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Phospholipase D development of a continuous spectrophotometric assay; kinetics with long chain phosphatidylcholines.

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PHOSPHOLIPASE D

I. DEVELOPMENT OF A CONTINUOUS SPECTROPHOTOMETRIC ASSAY

II. KINETICS WITH LONG CHAIN PHOSPHATIDYLCHOLINES

by

Jemmy Felix Takrama

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DEDICATION

TO SALLIE, OMINIK-MENSAH AND BOAFO-ALEX

ABSTRACT

Phospholipase D:

A. Development of a Continuous Spectrophotometric Assay

B. Kinetics with Long Chain Phosphatidylcholines

by

Jemmy Felix Takrama

A. A continuous spectrophotometric method for monitoring phospholipase D-catalyzed hydrolysis of long acyl chain phosphatidylcholines has been formulated at pH 8.0 in a mixed detergent system using the coupling enzymes choline oxidase and peroxidase. Major emphasis is placed on cabbage phospholipase D but the potential of the peanut enzyme is also indicated. Standard curves for phosphatidylcholine determination in both end-point and rate modes are presented and applied to the estimation of that phospholipid in a solubilized human erythrocyte membrane sample. In rate mode the method is suitable for kinetic study of phospholipase D with phosphatidylcholines in micellar form.

B. The rate method was used in the study of the kinetics of phospholipases D (cabbage and peanut) with long acyl chain phosphatidylcholines. Kinetic experiments were conducted at constant bulk concentration of detergents (D_o) and varying bulk substrate concentration (PC). Initial velocity and binding studies of PLD are consistent with a rapid random equilibrium mechanism where Ca^{2+}

interaction with enzyme constitutes an essential activating system.

In the presence of increasing amount of Ca^{2+}, the Vmax was proportionally increased for all phosphatidylcholines studied and Km^{app} was dependent on the amount of Ca^{2+}. The hydrolysis rate showed a cooperative dependence on Ca^{2+} concentration. The Ca^{2+}-induced enhancement of PLD activity was correlated with the increase in the concentration of Ca^{2+} associated with the phospholipids. Thus, activation of PLD is due to an increase in Vmax as well as alteration in affinity for phospholipid substrate.

The kinetic surves of initial rates versus substraate concentration were biphasic in the substrate concentration range of 0 to 2 mM with a high intial reaction rate phase followed by a phase of lower rates as the detergents to PC ratio decreased. Conversely, kinetics at constant detergents to PC ratio showed a phase of low initial rates followed by a second hyperbolic phase.

Observation of relatively large UV difference spectra due to Ca^{2+} interaction with PLD and/or PC mixed micelles suggest that some type of structural pertubations occurred in the protein and mixed micelles. Interpreted in terms of fluctuations in the phospholipid environment, its influence on PLD activity could partly explain some of the observed trends in the kinetics.

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ABBREVIATIONS

A	absorbance
AAP	4-amino antipyrene
BSA	bovine serum albumin
Ca^{2+}	calcium
CBBG	Coomassie Brilliant Blue G-250
CMC	critical micelle concentration
COD	choline osidase
D_o	total detergent concentration comprising L_o (total SDS concentration) and T_o (total Triton X-100 concentration)
DDPC	L-α-phosphatidylcholine, didecanoyl
DLPC	L-α-phosphatidylcholine, dilauroyl
DMG	β,β-dimethylgluturate
DMPC	L-α-phosphatidylcholine, dimyristoyl
DOC	deoxycholate
DOPC	L-α-phosphatidylcholine, dioctanoyl
DPPC	L-α-phosphatidylcholine, dipalmitoyl
DSPC	L-α-phosphatidylcholine, distearoyl
EDTA	ethylenediamine tetraacetic acid
HDCBS	2-hydroxy-3,5-dichlorobenzene sulfonate
Log	logarithm
uM	micromolar
mg	milligram
mL	millilitre
mM	millimolar

nm	nanometer
NMR	nuclear magnetic resonance
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PLD_c	cabbage phospholipase D
PLD_p	peanut phospholiapse D
PMR	proton magnetic resonance
POD	peroxidase
PS	phosphatidylserine
S_o	total substrate concentration
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TLC	thin-layer chromatography
UV	ultraviolet

CHAPTER I

INTODUCTION

The phospholipases catalyze the hydrolysis of phospholipids which may be considered as derivatives of glycerophosphate in which the two hydroxyls are esterified to long-chain fatty acids and the phosphoryl group forms a phosphodiester bond with a polar moiety [1]. The acyl esters at the sn-1 and sn-2 position are cleaved respectively by phospholipase A_1, (PLA_1), and phospholipase A_2, (PLA_2). The enzyme that cleaves the phosphodiester bond on the glycerol side is designated C, (PLC), and on the polar side phospholipase D (PLD). Enzymes that hydrolyze the remaining acyl group on lysophospholipids are commonly referred to as lysophospholipases.

The existence of the enzyme catalyzing the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA), plus choline was predicted over 50 years ago by Contrardi and Ercoli [2] and the first description of the enzyme in carrot roots and cabbage leaves was presented by Hanahan and Chaikoff [3,4].

Phospholipases D belong to a class of enzymes which function in a heterogeneous system, cleaving water insoluble substrates. In several studies, the adsorption of the enzyme at the interface of lipid and water or oil and water was shown to be a crucial step for the performance of the catalytic reaction [5]. Until recently, PLD was identified only in plant tissues, however, additional information has

been gathered to indicate that such an enzyme is present in microorganisms [6,7,8,9,10] as well as in mammalia [11,12,13].

Little is known about the physiological role of PLD in cellular metabolism. However, from studies of other enzymes characterized as having phospholipid requirements e.g., ATPase, glucose-6-phosphate and β-hydroxybutyrate dehydrogenase, it can only be speculated that PLD and the base exchange enzymes may provide a mechanisn for regulating the functional properties of membrane proteins by modulation of the polar portions of these phospholipids [14].

No systematic study has been published to indicate the efficiency of the catalytic reaction with well-defined substrate systems in which the actual form and concentration of the substrate and/or phospholipases D have been examined [5]. Careful studies on fatty acid chain length dependence have not been reported for these enzymes [1].

Earlier kinetic study on PLA_2 and PLD was based on a pH-stat assay which is limited in sensitivity and subject to sizable experimental error, particularly at low initial rates [15].

Phospholipases D From Cabbage and Peanut Seeds

PLD is found in a wide variety of higher plants [5] and especially in the genus Brassica [19,20]. Varying degrees of PLD activity have been demonstrated in either or both the soluble form or particulate form in carrot

seeds [20,21], cottonseeds [22,23] pea cotyledons [24], latex serum of Hevea brasiliensis [22,25], many bean cotyledons [26], peanut seeds [27] and cabbage leaves [19,21,22,24] to mention a few. Heller et al. [27] observed that the enzyme in dry peanuts was soluble and showed properties similar to those of the cabbage enzyme, but was found to also act on cardiolipin and on phosphatidylglycerol [28] in the absence of ether or anionic amphipaths.

Reports on substrate specificity of the phospholipases D have been at times contradictory since the physical state, and the source and purity of the enzymes are major factors governing substrate specificity. The soluble enzyme from cabbage leaves was capable of hydrolyzing naturally occurring as well as synthetic phospholipids under various conditions as tabulated by Heller [5]. In addition, O-lysine or O-alanine derivatives of phosphatidylglycerol [29,30] were also hydrolyzed. Cardiolipin resisted the cabbage enzyme as did phosphatidylglycerophosphate [31]. The plastid enzyme from cabbage required ether but not Ca^{2+}, although a crude mixture of soybean phospholipids was degraded even without ether [32]. Also degraded by the soluble cabbage enzyme are monoacylglycerophosphorylcholine (lysolecithin), 1-alkenyl-2-acylglycerophospholipids (plasmogens) as well as dialkyl derivatives of glycerophospholipids (1,2-ditetradecyl-, 1,2-dihexyl-, 1,2-dioctadecyl-sn-glycero-3-phosphorylcholines) [19,33,34]. Waku and Nakazawa [35] showed that the cabbage enzyme hydrolyzed

various synthetic 1-alkyl-2-acyl- and 1-alkenyl-2-acyl-*sn*-glycero-3-phosphorylcholines at rates one-tenth to one-third that of the 1,2-diacyl phosphatidylcholines. Furthermore, PC, PS and PE in rat liver microsomes were hydrolyzed [36], however PI hydrolysis has not been demonstrated with PLD_c [37,38].

A broad substrate specificity is also exhibited by the soluble peanut enzyme. Aqueous dispersions of PC, PE, PS, PG or diphosphatidylglycerol (cardiolipin) or as constituents of certain lipoproteins (e.g. red cell membranes) were degraded by the peanut enzyme [5]. The cabbage enzyme hydrolyzed SM in the presence of Triton X-100 or SDS but the peanut enzyme did not [39]. 1-Monoacyl-glycerophosphorylcholine was slowly degraded by the partially purified peanut enzyme [40].

There has been limited progress in the purification of cabbage phospholipase D (PLD_c), possibly due to the fact that the enzyme is very unstable and has not been purified to homogeneity. Following the first partial purification reported by Dawson and Long [19], further purification was achieved by Yang *et al*. [41] and by Allgyer and Wells [42] who reported a stable preparation and 680-fold purification in the presence of 50% ethylene glycol. The enzyme was found most stable between pH 6.5 and 7.0 [41]. The molecular weight of the purified cabbage enzyme was found to be 112,500 $\pm$7500 on SDS-PAGE and 116,600$\pm$6900 by sedimentation equilibrium ultracentrifugation. In addition, determination of the

molecular weight by the above methods in the presence of high concentrations of urea indicated that PLD_c is an oligomeric protein since smaller values were found in the latter case [42].

The peanut enzyme is a much larger protein than the cabbage enzyme. The catalytically active form has a molecular weight of about 200,000 by gel filtration on Sepharose-6B and smaller aggregrates are inactive [43,44]. Also, the peanut phospholipase D (PLD_p) is very sensitive to surfactants, inactivated by serum albumin [46] and inhibited by β-lipoproteins [28]. Unlike the cabbage enzyme the amino acid composition of the purified peanut enzyme has been reported [43].

With phosphatidylcholine as substrate, PLD from plant sources have a strong dependence on Ca^{2+} [19,20,33] which is far more effective as an activator than any ion tested [20]. The purified enzyme from cabbage was shown to have an absolute requirement for Ca^{2+} [19,47] and it was suggested that the optimal concentration of cation was proportional to the concentration of PC. The peanut PLD also had an absolute requirement for Ca^{2+} and Mg^{2+} could not replace it [27,45].

Organic solvents, particularly diethyl ether, ketones (n-dipropyl, methyl, pentyl), and esters (butyl acetate, ethyl butyrate) stimulate the activities of both the cabbage and peanut enzymes [19,20,28,33,49,50].

Anionic, cationic and nonionic detergents stimulate

both the cabbage and peanut enzymes frequently in a superior fashion compared to ether or other forms of activation [5]. The most potent and stimulating detergents of the soluble cabbage enzyme were SDS, monocytylphosphoric acid, phosphatidic acid, and triphosphoinositide, with SDS having the most dramatic effect [5]. SDS and taurocholate at PC/detergent molar ratios of 2:1 and 5:4, respectively, stimulated considerably the hydrolysis of the phospholipid by the peanut enzyme. PA and deoxycholate (DOC), were also effective [27,28,46].

Cationic amphipaths such as cetyltrimethylammonium bromide, cetylpyridinium chloride, stearoylamine or palmitoylamine, were potent inhibitors for the cabbage enzyme [5,49]. In contrast to the cabbage enzyme, the PLD from peanuts was activated by cetyltrimethylammonium bromide or by the nonionic Triton X-100 though their effects were only one-sixth of the optimal concentration of SDS [5].

SDS beyond a relatively narrow range of concentrations is inhibitory to the PLD hydrolytic reaction [7,27,28,34,46, 48]. Choline and ethanolamine inhibited the hydrolysis of ultrasonically treated particles of PC and SDS by the PLD from cabbage at pH 5.4-5.8 [48]. The plant enzymes, PLD_c and PLD_p have been shown to be inhibited by sub-stoichiometric concentrations of EDTA [51]. For instance, a homogenate of cabbage leaves which was shown to contain 40 mM Ca^{2+} was inhibited by only 10 mM EDTA [19]. Similarly, the purified enzyme from the same source, requiring 37.5 mM

Ca^{2+} for optimal activity, was totally inhibited only by 8.3 mM of EDTA [48].

p-Chloromercury benzoate (p-CMB) but not thiol alkylating reagents such as iodoacetate, iodoacetamide, or N-ethylmaleimide inhibited the impure plant enzymes [41]. The use of diisopropylfluorophosphate (DFP) to investigate the presence of an essential serine in PLD_c failed to confirm its existence [41].

A heat labile, dialyzable inhibitor isolated from cabbage leaves affected the activity of the enzyme from cabbage only and not that of cotton seeds [22]. However, Quarles and Dawson [24] could not detect any inhibitory substance in the leaf extracts.

Contrary to the observation of Dawson and Hemington [48] that serum albumin protected the cabbage enzyme and prevented its adsorption to charged surfaces such as glass, the purified peanut seeds PLD was inactivated by serum albumin [28,46].

It has long been recognized that phospholipases D in addition to their hydrolase activity, also exert a transphosphatidylase activity in the presence of a large variety of alcohols, thereby producing from PC compounds such as phosphatidyl ethanol, methanol, and isopropanol.

Recently, Kanfer [14] distinguished between transphosphatidylation catalyzed by PLD and base exchange activities of a class of base exchange enzymes which have hitherto been confused with the former. Accordingly, transphosphatidyla-

tion require much larger quantities of Ca^{2+} and water-soluble precursors than base exchange reactions. In addition, PLD will incorporate glycerol into phosphatidylglycerol and simple alcohols into unnatural phospholipids but the base exchange enzymes will not use these substrates. In the absence of a suitable alcohol PLD will catalyze a hydrolytic reaction but the base exchange enzymes will not modify the structure of a phospholipid and the former possesses acidic pH optima whereas the latter have alkaline pH optima.

Aggregation States of Phospholipid-Detergent Mixtures

Lipolytic enzymes have been repeatedly observed to act preferentially on substrates which form aggregates in aqueous environment, i.e., monomolecular films, micelles, liposomes, or other types of dispersions [51]. The aggregate nature of the substrate introduces uncertainties concerning the actual concentration of the substrate, and if more than one aggregate form are present, which may have different affinities for the enzyme, further complications are introduced [52]. Investigators have spent much time and effort characterizing phospholipids and phospholipid aggregates and relating these findings to observed lipolytic kinetics [1]. It must be noted that since the bulk of work was done on lipolytic enzymes other than PLD, approaches to elucidate the mode of action of PLD naturally followed certain paths paved by studies on similar heterogeneous

systems [5].

Detergent-phospholipid mixed micelles that have often been used in phospholipase assays offer the obvious advantage that the physical state of the phospholipid substrate can be controlled; in an excess of detergent, the micelle structure should be similar no matter which phospholipid is utilized. This offers the potential of standardizing the physical state of the substrate and lipid-water interface for comparing varying substrates [1]. On the other hand, detergents are not inert matrices and often inhibit or activate the phospholipases, which can interfere with any analysis. Though the non-ionic detergent Triton X-100 is less inhibitory when used in low concentrations, SDS, a potent activator routinely included in PLD assays [5,19,34] adds charge to the interface. Fortunately, the dispersive action of SDS due to the reduction of interfacial packing was not evidenced in the case of PLD, most probably due to the large concentration of Ca^{2+} ions required [5].

The surface charge is expressed in terms of zeta potentials, which influence the rates of hydrolysis of charged particles by phospholipase action [53,54,55]. Calcium reduced or eliminated changes in the negative zeta potential produced by negatively charged amphipaths leading to the proposal that Ca^{2+} promoted the adsorption of the enzyme onto the surface of the substrate and in its absence though adsorption still occurs it is accompanied by enzyme

denaturation [5].

Complete phase diagrams for all the commonly employed detergent/phospholipid/water systems as a function of temperature are unavailable, therefore a complete description of the aggregation states which occur in the mixture is limited. However, information on the formation of lipid detergent mixed micelles available to date allows us to generalize that above some effective detergent to phospholipid molar ratio, all of the phospholipid present as multibilayers vesicles, etc. is converted to mixed micelles [57]. This would normally correspond to the amount of detergent necessary to form an optically clear solution consisting of stable isotropic mixed micelles [56,57]. Thermodynamic arguments [57] suggest that the monomer concentrations of the individual detergents in a mixture remain below the CMC's of the respective pure detergents. Similarly the monomer concentration of phospholipid is lowered in detergent mixed micelles. For long-chain diacyl phospholipids, the monomer concentration is so low that it can be ignored for most purposes [57]. For example, Smith and Tanford [58] have measured the upper limit for monomer dipalmitoylphosphatidylcholine in water to be 4.7×10^{-10} M at 25°C.

Quantitative predictions about the thermodynamic properties of mixed micelles are impossible at the present time since knowledge of the repulsive interactions between like and unlike head groups is lacking [59]. Making the assumption that mixed micelles constitute a separate phase, the thermodynamic treatment is analogous to the treatment of

liquid-vapour equilibria for liquid solutions [59]. It should be noted that when mixed micelles are formed by amphipaths with different head groups, ideal mixing is not to be expected, however, when nearly ideal mixing occurs one can expect the mixed micelle to be intermediate in size between the corresponding pure micelles [59]. Mixed micelles differ greatly in size and structure depending on the substance solubilized, the detergent, and the detergent/solubilizate ratio [56].

