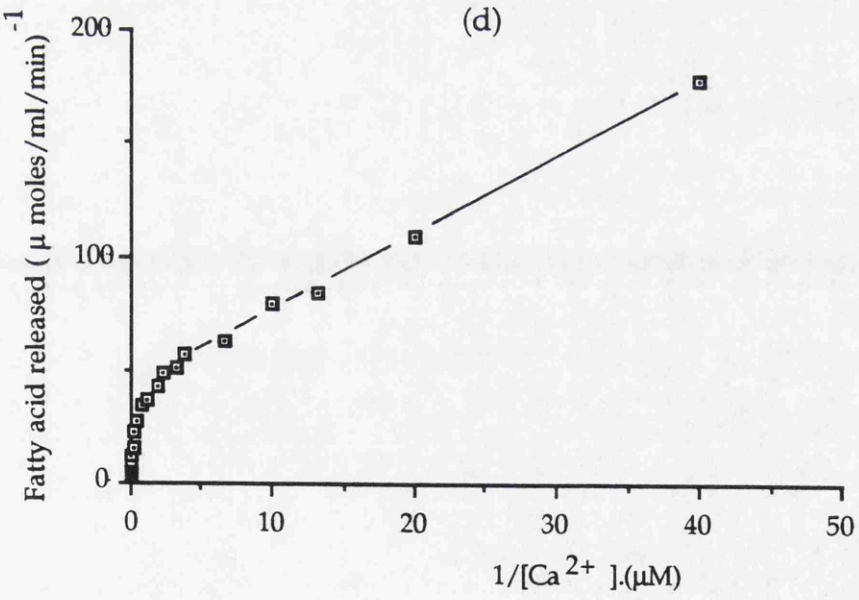

(b)
200
100
0
-1
Fatty acid released(μ moles/ml/min)
0
5
10
15
20
1/[Ca2+]. (μM)

Fig 3-2-3-1-1 c, d: Activation of the non-toxic basic isoform of PLA_2 from *Naja mossambica mossambica* by calcium.

The reaction rates were determined from the hydrolysis of 0.6mM DiC8PC as described elsewhere and the free calcium was calculated from the measured Kd of 8μM at pH 8.0.

d) The double reciprocal plot of the calcium dependent activity of *Naja mossambica mossambica* PLA_2,


(c)
2.0
1.0
0
Fatty acid released (μ moles/ml/min)
0
400
800
1200
[Ca 2+].(μM)


(d)
200
100
0
Fatty acid released (μ moles/ml/min) -1
0
10
20
30
40
50
1/[Ca 2+].(μM)

Nevertheless it supports, rather than conflicts with the other evidence given above.

3-2-3-2) Metal ion inhibition at high calcium concentration.

The most striking observation obtained here was the fact that zinc was a powerful inhibitor at high, but not at low calcium concentrations. Thus zinc was a good inhibitor under conditions where the reaction progress curves were hyperbolic. Theory predicts that inhibition of a metal binding enzyme by freely competing ions should give the same kinetic behaviour for all metals. Even though this did not appear to be true, nevertheless an attempt was made to compare the kinetic properties of a number of metal ion inhibitors at high calcium concentrations where the shape of the progress curves was hyperbolic. Under these conditions a new curve-shape phenomenon became apparent. The conventional hyperbolic curves seen at high calcium concentrations were seen to become much more strongly curved with some metal ions than others. This was tested by a computer facility that enabled progress curves to be altered on both the x and the y axes. Using this method it was possible to adjust curves for many metal ions to correspond in shape with those found at high calcium concentration. Inhibitory ions that behaved in this way are called type I below. In contrast another group of metal ions produced curves that could not be matched by this method these are called type II.

Metal ions	R_M/R *	R_M/R **	R_M^{**}/R_M^*
---	---	---	8.50
Zn^{2+}	0.370	0.13	2.80
Cd^{2+}	0.860	0.30	2.90
Mn^{2+}	1.000	1.00	8.50
Cu^{2+}	0.660	0.91	13.0
Co^{2+}	0.270	0.75	30.0
Pb^{2+}	0.097	0.52	42.0
Ba^{2+}	0.045	0.76	135

Table 3-2-3-2, The effect of divalent metal ions on the action of the basic non-toxic PLA_2 from *Naja mossambica mossambica* venom on 20µl of 40mg/ml DiC_8PC.

All metal ions were used at 0.5mM concentration, but only 25µM zinc.

R_M = Rate obtained in the presence of metal ion

* = reactions carried out with no added calcium (ie 2µM Ca^{2+})

** = Reactions carried out at 1mM Ca^{2+}

R_M^{**} = Rates obtained in the presence of metal ions at 1mM Ca^{2+}.

R_M^* = Rates obtained in the presence of metal ions at 2µM Ca^{2+} .

In the class of type I ions the inhibitory power was:-

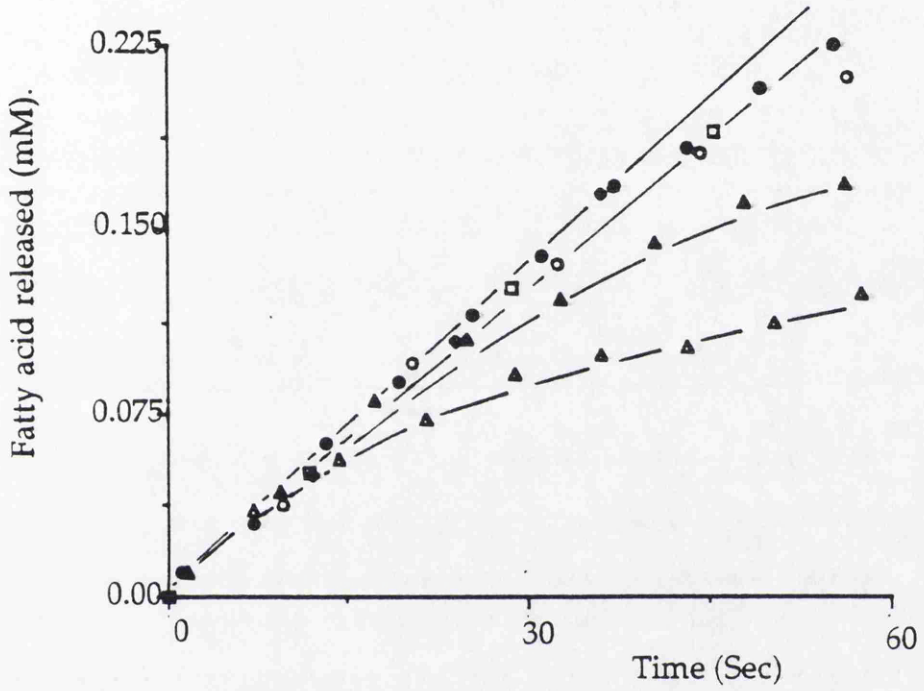
$Ba^{2+} > Pb^{2+} >> Co^{2+} > Cu^{2+} > Mg^{2+} > Mn^{2+}$.

In the class of type II ions the inhibitory power was:-

$Zn^{2+} > Cd^{2+}$.This was a very clear cut distinction and was of particular interest because of the close chemical relationship between Zn^{2+} and Cd^{2+} despite the quite big difference in crystal radii (**Fig 3-2-3-2 a**).

A further test of the inhibitory mechanism was to determine the effect of the metal ions on substrate affinities. This was attempted by comparing data for the dependence of rate on DiC_8PC concentration at high and low calcium concentrations with the effect of zinc at high calcium levels. the results (**Fig 3-2-3-2 b**), show that substrate affinity is not decreased by decreasing calcium as predicted from the single site model, but there is some indication that affinity might increase at low calcium concentration,

Fig 3-2-3-2 a, The effect of different divalent cations on the hydrolysis curve shape of DiC_8PC.

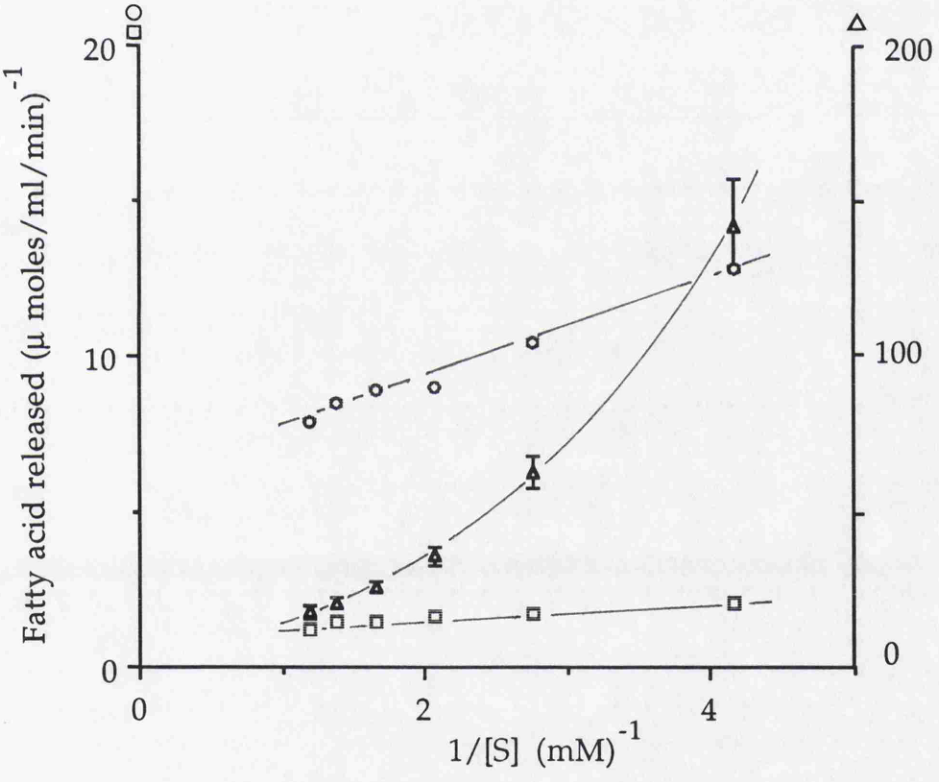


Normalised reaction progress curves for the hydrolysis of 0.3mM dioctanoyl phosphatidyl choline by the basic non-toxic PLA_2 from *Naja mossambica mossambica* under the conditions described elsewhere but in the presence of :-

a) 2μM Ca^{2+} (□), b) 1mM Ca^{2+} (■) or with 1mM Ca^{2+} and c) 0.5mM Ba^{2+} (○) , d) 0.5 mM Pb^{2+} (●), e) 20μM Zn^{2+} (△) and f) 0.5 mM Cd^{2+} (▲).

The initial rates were measured as described previously and time scales were normalised by a multiplying factor proportional to each initial rate.

Fig 3-2-3-2 b, The effect of zinc on the hydrolysis of different concentrations of dioctanoylphosphatidylcholine by the basic non-toxic PLA_2 from *Naja mossambica mossambica* venom.



Initial rates of the hydrolysis were measured by conductimetric analysis of reactions carried out in the absence of calcium (○) and in the presence of 1mM $CaCl_2$ (□) and 1mM $CaCl_2$ and 25μM zinc (△)

which would not agree with the simple model. The effect of zinc was quite clear. Inhibition increased very rapidly as substrate concentration fell, consistent with a major effect on the K_m term. It was therefore clear that zinc inhibition was quite unlike inhibition by lowering calcium concentrations and this provides further evidence that an active form of the enzyme binds both calcium and zinc.

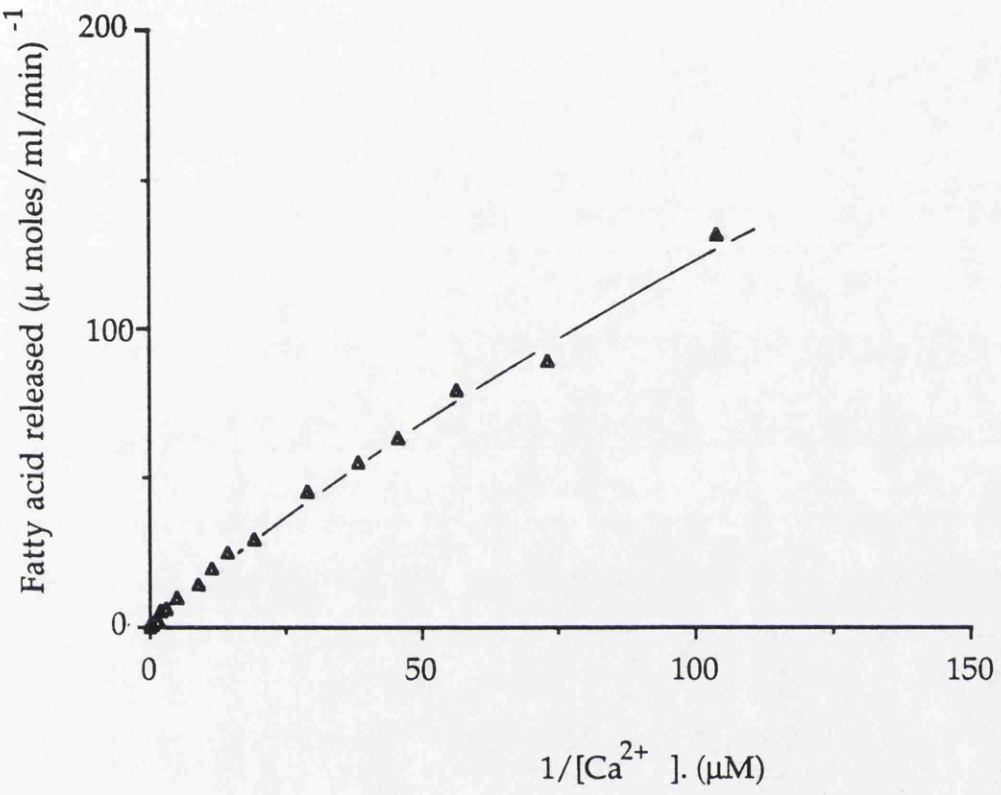
Finally the effect of metal ions was investigated on a substrate present in both monomeric and micellar forms to see if an interfacial phenomenon might be involved. The effect of calcium was determined both above and below the CMC for DiC_6PC together with inhibition data for both Zn^{2+} and Ba^{2+}. Table **3-2-3-2 b** shows the collected inhibition data for these metal ions.