Despite the preference of phospholipases for micellar over monomeric substrates which are poorly attacked by these enzymes, many workers [42,60,61,62], with the anticipation of extrapolating classical enzyme kinetics to the lipolytic enzymes which function in heterogeneous systems, resorted to the use of short-chain phospholipids which are water soluble. The diacetyl-, dipropanoyl-, and dibutanoyl-, phosphatidylcholines are water soluble as monomers [63]. The dihexanoyl-, diheptanoyl-, and dioctanoylphosphatidylcholines form micelles when dissolved in water at concentrations above their critical micelle concentration (CMC) [64,65,66]. For these short-chain phospholipids, whether monomeric or micellar forms prevail in solution depend on the phospholipid concentration and in addition, the transformation between them may not be sharp [67]. Furthermore, while the conformation of the phospholipid in micelles is similar to that in other aggregate forms of the phospholipid it is probably not exactly the same for monomeric phospholipids [68,69]. Also, since monomeric micelles are each

characterized by their own kinetic constants [62], another problem of phase separation is introduced though this can be avoided by selecting conditions that either maximize or minimize monomer concentration which is negligible for normal phospholipids.

Notwithstanding the above, micelles of synthetic short-chain phospholipids have the limitation that the physical state of the aggregate and the lipid-water interface is totally controlled by the phospholipid composition, so there is little to independently vary or control. For normal long-chain phospholipids, either isolated from natural sources or synthesized, small unilamellar, large unilamellar, or multilamellar vesicles can be formed, but again their characteristics are determined by the particular phospholipid studied [1].

Obviously, the relevance of any kinetic conclusions obtained from studies with synthetic monomeric substrates to natural systems can be questioned [1]. Admittedly, Allgyer and Wells [42] in their work with the cabbage PLD concluded that their anticipated simplifications in the kinetics by using short-chain lecithins as substrates were not realized and one should be cautious in extrapolating studies with short chain substrates to an interpretation of catalytic events that occur with natural substrates [70].

Interactions Between Phospholipid Micelles, Proteins and Cations

In a lipid/detergent dispersion, a mixed micelle is

most probably the form which interacts with the enzyme; such systems were analyzed by constructing ternary phase diagrams [71,72,73,74].

Complexes of PC-SDS-Ca^{2+} (or Mg^{2+}) adsorb PLD purified from cabbage leaves [34,48]; peanut seeds [37-39] or cottonseeds [75,76]. The enzymatic activity was associated with these complexes. However, if the PC (egg lecithin) was replaced by di-C_{14} alkyl analogue of PC, no adsorption to such complex occurs [34]. Heller et al. [51] studied the properties of PC-SDS-Ca^{2+}, complexes using proton magnetic resonance (PMR), and analytical ultracentrifugation with 20 mM egg yolk PC. Their results indicated that in the absence of Ca^{2+}, non-homogeneous mixtures of PC-SDS aggregates existed when the SDS:PC molar ratio varied from 0.25 to 2, and aggregates having high molecular weights coexisted with much smaller aggregates. PMR spectra obtained in the presence of Ca^{2+} indicated that Ca^{2+} forms complexes with PC-SDS micelles and the arrangement of PC molecules in unprecipitated ternary complexes was similar to their packing in the PC-SDS mixed micelles [34]. Heller et al. [34] further observed that PLD (peanut) was sedimented quantitatively with PC-SDS-Ca^{2+} aggregates, but not with the Mg^{2+} containing complexes, suggesting that Ca^{2+} conferred a spatial organization and/or surface charge density upon the aggregated micelles allowing appropriate adsorption of the enzyme. Using radioactive PC, it was observed that heavy aggregates composed of PC-SDS-Ca^{2+}, which sedimented at very low centrifugal forces were

unreactive as substrates for hydrolysis by the enzyme and only the 'lighter', 'soluble' aggregates were degraded.

In recent studies, Rakhimov et al. [77] showed that Ca^{2+} participates in the formation of a catalytically active intermediary enzyme-substrate complex with a strict stoichiometric ratio of micellar substrate, SDS and Ca^{2+}. They further maintained that Ca^{2+} is also necessary for neutralization of the extra charge on the surface of substrate micelles, production of an appropriate ionic strength, and modification of the substrate phase surface at the interface [77].

Approaches to the Solubilization of Phosphatidylcholines and the Determination of Phospholipase D Activity

Various procedures are used for solubilization of complex lipids, such as addition of detergents or organic solvents, emulsification, ultrasonic irradiation or coating on a solid surface [71,78-80].

Simple mechanical dispersion of 'purified' egg yolk lecithin to form coarse liposomes, which are hardly affected by the enzymes were used in many of the earlier studies.

The ether system whereby the hydrolysis has been demonstrated to occur at the water-ether interface has also been used by some investigators [28,34]. Here, a typical reaction mixture contained buffer, $CaCl_2$, PC (alone or mixed with labelled substrate, 1 to 7 mM), and enzyme. Ether was added in 0.1 to 1 vol. of the aqueous phase resulting in a biphasic system. Shaking was carried out at 25-30°C for

10 to 60 min [41,45,48,81,82]. The reaction was then terminated with acid ($HClO_4$ or Cl_3CCOOH), and the aqueous phase extracted with ether and products identified by radioactivity counts, TLC or chemical analysis.

The monolayer system consists of a quantity of PC ([^{14}C]methylcholine) alone or in a mixture with an appropriate amphipath spread in petroleum-ether (b.p. 60- 80°C) - chloroform (4:1 by vol.) on a clean water surface containing $CaCl_2$ and dimethylglutarate buffer. The solvent is allowed to evaporate, a magnetic stirrer is started for 1 min, stopped, and the surface pressure adjusted to a desired value by moving the barrier. The enzyme is then injected through the film, and stirring is started again. The change in radioactivity due to the removal of the [^{14}C]choline from the film in free, subphase-soluble form, is recorded continuously either alone or in conjunction with alterations in surface pressure [83,84]. This procedure has three principal disadvantages - it requires sophisticated instrumentation that is not commercially avialable; the two-dimensional state of the substrates makes data analysis and interpretation complex, and finally, the substrate concentrations or actual amounts of enzyme molecules involved in the catalysis are also uncertain.

Currently, the most widely used procedure is the detergent system. SDS, a potent activator of phospholipases D and a solubilizing agent for PC has been routinely added to the reaction mixture [27,28,45,46,48]. Some typical

assay protocols (an extension of Heller's [5] list) are:

(a) An aqueous mixture of PC and SDS and Ca^{2+} with enzyme added to initiate the reaction [45,46,48].

(b) Same as (a) except that PC suspension is ultrasonically irradiated prior or after addition of SDS followed by addition of enzyme [48].

(c) Mixing of solutions of PC and SDS in chloroform and methanol, in appropriate molar proportions, removing the organic solvents completely by N_2 and freeze drying, then adding buffer, Ca^{2+}, and enzyme in that order [44]. Here also the reaction products were analyzed as in the ether system.

(d) More recently, complete enzymatic systems have been developed to assess PC concentration [85,86,99] and to determine PLD activity [87]. Carman et al. [87] developed a continuous spectrophotometric assay for PLD from cabbage using choline kinas, pyruvate kinase, and lactate dehydrogenase to couple the release of choline with the oxidation of NADH. The reaction mixture, in addition to the above, enzymes contained Triton X-100 at a final concentration of 1 mM $CaCl_2$, $MgCl_2$, KCl but no SDS. Though these studies show that initial rates were being measured, the presence of Mg^{2+} which is obligatory for the system, could further complicate the Ca^{2+}-dependence of the enzyme in any rigorous kinetic analysis.

Kinetic Studies

Kinetic analysis has always played a key role in studies of the mechanism of enzyme action. The Ca^{2+} requirement for PLD from cabbage is complicated and the concentration of Ca^{2+} used in an experiment affects the pH optimum of the enzyme. With PC and lysoPC, the optimum Ca^{2+} concentration was found to be 25-40 mM for the partially purified enzyme [24,48]. With dihexanoyl-PC as a substrate, the more Ca^{2+} added, the lower the pH optimum, an apparent Km for Ca^{2+} was found to be only 0.21 mM at pH 7.5 and rates plotted against substrate concentration showed regions of parabolic and hyperbolic kinetics [42].

PLD purified 240-fold from rat brain microsomes has been shown to hydrolyze sonicated PC and PE with Km values of 0.75 and 0.91 mM in the presence of the detergents miranol 2HM and cholate and about 4 mM Ca^{2+} at 37°C, pH 6.0 [11,89].

Numerous papers which deal with enzymes that act on complex lipids contain irregular kinetic curves [90]. Chen and Barton [34], using dialkyl analogues of PC containing C_{14}, C_{16} or C_{18} as substrate for PLD_c, found that though higher concentrations of SDS were required than for the diacyl counterparts to obtain optimal stimulation, the curves of rate against concentration of the ether analogues in both aqueous (in presence of SDS) and organic (chloroform-ether, 1:10) systems were biphasic, indicating an inhibition by excess substrate.

Leibovitz-Ben Gershon *et al*. [91], investigating the

kinetic properties of lysophospholipase D from rat brain, found that the soluble form exhibited hyperbolic kinetics only to a certain substrate concentration, above which it became parallel to the abscissa, for the soluble enzyme, and for the particulate enzyme the curves were biphasic. The biphasic kinetics have been observed by several investigators with lysophospholipase D from various sources [90, reviewed in 92].

Mechanism of PLD Action

The catalytic mechanism of PLD has not been well characterized. Due to the limited progress made in the purification of this enzyme coupled to the heterogeneity of the reaction mixture, kinetic studies have been very difficult and therefore only fragmentary mechanistic information is available.

Reduction of a labile sulfhydryl group presumed as the basis of dithiothreitol activation of PLD_c [42] and the inhibition of the impure enzyme by p-chloromercurial benzoate (but not other thiol reagents) [41] was suggessive of the presence of an 'essential' SH. However, direct measurements of SH in the highly purified peanut PLD failed to show its presence [43]. Whether or not the thiol group plays a catalytic role is unclear [1]. The exchange reaction between the choline group of PC and free [^{14}C]choline has been used to support the possible existence of a phosphatidyl-enzyme complex [41].

Based on recent stereochemical studies using diastereo-

mers of PC that contain ^{18}O [94,95] and S [96], Bruzik and Tsai [94,95] and Jiang et al. [96] concluded that both the transphosphatidylation and hydrolysis reactions catalyzed by the cabbage PLD proceed with an overall retention of configuration and without appreciable exchange of oxygen isotopes; a mechanism whereby a phosphatidyl-enzyme intermediate is formed initially, which can then be transphosphatidylated to form phosphatidylethanolamine or hydrolyzed to phosphatidic acid seems most probable.

Interfacial issues pervade the study of all phospholipases, and the mechanism(s) responsible for interfacial activation continue to be actively investigated but presently no consensus has been reached on a single explanation. Apart from the importance of understanding the physical basis for this phenomenon, interfacial activation can represent an experimental complication of some magnitude when studying the mechanism of catalysis [70]. The determination of interfacial area of an emulsion is difficult and the representation of kinetic data in this manner has not been widely used [97]. In addition, if detergents or other additives are present the surface area of the emulsion does not represent the true 'substrate' concentration since part of the surface is occupied by the detergent or the additive. For these and other reasons [97] it is apparent that interpretation of Michaelis parameters is ambiguous when using lipolytic enzymes and insoluble substrates [70].

Scope of Study

A continuous assay of very high sensitivity for monitoring the action of PLD on phosphatidylcholines of varying chain lengths is developed at fixed total detergent concentration. With this assay, the dependence of the rate of the PLD-catalyzed reaction on substrate and calcium ion concentrations is determined, with the aim of gaining some information regarding the mechanism of action of this enzyme.

To supplement the kinetic studies several physical measurements are utilized: (a) UV difference spectroscopy provides information on the interaction of calcium ion with the enzyme and with the substrate micelles; (b) light scattering measurements are employed to ensure that optically clear phospholipid solutions are being studied; (c) critical micelle concentrations of Triton X-100 in various mixtures are determined to ensure that all kinetic studies are carried out with substrate in the same phase.

CHAPTER II

EXPERIMENTAL

Materials

Phospholipase D from cabbage and peanut [phosphatidylcholine phosphatidohydrolase, E C 3.1.4.4.] Type I, (634 U/mg protein, cabbage), Type IV (550 U/mg protein, cabbage) and Type II (200 U/mg protein, peanut), were purchased from Sigma Chemical Co., St. Louis, Missouri 63178. Some preparations of cabbage PLD (Lot 1112103, 0.3 U/mg solid, Boehringer and Lot 102634, 0.52 U/mg solid, Calbiochem) were also obtained initially from Boehringer Mannheim Canada Ltd., 1475 Begin, St. Laurent, Que H4R 1V8 and Calbiochem-Behring, P.O. Box 12087, San Diego, CA. 92112.

Choline oxidase (COD) from *Alcaligenes* species [choline: oxygen 1-oxidoreductase, E C 1.1.3.17] and bovine serum albumin (BSA, Cohn fraction IV crystalline) were Sigma Products.

Horseradish peroxidase (POD), [hydrogen peroxide oxidoreductase, E C 1.11.1.7] Grade II, 100 U/mg solid was obtained from Boehringer-Mannheim.

Enzyme units quoted in the text are those defined by the respective suppliers. Unit definitions are as follows: PLD (Sigma), one unit is the amount of enzyme which will liberate 1.0 μmole of choline from L-α-phosphatidylcholine ([^{14}C]-labelled) per hour at pH 5.6 at 30^{0}C. PLD (Boehringer and Calbiochem) one unit is defined as the

amount of enzyme that will hydrolyze 1.0 µmole of lecithin per minute at 25°C, pH 7.0. For choline oxidase, one unit is that amount of enzyme which catalyzes the formation of 1.0 µmole of H_2O_2 from choline and H_2O per minute at 37°C, pH 8.0. One unit of peroxidase is defined as that amount of enzyme which catalyzes the oxidation of 1.0 µmole ~~of guai~~acol by H_2O_2 per minute at 25°C, pH 7.0.

L-α-phosphatidylcholine, didecanoyl, dimyristoyl, dipalmitoyl and distearoyl (synthetic), sodium dodecyl-sulfate (SDS) and Triton X-100 were Sigma products. 4-Aminoantipyrene (AAP) and choline chloride were purchased from ICN Pharmaceuticals, Inc., Plainview, N.Y. Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) was synthesized initially as described under methods but later consignments were obtained from Aldrich Chemical Company Inc. P.O. Box 2060, Milwaukee WI 53201. Coomassie Brilliant Blue G-250 (CBBG) (Lot B9J) was obtained from Eastman Kodak Co., Rochester N.Y. 14650. All other reagents were standard reagent grade.

Apparatus

Spectrophotometers, Model 35 and Acta MVI, Beckman Instruments Inc., Analytical Instruments, Fullerton California; Shimadzu UV-240, Shimadzu Corporation, Spectrophotometric Instruments Plant, Analytical Instruments Division, Kyoto, Japan; and Hewlett-Packard 8451A Diode Array, Hewlett-Packard Scientific Instruments Division, 1601

California Avenue Palo Alto, CA 94304, were all used in the studies.

All spectral scans and time vs colour development studies were performed in both Beckman 35 and Acta MVI. The former is equipped with an electrical heating module while the temperature of the cell compartment of the latter was maintained constant with a circulating water bath. Virtually identical results were obtained on both instruments. Single wavelength and two-wavelength readings were performed with the Shimadzu UV-240, equipped with linear regression autocalculating mechanism.

UV-difference spectra were recorded using the single beam Hewlett-Packard Instrument which is equipped with a diode array detector. All absorbance measurements were made using a standard quartz semimicro cuvettes (1 mL, 1-cm path-length).

Reagents

All solutions were prepared using distilled deionized water and all chemicals and enzymes were used without further purification.

The buffers used include: (a) Sodium acetate buffer (0.2 M, pH 5.0 to pH 6.5); (b) β,β-dimethylglutarate buffer (DMG buffer) (10 mM, 0.2 M, pH 7.0) and (c) Tris-HCl buffer (50 mM, 0.4 M, pH 7.0 to pH 8.0). These buffer solutions were made using a Radiometer model 26 pH meter equipped with radiometric semi-microcombination electrode number Gk

2301-C. The pH meter was standardized with buffer solutions of pH 9, 7 and 4 supplied by Fisher Scientific. All pH measurements were done at room temeprature.

PLD (cabbage) solutions were prepared in 10 mM DMG buffer, pH 7.0 containing 1 mg/mL BSA and used at room temperature on the day of preparation. PLD (peanuts) solutions were prepared in 10 mM Tris-HCl buffer, pH 8.0 but without BSA.

Working acetate buffer (stepwise assay): This was prepared to contain 0.3% Triton X-100 (w/v), 6 mM SDS, and 24 mM AAP in 0.2 M acetate buffer, pH 5.5.

Tris-HCl Reagent (Tris Reagent) (stepwise assay): This reagent was made to contain 0.2% Triton X-100 (w/v), and 18 mM HDCBS in 0.4 M Tris-HCl buffer pH 8.0, as well as 2.8 U/mL COD and 6.0 U/mL POD and stored in a brown bottle.

Tris Reagent (continuous assay): This was prepared to contain 18 mM HDCBS, 4.8 mM AAP, 10 U/mL COD and 20 U/mL POD in 50 mM Tris-HCl buffer, pH 8.0. This reagent is stable for at least four days when stored in a brown bottle at 4°C. There is no nonenzymic coupling of AAP and HDCBS at this pH compared to that observed when this reagent is prepared at pH 7.0 where the stability also reduces to less than 24 hrs. when stored under the same conditions.