Substrate	Metal ion	R_M/R *	R_M/R **	R_M^*/R_M^{**}
	----	----	----	6.25
(4mM)	Zn^{2+}	0.470	0.41	5.49
DiC6PC	Ba^{2+}	0.085	0.63	46.9
12mM	----	----	----	17.9
DiC_6PC	Zn^{2+}	0.41	0.50	21.25
	Ba^{2+}	0.09	0.86	160.2

Table 3-2-3-2 b, The effects of barium and zinc on the hydrolysis of DiC_6PC by the basic non-toxic PLA_2 from *Naja mossambica mossambica.*

All of these data provide compelling evidence for the two site model, but structural and analytical data to support this model are lacking. However crystallographic data for the PLA_2 enzyme from the formosan cobra, *Naja naja atra,* has shown the presence of a second calcium ion that seems to be involved in the catalytic reaction. The experiments were therefore repeated with this enzyme and substantially identical results were obtained. This included direct kinetic evidence for two calcium binding sites **(Fig 3-2-3-2 c).** Biphasic curves at low calcium concentration

Fig 3-2-3-2 c, Calcium dependence of *Naja naja atra* phospholipase A_2



Initial rates of the hydrolysis of 0.4mM diocatonoyl phosphatidyl choline by *Naja naja atra* PLA_2 were measured by conductimetric analysis of the results obtained by the addition of different concentration of $CaCl_2$ in the presence of 1mM NTA buffer.

that were sensitive to Zn^{2+} and most significantly the fact that Zn^{2+} was a more powerful inhibitor at high than at low calcium concentrations (**Fig 3-2-3-2-d**).

These data suggest that the enzymes from *Naja naja atra* and *Naja mossambica mossambica* have very similar catalytic and modulatory properties.

Further studies with other PLA_2 enzymes showed some quite significant differences. The simplest and most direct test for the two site mechanisms is to examine progress curves in NTA/Ca^{2+} buffers for sigmoidal character. This was done for five phospholipase A_2 enzymes, the basic toxic and the acidic forms from *Naja mossambica mossambica* , the 11' PLA_2 from *Notechis scutatus scutatus* , the dimeric PLA_2 from *Crotalus adamanteus* and the *Naja naja atra* phospholipase A_2. Of these enzymes only the basic toxic form from *Naja mossambica mossambica* and the 11' isoform from *Notechis scutatus scutatus* gave hyperbolic curves (**Fig 3-2-3-2 e**).

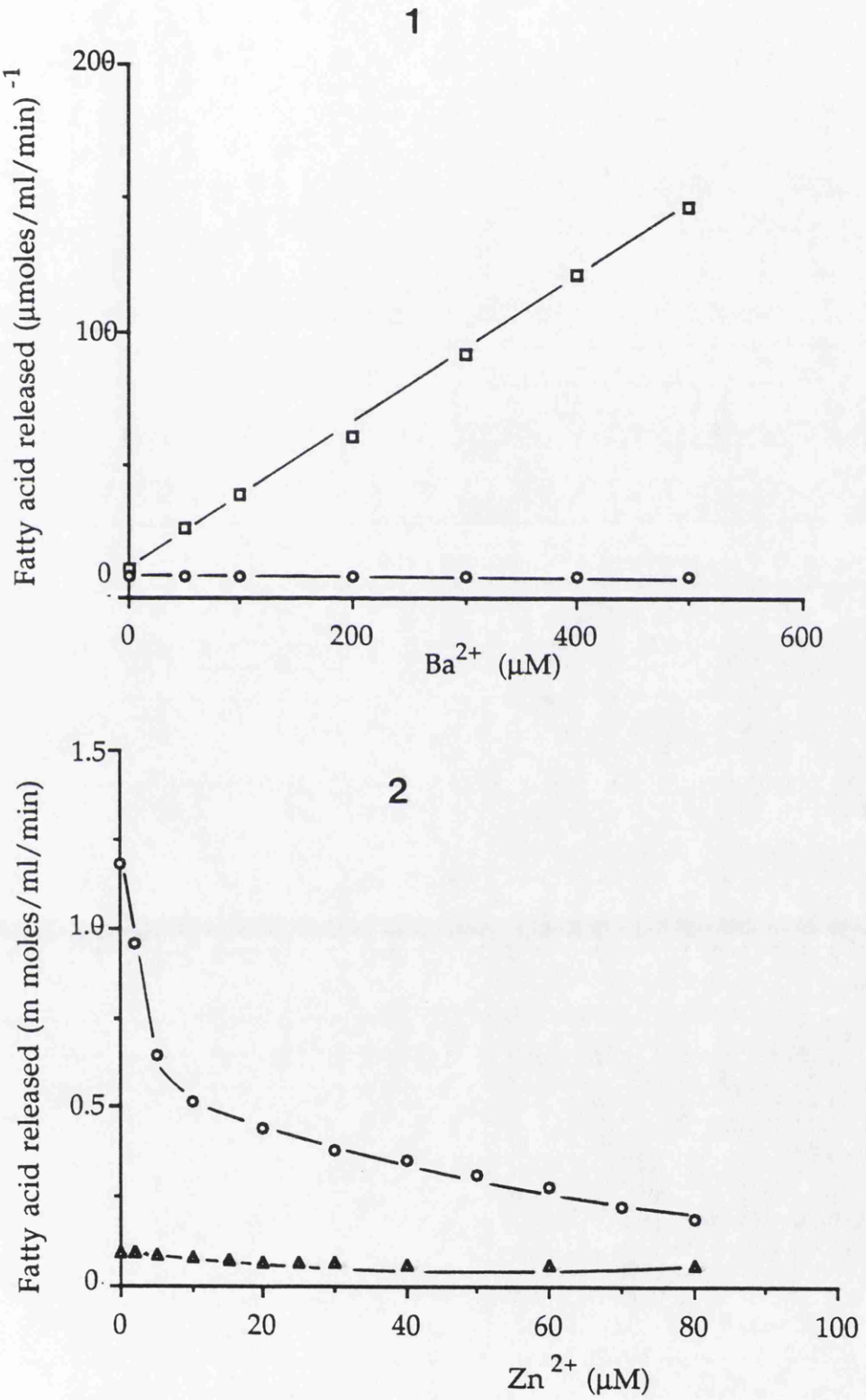
To study this further, the inhibition of these two enzymes by Zn^{2+} and Ba^{2+} was measured at high and low calcium concentrations. In contrast to the results presented above Zn^{2+} was almost equally inhibitory at both high and low calcium concentrations (**Fig 3-2-3-2 f**). One model to explain this result is that occupation of the second calcium site has much less effect on the activity of these enzymes than on the other enzymes.

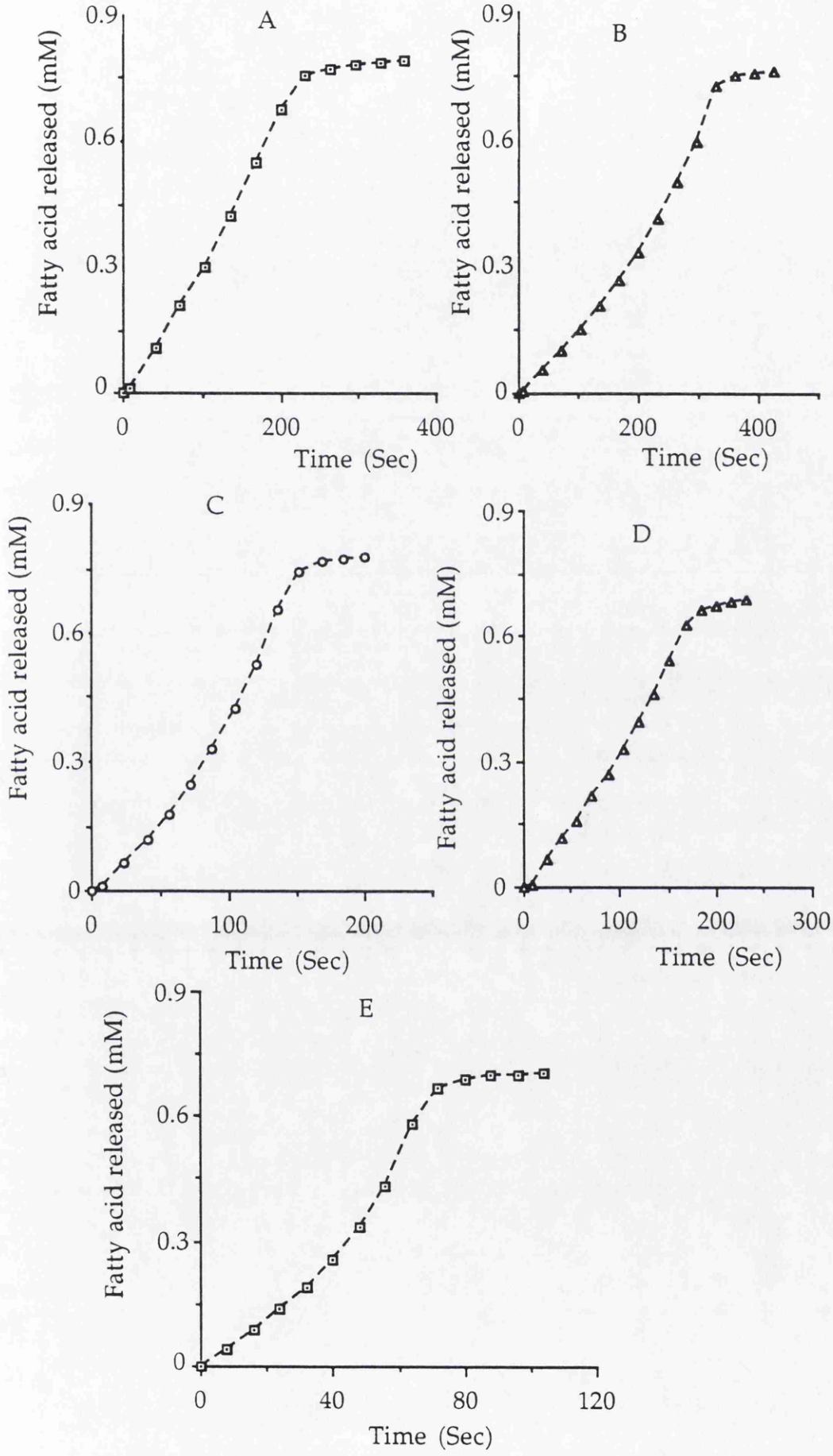
Fig 3-2-3-2 d, The inhibition of *Naja naja atra* phospholipase A_2 by barium (1) and zinc(2).

1) The reciprocal plots of barium inhibition were obtained from the hydrolsis of 0.6mM DiC_8PC by 2μg PLA_2 in 10mM triethanolamine/HCl buffer pH 8.0 in the absence (□) and presence of 1mM $CaCl_2$ (○).

2) The inhibitory effect of zinc on the hydrolysis of 0.6mM DiC_8PC by 2μg PLA_2 in absence (△) and presence of 1mM $CaCl_2$ (○).
The reactions and initial rate measurements were carried out under the conditions described above.

Fig 3-2-3-2 d, Inhibition of *Naja naja atra* PLA_2 by barium and zinc.




A
B
C
D
E
Fatty acid released (mM)
Time (Sec)
0.9
0.6
0.3
0
0
200
400
100
300
40
80
120

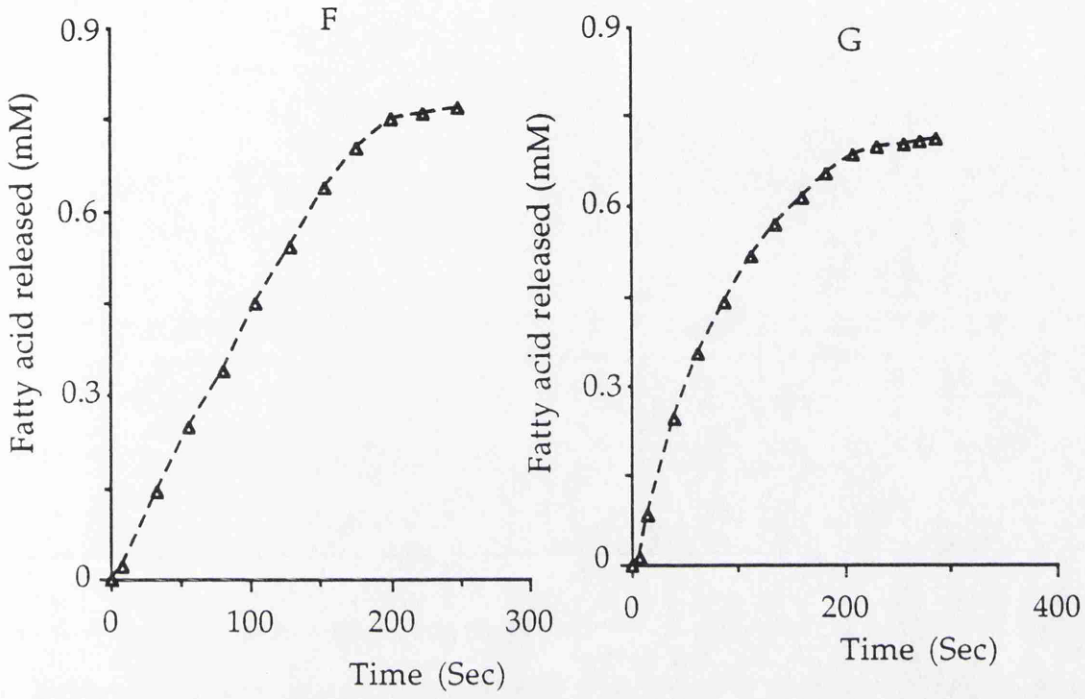


Fig 3-2-3-2 e, The hydrolysis of didecanoyl phosphatidylcholine by different PLA_2 enzymes.

The hydrolysis reactions were carried out by injecting the enzymes into conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 at 37 °C and 20µl of 45mg/ml $DiC_{10}PC$ In the presence of 1mM NTA (nitrilotriacetic acid) and 160µM $CaCl_2$.

A) The hydrolysis progress curve catalysed by 1µg bee venom PLA_2

B) with 1.2µg of PLA_2 from *Crotaleus adamanteus*

C) with 1.8µg of *Naja naja atra* PLA_2

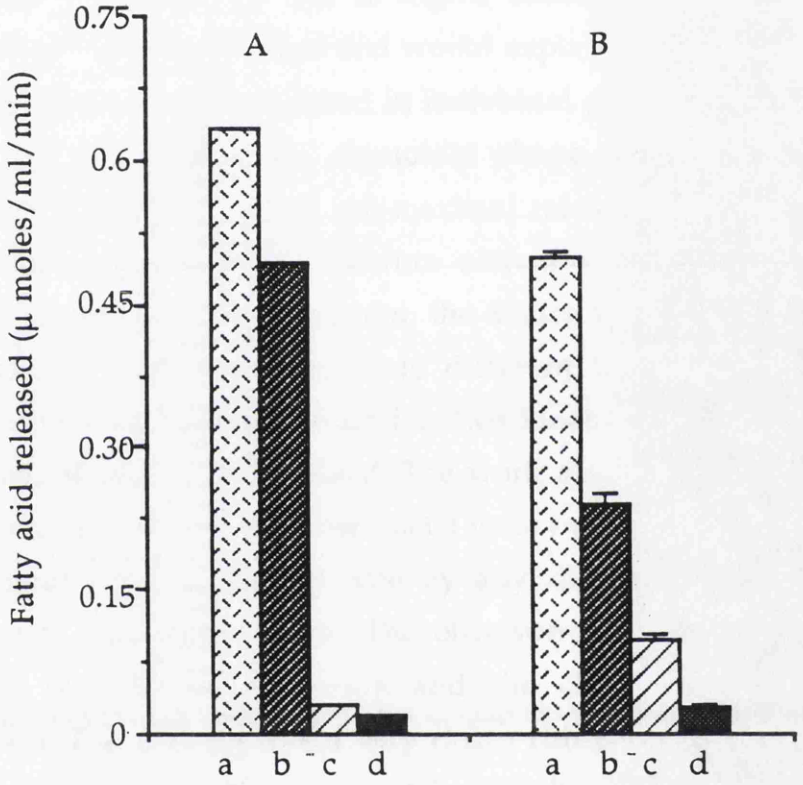
D) with 0.5µg of the basic non-toxic PLA_2 from *Naja mossambica mossambica.*

E) with 2.4µg of the acidic isoform of PLA_2 from *Naja mossambica mossambica.*

F) with 1.2µg of the basic toxic isoform from *Naja mossambica mossambica.*

G) with 1.5µg of *Notechis scutatus scutatus* PLA_2.

Fig 3-2-3-2 f, The effect of zinc on the hydrolysis of DiC_8PC by 11′ PLA_2 from *Notechis scutatus scutatus* and the basic toxic PLA_2 from *Naja mossambica mossambica.*



Initial rates of the hydrolysis reactions were measured as described elsewhere, but by adding 2μl of 1mg/ml of (A) *Notechis scutatus scutatus* PLA_2 and (B) the basic toxic isoform from *Naja mossambica mossambica* to conductivity cells containing 2ml of 10mM triethanolamine/HCl buffer pH8.0 and 20μl of 40mg/ml DiC_8PC with (a) 1mM $CaCl_2$, (b) 1mM $CaCl_2$+ 25μM zinc, (c) no added $CaCl_2$, and (d) No added $CaCl_2$ but with 25μM zinc.

Summary.

Earlier studies of PLA_2 kinetics, carried out in this laboratory, identified a new phenomenon, that the attack on micellar substrates showed sigmoidal progress curves with a relatively sharp rate increase near the end of reaction. Because these substrates undergo the monomer/micelle transition this could be due to higher susceptibility of the monomeric rather than the micellar form and would explain why the expected rate fall at the CMC was never observed in individual progress curves. The present work has shown that the sigmoidal shape is due to the absence of transition metal ions and to sub-maximal calcium concentrations and it is completely absent at full calcium activation. Because the enzyme is inactive in the absence of calcium, the ability to distinguish an activity at low calcium that is qualitiatively different from that at high calcium shows very clearly that calcium has two kinetic roles and that more than one calcium affinity is involved. The work also shows that the sigmoidal shape is abolished by transition metal ions, thus indicating that occupation of a lower affinity calcium site by any divalent ion gives normal as opposed to sigmoidal curves. This observation was extended to study the kinetics of calcium activation and the characteristics of metal ion inhibition. The data provided very clear evidence that metal ion inhibitors fall into two groups that act at different sites namely, barium and large cations group, and zinc/cadmium group. Although the literature shows that some of these enzymes have two calcium binding sites, none has raised the possibility that they may have different affinities for inhibitory ions, or even, in most cases, that there are two kinetically significant sites.

Part Three

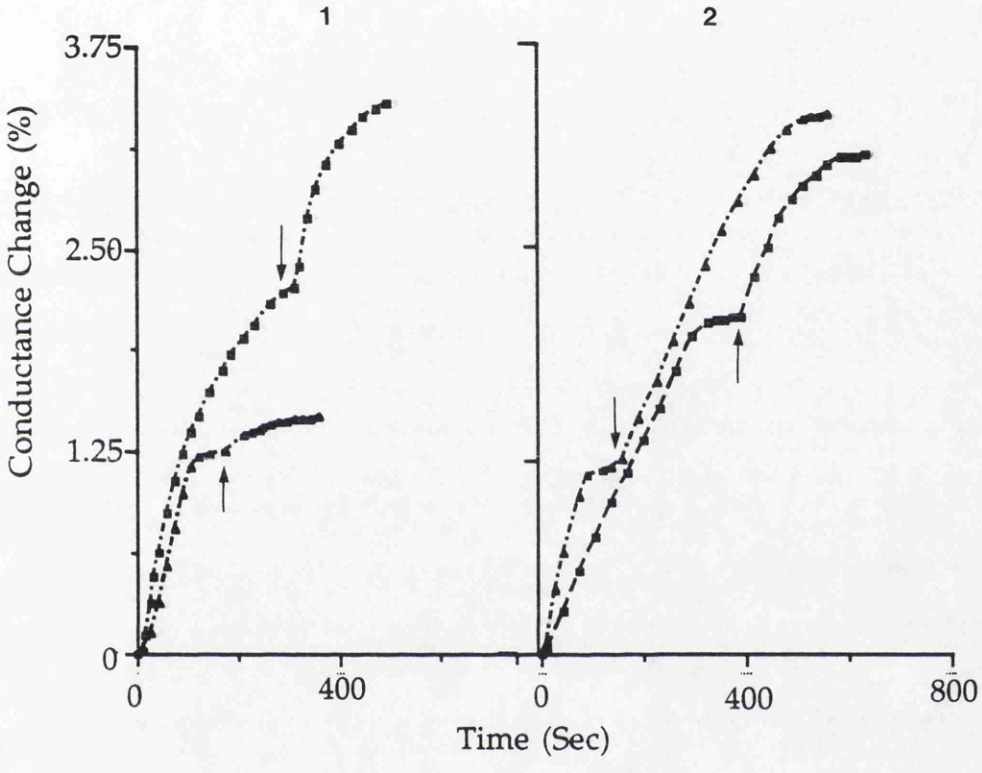
3-3-1) Studies of phospholipase D.

There is only a single report of the use of the conductimetric assay for PLD (Lawrence, 1971) and no account of its application to the study of the PLD catalysed transferase reactions. This work was undertaken to see if the prediction of ion changes would lead to the development of useful assay methods. Although the major interest was in the synthesis of different phospholipid types as substrates for PLA_2 by means of the transferase enzyme, the assay was initially characterised using a non-transferring PLD enzyme from *Streptomyces chromofuscus* using DiC_8PC as substrate in the standard PLA_2 assay conditions. It was clear that a very satisfactory assay could be run. Measurement of substrate conversion allowed the activity to be determined in IUs. Measurement of the rate of conversion of DiC_8PC in comparison with the rate of release of choline from egg lecithin (Sigma catalogue) the enzyme appeared to be 2-3 times more active on the former than the latter compound. However, the sensitivity of the assay was good because the conductance changes were twice those given by the PLA_2 catalysed reaction showing a detection limit in the order of 0.01 IU of enzyme.

This result indicated that the conductimetric assay method is a very useful and a unique method for the measurements of PLD catalysed reactions. Attempts to run PLD and PLA_2 assays sequentially were successful and (**Fig 3-3-1 a**) confirmed that PLD gave conductance changes approximately twice those seen for PLA_2 and that it can be used either before or after PLA_2. Thus PLD can attack lysophospholipids and PLA_2 could attack phosphatidic acid derivatives.

Comparative studies with phospholipase D purified from savoy cabbage by the protocol given in **Scheme 2-6** (see Material and methods) showed some differences in the characteristics of the two enzymes. Firstly, the cabbage enzyme was inactive after PLA_2 showing that it does not attack LPC and secondly, more surprisingly, whilst the bacterial enzyme was totally inhibited by excess EDTA, the cabbage enzyme was only partially

Fig 3-3-1-a, Conductimetric assays for phospholipase D and A_2 enzymes.



The reactions were carried out injecting 20µl of 0.3 mM dioctanoyl phosphatidylcholine into 2ml of 10 mM triethanolamine/HCl buffer pH 8.0 followed by the sequential addition of 2µl of 1 mg/ml bee venom PLA2 or PLD enzymes, when the reactions were over, either 10 µl of cabbage PLD (1) or 2µl of bacterial PLD (2) were added.

The arrows indicate the addition of the second enzyme.

inhibited (**Fig 3-3-1 b, c**), suggesting that the enzyme has a calcium sensitive and a calcium insensitive component of activity. Because the degree of calcium sensitivity decreases throughout the purification it seems most probable that there are two separate enzyme species, or else that calcium sensitivity might be confered on the enzyme by a labile sub-unit.

3-3-2) Calcium dependence of PLD enzymes.

Results obtained at high calcium concentrations showed a feature specific to the conductimetric method, It was very clear that phosphatidic acid derivatives are insoluble in the presence of calcium. Precipitate formation could be seen in the conductivity cells and when it occurred the conductance changes tended to reverse to give sharp conductance falls, This appeared to be due, in part, to hydrophobic interaction between acyl side chains, demonstrated by the fact that the effect was partially reversed by detergent, that it did not occur when lysophospholipids were used as substrates for the bacterial PLD and was greatly reduced when DiC_6PC was used as a substrate. This effect of calcium limited the range of applicability of the assay and it was first necessary to determine the true affinity of the enzymes for calcium.