Stock Phosphatidylcholine Solutions:

(a) Low concentration PC standards

This solution was made to contain 70 μM dipalmitoyl

phosphatidylcholine (DPPC) solubilized in 0.09% SDS-0.2% Triton X-100 detergent mixture. Only these DPPC standards were used in the development of the continuous assays.

(b) Intermediate Range PC Standards

These solutions, also made up in 0.09% SDS-0.2% Triton X-100, contain 0.83 mM or 1.67 mM of either didecanoyl phosphatidylcholine (DDPC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC).

(c) High PC Standards

For these solutions, about 6.67 mM of the respective phosphatidylcholines - DDPC, DMPC, DPPC and DSPC - were solubilized in 0.09% SDS-0.2% Triton X-100.

All the PC standards were prepared at room temperature and twelve to eighteen hours of constant stirring was necessary to effect solubilization of DPPC and DSPC while less time (4 hr) was required for DMPC and DDPC to obtain optically clear solutions. The low and intermediate range standard solutions so made, remain clear indefinitely when stored at 4°C. However, the high standards were clear only for 3 days when stored at room temperature, and slight cloudiness which does not disappear even when further stirring occur thereafter. These high standards were not stored at 4°C since this permanent opalescence develops even faster. The intermediate and high standards were used

for the kinetic studies. Only optically clear solutions were used in all the studies. Serial dilutions of the PC standards were made in 0.09% SDS-0.2% Triton X-100.

(d) Choline Chloride Standard

Fresh solutions were prepared to contain 70 μM choline chloride in 0.09% SDS-0.2% Triton X-100 and serial dilutions were made in this detergent mixture.

(e) Calcium Chloride Solutions

A stock solution of 1.0 M $CaCl_2$ was made in water from which other stock solutions of required concentrations were made.

Although stability guidelines have been given previously [86] for our stepwise assay, all enzyme containing solutions and choline chloride solutions were used only on the day of preparation.

Methods

(a) Synthesis of Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate

Synthesis of sodium 2-hydroxy-3,5-dichlorobenzenesulfonate was carried out by a modification of the method of Artiss [98].

Five grams of 2-hydroxy-3,5-dichlorobenzenesulfonyl chloride were dissolved in 20 mL of water in a 25-mL round bottom flask and warmed on a steam bath. Then 1.53 g (2

equivalents) NaOH pellets were added then heated for 30 min on the steam bath followed by suction filtration while hot on a Whatman No. 2 filter paper. The filtrate was allowed to stand at room temperature overnight. The crystals formed were filtered off and dried. The yield based on 2,4-dichlorobenzenesulfonyl chloride was 21%. NMR spectra were obtained on an EM 360 NMR spectrometer (Varian Industrial Products) in deuterium oxide and are reported in ppm from sodium trimethylsilylpropanesulfonate as internal standard. The product was an off-white solid, m.p. > 270°C, NMR 7.67 (d, J = 2.7 Hz, 1H), 7.74 (d, J = 2.7 Hz, 1 H). Experimental results with our product was identical to those obtained using the preparation purchased from Aldrich.

(b) Enzyme Activities

In order to obtain a suitable activity of PLD, the units of the enzyme were adjusted until an absorbance at 510 nm greater than 0.9 was obtained upon completion of the colour reaction using the 70 uM PC standard as sample. This was routinely checked for each purchase using the stepwise assay and later the continuous rate method using two or three representative time scans. This procedure was necessary since we observed a change in suppliers cited enzyme activities since the two previous publications [86,99] from this laboratory under identical conditions.

The activity of the coupling enzymes were also checked routinely with 70 uM choline chloride as sample.

(c) Measurement of Optical Clarity of PC Solutions

The ratios of the absorbance values at 700 nm to absorbance values at 500 nm of solubilized lipid solutions were used to measure light scattering by these solutions [100]. According to the Raleigh Rule for Light Scattering, ($K = c/\lambda^4$, K = extinction due to light scattering; c = scattering constant; λ = wavelength in nm), for scattering light the ratio of A_{700}/A_{500} should equal $(500)^4/(700)^4 = 0.26$. All our stock solutions showed no absorbance at both 500 nm or 600 nm. On the other hand, PC solutions deliberately prepared in the presence of excess $CaCl_2$ having observable turbidities gave ratios greater than 0.45. To check whether light scattering effects were present in our assay protocol in the presence of $CaCl_2$, 300 µL of serially diluted PC solutions, 50 µL of the highest concentrations of $CaCl_2$ normally encountered were mixed with 650 µL of Tris-HCl buffer pH 8.0 and the absorbance at 500 nm and 700 nm were measured. All solutions in our range of PC and $CaCl_2$ concentrations do not give any evidence of light scattering by the Raleigh method.

(d) The Stepwise Assay

The assay protocol was identical to that reported previously [86,99]. In brief, 100 µL of working acetate buffer, 300 µL of standard or sample and 50 µL of 0.3 M

aqueous calcium chloride were mixed. The hydrolysis reaction was initiated with the addition of 50 µL of PLD solution. The reaction was allowed to proceed at 30°C in a heating bath (dri-bath) for 15 min. at which time 500 µL of Tris Reagent was added and mixed. The reaction mixture was incubated for 12 min. at 37°C and then absorbance at 510 nm was measured in 1 cm cuvettes. A reagent blank in which the sample was replaced by the appropriate detergent mixture and PLD solution was replaced by DMG buffer containing 1 mg/mL BSA was used to zero the instrument. A phospholipase D blank in which only sample was replaced by the detergent mixture and a sample blank in which only PLD solution was replaced by DMG/BSA buffer were run at the same time. The absorbance of the PLD blank was subtracted from all tests and standards while the absorbance of the sample blanks was subtracted from the readings of their respective samples.

The Sigma Type IV PLD used for the bulk of this work showed a null blank reaction. However, the Sigma Type I, the Boehringer and the Calbiochem PLD showed minimum blank absorbances of 0.022, 0.100 and 0.700, respectively.

(e) <u>The Continuous Assay</u>

Unless stated otherwise the procedure was as follows: 100 µL of 50 mM Tris-HCl buffer, pH 8.0, 300 µL of sample or standard or serial dilutions thereof, 500 µL of Tris Reagent, and 50 µL $CaCl_2$ solution added according to that

order and mixed. A baseline was recorded for 3 min. at 37°C then the reaction was initiated by addition of 50 uL of the appropriate dilution of PLD, mixed and allowed to proceed at 37°C in the recording spectrophotometer. Baseline corrections were negligible except at high substrate concentrations encountered in the kinetic study. Blanking was performed as in the stepwise assay. For rate measurements, the velocity of red dye formation was monitored at 510 nm for times up to 5 min. For end-point determinations the absorbance at 510 nm was measured after 20 min.

(f) Critical Micelle Concentration Determinations

CMC for Triton X-100, and various mixtures of Triton X-100, SDS, DPPC and Ca^{2+} was determined according to the method of Rosenthal and Koussale [101] based on the change in absorption spectrum of Coomassie Brilliant Blue G-250. The CBBG reagent was prepared as described by Bradford [102]. Briefly, 100 mg of CBBG was dissolved in 50 mL 95% ethanol. To this solution 100 mL 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. Final concentration of CBBG in the reagent was 0.01% (w/v) and in the assay was 40 ppm (w/v). Concentrations of the detergents and PC stock solutions were maintained as in our continuous assay described above.

The assay procedure was as follows: Varying concentrations of detergent or mixtures were added in a volume of 0.2 mL to 4.8 mL of CBBG reagent. The solution was mixed

briefly and an absorption spectrum was obtained against a water blank. Alternatively, only absorptions at 470 nm and 620 nm were obtained simultaneously (dual-wavelength measurements). The A_{470} and A_{620} were plotted independently against detergent concentration. The CMC can readily be determined from this plot.

(g) UV - Difference Spectroscopy

Difference spectra were recorded by using the single beam Hewlett-Packard Instrument described above. The data analyzer was used to store the reference spectrum. Difference spectra were obtained by direct automatic subtraction of the stored spectrum from the actual measured one. To start with, the instrument is blanked with no cuvette in the cell compartment in the relevant wavelength range. Then the quartz cuvette containing e.g. for calcium titrations with enzyme, 50 mM Tris buffer, buffered enzyme solution, and water were mixed and placed in the cell compartment. The reference spectrum was then recorded and stored in the memory. The same cuvette was then washed and equal volumes of Tris buffer, enzyme solution and calcium solution as was in the reference was placed in the cell compartment and the difference spectrum recorded automatically using the alphanumeric keyboard for the command. The solutions were not stirred during recording of the actual difference spectrum. In all cases, recordings were repeated to check for the stability of the measured signal. The observed difference

signal was routinely taken from the value of the absorption maximum relative t the baseline. Difference spectra obtained in either the presence or absence of 0.05 M NaCl in the buffers to eliminate ionic strength changes were identical.

(h) SDS-PAGE

SDS-polyacrylamide gel electrophoresis was performed with the Laemmli [88] gel system using 8% polyacrylamide gels (14 x 10 x 0.15 cm) with dithiothreitol as protein reductant.

To test for presence or absence of Ca^{2+}-activated or other proteases, SDS-PAGE was conducted on the enzyme proteins in the presence and absence of 2.5 mM Ca^{2+}. Both the Ca^{2+}-treated and non-treated enzymes were incubated for times of 0, 30 and 60 minutes at 37°C before being mixed with an equal volume of sample buffer, boiled 1 min and loaded on to the gel.

(i) Preparation of Human Erythrocyte Ghosts

Human erythrocyte ghosts were prepared as previously described [99] by the method of hypotonic lysis [106] and washed until white by multiple centrifugation-resuspension cycles initially in 5 mM sodium phosphate buffer, pH 7.4, containing 15 mM sodium chloride and 0.1 mM EDTA. Finally, the ghost pellet was washed twice with 1 mM Tris-HCl buffer pH 8.0, containing 15 mM sodium chloride and 0.1 mM EDTA.

The final pellet was diluted twofold with 0.9% SDS. Aliquots of this stock solution were taken for total protein determinations and the balance was diluted tenfold with 10% Triton X-100 and water so that the final detergent concentrations were 0.2% Triton X-100 and 0.09% SDS. This solution and serial dilutions in the same detergent mixture were assayed for PC content.

(j) Protein Determination

Protein determination was performed by the method of Peterson [107] without sample precipitation based on BSA standards.

CHAPTER III

RESULTS AND DISCUSSION

A. Development of the Continuous Lecithin Assay

Various chromogen systems have been utilized for the POD catalyzed reduction of hydrogen peroxide to make the reaction amenable to spectrophotometric analysis. Phenol and AAP have been used extensively for this purpose [85,108-111]. Barham and Trinder [112] reported that the use of a crude sulfonic acid derivative of 2,4-dichlorophenol increased the sensitivity of their system four-fold over the corresponding phenol reaction. Artiss *et al*. [86] found that the unreacted sulfuric acid in the Barham and Trinder [112] system had detrimental effects on the buffering capacity of the Tris-HCl buffer employed. They isolated and characterized the sulfonated dichlorophenol as its sodium salt, 2-hydroxy-3,5-dichlorobenzenesulfonate. The use of HDCBS has been shown to produce a several-fold increase in sensitivity over that of phenol [86,113]. From comparative data of various ratios of AAP to HDCBS, Artiss *et al*. [86] decided on a molar ratio of 2.4:9.0, AAP:HDCBS. Thus AAP and HDCBS have been kept at 2.4 and 9 mM, respectively, in the development of our continuous assay. Similarly the optimization procedures were carried out using L-α-dipalmitoyl phosphatidylcholine based on a sample volume of 300 uL equivalent to about 70 uM substrate and using the cabbage phospholipase D.

$$\text{PHOSPHATIDYLCHOLINE} \xrightarrow{\text{PHOSPHOLIPASE D}} \text{PHOSPHATIDIC ACID} + \text{CHOLINE}$$

$$\text{CHOLINE} + 2\,O_2 + H_2O \xrightarrow{\text{CHOLINE OXIDASE}} \text{BETAINE} + 2\,H_2O_2$$

$$2\,H_2O_2 + \text{HDCBS} + \text{AAP} \xrightarrow{\text{PEROXIDASE}} \text{RED CHROMOGEN}$$

THE ASSAY SYSTEM

SDS is a known activator of PLD [7,27,28,34,45,46,48] and a powerful membrane solubilizing agent [106], while Triton X-100 has been reported to inhibit PLD activity [114]. The use of SDS in our reaction mixture is obvious, and Triton X-100, however, was found necessary in order to prevent turbidity upon addition of calcium, an essential activator of PLD [5] due to formation of calcium dodecylsulfate.

Taylor et al. [99] studied the effect of various ratios of SDS to Triton X-100 in the solubilization of PC and their effect on the hydrolysis rate of PLD_c using the stepwise assay developed in this laboratory [86]. Their results showed that best results were obtained when PC was made up in 0.09% SDS-0.2% Triton X-100. Consequently, we solubilized all our PC samples in a detergent mixture of 0.09% SDS-0.2% Triton X-100. We, however, found that for the continuous assay, omission of Triton X-100 from the Tris reagent component resulted in very large increases in rates. Therefore, SDS and Triton X-100 were only included in the samples at the levels used previously.

Cabbage PLD has been reported to exhibit optimum activity at pH 5.5 but to be less stable at that pH than at pH 7.0 [115]. In previous stepwise assays [86,99,110,116, 117], reagents containing COD and POD were prepared at pH 8.0 since these two enzymes show optimal activities in the range of pH 7-8 [118]. The PLD hydrolysis step was carried

out separately at some pH around 5.5. However, it has been shown that the pH optimum of PLD_c can be shifted to 6.6 with the addition of SDS [119]. Calcium has been demonstrated to modulate the pH-dependence of the cabbage enzyme; a pH optimum of 6.25 was exhibited at high $CaCl_2$ concentration and an optimum of 7.25 was displayed at very low calcium concentrations using dihexanoyl phosphatidylcholine as substrate [42].

Optimization Strategy

Following these reports and in addition to our preliminary studies showing that PLD_c hydrolysis is possible between pH 7 and 8, we thought it expedient to develop a continuous assay for cabbage PLD by judiciously controlling the amounts of these modifiers in the reaction mixture. The cost of choline oxidase and its steep loss in relative activity below pH 8.0 made the latter pH seem appropriate for formulation of a continuous assay.

In our optimization procedures for both the continuous end-point and rate methods, the concentration of Ca^{2+}, SDS, and Triton X-100 were chosen such that levels adequate for PLD activation but simultaneously low enough to avoid calcium dodecylsulfate precipitation, and Triton X-100 inhibition of the PLD activity were maintained. Under these circumstances, it was possible to combine enzymes and modulators into one continuous monitoring system at pH 8.0 with comparable sensitivity as the most recent stepwise assay

[99]. See Table Ia and b.

Variation of COD and POD Activities

To optimize the activities of the coupling enzymes, each coupling enzyme was assayed using the same buffer, pH, temperature, and substrate concentrations that were employed to determine the primary enzyme as indicated by McClure [120]. Furthermore, we found the effect of varying the activities of the two coupling enzymes preferable to the usual practice of adding excess coupling enzymes in order to minimize cost.

The effects of COD and POD were individually examined on the complete reaction system such that the concentration of all the reaction components were held constant at their level normally encountered while the activities of the respective coupling enzymes were varied. From the results of these experiments, Table IIa, b, Figure 1a, b and Figure 2a, b, optimal values of 10 units and 5 units per assay of POD and COD respectively were estimated and used in our final assay protocol.

Variation of Ca^{2+} Levels

The data in Table III and Figure 3a, b show the effect of Ca^{2+} on the rate of hydrolysis of PC. From those results, we decided on 10 mM Ca^{2+} for the quantitation of PC for both end-point (Figure 3a) and rate (Figure 3b) assays. Calcium concentrations greater than 12.5 mM lead to

Table Ia

Standard Curve Data for Phosphatidylcholine or Choline[a]

Method	Substrate	Least Square Parameters[b] A	B	r^2
Continuous	Choline	13.00±0.01	0.012±0.000	0.9999
Stepwise[c]	PC	13.21±0.27	-0.040±0.002	0.9989
Stepwise[d]	PC	12.04±0.07	-0.012±0.000	0.9999
Continuous (Endpoint)	PC	14.91±0.15	0.010±0.001	0.9998
Continuous (Endpoint)	PC	14.19±0.09	-0.041±0.001	0.9999
Continuous (Rate)	PC	3.50±0.09	0.000±0.001	0.9987
Continuous (Rate)	PC	3.99±0.20	-0.002±0.001	0.9967

[a]Runs were formulated as in standard procedures, see text.

[b]Data were fitted to the equation $A_{510} = A[\text{Substrate}] + B$ by least analysis with the coefficient of regression shown in column 5. Uncertainties in A and B are root mean square deviations. Units for the least square constants for A are, mM^{-1} and $mM^{-1}\,min^{-1}$. Similarly, the units for the intercept B, are expressed in absorbance and absorbance/min for the endpoint and rate modes respectively.

[c]PLD (cabbage) from Sigma. Hydrolysis at 5.5.

[d]PLD (cabbage) from Boehringer. Hydrolysis at 5.5.

Table Ib

Absorbances of Phosphatidylcholine or Choline Standards[a] used for Regression Data Shown in Table I(a)

[SUBSTRATE]	A	A	A	A	A	ΔA/min	ΔA/min
μM	1	2	3	4	5	6	7
70.00	0.922	0.888	0.858	1.047	0.958±0.010		
58.33	0.770	0.727	0.712	0.890	0.785±0.004	0.200±0.000	
46.67	0.620	0.570	0.570	0.704	0.617±0.001	0.168±0.001	
35.00	0.466	0.418	0.437	0.528	0.452±0.002	0.123±0.000	0.135
23.33	0.315	0.293	0.296	0.366	0.291±0.006	0.080±0.002	0.095
11.67	0.164	0.100	0.151	0.179	0.129±0.009	0.040±0.000	0.042

[a]Each standard was measured only once except where uncertainties are indicated for duplicate runs.