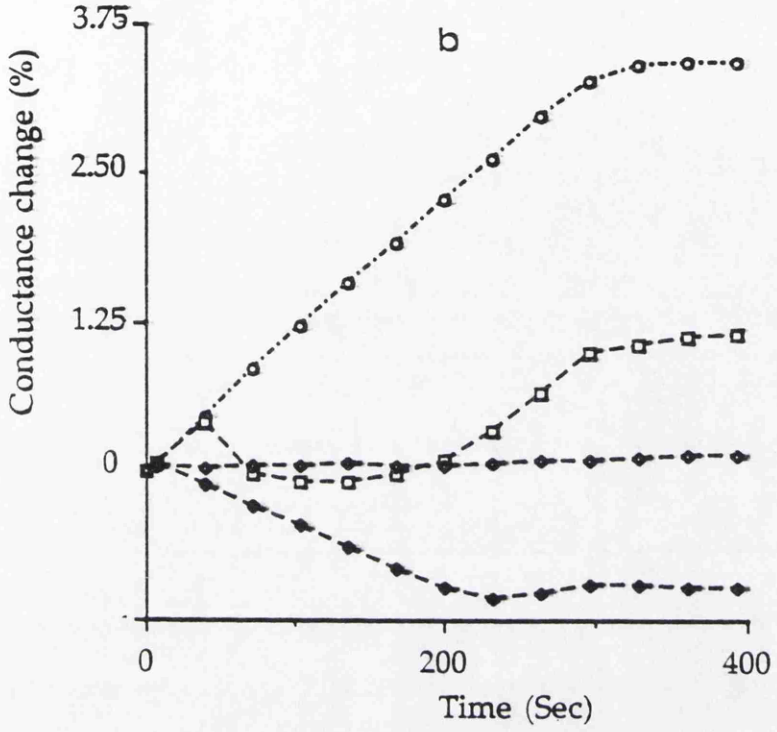
High calcium concentrations limit the extent of the initial phase of reaction that can be measured by conductimetry and this limitation increases with substrate hydrophobicity. Advantage was taken of the fact that lyso-octanoyl phosphatidylcholine was a good substrate for the bacterial enzyme in order to measure the calcium dependence. It was quite clear that calcium levels above those found in distilled water gave no additional activation. Use of NTA buffers to control calcium concentrations showed that the Kca^{2+} was in the order of 2μM. From this result it was concluded that high levels of calcium were not necessary to support enzymic activity. It was clear however that there was a further problem. Because the product PA compounds precipitate calcium, the long-chain derivatives should cause this precipitation when present at very low concentration. This would have two effects; firstly it would limit

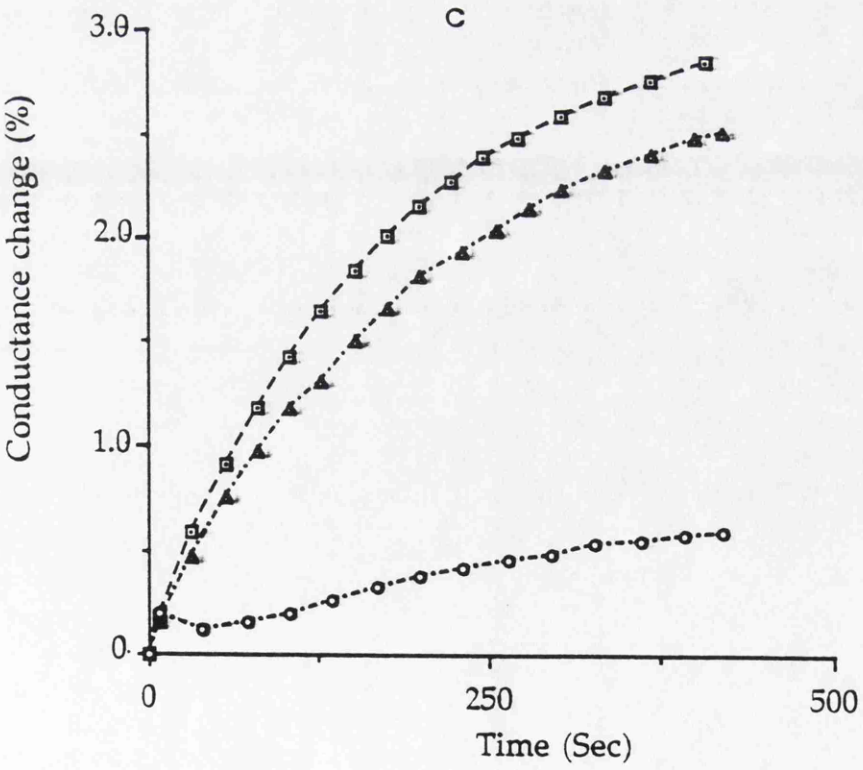
Fig 3-3-1, The effect of Ca^{2+} and EDTA on the action of bacterial and cabbage phospholipase D enzymes on the hydrolysis of dioctanoyl phosphatidylcholine .

b) The reactions were carried out in 10mM triethanolamine /HCl buffer pH 8.0 by injecting 2µl of 1mg/ml of bacterial PLD into cells containing 20µl of 40 mg/ml substrate in the absence (control) of Ca^{2+} and EDTA (○) and in the presence of 1mM EDTA (◇) , 0.1 mM $CaCl_2$ and 1mM $CaCl_2$ (□ ■).

c) Hydrolysis reactions of DiC_8PC by cabbage PLD were carried out as above but by adding 10µl of the enzyme to cells containing the substrates in the absence of added $CaCl_2$ or EDTA (⊡) and with 1mM EDTA(△) and 0.1mM $CaCl_2$ (○)

Fig 3-3-1 b, c. The effects of calcium and EDTA on bacterial and cabbage PLD enzymes.





the linearity of the assay very severely and secondly it would eventually deplete the enzyme itself of the level of calcium necessary for activity.

Because of these limitations, it was very significant that the cabbage enzyme retained activity in the presence of EDTA (see below) and was therefore free from all artefacts of metal ion binding.

3-3-3) The effect of EDTA on the action of cabbage PLD on DiC_8PC.

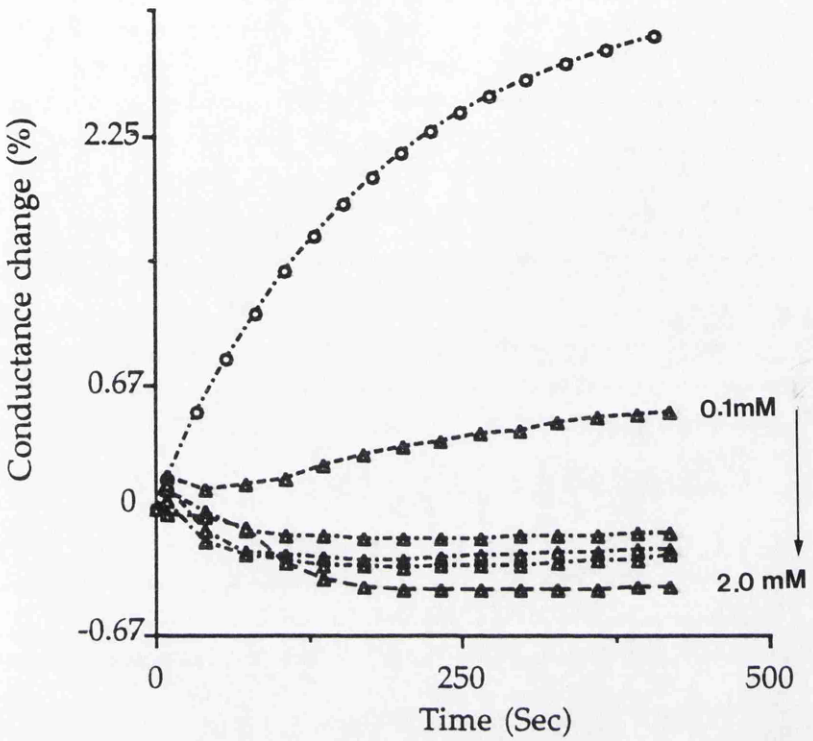
The effect of EDTA was examined using two samples of cabbage enzyme from the third stage of purification (see scheme 2-6-1), ie after acetone precipitation and centrifugation at 13000g. Both the supernatant and the precipitate were assayed for activity in the presence and absence of Ca^{2+} and EDTA. It was found that the supernatant, which had most of the activity, was slightly inhibited by EDTA, whereas Ca^{2+} had a very clear effect on the reaction progress, In the presence of added Ca^{2+} the conductance change was reversed instantly to give a decrease followed by a negligible rise in conductance values , this phenomena was accompanied by the formation of an insoluble compound which is believed to be due to Ca^{2+} binding to the reaction product phosphatidic acid. **Fig 3-3-3 a**, shows the hydrolysis of DiC_8PC by the soluble fraction of the purified cabbage PLD in the presence of EDTA and Ca^{2+}, and **Fig 3-3-3 b**, demonstrates the effect of varying EDTA concentrations. It is very clear that even higher concentrations of EDTA (2mM) did not inhibit the enzyme completely.

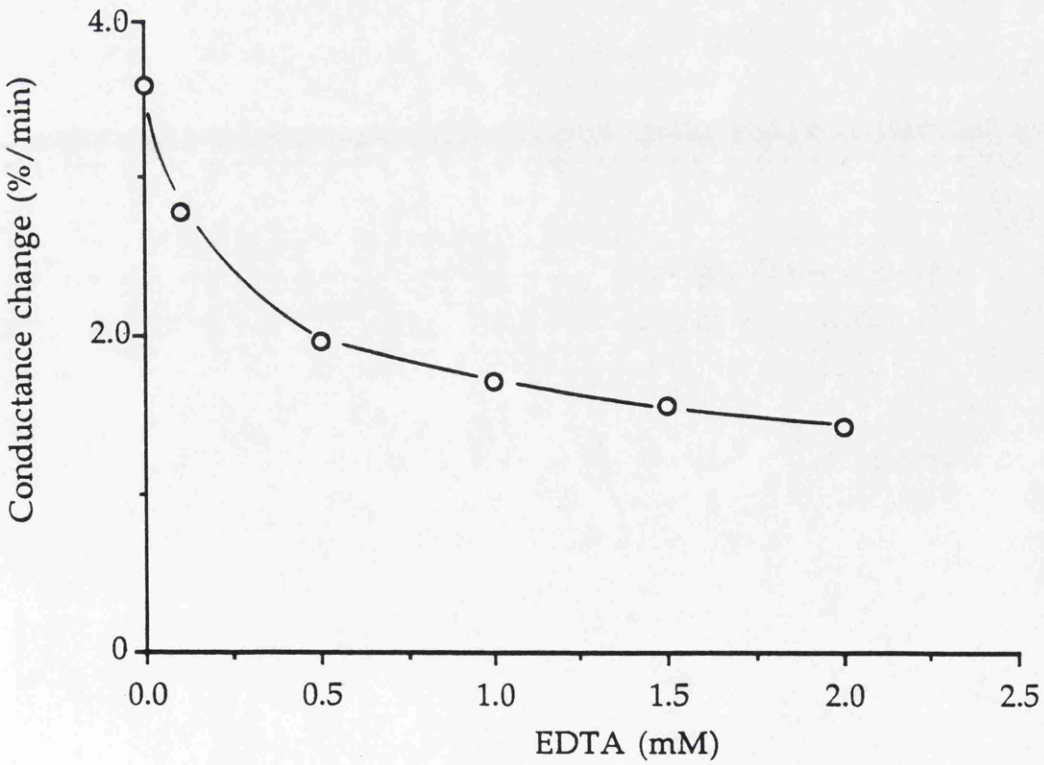
Fig 3-3-3 a, The effect of different Ca^{2+} concentrations on the hydrolysis of DiC_8PC by cabbage phospholipase D.

The reactions were carried out as described before but in the absence (○) and presence of 0.1, 0.5, 1.0, 1.5, and 2 mM Ca^{2+}(△) respectively.

Fig 3-3-3 b, The effect of different concentrations of EDTA on the hydrolysis of dioctanoyl phosphatidyl choline by savoy cabbage phospholipase D.

The reactions were carried out as described elsewhere and the initial rates were measured by the conductimetric analysis of the response as a function of EDTA concentrations.


Conductance change (%)
2.25
0.67
0
-0.67
0.1mM
2.0 mM
0
250
500
Time (Sec)


Conductance change (%/min)
4.0
2.0
0
0.0
0.5
1.0
1.5
2.0
2.5
EDTA (mM)

3-3-4) The effect of pH on PLD catalysed reactions.

Calcium binding to the reaction product PA was a major problem and an unavoidable limitation of assays where enzymes such as PLD require calcium for their activity. This was eventually overcome by carrying out the reactions either with LPC as described above or else by working at low pH where the second phosphate ionization is suppressed. Experiments of this kind were therefore carried out with imidazole buffer pH 5.5 which is near the pK of the second phosphonate oxygen. The results showed good activity at this pH with neglegeble Ca^{2+}/PA formation, but because PLA_2 enzymes show very low activity at this pH, this was not an ideal system for study.

3-3-5) Transesterfication reactions:

These reactions were investigated by the addition of different alcohols to the reaction medium to see whether or not this enzyme could exchange the choline head group for any other acceptor. The equation for the transferase reaction indicates that it should give approximately half the conductance change of that seen in the hydrolytic reaction at high pH and in the presence of a cationic buffer.

Both cabbage and bacterial phospholipase D enzymes were examined for their ability to transfer the phosphatidyl residue to alcohols. The results confirmed that in the case of the bacterial enzyme, alcohols had a very little effect on both, total conductance change and reaction rate. In contrast, alcohols reduced the total conductance change for reactions catalysed by the cabbage PLD enzyme to approximately half of that given by the control hydrolysis reaction, showing that this enzyme is an effective transferase (**Fig 3-3-5**).

These results therefore present criteria for establishing the occurrence of the transferase reaction and show that the method would be useful for studying the effects of a variety of alcohols on the transesterification.