Columns 1-7 above correspond to lines 1-7, respectively, in Table I(a).

Table IIa

The Hydrolysis Reaction Rates of Various POD Activities

Units of POD per assay	Activities[a] A	ΔA/min
2	0.858±0.003	0.103±0.003
5	0.965±0.005	0.105±0.002
7	-	0.116±0.001
10	1.023±0.003	0.121±0.000
15	0.977±0.013	0.128±0.003
20	0.990±0.000	0.127±0.000

[a]Conditions: PC: 70 μM, Ca^{2+}: 9 mM, COD: 4 Units, PLD (Type IV): 35 Units, pH 8.0. All are continuous time scans which levelled off after 20 min.

Table IIb

The Hydrolysis Reaction Rates of Various COD Activities

Units of COD per assay	Activities[a] A	ΔA/min
2	0.950±0.005	0.109±0.001
4	1.020±0.005	0.135±0.005
5	1.027±0.008	0.146±0.011
6	0.972±0.011	0.139±0.005

[a]Conditions: PC: 70 μM, Ca^{2+}: 9 mM, POD: 10 Units, PLD (Type IV): 35 Units, pH 8.0. All are continuous time scans which levelled off after 20 min.

Figure 1

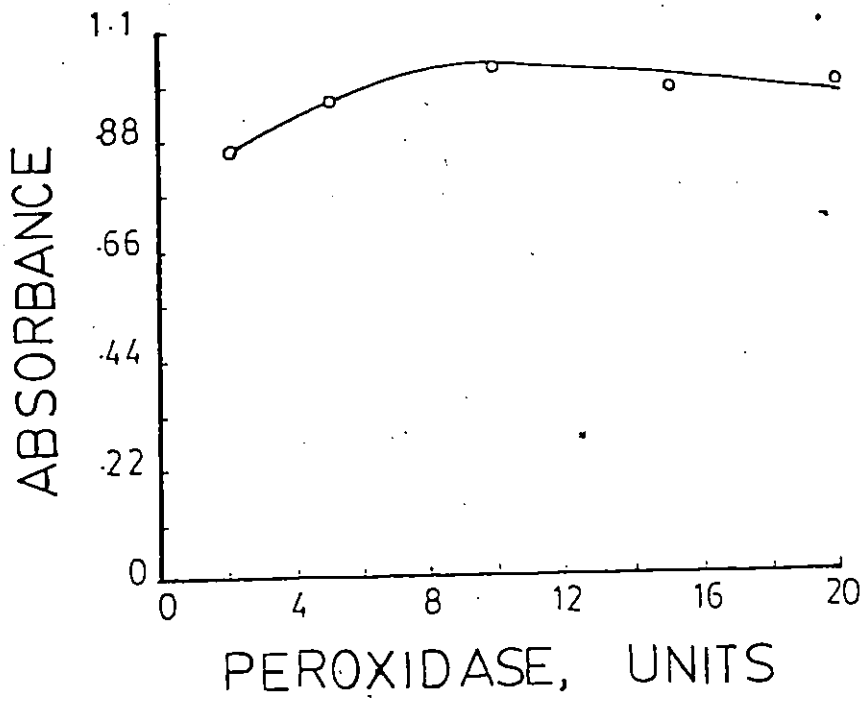
Variation in Peroxidase Activity

Legend;

Enzyme activity was varied using 70 µM PC sample at 4 Units choline oxidase, 9 mM Ca^{2+} and 35 Units PLD (Type IV). Error estimates are given in Table II(a). Top graph represents the continuous endpoint method and bottom diagram represents the rate method. Both determinations were made under same conditions.

Figure 1

a)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.66
.44
.22
0
ABSORBANCE
0
4
8
12
16
20
PEROXIDASE, UNITS

b)

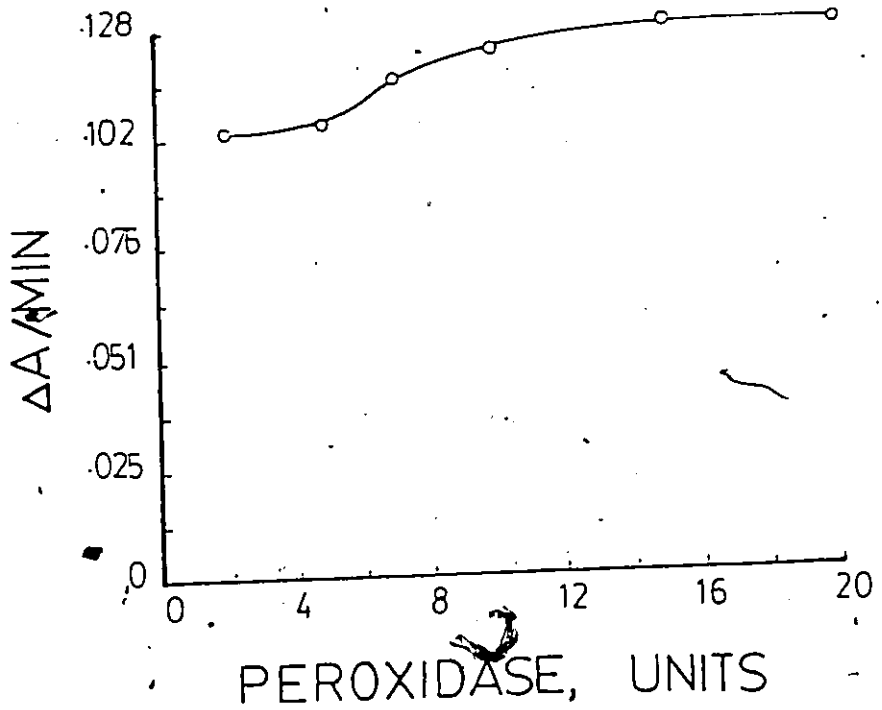

.128
.102
.076
.051
.025
0
ΔA/MIN
0
4
8
12
16
20
PEROXIDASE, UNITS

Figure 2

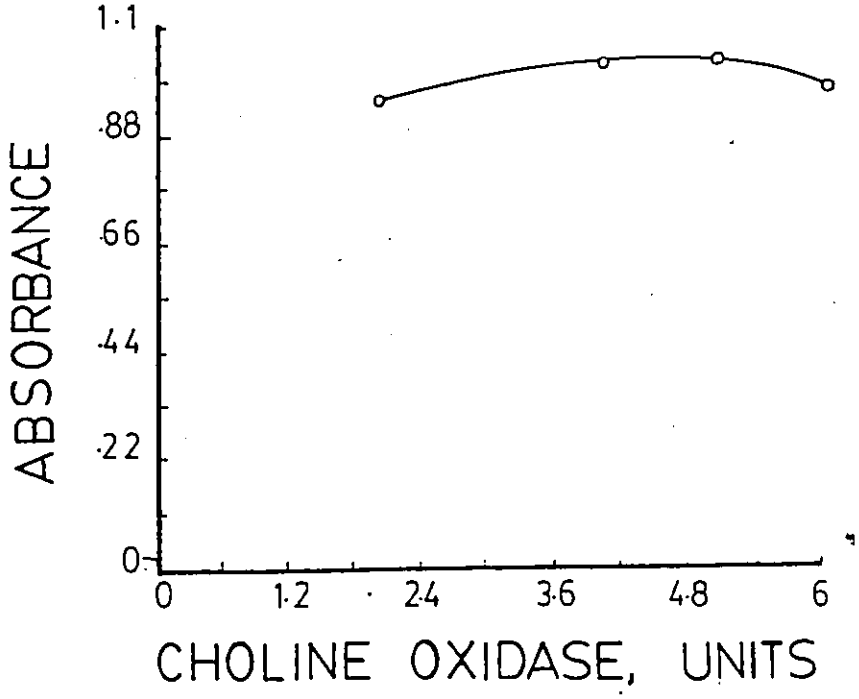
Variation of Choline Oxidase Activity

Legend:

Enzyme activity was varied using 70 µM PC sample at 10 Units peroxidase, 9 mM Ca^{2+} and 35 Units PLD (Type IV). Error estimates are given in Table II(b). Top graph represents the continuous endpoint method and bottom graph represents the rate method. Both determinations were made under same conditions.

Figure 2

a)


1·1
·88
·66
·44
·22
0
ABSORBANCE
0
1·2
2·4
3·6
4·8
6
CHOLINE OXIDASE, UNITS

b)

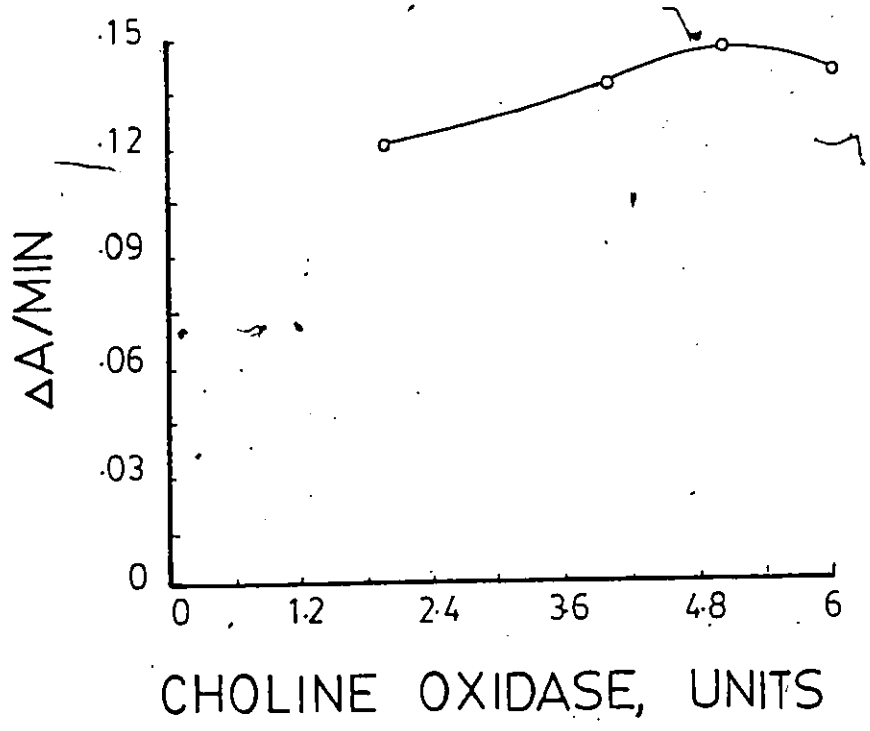

·15
·12
·09
·06
·03
0
ΔA/MIN
0
1·2
2·4
3·6
4·8
6
CHOLINE OXIDASE, UNITS

Table III

The Hydrolysis Reaction as a Function of Calcium Concentration[a]

Concentration of Calcium (mM)	Absorbance[b]	ΔA/min[c]
0.0	0.042±0.005	0.000±0.000
2.5	0.830±0.008	0.080±0.003
5.0	0.952±0.004	0.173±0.003
7.0	0.969±0.001	-
7.5	-	0.226±0.003
9.0	0.967±0.004	-
10.0	0.984±0.004	0.241±0.001
12.5	0.983±0.016	0.240±0.006

[a]Conditions: PC: 70 μM, COD: 5 Units, POD: 10 Units, PLD (Type IV): 35 Units, pH 8.0.
[b]Endpoint method.
[c]Rate method.

Figure 3(a)

Determination of Optimum Concentration of Ca^{2+} (End-Point Method)

Legend:

Optimum Ca^{2+} concentration was determined using 70 µM PC sample at 10 Units peroxidase, 5 Units choline oxidase and 35 Units PLD activity. The reaction mixture was incubated at 37°C/20min. and absorbance read at 510 nm. Error margins are given in Table III.

Figure 3(a)

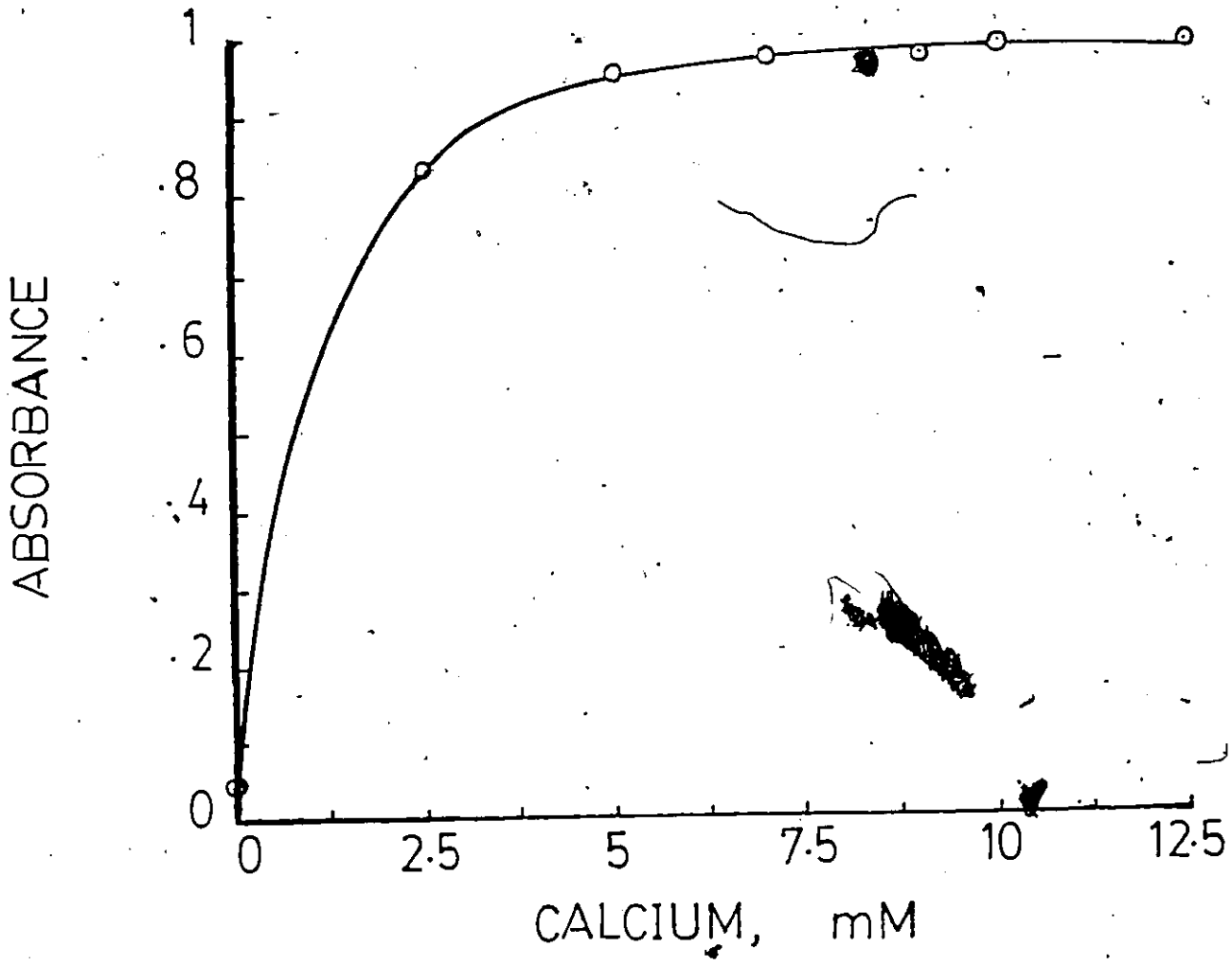


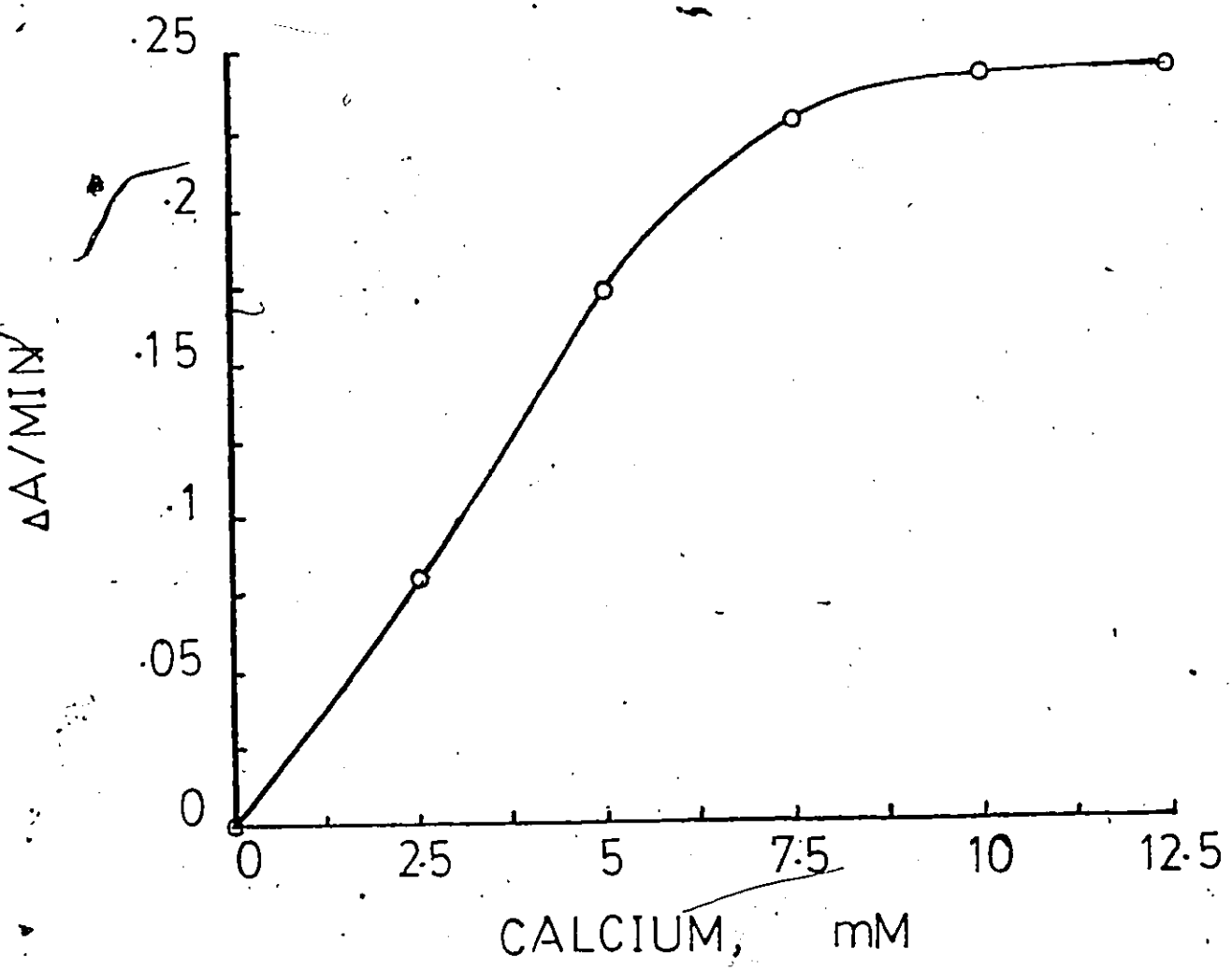
Figure 3(b)

Determination of Optimum Concentration of Ca^{2+} (Rate Method)

Legend:

Optimum Ca^{2+} concentration was determined using 70 µM PC sample at 10 Units peroxidase, 5 Units choline oxidase and 35 Units PLD activity. The reaction rates were estimated from the slopes of the time scans up to 5 mins. Error margins are given in Table III.