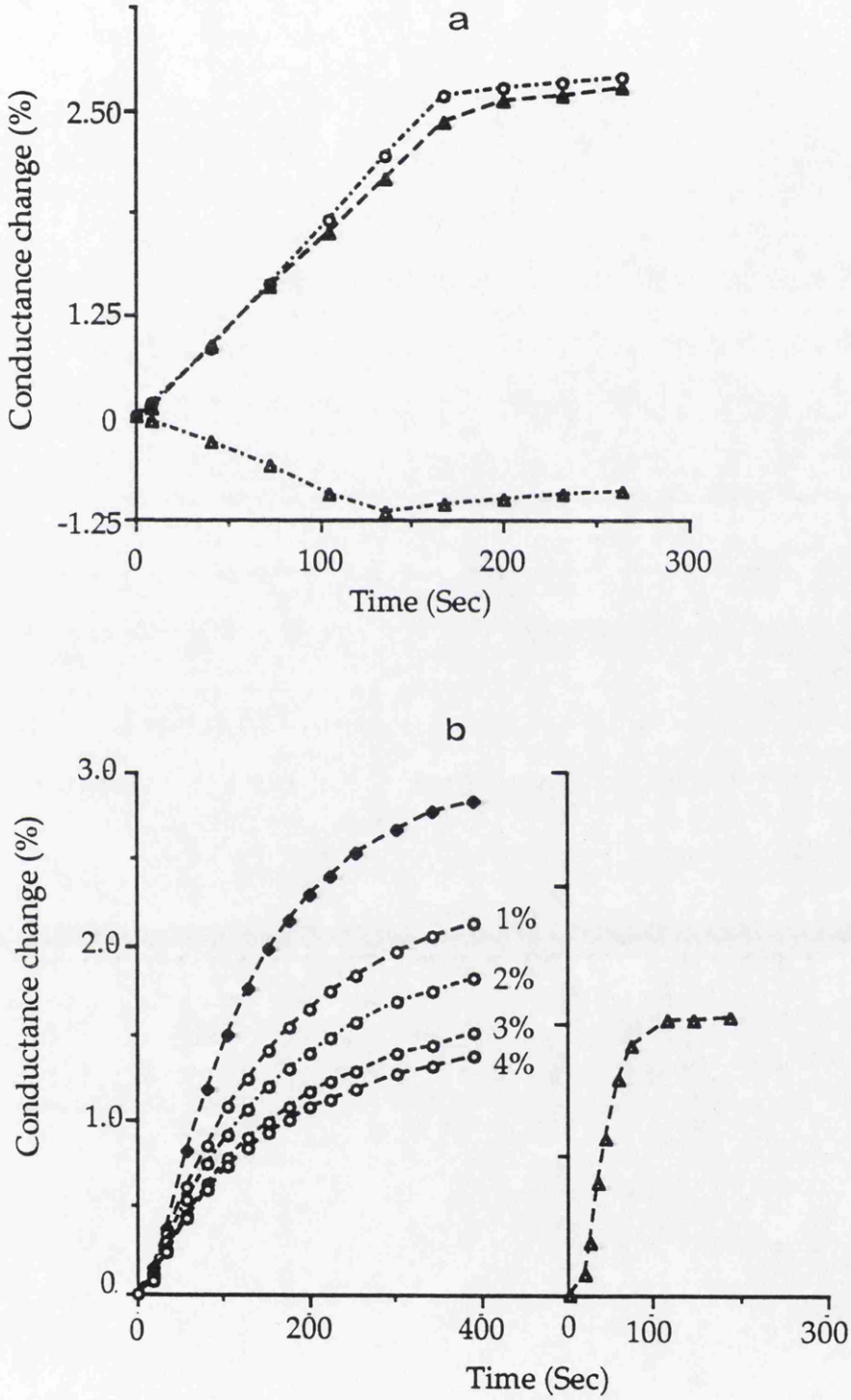
Fig 3-3-5.

a) The reactions were carried out by injecting 2μl of 1mg/ml bacterial PLD into cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20μl of 30mg/ml DiC_8PC in the absence (○) and presence of 0.1mM $CaCl_2$ (△) and 2% ethanol(▲).

b) The catalysis of the transferase reactions by cabbage phospholipase D

Reactions were run as above in the absence (◆) and presence of different concentrations of ethanol. The result was assesed by direct comparaison with the hydrolysis of DiC_8PC by bee venom PLA_2 (△).

Fig 3-3-5- , The effect of ethanol on the hydrolysis of DiC_8PC by bacterial and cabbage phospholipase D enzymes.



3-3-5-1) The effect of different alcohols on the transferase reactions catalysed by cabbage phospholipase D.

The results presented above show that PLD and PLA_2 can be carried out in sequence on the same reaction system. Use was made of this to synthesise different phospholipids by head group substitution using PLD and examine these compounds as substrates for PLA_2, in the first instance without isolating or purifying the derivatives.

Preliminary studies with ethanol confirmed that it supported optimal transferase activity of the cabbage PLD enzyme at a concentration of 2-3%. (**Fig 3-3-5**). Similar results were obtained for other primary alcohols (**Fig 3-3-5-1 a**), but secondary alcohols were found to be very poor acceptors. On the other hand compounds which contained both primary and secondary hydroxyl groups were as effective as the primary alcohols. The use of compounds with varied alkyl chain length showed that long-chain acceptors were a better acceptors than the shorter chain ones (**Fig 3-3-5-1 b**). Ethanolamine was excluded from these tests simply because of the difficulty in controling the pH of the reaction medium .

3-3-5-2) The effect of Ca^{2+} on the transesterification reactions.

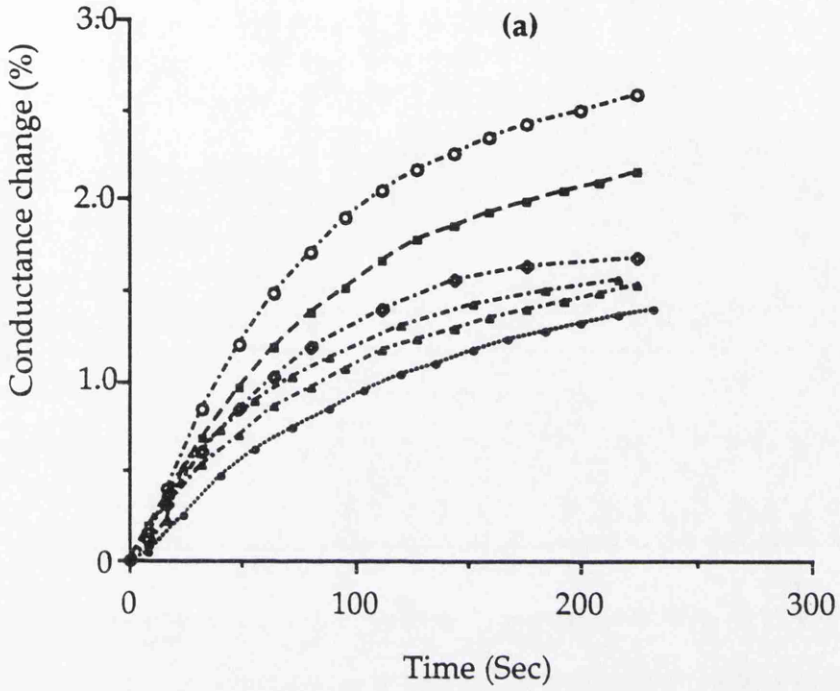
The effect of Ca^{2+} on the transesterification reactions provided further evidence that the cabbage PLD does indeed catalyse the transferase reactions in addition to the hydrolysis reactions. With this enzyme, but not the bacterial enzyme, the presence of alcohols that supported transferase activity greatly reduced the tendency for precipitates to form, or conductances to fall in the presence of high calcium concentration. This is in accord with the model that calcium binds only weakly to the monobasic anions of phosphatidic acid in contrast with the dibasic anions (**Fig 3-3-5-2 a**). This means that the kinetics of the transferase reactions can be followed by the conductimetric method even at elevated calcium concentrations. It is possible to use the results obtained in the presence of high calcium levels to estimate the relative proportions of transferase and hydrolase

Fig 3-3-5-1 a, The hydrolysis of dioctanoyl phosphatidylcholine substrate by cabbage phospholipase D in the presence of alcohols.

The reaction were carried out as decribed elsewhere , but in the absence

(○) and presence of 2% of methanol (▲), ethanol (◆),n-propanol (▲), 2-propanol (●) and butanol (●) .

Fig 3-3-5-1 b, The same as the above but in the absence (○) and presence of 2% of 1,2-propane diOL (●), thiodiglycol (■), 1,3-diethylhexanediOL( ) and 20µl of 100mg/ml serine (▲).


(a)
Conductance change (%)
3.0
2.0
1.0
0
0
100
200
300
Time (Sec)

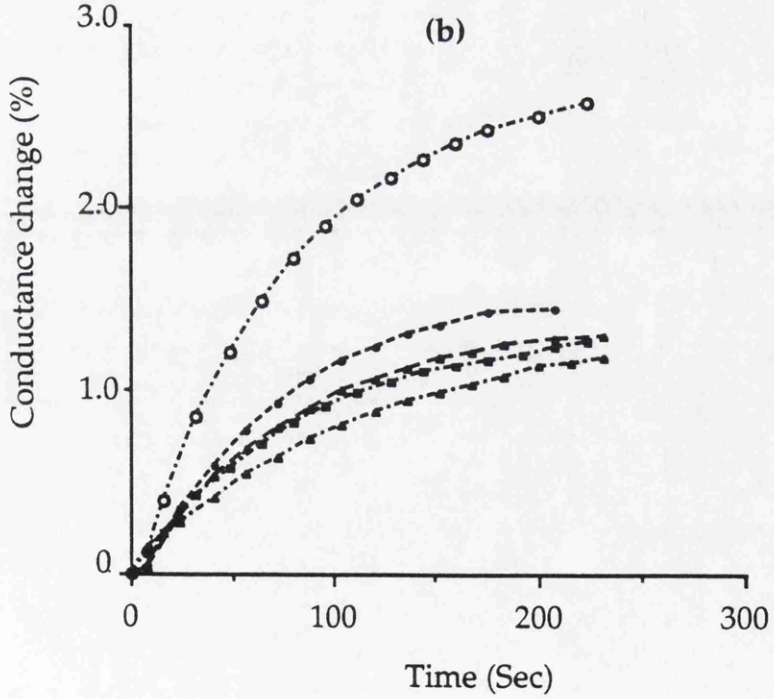
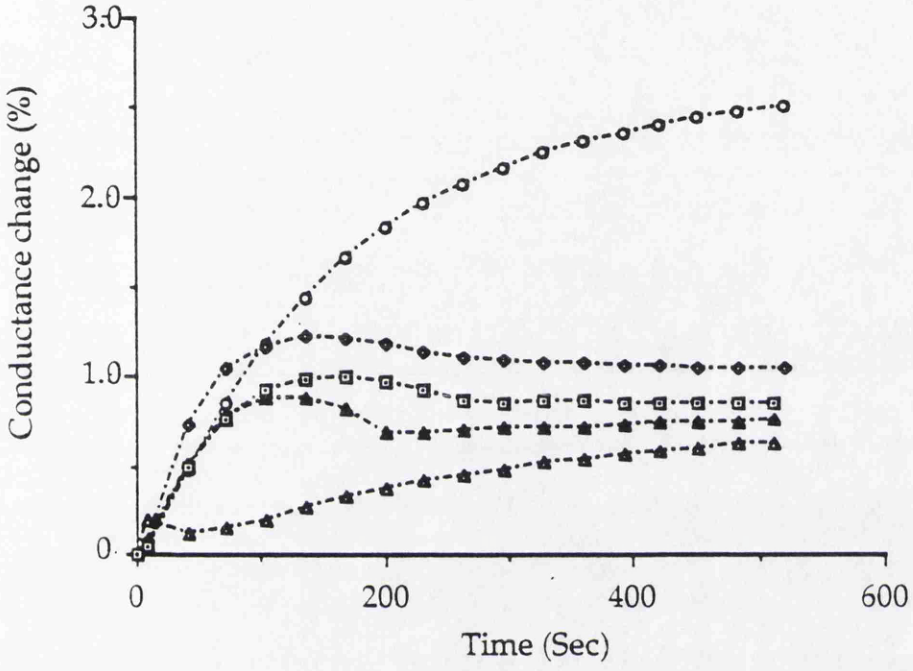

(b)
Conductance change (%)
3.0
2.0
1.0
0
0
100
200
300
Time (Sec)

Fig 3-3-5-2 a, The effect of calcium on the cabbage phospholipase D transphosphatidylation catalysed reactions.



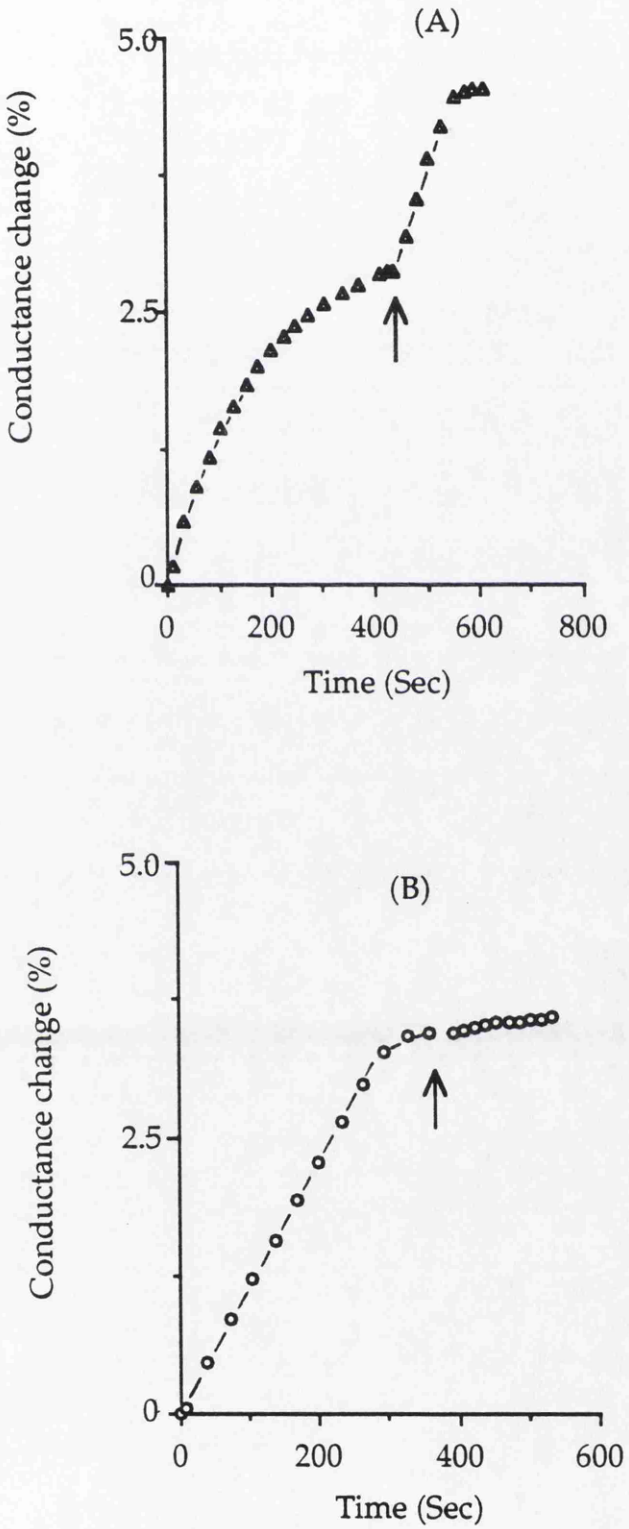
The reactions were carried out by injecting 10µl of PLD into cells containing 2ml of 10 mM triethanolamine/HCl buffer pH 8.0 and 20µl of 0.3mM dioctanoyl phosphatidylcholine in the absence (○) and presence of:

0.1mM $CaCl_2$ (△) and 0.1mM $CaCl_2$ with 1% (▲), 2% (◙) and 3% (◇) ethanol.

activities occurring. The data make it clear that both reactions occur together in a proportion determined by the alcohol concentration, but after the phosphatidyl alcohols are formed they remain stable in the presence of the enzyme. This stability means that they are poor substrates for hydrolysis by the same PLD enzyme.

In this respect it was of interest to generate phosphatidyl alcohols by using the cabbage enzyme and then to test their rate of hydrolysis by the bacterial enzyme. The results, **Fig 3-3-5-2 b,** show that the bacterial enzyme is indeed able to hydrolyse these compounds, indicating that it is not an effective transferase possibly only because it also hydrolyses these products.

Fig 3-3-5-2 b, The effect of the addition order of bacterial and cabbage PLD enzymes on the hydrolysis of DiC_8PC.



The hydrolysis reactions were carried out by injecting 10µl of cabbage PLD and 2µl of 1mg/ml bacterial PLD into cells containing 2ml of 10mM triethanolamine/HCl buffer pH 8.0 and 20µl of 40mg/ml DiC_8PC.

A) Cabbage PLD followed by bacterial PLD.

B) Bacterial PLD followed by cabbage PLD.

The arrows in each case indicate the addition of the second enzyme.

Summary.

Phospholipase D activity was investigated in order to extend the range of substrates available for PLA_2 studies and in particular to use compounds where the physico-chemical properties would be greatly changed. The conductimetric method provides (in principle) an ideal assay for both the hydrolytic and alcohol transferring functions of this enzyme, but no detail investigation of the conductimetric method had been reported in connection with this enzyme.

The assay was shown to be very suitable for monitoring purifications and it lead to some very interesting observations about the specificity and ion dependence of these enzymes.

Firstly it showed that the cabbage enzyme could apparently convert to a calcium independent form during purification and that this enzyme could not attack lysophospholipids. A reason was proposed to explain why the bacterial PLD enzymes could not act as transferases.

The results show very clearly that conductimetry has a major role to play in the investigation of these enzymes.

The study showed that mono-methyl dioctanoyl phosphatidate was a better substrate for PLA_2 than the parent compound and it would be highly desirable to repeat the studies of substrate morphologies with the equivalent methyl-PA series, especially because these series should differ quite considerably in their CMC values.

Chapter Four

Discussion

4-1) Conductimetric enzyme assays.

The kinetic aspects of phospholipase A_2 enzymes have been studied by many different groups and the consensus opinion is that the properties of the enzymes in this very large family are differ only in minor details. Thus the kinetic behaviour of the porcine pancreatic enzyme has served as a model for enzymes from venoms and other sources. There has been general agreement on the role of the metal ion at the active site and in the effect of the substrate-water interface in modulating the behaviour of the enzyme. Nevertheless, none of these groups has described some of the phenomena seen in this present study and which question many of the earlier interpretations. The most striking of these phenomena are the non-competitive aspect of zinc inhibition and the unusual progress curve shapes seen at low calcium levels in the absence of inhibitory transition metal ions. The major difference between our studies and those of other groups is that we alone use the conductimetric assay. It is therefore possible that the present results could be due to artefacts of the method and it has been necessary to assess the method in comparison with other techniques to justify its use and to attempt to confirm that the results it produces represent real phenomena.

In comparison with other applicable assays, such as the titration method, the conductimetric assay has a rapid response time and can respond to very early events. Furthermore, the multi-channel feature allows many reactions, including a range of controls, to be run in the same time and under the same conditions within one single experiment. Assay sensitivity was such that conductance changes as small as 0.01% could be resolved, corresponding to changes of ca 3 μ molar ie (3n moles per ml).

4-2) Kinetic studies of phospholipase A_2 enzymes.

The kinetic behaviour of phospholipase A_2 enzymes is complicated because they act at a lipid-water interface and because the nature of the substrate can be very varied. Most of the definitive studies have used simple water-soluble substrates where interfacial phenomena are avoided. These have given useful information about the nature of the enzyme, but

they cannot give information about control mechanisms that may apply when the enzyme is acting at an interface. At the next level of complexity the action of the enzyme has been studied against simple micelle-forming substrates and these have the added advantage that the enzyme has highest catalytic activity when the substrates are in this state, but again many regulatory mechanisms may not apply. Work with liposome-forming substrates is very much more complicated and one of the aims of this study has been to use substrates on the borderline of the stability of the bilammelar state in order to study the action of the enzyme on both sides of the micelle/liposome transition and its relevance to the phenomenon of acyl group activation.

In using this wide range of substrates, it became clear that some of the basic aspects of PLA_2 kinetics had not been resolved. Thus it was not certain why there was no kinetic response reflecting the monomer/micelle transition for dioctanoyl phosphatidylcholine (DiC_8PC). There were no studies that related metal ion activation to substrate morphology and there were no good data to indicate which of the kinetic parameters are affected by calcium activation. Part of the aim of this work was to use the conductimetric assay to provide the kinetic basis for understanding the mechanism of acyl group activation of a number of PLA_2 enzymes. In the course of these investigations, it became clear that certain kinetic phenomena had not been reported elsewhere. These included unusual aspects of reaction progress curves and responses to metal ion activation and inhibition. It was therefore important to determine whether or not the results of the conductimetric assay were susceptible to experimental artefacts. Particular attention was paid to assay linearity, especially in the presence of divalent cations which could bind to fatty acids. In all conditions that were used here the assay was found to be highly linear for total conductance change <5%. The method is however restricted to the use of fatty acids of chain length <12C in the absence of organic solvents or solublising agents such as albumin. The present work largely stayed within this limitation by using synthetic substrates with short-chain fatty acids in the 2-position.

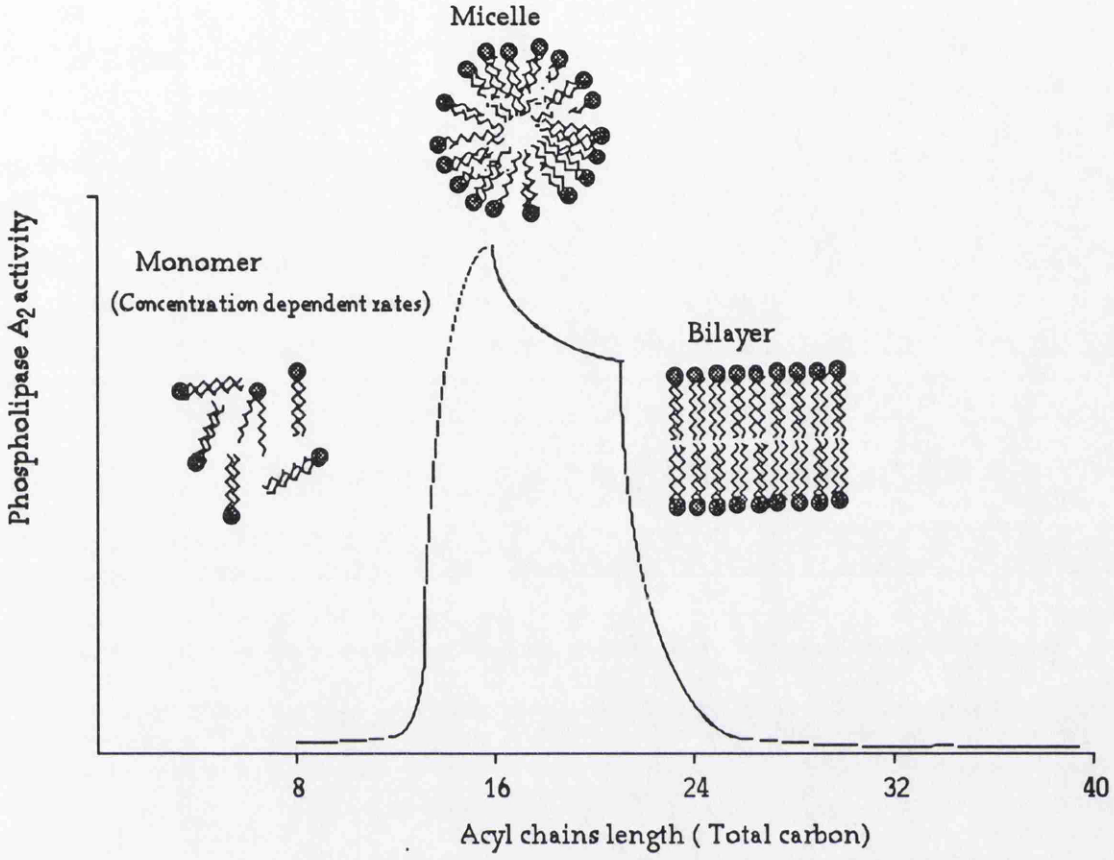
The first aspect of this investigation centred around the problems of the monomer/micelle transition. Shipolini *et al.* had shown that for the bee venom enzyme there was no sharp change in susceptibility of DiC_6PC at the CMC and proposed that the attack might be exclusively against monomer. The present results confirm Shipolini's data that there is no transition and that this enzyme at least is as active against the monomer as the micelle. Indeed these data could be interpreted as showing that the enzyme does not act on the micelle. In contrast, the *Naja mossambica mossambica* enzyme shows clear distinction between the monomer and micelle forms, resembling the pancreatic enzyme in this respect. However, despite an extensive search, no similar effect could be seen for DiC_8PC with either enzyme, but it is quite clear that under the present reaction conditions the enzyme must encounter monomer alone as well as the monomer/micelle mixture. No feature of the results enables any distinction to be made between attack on monomer and attack on micelle. Results for other compounds of this series suggest the following:-

1) V_{max} for the enzyme acting on PC derivatives increases rapidly with chain length whilst K_m decreases.

2) At the CMC there is no further increase in monomer concentration and monomer related phenomena do not change thereafter.

3) Short-chain, low-affinity substrates show substrate-concentration dependent changes above the CMC.

These results indicate that the main change produced by increasing acyl chain length is to increase the susceptibility of the individual substrate molecule within the active site. This increase in susceptibility was opposed by the falling CMC. For longer chain substrates there was no increase in rate once the CMC was reached and the plateau rate fell continuously with chain length. When the bilammelar morphology was adopted ($DiC_{12}PC$), there was a further dramatic fall in susceptibility.



The difference in the action of bee venom and other PLA_2 enzymes is obscure. One point of contrast in the structure is that the calcium binding loop starts at the 10^{th} amino acid from the N-terminus.

Comparison of the sequences aligned at the calcium binding loop emphasises this difference,

1) I I Y P G T L W C **G**

2) N L Y Q F K N M I H C T V P S R P W W H F A D Y G C Y C **G**

The relationship of the active site to any N-terminal interfacial binding site must be very different for this than for the other enzymes.

The major problem with these results is not to understand the apparently anomalous behaviour of the bee venom enzyme, but to

understand why the other enzymes do not show the interfacial phenomenon when attacking DiC_8PC and higher derivatives, especially as these are nearer to the physiological state of the enzymes normal substrates than is DiC_6PC. This tends to question the significance of the proposed surface attachment site for enzymic function. The data for *Naja mossambica mossambica* and other snake venom enzymes indicates that there is no difference in the rate of attack of the enzyme against monomeric and micellar substrates. This is shown in the plot of rate against substrate concentration both at high and low calcium concentrations, (see figure 3-2-3-2 b). In each case the double reciprocal plot is highly linear. In contrast the plot obtained in the presence of inhibitory metal ions, such as zinc, is non-linear, indicating that interfacial recognition might be important in the presence of metal ion inhibitors. The kinetic effects of a change from attack on the micelle to attack on the monomer should be apparent during an individual progress curve, but the anticipated response pattern has only been seen in reactions where inhibitory concentrations of zinc are present (Fig 3-2-3-2 b).

In summary, these results suggest that the difference between short and longer-chain substrates is that the requirement for an interface disappears as chain length increases, so that attack is equally effective on monomeric or micellar substrates. In view of the qualitative difference between the micelle surface and the free monomer state, this seems to be quite improbable. One mechanism that has been suggested is that the enzyme nucleates a micelle and therefore does not see the monomeric state.

The use of mixed chain length substrates has shown that a marked change in response to the enzyme occurs which correlates with a sharp fall in solution viscosity and the onset of sensitivity to detergent stimulation of PLA_2. Substrates shorter than $1\text{-}C_{16}$, $2\text{-}C_7PC$ are very sensitive to PLA_2 action. In contrast to the double short chain compounds, their solutions in water are quite viscous, suggesting an extended configuration, but their susceptibility is not sensitive to detergents. Above $1\text{-}C_{16}\text{-}2\text{-}C_9PC$, the aqueous solutions are non-viscous, sensitivity to PLA_2 is sharply reduced and was detergent sensitive, suggesting the formation of a liposomal,

bilayer structure. During hydrolysis these latter compounds show a very sharp rate transition that probably correlates with the transition from bilammelar to micellar morphology. Whatever the nature of the transition, it represents a point at which the monomer is in equilibrium with two other substrate morphologies, as there is no good reason to suppose that the monomer concentration will be greatly affected by the transition in the bulk phase.