Figure 3(b)



precipitation in the end-point method on cooling after full colour development. It is evident from these studies that the combined use of SDS and Ca^{2+} as activators of PLD has drastically cut down the calcium requirement for the enzyme. Thus a 3- to 6-fold lower calcium concentration was used in this work compared to 30 mM [99], 40 mM [87] and 55 mM [86] that have previously been used by us and others.

Time for Hydrolysis in End-Point Method

The time for complete hydrolysis was determined from replicate time scans, using the high standard as a sample, an assessment similar to the criterion used by Takayama *et al.* [109]. Figure 4 shows that the reaction was complete for both highest and lowest level standards within 15-20 min. The trace on this graph were taken from single time scans for the respective concentrations.

Summary

Based on the foregoing considerations and results our Tris Reagent was made up to contain 20 U/mL POD, 10 U/mL COD, 18 mM HDCBS, and 4.8 mM AAP in 50 mM Tris-HCl buffer, pH 8.0. The final concentrations of components in a reaction mixture for both end-point and continuous assays is as follows: 5 U COD; 10 U POD, 9 mM HDCBS, 2.4 mM AAP, 10 mM Ca^{2+}, 0.94 mM SDS and about 0.96 mM Triton X-100 (based on average formula weight [56]) and an appropriate dilution of PLD added last to initiate the reaction. A short lag period

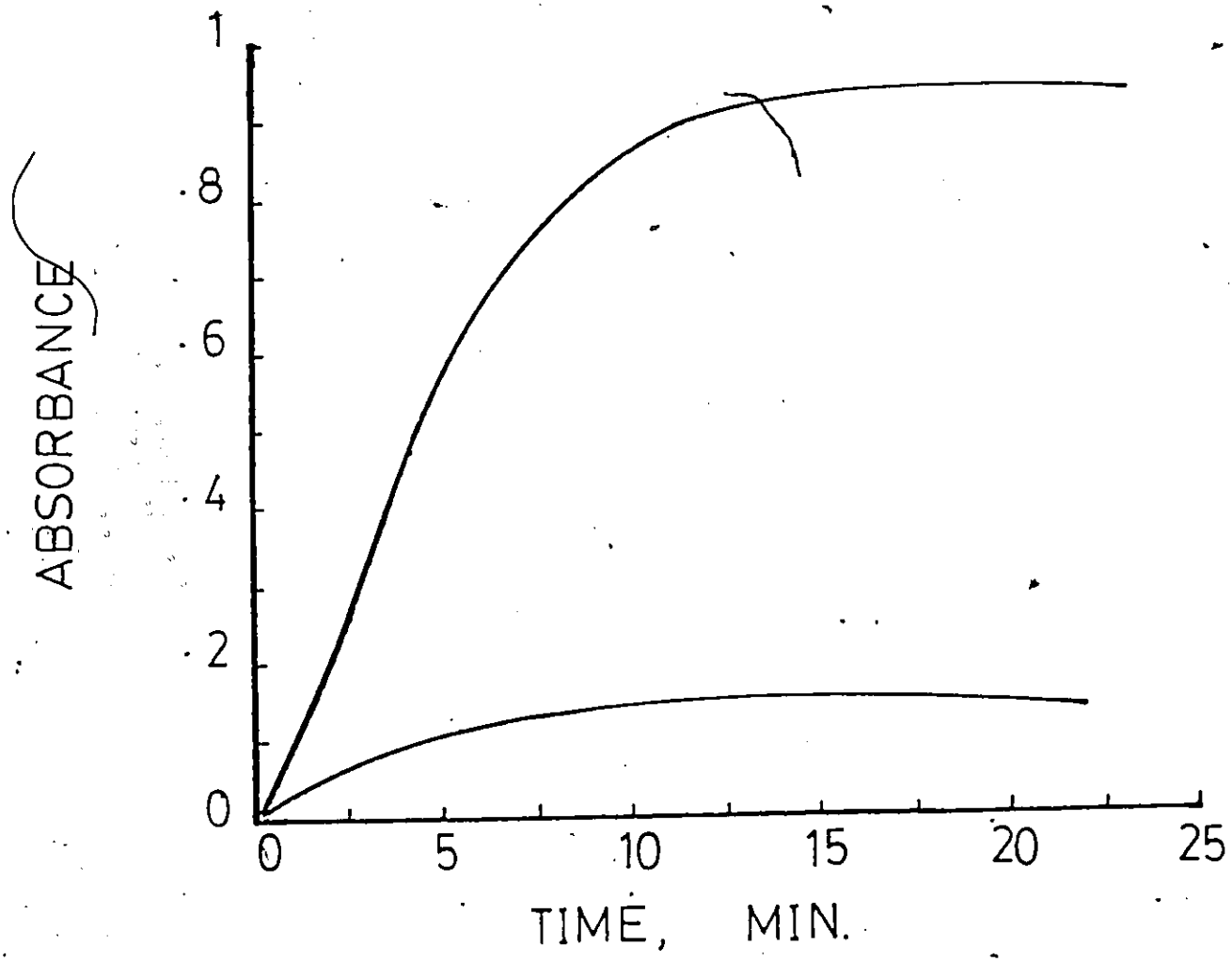
Figure 4

Time Dependence of PC Hydrolysis

Legend:

Shown are representative rise curves at 37°C at 510 nm, for two levels of phosphatidylcholine, 11.67 µM (Lower) and 70 µM (Upper curve).

Figure 4



of less than 30 seconds was followed by a constant rate of colour development which was linear for at least 5 min and gradually levelled off as substrate was being depleted (see Figure 4). It should be noted that SDS and Triton X-100 concentration have been cut to half from their previous values of 1.9 mM and 1.73 mM respectively reported in our stepwise assays [99,117].

Typical standard curves are presented in Figure 5 and Figure 6 for both end-point and rate determinations, respectively, the data for which are presented in Table Ib (col. 5 and 6, respectively). The best fit lines were obtained through linear regression analysis with the least square constants shown in Table Ia lines 5 and 6, respectively. Error limits on the parameters are root mean squares [123].

A comparison between the molar extinctions and absorbances produced by PC standards and choline chloride standards (Table Ib, col. 1 and 5; and Table Ia, col. 3) for the same amount of PC or choline chloride for the end-point method suggest that all the PC was hydrolyzed by PLD. Artiss *et al*. [86] and Artiss [98] observed about 80% hydrolysis in their stepwise assay, this may probably be due to the high concentrations of detergents included in the reaction mixture as noted by Taylor *et al*. [99].

Initial Rates Mesurement for PLD

The continuous rate assay was also linear with phospholipase D concentration for substrate concentrations greater

Figure 5

Standard Curve for End-Point Determination of PC

Legend:

Shown is a typical standard curve for end-point determination of PC at standard assay conditions. Each standard was measured in duplicate.

Figure 5

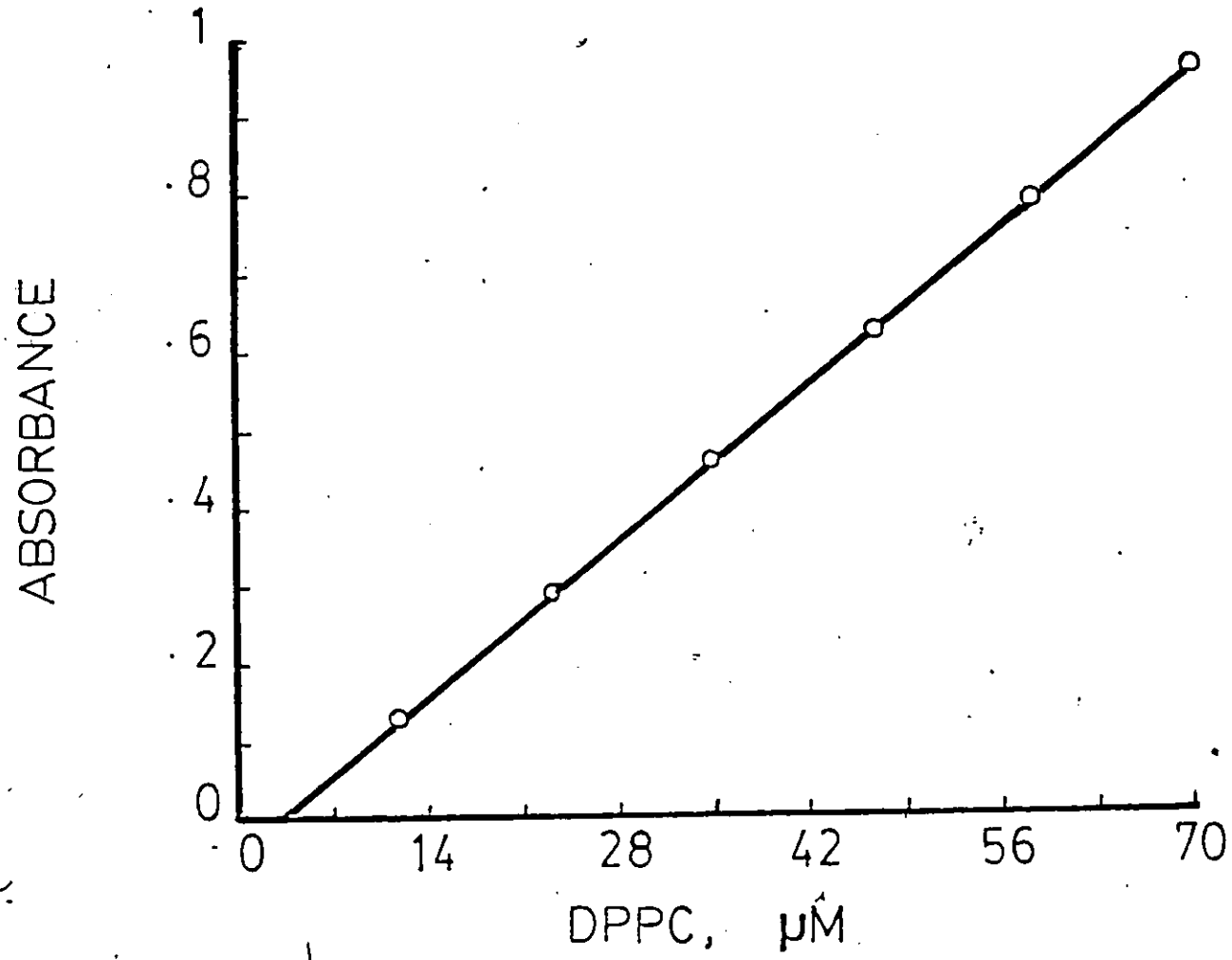


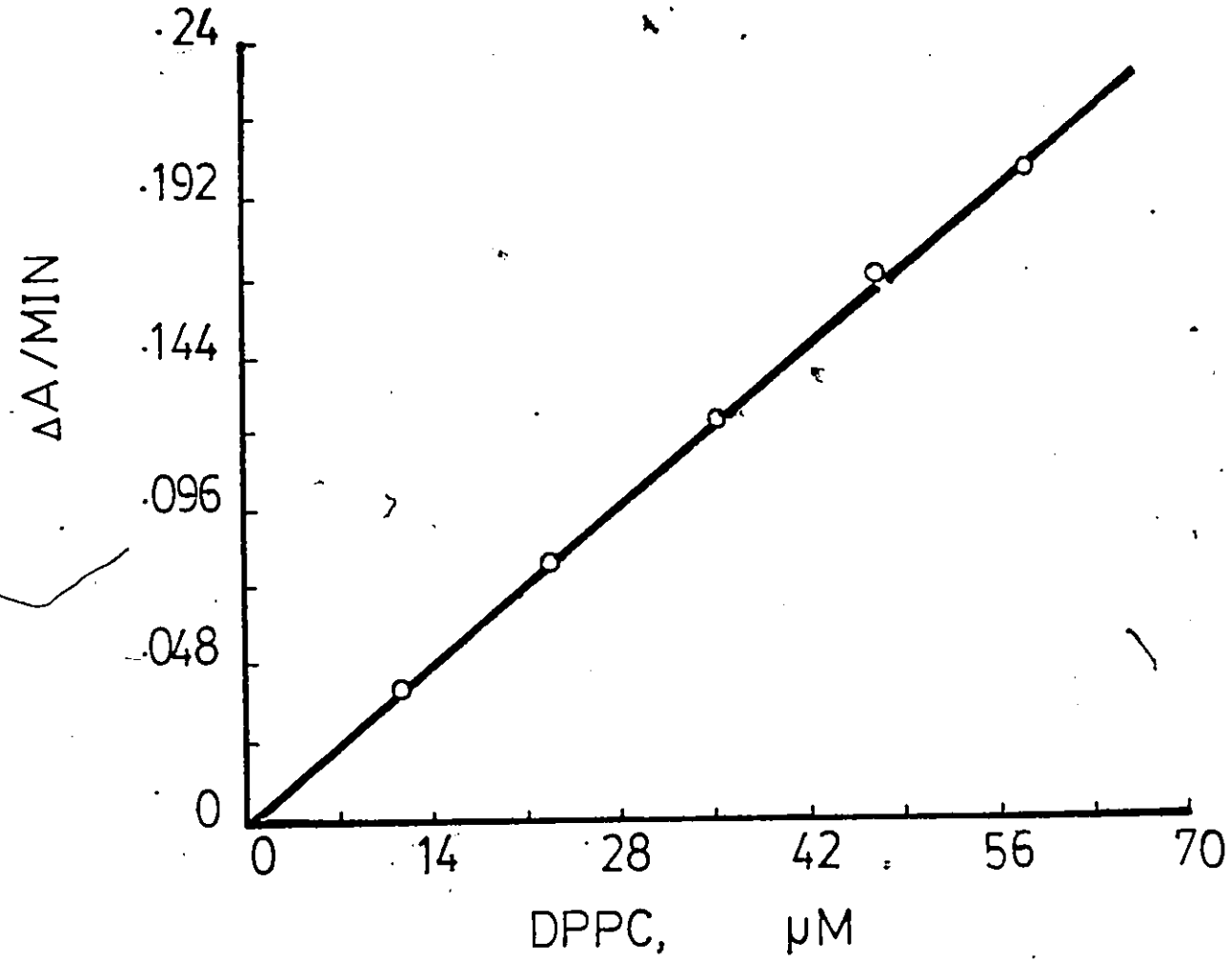
Figure 6

Standard Curve for Rate Determination of PC

Legend:

Shown is a typical standard curve for rate determination of PC at standard rate assay conditions. Each standard was measured in duplicate.

Figure 6



than were used in the development of the method.

Figure 7 (upper line), illustrates the rate of hydrolysis with increasing enzyme concentration with 0.125 mM PC as substrate. The linearity shown on this plot indicates that initial rates of reaction are being measured. By doing such plots, one can also get an estimate of enzyme activity under these conditions using the rate assay. Data for Figure 7 are posted in Table IV.

Though the assay was designed and optimized for the cabbage enzyme, Figure 7 (lower line), shows that it can equally be applied to the peanut enzyme. The lower rate observed for the peanut enzyme is not surprising since the enzyme has been reported to have a lower specific activity than its cabbage counterpart [46], although the specific activity quoted by the maufacturer, was 36% of that of the cabbage enzyme at pH 5.5. The peanut enzyme could not be used in the end-point method due to its lower specific activity plus the fact that very high concentrations of this enzyme could not be prepared since such solutions are visibly opalescent. This limits the amount of enzyme that can conveniently be utilized in our assay protocol without seriously jeopardizing optical clarity of the reaction mixture.

Determination of Lecithin in Human Erythrocyte Membranes

The sensitivity and versatility of the end-point and rate methods are illustrated by application to the determi-

Figure 7

PC Reaction Rate as a Function of PLD Concentration

Legend:

Shown is the rate of hydrolysis as a function of cabbage PLD (□) and peanut PLD (O) concentration with 0.125 mM PC concentration.

Figure 7

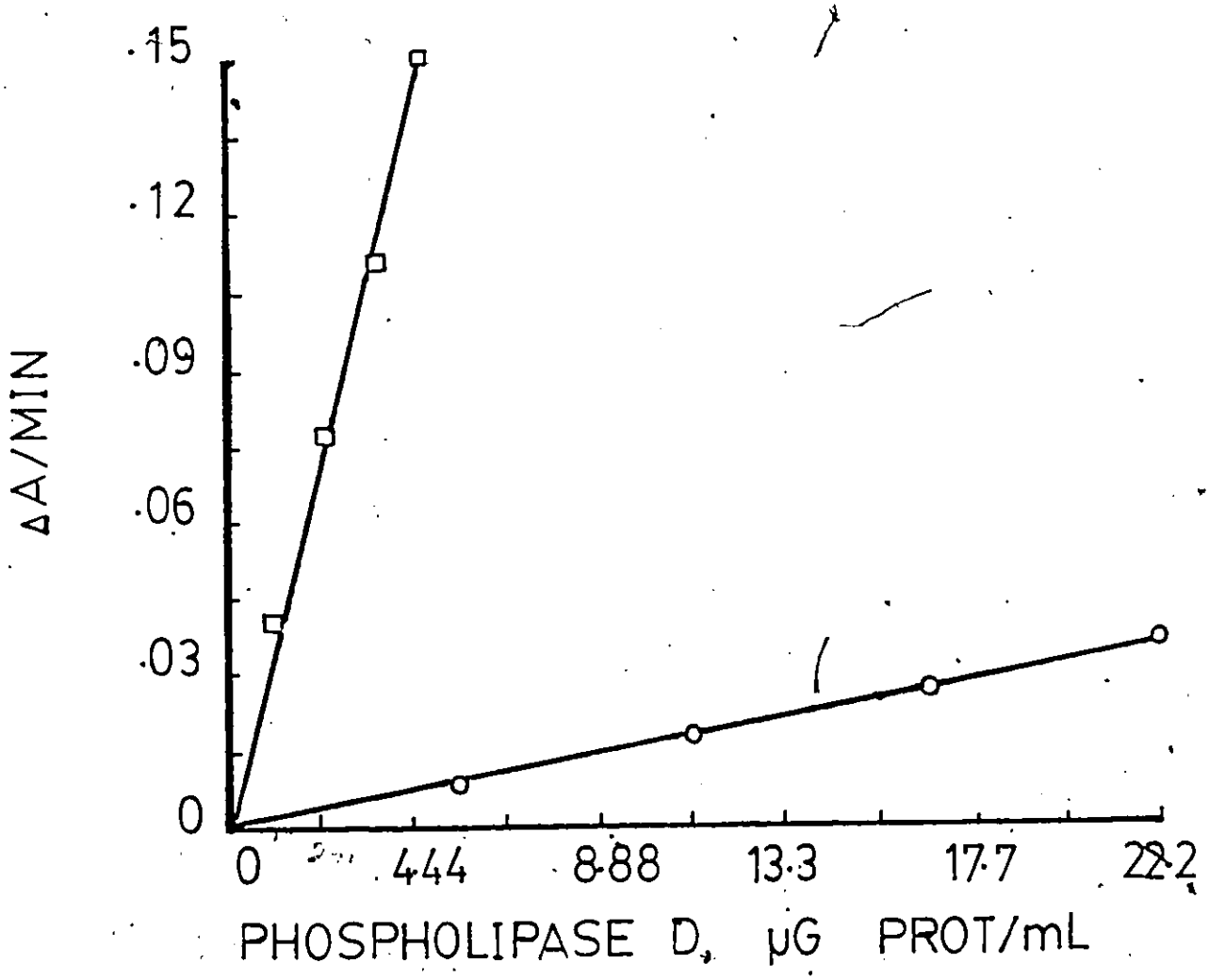

·15
·12
·09
·06
·03
0
ΔA/MIN
0
4·44
8·88
13·3
17·7
22·2
PHOSPHOLIPASE D, μG PROT/mL

TABLE IV

The Hydrolysis Rate as a Function of PLD Concentration[a]

PLD_c µg protein/mL	Reaction Rate ΔA/min	PLD_p µg protein/mL	Reaction Rate ΔA/min
1.22	0.034+0.002	5.5	0.008+0.000
2.44	0.068+0.001	11.1	0.017+0.000
3.66	0.110+0.000	16.6	0.025+0.000
4.66	0.150+0.000	22.2	0.035+0.000

[a]Conditions: PC: 0.125 mM, Ca^{2+}: 9 mM, COD: 5 Units, POD: 10 Units, pH 8.0. Standard rate method.

nation of PC content of solubilized human erythrocyte membranes. PLD from microbial origin has been used to determine total choline-containing phospholipids in various biological samples [86,109,111]. In contrast, the cabbage PLD used in the present determination is specific for PC under the conditions of the assay [86,99,117].

In these experiments with the erythroycyte membranes, the washed packed ghosts were solubilized to contain a final detergent concentration of 0.09% SDS-0.2% Triton X-100 (see Methods) and serial dilutions in the same detergent mixture were used for the PC assays. Table V shows the results with both end-point and rate methods. Based on the standard curves (Table Ia lines 4 and 7) run alongside the membrane samples, values of 856±14 and 857±70 nmol/mL packed ghosts were estimated respectively for the end-point and rate determinations for a ghost suspension containing 4.7±0.1 mg protein per millilitre.

These results could also be expressed as 182±7 and 182±19 nmol/mg membrane protein respectively. These values compare very well with those obtained under otherwise similar conditions but using the stepwise assay by Taylor *et al.* [99] which was 184±11 nmol/mg membrane protein. The striking agreement with the latter determination [99] indicates that the full potential of the stepwise reaction has been realized in our continuous methods.

The slightly larger uncertainity margin in the rate method compared to the end-point method may be due to the

Table V

Phosphatidylcholine Content of Human Erythrocyte Ghosts

Fold-Dilution of Membrane Samples[a]	End-Point Method		Rate Method		
	A[b]	PC content[c]	ΔA/min	PC content	
20	0.642, 0.657	848, 868			
30	0.426, 0.428	837, 841	0.102, 0.104	785	800
40			0.083, 0.075	856	776
60	0.229, 0.224	881, 861	0.061, 0.062	953	969

[a]Fold-dilution was calculated from the packed ghost stage.
[b]PLD and sample blanks were null.
[c]PC concentrations was expressed as: nmol/mL packed ghost.

heterogeneity of the phospholipids in the membrane sample and more importantly the fact that only a small fraction was hydrolyzed in the former case.

It may be interesting to note that PC determination in erythrocyte membranes had hitherto involved tedious procedures including initial phospholipid extraction into organic solvent, thin-layer chromatographic separation of components, elution of the separated zones and phosphate determination on the eluents [121,122]. This work thus represents the first rate method for PC determination.

Initial Problems with PLD

It may be worthwhile at this point to mention some initial problems encountered in the use of PLD.

Earlier phospholipases D obtained from Sigma showed about 7-fold less activity than expected (using manufacturer's protocol) as such new orders were suspended. New enzymes were obtained from Calbiochem. Those enzymes exhibited very poor solubility, had a strong rotten cabbage smell with very high blank reaction. Dialysis for 17-24 hrs followed by bench-top centrifugation were sufficient to correct for the blank readings, but much activity was lost that way. The use of the Calbiochem enzyme was discontinued.

Enzyme purchases from Boehringer Mannheim showed tolerable blank absorbances, though the preparation was not particularly soluble and moderate concentrations had to be used for good results. Some work was done with this enzyme

(Table Ia line 3).

Purchases of enzymes from Sigma were resumed when we were sent a free test enzyme (Type I) which we found to show twice the activity quoted. Type I enzyme was purchased until it was withdrawn from the market then substituted with the Type IV. The Type IV enzyme was very soluble, easier to handle, and showed no blank absorbances even at high concentrations. Assay optimization was carried out with this enzyme.

The peanut PLD was also a Sigma product. Type II was found active while the Type III inappropriate since very long lag phases, never encountered with any of the PLD_c or the Type II PLD_p, were displayed. Though the Type II and Type III solutions were slightly opalescent, the Type II was used for all reactions involving the peanut enzyme.

In all kinetic studies (next section) carried out with these enzymes, the activities were normalized as per mg protein.

B. <u>Kinetics With Long Chain Phosphatidylcholines</u>

<u>Kinetic Approaches</u>

An overview of our kinetic studies of phospholipase D with phosphatidylcholine in the presence of calcium illustrate a key role by the metal modifier. Based on the observed kinetic and supplimentary physical evidence, a rapid random equilibrium mechanism is the simplest model that adequately describes the data.

Monomeric substrates would have been the most appropri-

ate system to test the general modifier model. Unfortunately, such an approach is not feasible due to the myriad of limitations of this substrate mentioned earlier. This necessitates the use of a micellar system. By the nature of the micellar system, binding of enzyme to the substrate involves two ordered steps: initial binding to the interface and secondary binding within the interface [15]. There are three variables involved in experiments with detergent/phospholipid mixed micelles [15]: D_o (total detergent concentration comprising L_o (SDS concentration) and T_o (total Triton X-100 concentration)), S_o (total substrate concentration and X_s (surface substrate concentration, PC as mole fraction of PC plus detergents). If one is fixed and the other two are varied, three types of experiments can be performed.

In case I, the surface concentration of substrate, X_s, is fixed and the bulk concentration of substrate, S_o, and of detergents, D_o are varied. This is accomplished experimentally by increasing the concentrations of phospholipid and detergents proportionally. In case II, the bulk concentration of substrate, S_o is fixed but the surface concentration of substrate, X_s and the bulk concentration of detergents, D_o are varied. This is accomplished experimentally by just increasing the concentration of detergents. In case III, the bulk concentration of detergents, D_o is fixed and the bulk concentration of substrate, S_o and its surface concentration, X_s are varied. This is accomplished experimentally by just

increasing the phospholipid concentration.

Case I and II are not feasible in our assay system since SDS and Triton X-100 are activator and inert matrix respectively only in a narrow range of concentrations and neither can be added in arbitrary excess. For example, doubling or tripling the Triton X-100 level from that used in the standard rate assay resulted in a 5- or 20-fold, respectively, lower rate for the 70 uM PC standard. With SDS, similar but less dramatic differences in rates were observed between 0.1 and 1.5 mM detergent in the cuvette. In addition, calculation of surface concentration of substrate (as in case I) should really utilize the concentration of micellar Triton X-100 and SDS under the conditions of the experiments. In fact, these cannot be assumed to be equal to the CMC [15]. Furthermore, any values obtained at constant bulk concentration of substrate (case II) may not be completely valid as the surface areas may actually change as the surface concentration of substrate changes. Finally, calcium modification of the substrate may introduce further uncertainties with regard to the surface concentration.

Case III is the simplest approach and circumvents obvious pitfalls due to experimental design. This last case is the approach adopted in all our kinetic studies. All experiments were conducted only once where qualitative observation of kinetic trends were the objective but in duplicates or triplicates, where uncertainties are indicated, for estimation of kinetic constants. Replications of individual experiments were not conducted on different days

since about four calcium ion concentrations were used to span similar substrate concentration ranges with similar trends being observed. In addition, measured velocities were dependent on the enzyme preparations used.

Kinetics of PLD Catalyzed Hydrolysis of Phosphatidylcholines of Varying Chain-Lengths

Two different ranges of substrate concentration have been used in this kinetic study: (1) High substrate, low molar detergent ratio stocks (D_o:PC $\geqslant$ 1:1) where the highest sub-substrate cuvette concentration was 2 mM. (2) Intermediate substrate, medium molar detergent ratio stocks (D_o:PC $\geq$ 8:1) where the highest substrate cuvette concentration was 0.25 mM. A low substrate, high molar detergent ratio stocks (D_o:PC $\geq$ 90:1) was used in the development of the assay.

Hydrolysis as a Function of High Phosphatidylcholine Concentrations

Figure 8 shows the DPPC concentration dependence of intial velocity at various fixed concentrations of calcium (2.5, 5, and 10 mM). The family of curves generated show similar characteristics: The velocity rises to a maximum (peak) and falls again at higher concentrations of substrate tending to zero. Also, the peak velocities of the curves move to higher v' and S_o values as Ca^{2+} is increased. Similar velocity-substrate profiles were obtained in the PLD hydrolysis of the other phosphatidylcholines employed under identical conditions. Unless otherwise stated all reactions were conducted using the cabbage enzyme. The results for

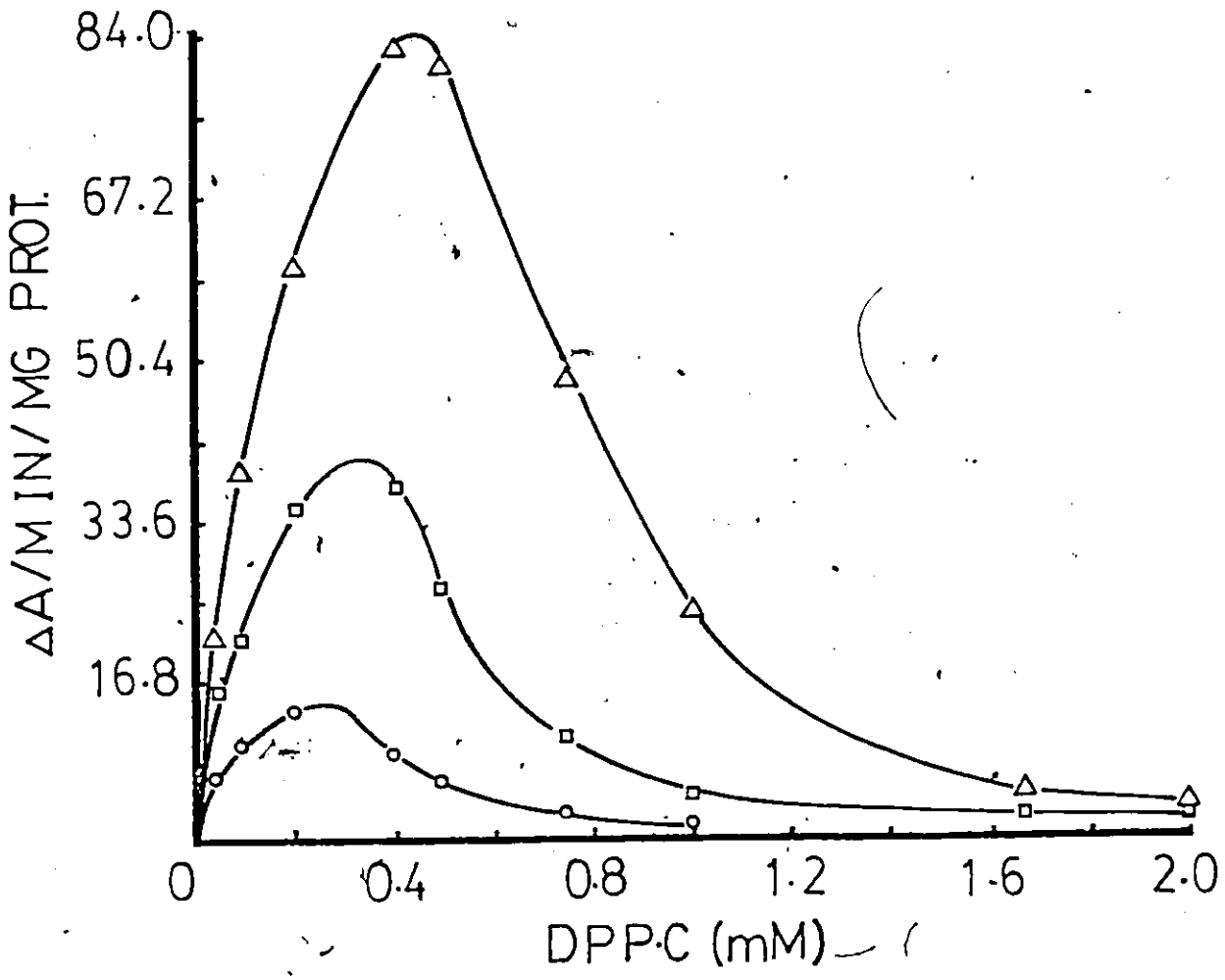
Figure 8

Initial Rates as a Function of High Concentration of DPPC at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for high substrate concentration (D_o:PC ≥ 1:1) at Ca^{2+} concentrations of 2.5 mM (O), 5 mM (□) and 10 mM (△).

Figure 8



these other phosphatidylcholines are illustrated in Figure 9 (DDPC); Figure 10 (DMPC) Figure 11 (DPPC, PLD_p). Data for Figures 8-11 are given in Tables VIa, b, c. DSPC solutions at this detergent/phospholipid ratio were turbid and not used in this case.

In order to eliminate the possibility that the coupled rate assay had any influence on the unexpected kinetic behaviour obtained using the high substrate concentration samples, similar rate measurements were conducted but by the stepwise assay using short time incubation followed by 1-min boiling to terminate the reaction. Similar results were obtained. Figure 12 compares the results of both stepwise and rate assays. For the stepwise assay, the peak velocity occurs at a substrate concentration which was two times higher than for the continuous rate assay. It must however, be recalled that at hydrolysis, the detergent concentration for the stepwise assay is twice as high as for the continuous assay. It should not therefore come as a surprise when these two peak velocities and practically both curves coincide when the DPPC concentration was expressed as mole fraction of the micellar substrate (Figure 13, Table VI(d)). Therefore, it is concluded that the rates measured in the continuous assays are not artefacts of the coupling enzymes.