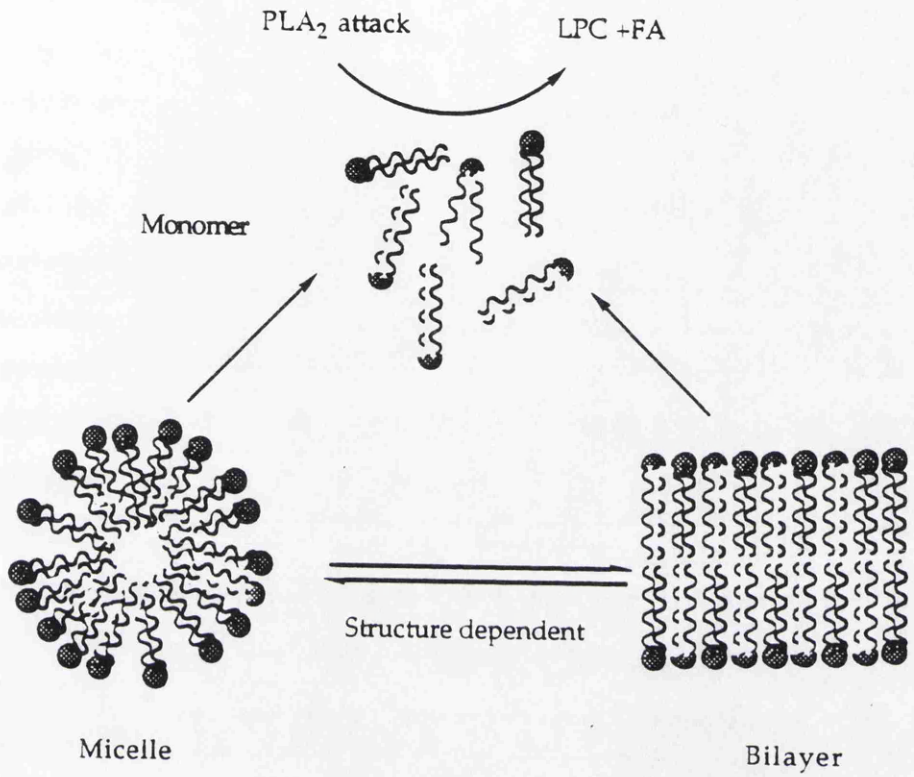
Therefore it seems quite unlikely that for these compounds there could be any significant attack on the substrate free monomer. Furthermore because detergent has no significant effect on the rate of attack on either monomer or micelle, the low rate must indicate direct action against the bilammelar state. Thus the conclusion is that these enzymes attack all forms of the substrate directly, but there is a problem in interpretation. Monomer is attacked more slowly than micelle for substrates at the bilammelar/micelle transition point, but not for the short chain micellar substrates. The cross-over point where monomer, in the concentrations present at the CMC starts to be less susceptible than the micelle which was seen for DiC_6PC but not for DiC_8PC, must reappear at higher chain lengths. The sensitivity of the assay method indicates that such a transition would be seen if it occurred for compounds of chain length greater than $DiC_{11}PC$.

In the present study, the interest in the monomer/micelle transition lay in its possible effect on reaction progress curve shape. Many of these curves showed an increased rate as substrate was depleted and this could have been explained by the possibility that some of these enzymes attack the monomer at a higher rate than the micelle, but preferentially bind to the micelle if it is present. However, this explanation for sigmoidal progress curves was ruled out by two observations:-

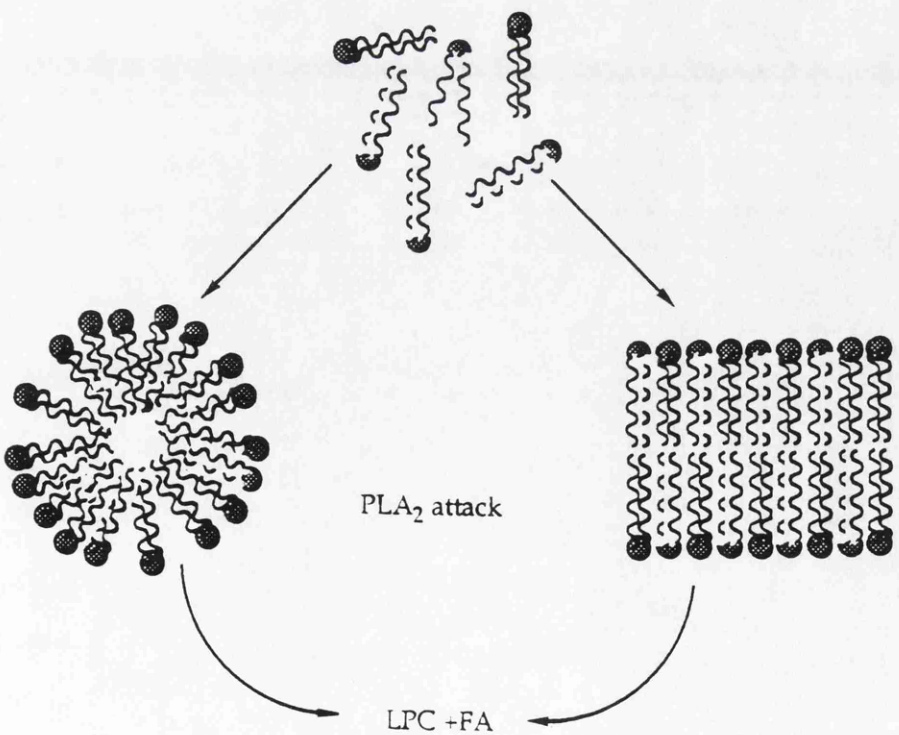
1) That the rate transition occurred at a concentration which was not very sensitive to substrate structure and

2) For a given substrate, the rate transition did not occur for a constant amount of residual substrate, but for a constant amount depleted.

Action on monomer



Action on aggregates



4-3) Effect of substrate morphology on susceptibility to activation.

The results for activation studies confirmed the work of others that long-chain fatty acylation did not affect the action against monomeric substrate. It did, however, show that the degree of rate enhancement against micellar substrate could be quite considerable and depended on the calcium concentration. Thus at high calcium concentration, very little activation was apparent. But at low calcium levels a 4-fold activation could be obtained. This supported Drainas's observation that activation by calcium and by acylation could be complementary. The simplest explanation for this result is that acylation increases the affinity of the enzyme for calcium. Detailed examination of the data showed that reaction rates were not strongly affected by calcium at low concentration, but diverged at an intermediate range. This result strongly suggested that two distinct calcium sites are present, only one of which is sensitive to activation and was the starting point for the subsequent investigation of the metal ion binding properties of these enzymes.

4-4) Activation of bee venom PLA_2 by long-chain fatty acylation.

Lawrence and Moores (1975) showed that the activation of this enzyme by aliphatic anhydrides was irreversible and also that similar activation could be produced by the cross-linking agent glutataraldehyde in the presence but not the absence of long-chain fatty acid. This indicated that activation was due to stabilisation of an active conformation. Camero-diaz *et al* (1985) demonstrated that activation by long-chain fatty acylation was irreversible and that the binding of the acyl residue to enzyme resulted in a form of the enzyme that was highly resistant to proteases and thiols. Both of these results emphasise the possibility that activation is due to conformation change, in contrast to the generation of a hydrophobic attachment site. This model however, does not readily accommodate the observation that activation is much greater for long-chain than for short-chain substrates. Lawrence introduced the modification that bilammelar substrates induce the inactive conformation and the action of the activator

is to prevent this change. By implication, the free enzyme would adopt the active rather than the inactive conformation. The starting concept of the present work was that there would be a requirement for the substrate to adopt bilammellar morphology and that there would be an involvement of metal ion activation.

The main requirement for this work was to obtain a range of substrates that could be investigated in a pure aqueous medium and which covered the full range of substrate morphologies. This was possible using the symmetrical diacyl PC derivatives where the major micelle-bialyer transition occurs at C_{11}-C_{12}. However, the range of asymmetric compounds based on 1-long-chain PC gave much more discrimination around the transition region. Thus for $1C_{16}$PC derivatives the change from apparently micellar to apparently bilammelar structures occurred over the range $2C_6$-$2C_9$. It should be pointed out that the morphology is determined by both chains and with the symmetrical derivatives this means that changes equivalent to two carbons are made at each step.

Use of these compounds enabled the factors that changes the bilayer micelle transition to be studied in detail. It was clear that even the early phase of attack was quite complex. All reactions started with a rapid initial burst followed by a more or less constant rate which then tended to fall slightly before the sharp transition to high rates characteristic of micellar substrates. Thus there appeared to be at least one pre-transition before the major transition. The early fast rate may be due to dislocations in the bilayer. Prolonged sonication to remove such defects resulted in unexpected behaviour, where initially the rate in the early phase increased with sonication and the time to transition fell, but after an optimum period these changes gradually reversed. There was no evidence for significant phospholipid hydrolysis during the sonication period.

Use of these substrates allowed a simple comparison to be made of the effect of normal and activated enzyme on the two major condensed substrate morphologies, The results showed that activation increased the rate in the first (bilayer) phase by ca 4-5-fold, whilst only increasing that for the second (micellar) phase by ca 2-fold. These differences were maintained even in the presence of calcium or albumin, both of which agents changed the basal rate quite significantly. The most interesting

effect of activation was on one of the characteristics of the pre-transition curve. Here the initial rate in response to activated enzyme was quite large, but decreased almost to control level before the true transition. When high calcium concentrations were used, this rate decceleration did not occur.

Taken together these results show a small increase in susceptibility to the activated enzyme as the morphology changes from monomer to micelle to bilayer, but the largest observed effects are seen at the start of attack on the bilayer form. There is a very clear indication that the most rapid rates seen with the activated enzyme occur when the initial rate is fast enough to pass through the intermediate slow phase. One model is that the enzyme attacks certain discontinuities in the bilayer very rapidly but the products can reorganise the structure to remove these 'hot-spots'. If attack is very rapid the reorganisation cannot occur before the product concentration becomes high enough to trigger the bilayer micelle transition.

4-5) The role of organic solvents.

Organic solvents were used in the buffering systems of PLA_2 catalysed reaction in order to increase the solubility of long acyl-chain phosphatidylcholine derivatives, but the structure of the substrates under these conditions are not defined. There seemed good reason to suppose that solvents might increase the CMC by stabilising the monomer and might destabilise the bilayer in favour of the micelle. The kinetic results are consistent with this interpretation. The most significant result of this study was the demonstration that for the bilayer forming substrates n-propanol abolished the action of the native enzyme at much lower concentrations than the activated enzyme.

This suggests that the interaction of enzyme with the substrate is weakened by the solvent but stabilised by the acyl chain. As such, the result is consistent with both the hydrophobic anchor and the stable conformation models. Nevertheless the system provides a simple and sensitive assay for activation.

4-6) Erythrocyte membrane models.

The activation phenomenon was also examined here using biological membranes of rat and rabbit erythrocytes The results were in good agreement with those obtained by different workers in this laboratory and confirmed that the lytic activity of the acylated enzyme increases by ca 65-fold in the presence of albumin. In the absence of albumin both native and activated forms of the enzyme showed no significant activity. Drainas *et al* (1981) proposed that albumin mediates the enzymic activity by preventing the strong product inhibition caused by lysophospholipid. This compound was found to play a very important role in the regulation of the enzymic activity. It is obvious in this case that the extraction of the reaction products, fatty acid and lysophospholipid by albumin increases the fragility of the membrane which then becomes more susceptible to PLA_2 attack. The results of these studies are very complicated because they involve many factors each of which acts in different ways depending on the precise conditions. As far as possible all of the results have been summarised in the following table.

Additions	Response
Fatty acid	Very fast partial leakage
Fatty acid + LPC	Strong inhibition
LPC + fatty acid	Weaker inhibition
Albumin	Slow complete leakage
Albumin +fatty acid	As above
Albumin + LPC	Concentration dependent inhibition
LPC + albumin	As above
Fatty acid + albumin	Rapid complete leakage
Fatty acid + LPC + albumin	Weak inhibition, partial leakage
LPC + fatty acid + albumin	Strong inhibition, partial leakage.

These results emphasis the effect of addition order. In the case of albumin added before fatty acid the distinction is trivial because it means that fatty acid never enters the cell membrane, by where the fatty acid is added after albumin the effects are clearly due to re-extraction of the fatty acid from the membrane. When LPC and FA are used together all of the evidence suggests that both enter the membrane, but the results that they produce still depend on addition order. LPC is much more inhibitory added after fatty acid. When these reagents are re-extracted form the cells by albumin the effetcs of the original addition order persist, but in the opposite sense. LPC has less effect when added after than before FA. These results strongly suggest that non-random processes occur in the membrane, but to investigate the nature of these processes would be a separate and major project. In terms of the response to activated enzyme it is clear that FA treated cells, do not sense the activation very strongly, but LPA inhibits activated enzyme as effectively as the normal enzyme. All of these results serve to emphasise the inhibitory role of LPC in the action of PLA_2 enzymes on erythrocyte membranes. In contrast none of the studies of defined substrates show any comparable inhibitory action. Thus it seems that artificial lipid structures are poor models for studying the action of these enzymes on real membranes.

4-7) Fatty acyl activation/Interfacial activation relationship.

All of these results indicate that the effect of activation is to bring the rate of attack on bilayer surfaces close to that on micelles. Lawrence proposed that the inhibitory action of LPC in the RBC system was due to the fact that the surface bilayer had regions the were micelle-like due to high induced curvature, these were proposed to be very sensitive to PLA_2 attack and inhibition occurred because these sensitive regions were preferentially occupied by added LPC or were filled by it during the initial stage of attack of PLA_2 on the cells. This model is not supported by the present data which emphasises that activated enzyme attacks the bilayer structure more rapidly than native enzyme but is less effective when the substrate is micellar. Nevertheless the studies of bilayer-forming substrates emphasise the heterogeniety of attack on the bilayer structures

and the possibility that hydrolysis rates are strongly affected by the presence of discontinuities. It is very clear from the above studies that certain features of the bilayer are very susceptible to the activated enzyme, but they do not seem to represent the bulk bilayer structure. If such discontinuities exist, they might also be good candidates for occupation by LPC.

4-8) Metal ion activation/inhibition.

All of the previous studies from other laboratories have been directed to the simple model that these enzymes have one calcium ion associated with the active site. Kinetic analysis has tended to show that the addition order is Ca^{2+} adding before the substrate. This leads to a simple kinetic equation:-

$$E + Ca \rightleftharpoons ECa + S \rightleftharpoons ECaS \rightleftharpoons ECa + P \longrightarrow E + Ca$$

$$1/v=1/Vmax(1+K_{Ca}/[Ca])(1+[M]/K_M)+ Ks/[S](1+K'_{Ca}/[Ca])(1+[M]/K_M))$$

From which it can be seen that for a simple reversible activating ion (Ca^{2+}) inhibition by an inhibitory ion acting reversibly at the same site (M) increases as [Ca^{2+}] falls or [M] rises. Linear reciprocal plots are the main tests of this mechanisms. Few workers have carried out detailed analyses aimed to obtain all of the kinetic parameters of activation and inhibition. Wells, working with *Crotaleus adamanteus* gave the most complete analysis using the substrate dibutyryl phosphatidylcholine. In this case the data was consistent with a competition at a single site.

Tsai *et al,* gave a complete analysis for the bee venom enzyme based on competition between calcium and cadmium, ions with almost identical radii. they used spectrophotometric and kinetic methods and obtained a Km of 2.5mM for Ca^{2+} by both methods. This seems a remarkable results in view of other data that indicates K_{Ca}^{2+} is ca 2.5μM and tends to be independent of substrate type. this tends to cast doubt on the interpretation of the spectrophotometric results which were obtained under conditions virtually identical to our present kinetic conditions. None of these workers have asked pertinent questions about the ability of Ba^{2+} (1.34Å) and Zn^{2+} (0.70Å) to bind at the site for Ca^{2+} (0.99Å) when many ions of intermediate size could not.

The study of calcium dependence of the bee venom and the basic non-toxic isoform from *Naja mossambica mossambica* enzymes showed

some very complicated results which indicated that these enzymes might possess more than one calcium binding site. This is clearly shown from the reciprocal plots. The use of other metal ions show very clearly that a range of divalent cations and Ba^{2+} in particular were true competitors for Ca^{2+} consistent with action at a single site, but the paradoxical results involving Zn^{2+} have lead to a different conclusion.

The most important of these is the observation that inhibition by zinc is low at low calcium concentrations and increases as calcium concentration is increased. The only cation that behaved in a similar manner to zinc is its chemical twin cadmium. The obvious explanation for such an effect is that zinc inhibits a calcium-dependent action of the enzyme that is not present at low calcium concentration. This lead to the concept that the enzyme has two calcium sites, one being a high affinity essential site that supports only 10% of the total activity, can bind barium class cations and the second is a lower affinity site that modulates the activity of the enzyme but need not to be occupied by calcium for activity. This second site appears to be a zinc binding site, Its occupation by zinc abolishes the enzymic activity supplemented by the second calcium ion.

The failure of zinc and cadmium to inhibit the enzyme completely at low calcium levels indicate that the enzyme under these condition can bind both calcium at one site and zinc at the other site, the occupation of this site by zinc probably produces << 5% of the total activity. In view of the possibility that the metal ion inhibition might be affected by physico-chemical state of the substrate, some measurements were carried out using dihexanoyl phosphatidylcholine. Measurements of rate of hydrolysis as a function of substrate concentration showed a very clear activation consistent with the preferred attack on the micellar morphology, but the effect of zinc both below and above the CMC was very similar and non-competitive in nature although this effect was not as clear as with the dioctanoyl phosphatidylcholine.

The two-site model was further examined by the use of a better characterised enzyme from the type 1, *Naja naja atra* , as this enzyme was shown to have two calcium binding sites, The results confirmed the

non-competitive inhibition by zinc and cadmium. However, it could be argued that the effect of zinc could be due to some artefacts of the assay method occurring because zinc binds to the reaction product and produces serious inhibition under some conditions but, not others. This seems to be very unlikely, simply because of the failure of zinc to inhibit at low calcium levels, also the calibration of the assay in the presence of high zinc concentrations showed no departure from linearity and the conductance changes produced were similar both in the presence or absence of zinc. Further support of the two-site concept came from the results obtained when these experiments were repeated using other enzymes, namely the basic toxic isoform from the same venom of *Naja mossambica mossambica* and the 11' isoform from *Notechis scutatus scutatus*. These two enzymes showed a competitive zinc inhibition indicating that this phenomenon is real and rules out any possible doubt about the validity of the assay method.

Once this model has been accepted it leads to some very interesting consequences for the interpretation of interfacial phenomena. The 2-site model is fully consistent with the data on curve shape changes that have been revealed by this work. The sigmoidal curves obtained at low calcium in the absence of transition metal ions would seem to be the basic kinetic form of the enzyme when activated by the single essential calcium ion. When the enzyme has two calcium ions the kinetic form is of the conventional hyperbolic type. This can be analysed in terms of two contributions to the kinetics, and the important parameter is to know what the maximum activity of the enzyme with one bound calcium ion is?.

One simple approach to this is to use the curve shape parameter which we define as Csp = (Mr - Ir)/Ir, which varies from 3 to 1 to find out the concentration of calcium at which both species make equal contributions to the rate. ie The curve shape parameter is halved. To a first approximation the curve at high calcium concentration is linear for 80% of the time course whilst the curve at low calcium has a slope difference of 3/1 where the maximum rate was measured at 70% conversion. Thus where the two curves make an equal contribution to

the initial rate the ratio of the maximum to the initial rates is (3+1)/2. This is very clearly shown in Fig 3-2-2-1 d, where the shape parameter at low calcium plateaus to a level of ca 2.5. As calcium increases it starts to fall and decreases to 1.0. From this an accurate estimate of K_{Ca2+} for the binding of a second calcium ion can be made. At this concentration the initial rate is ca 10-15 % of the initial rate at saturating calcium concentrations.

All the results presented in this work conclude the existence of at least two calcium binding sites in some phospholipase A_2 studied here, nevertheless, this model remains an open question in the absence of confirmative crystallographic studies. In view of simple comparison of the primary structure of these enzymes indicate that it is very hard to speculate where the second calcium binding loop could be situated, for example *Naja naja atra* PLA_2 and the basic non-toxic isoform *Naja mossambica mossambica* showed a difference of 21 amino acids spread all over the sequence. This unfortunately allowed no conclusion to be made from this analysis.

```
1N L Y Q F K N M I Q C T V P S R S W W D F A D Y G C Y C
2* * * * * * * * * H * * * * * * P * * H * * * * * * * *

G R G G S G T P V D D L D R C C Q V H D N C Y N E A E K
* * * * K * * A * * * * * * * * * * * * * * G * * G *

I S G C W P Y F K T Y S Y E C S Q G T L T C K G G N N -
L - * * * * * L T L * K * * * * * - K * * * S * * * * K

C A A A V C D C D R L A A I C F A G A P Y N D N D Y N I
* * * * * * * N * * L V * * N * * * * * R * I * A N * * *

N L K A R C Q E
* * * E * * * -
```

Interestingly, the amino-acid sequence of bee venom PLA_2 on the other hand, show a new possible calcium binding loop in the region between 100-110 residues.

^{3}I I Y P G T L **W** C **G** H **G** N K S S G P N L L G R F K H T

D A C C R T **H** **D** M C P D V M S A G E S R H G L T N T A S

T R R L S C D C **D** D K F Y D C L R N S A D T I S S Y F

V G K M **Y** F N L I A T K C Y K L E H P V T G C G E R T

E G R C L H Y T V D K S K P V Y Q W F D L R K Y.

The amino acid residues in bold format represent those involved in Ca^{2+} binding and catalysis.

1) *Naja naja atra* PLA_2.

2) The basic non-toxic PLA_2 from *Naja mossambica mosambica* venom.

3) Bee venom phospholipase A_2.

4-9) Phospholipase D study.

The aim of the present study of phospholipase D was simply to try to generate other series of phospholipase A_2 substrates based on the acyl side chain configurations already discussed above. The hydrolytic activity of this enzyme would generate the phosphatidic acid derivatives, which having a dibasic phosphate group, would be unlikely to from micelles or bilayer structures as readily as the PC derivatives. Similarly the large number of derivatives that could be made by transphosphatidylation using alcohol acceptors should enable many PLA_2 substrates to be prepared that should have very different physico-chemical characteristics.

This study was greatly facilitated by the fact that the conductimetric assay could be run for both enzymes in series and it was therefore a simple matter to generate a new phospholipid by PLD attack and to test it as a PLA_2 substrate.

The first observation concerning phosphatidic acid (PA) derivatives was their extreme insolubility in the presence of calcium, this was probably well-known, but it was extremely obvious when the formation of these compounds was measured by conductimetry. It tended to limit the range of acyl chain lengths that could be used in this work. PA derivatives were all shown to be substrates for PLA_2, although not as good substrates as the parent PC compounds. In contrast the use of alcohols as acceptors for transphosphatidylation enable substrates to be generated that were more susceptible than the parent PC compounds. The most interesting and potentially most useful compounds were the methanol adducts. These were the only compounds that appeared to be more susceptible than the parent PC, but the interesting characteristic was that the progress curve showed a clear indication of low binding affinity, ie high K_m. This was reminiscent of DHPC and it may indicate that the substrate was indeed present as free monomer. Clearly the negative charge of this molecule should raise the CMC quite considerably and it would be expected that this substrate, unlike its parent, would be present in the monomeric form. The aim of this work would be to purify the methanol adducts derived from the various PC

derivatives and to carry out detailed studies of the effect of morphology on kinetics. This work showed one rather unexpected result, that the PS derivative was very weakly susceptible to the enzyme.

These studies established some very interesting facts concerning PLD enzymes. Firstly, they showed that the conductimetric assay could be used to measure the activity from a variety of tissue sources. The results confirmed the predicted conductance changes and showed that PLD and PLA_2 assays could be run sequentially. Secondly, they showed that a conductimetric assays for the transferase activity was possible. This is the first direct assay method able to measure the transferase activity. All of the conductimetric results showed that the interactions of PA derivatives with calcium dominated the conductance changes. These results established that the major factor in calcium binding by PA species was the degree of hydrophobicity of the side chains. Thus LPA derivatives were very much less prone to bind Ca than the corresponding PA derivatives. This tendency to bind calcium had important consequences for enzyme activity.

4-9-1) Calcium dependence of PLD enzymes.

Most PLD enzymes are thought to be calcium dependent. This was shown to be true for the *Streptomyces chromofuscus* enzyme, but rather unexpected results were seen with the enzyme from savoy cabbage. The enzyme in crude supernatants was mostly calcium requiring, with less than 1/3 being insensitive to inhibition by EDTA. However as the enzyme was purified the EDTA resistant activity increased in proportion. This was unexpected because heating crude enzyme extracts tended to destroy the EDTA resistant component faster than the EDTA sensitive component. Nevertheless the most purified cabbage enzyme was almost totally insensitive to EDTA. A very good control for this sensitivity was to run PLA_2 and PLD in the same solutions. It was easy to demonstrate that the cabbage enzyme was active under conditions of low calcium where bee venom PLA_2 was inactive.

In order to measure the true calcium dependence of the bacterial enzyme it was necessary to use a substrate whose product PA was not calcium-binding. This was done using octanoyl-LPC and showed a very convincing affinity curve. The interest in this was the fact that it demonstrated simple calcium dependence under conditions where the dependence of PLA_2 was complex.

In comparison with the bacterial enzyme, the cabbage enzyme was far more interesting. The change from being largely calcium dependent to essentially calcium independent during purification has not been clarified although several explanations are possible. The most likely model is that two different activities co-exist, but the calcium independent activity is more stable and survives purification. Nevertheless, it seems very peculiar that two enzymes with similar characteristics should differ in this one very significant feature, especially as Ca^{2+} figures so prominently in the active sites of other phospholipases. Nevertheless there are calcium independent PLA_2 enzymes, so that there is a good precedent for this.

Using this enzyme it was quite easy to show that the transferase activity could be assayed by the conductimetric method and that there were great advantages in being able to work with a calcium independent enzyme. It was also possible to address the problem of why the bacterial enzyme was not a transferase. This was done by generating phosphatidyl alcohols using the cabbage enzyme and investigating their susceptibility to the bacterial enzyme. The results showed that the bacterial enzyme hydrolysed these compounds very rapidly. Thus if transferase activity occurred, the product could not accumulate.

The studies of PA derivatives made in this were essentially preliminary. It was clear that the methanol derivative of DiC_8PA was a better substrate for PLA than DiC_8PC. The hydrolysis curve indicated a high V_{max} and a high K_m typical of a short-chain monomeric substrate. It would now be extremely interesting to prepare these compounds in large quantities and with different acyl chain dispositions to further investigate the relationship between side chain length, substrate morphology and enzyme activity.

Chapter Five

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