From the results of Figure 8-13, it is evident that maximal PC hydrolysis depends upon the relative concentrations of both Ca^{2+} and PC. A characteristic feature of these curves is that the velocity curves obtained with

Figure 9

Initial Rates as a Function of High Concentration of DDPC at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for high substrate concentration (D_{σ}:PC $\geq$ 1:1) at Ca^{2+} concentrations of 0.5 mM (□), 2.5 mM (△) and 10 mM (○).

Figure 9

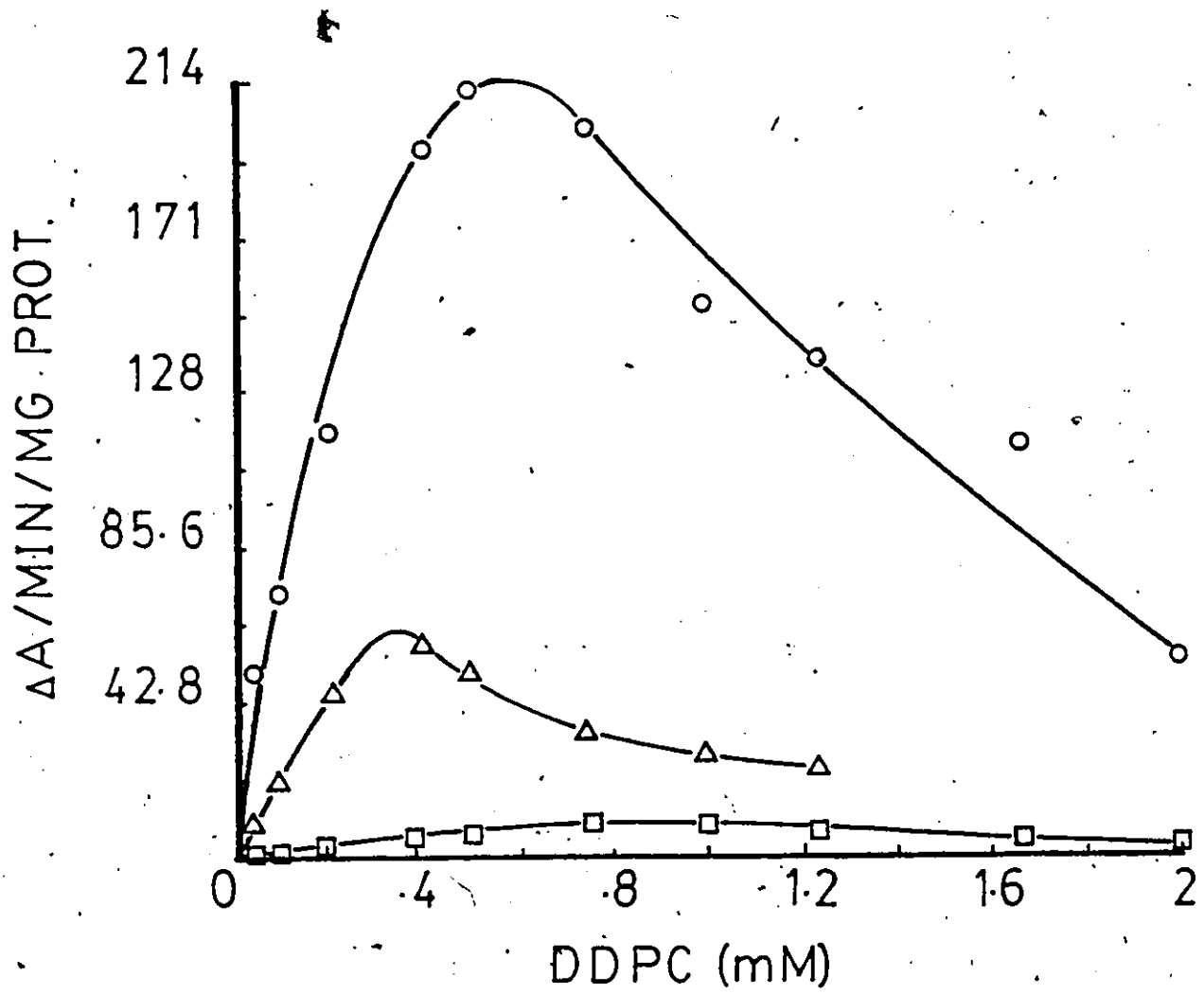


Figure 10

Initial Rates as a Function of High Concentration of DMPC at Ca^{2+} Concentration of 5 mM.

Legend:

Initial rates were determined by standard rate assay for high substrate concentration (D_o:PC ≥ 1:1) at Ca^{2+} concentration of 5 mM.

Figure 10

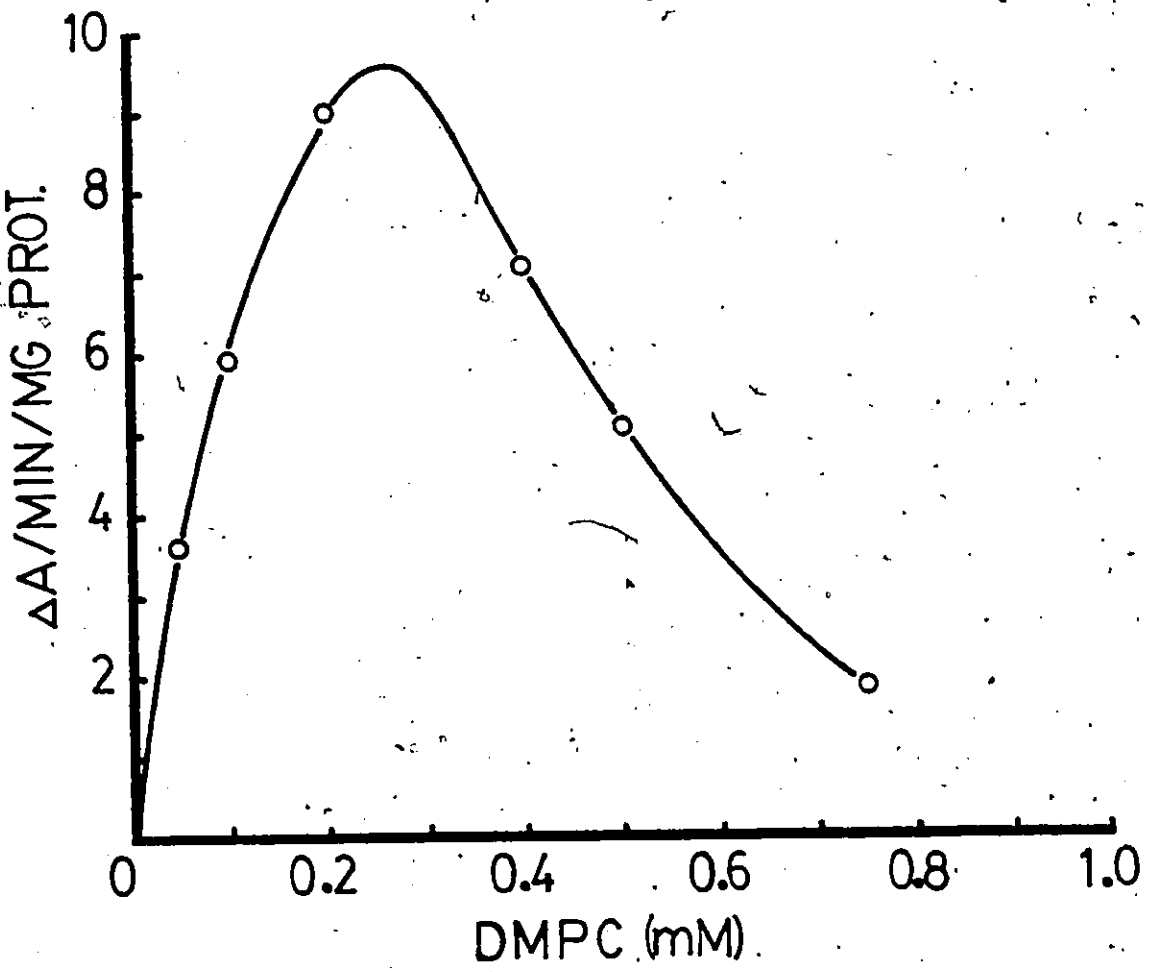


Figure 11

Initial Rates as a Function of High Concentration of DPPC at Various Ca^{2+} Concentrations for the Peanut PLD.

Legend:

Initial rates were determined by standard rate assay for high substrate concentration (D_o:PC $\geq$ 1:1) at Ca^{2+} concentrations of (a) 10 mM and (b) 0.5 mM using the peanut PLD.

Figure 11

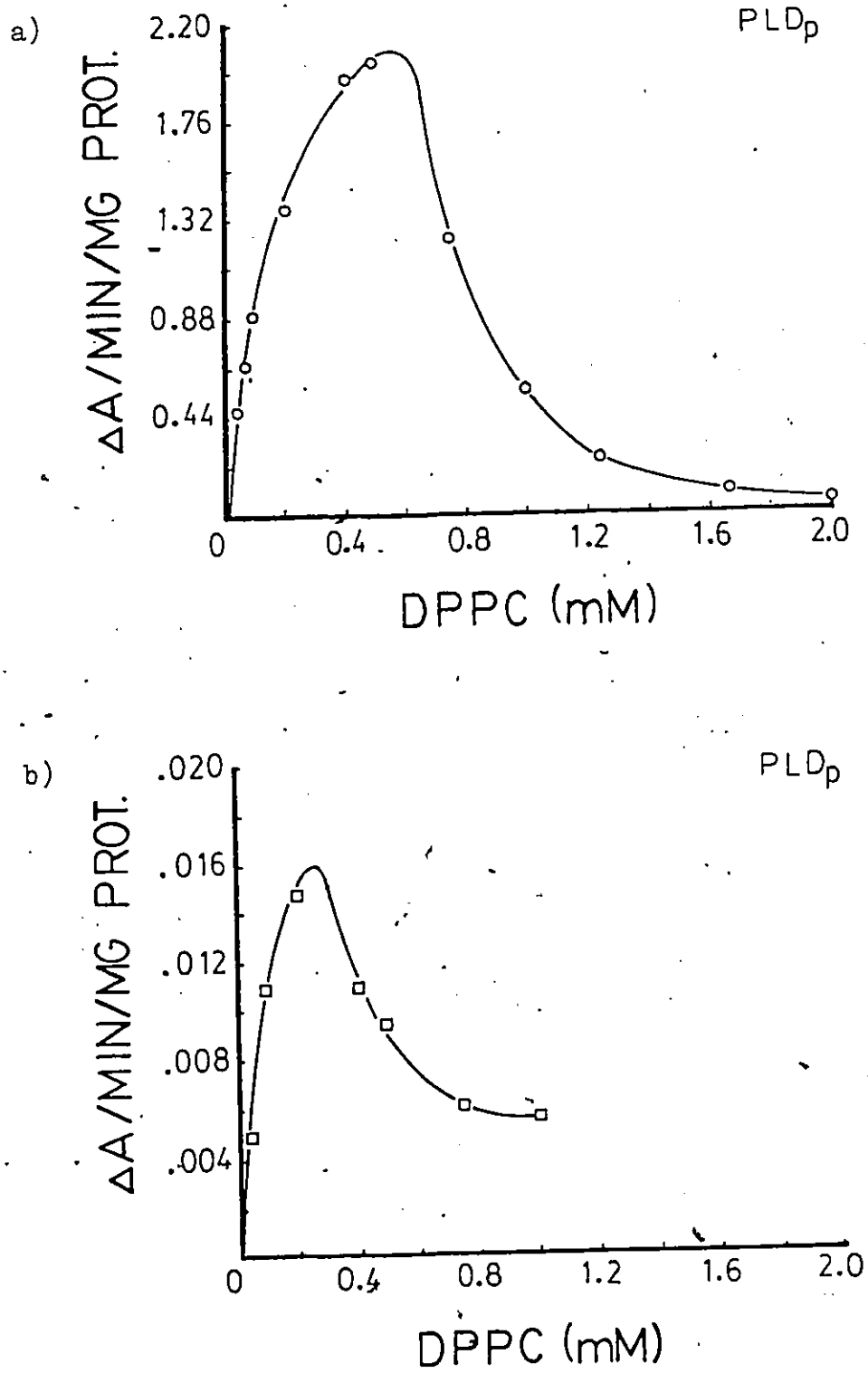

a)
PLDp
ΔA/MIN/MG PROT.
2.20
1.76
1.32
0.88
0.44
0
0.4
0.8
1.2
1.6
2.0
DPPC (mM)
b)
PLDp
ΔA/MIN/MG PROT.
.020
.016
.012
.008
.004
0
0.4
0.8
1.2
1.6
2.0
DPPC (mM)

TABLE VI(a)

PLD_c Hydrolysis of High Concentration of DPPC

(D_o:PC ≥ 1:1)

[DPPC][a]	ΔA/min/mg protein			
mM	Ca = 0.5 mM	Ca = 2.5 mM	Ca = 5 mM	Ca = 10 mM
0.050	0.307	6.660	14.754	21.189
0.100	0.350	9.836	20.328	38.320
0.200	0.372	12.910	34.426	59.836
0.400	0.410	8.566	36.721	81.967
0.500	0.256	5.799	26.230	80.574
0.750	0.246	2.521	16.082	48.036
1.000		1.516	4.344	23.771
1.670			2.172	4.713
2.000			1.762	3.852

[a]Standard rate assay conditions and concentrations expressed as final cuvette concentrations. Data for Figures 8, 14, 17a, b.

TABLE VI(b)

PLD_c Hydrolysis of High Concentration of DDPC (D_o:PC ≥ 1:1) and DMPC (D_o:PC ≥ 1:1)[a]

[DDPC][a]	ΔA/min/mg protein			DMPC
mM	Ca = 0.5 mM	Ca = 2.5 mM	Ca = 10 mM	Ca = 5 mM
0.050	0.399	8.471	50.823	3.576
0.100	0.896	18.635	71.636	5.922
0.200	1.377	42.836	116.166	8.984
0.400	3.176	56.631	195.305	7.026
0.500	3.993	49.129	212.246	5.019
0.750	7.260	33.882	199.903	1.857
1.000	7.260	27.590	152.348	
1.250	4.526	24.201	136.084	
1.670	3.146		113.021	
2.000	1.876		54.696	

[a]Standard rate assay conditions and concentrations expressed as final cuvette concentrations. Data for Figures 9, 10, 15 and 16.

Table VI(c)

PLD_p Hydrolysis of High Concentration of DPPC (D_o:PC ≥ 1:1)

[DPPC] mM	ΔA/min/mg protein[a] Ca^{2+} = 0.5 mM	Ca^{2+} = 10 mM
0.050	0.0048	0.465
0.067		0.662
0.100	0.0109	0.887
0.200	0.0147	1.360
0.400	0.0109	1.940
0.500	0.0094	2.013
0.750	0.0061	1.220
1.000	0.0056	0.544
1.250		0.237
1.670		0.094
2.000		0.050

[a]PLD peanuts used. Standard rate assay conditions. DPPC and Ca^{2+} concentrations are final cuvette concentrations. Data for Figure 11.

Figure 12

Comparison of DPPC HYdrolysis by the Standard Rate Assay and the Stepwise Assay at 10 mM Ca^{2+} Concentration.

Legend:

The initial rate measurement curve (□) presented here is the same as in Figure 8 for $[Ca^{2+}]$ = 10 mM. The stepwise assay (O) was conducted by short time incubations (3 min) followed by 15 min colour development at $[Ca^{2+}]$= 10 mM.

Figure 12

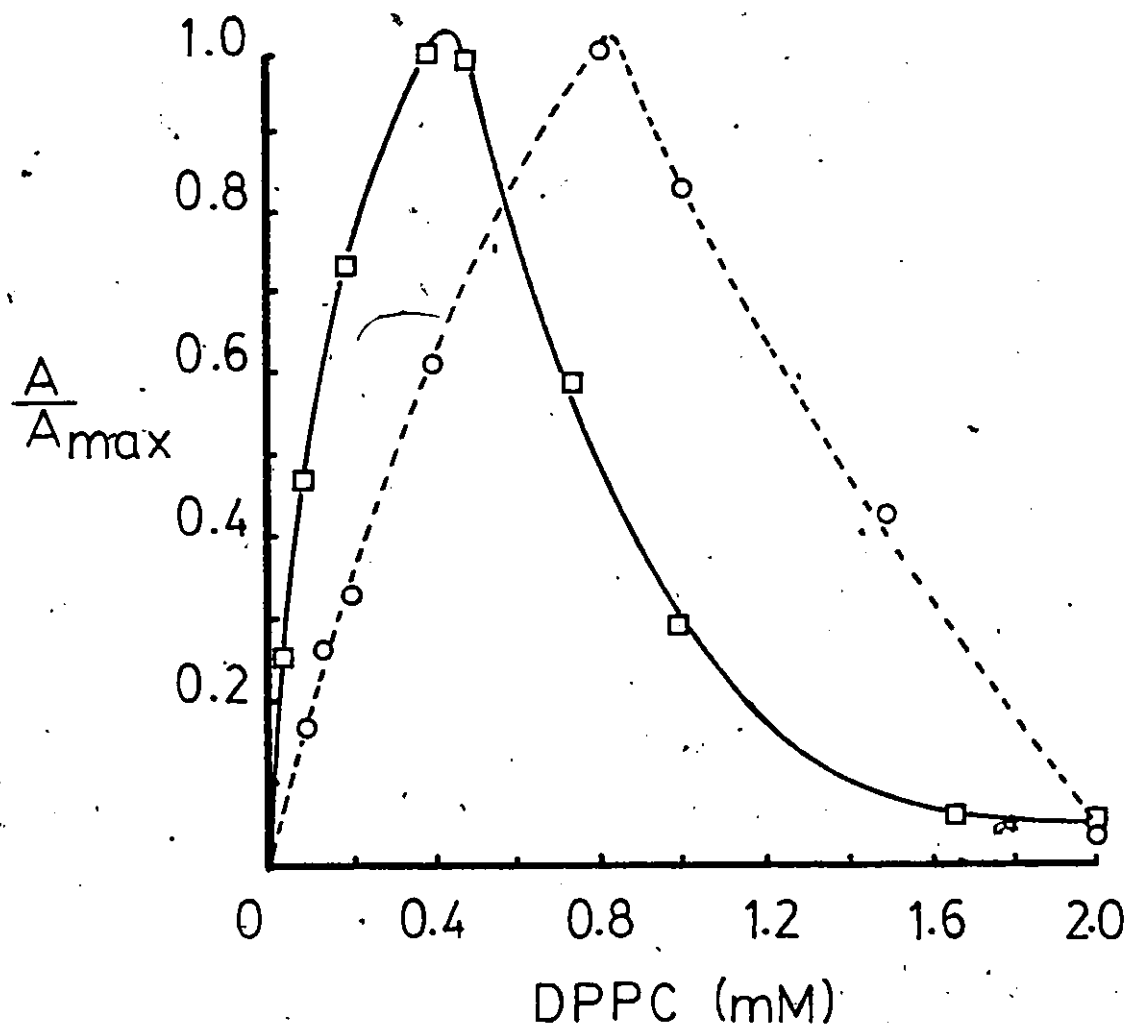


Figure 13

Comparison of the Standard Rate Assay and the Stepwise Assay with DPPC Concentration Expressed as Surface Concentration.

Legend:

Conditions are same as in Figure 12. Symbols stand for rate (□) and stepwise (O).

Figure 13

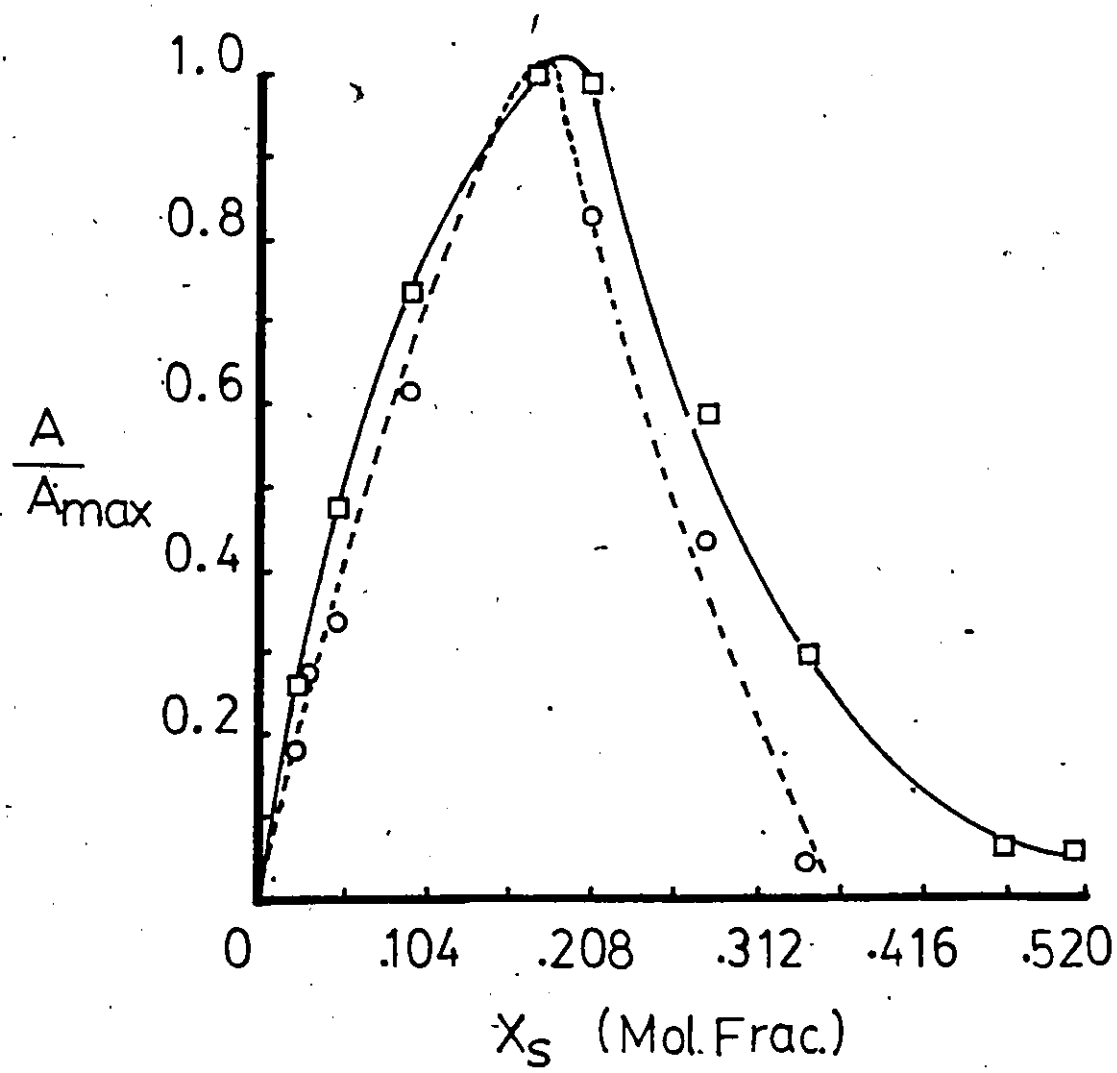


Table VI(d)

Stepwise[a] Hydrolysis of High PC Standards (D_o:PC ≥ 1:1) at [Ca^{2+}] = 10 mM.

DPPC mM	mol. fraction X_S	A_{510}[b]	A/Amax
0.100	0.026	0.065	0.165
0.134	0.034	0.100	0.255
0.200	0.050	0.127	0.323
0.400	0.095	0.238	0.606
0.800	0.174	0.393	1.000
1.000	0.209	0.324	0.824
1.500	0.283	0.166	0.422
2.000	0.345	0.013	0.033

[a]Stepwise assay using short time incubation for 3 min, followed by boiling for 1 min, then colour development carried out for 15 min. Cuvette concentrations of DPPC posted.

[b]Net absorbances posted, blanks were run for each DPPC concentration.
Data for Figures 12 and 13.

higher fixed Ca^{2+} concentration are shifted upward and are dependent on total Ca^{2+} concentration even at very low values of PC. These results would be consistent only if the concentration of the reactive species were proportional to the amount of Ca^{2+} in the system. This means that the reactive species is not free PC (micellar but uncomplexed). The inhibitory effect at high PC concentration seen in Figure 8-13 could arise in various ways: (1) uncomplexed PC might act as a competitive inhibitor by binding to the catalytic site of the enzyme (2) uncomplexed PC, by complexing free Ca^{2+}, might reduce the concentration of an essential activator as well as form inactive enzyme-Ca^{2+}- PC complexes, (3) Since the enzymes used were impure, presence of proteases in these preparation could cause digestion of the phospholipases, with consequent loss in activity, or (4) a decrease in water activity as a consequence of increase in the molar fraction of phospholipid might cause a change in the state of aggregation of the substrate possibly by formation of more extended micellar forms, which are poorly attacked by the phospholipases.

The last factor, documented by Winsor [124] for micellar transitions in isotropic aqueous detergent solutions, is not sufficient to explain the observed inhibition of enzyme activity at high substrate concentration. Furthermore, light scattering experiments conducted on our reaction mixture, but excluding the enzymes, indicated that there was no particles detectable within the concentration ranges of PC and Ca^{2+} used. The third factor above, influence of protease, was tested on SDS-PAGE (see Methods). The enzyme preparations

were found to be free of any proteases as evidenced by similar gel patterns in the presence or absence of calcium over a range of incubation times at 37°C. Both alternatives (1) and (2) seem plausible on the basis of existing data. In previous studies with phospholipases D [51,77], Ca^{2+}, detergent, PC and enzyme have been implicated in the catalytic reaction. Chen and Barton [34] have also documented the inhibition of PLD by excess substrate. In a similar investigation, Nakagaki et al. [125] analysed the interfacial reaction of PLD by measuring the fluorescence change of 8-amino-1-naphthalenesulfate present in the micelle solution. Their results indicate that with increasing initial molar fraction of lecithin, the rate constant of the enzymic process decreased, and an initial slow reaction occurred in the mixed micelle solution with large initial molar fraction lecithin [125].

In order to gain further information about the inhibitory effect at high PC concentration, plots of the reciprocal velocity against total PC (Dixon plot) were made. Figure 14 (DPPC), Figure 15 (DDPC) and Figure 16 (DMPC) demonstrate that inhibition by PC was markedly biphasic indicating among other factors that inhibition at the catalytic site almost certainly takes place. As is usual in the case of substrate inhibition, the reaction rate approaches the Michaelis-Menten value when substrate concentration is small, but approaches zero instead of Vmax when substrate concentration is large [126].

To gain more insight into the roles of Ca^{2+} in the hydrolysis the reaction rates were plotted as a function of Ca^{2+} concentration at various fixed values of PC concen-

Figure 14

A Plot of Reciprocal Initial Rates as a Function of DPPC Concentration (Dixon Plot) at Various Ca^{2+} Concentrations.

Legend:

Reaction conditions same as for Figure 8. Symbols stand for Ca^{2+} concentrations of 2.5 mM (O), 5 mM (□) and 10 mM (△).

Figure 14

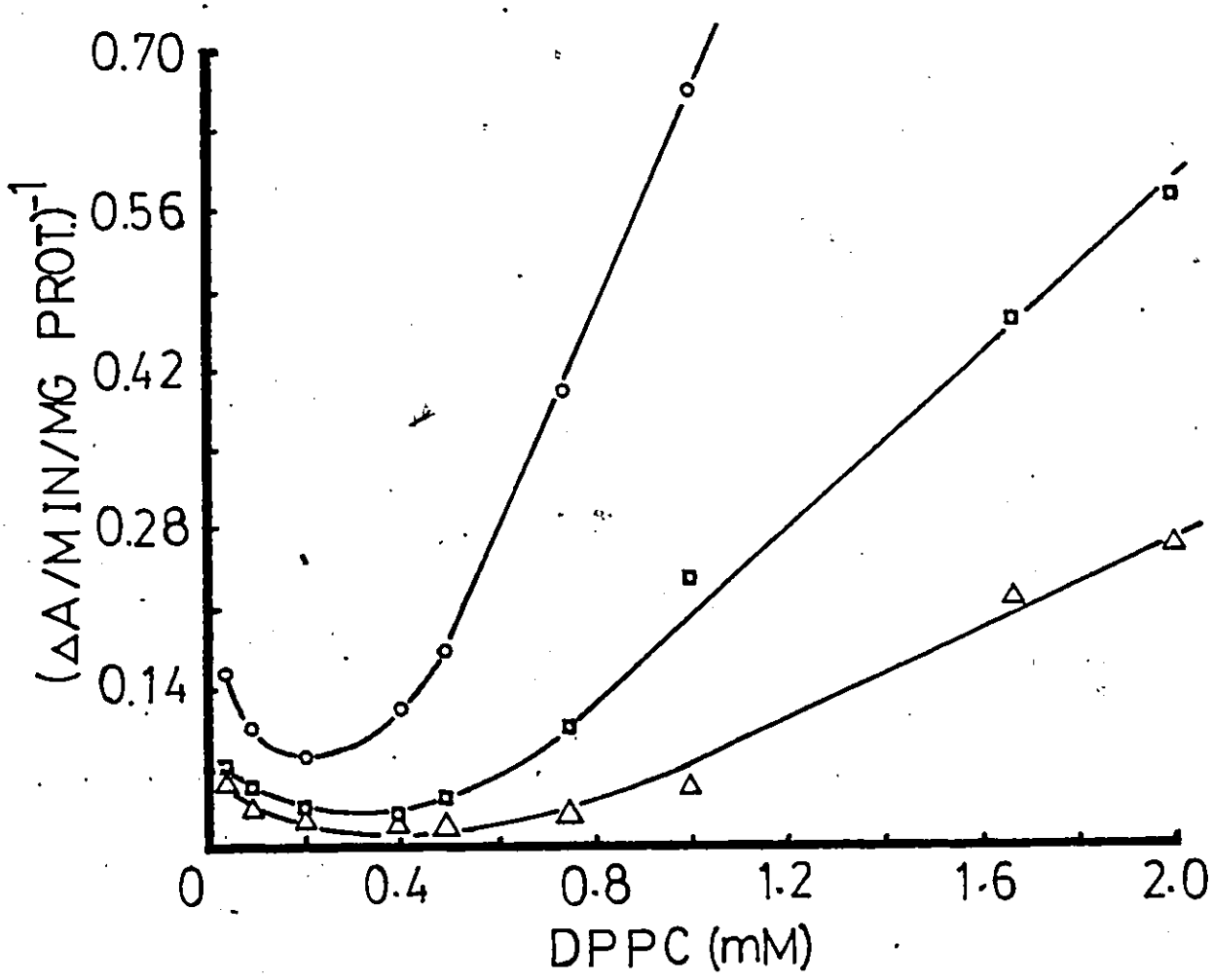


Figure 15

A Plot of Reciprocal Initial Rates as a Function of DDPC Concentration (Dixon Plot) at Two Concentrations of Ca^{2+}.

Legend:

Reaction conditions same as for Figure 9. Symbols stand for Ca^{2+} concentrations of 2.5 mM (△) and 10 mM (○). Plot for Ca^{2+} concentration of 0.5 mM not shown due to wide scale disparity.

Figure 15

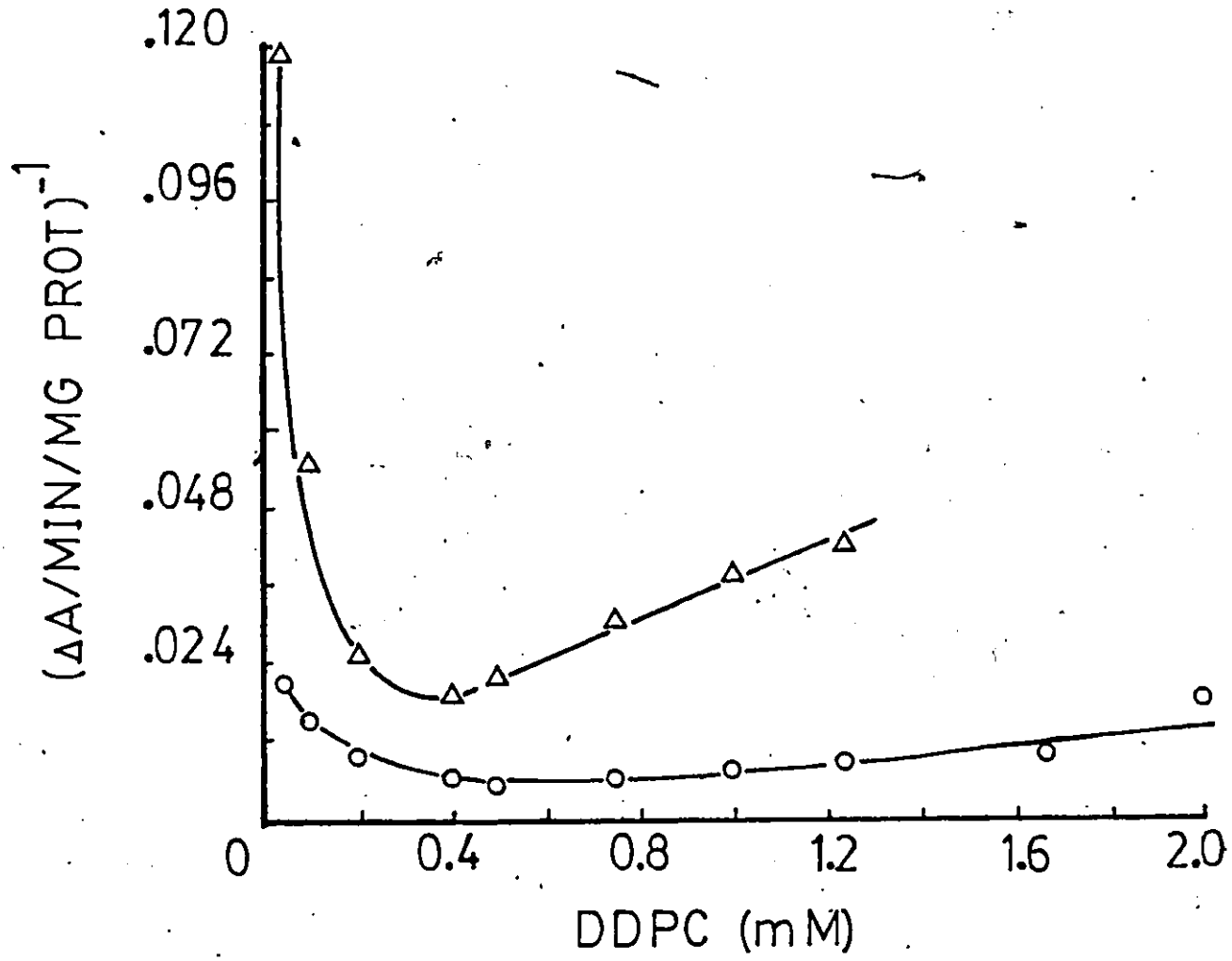


Figure 16

A Plot of Reciprocal Initial Rates as a Function of DMPC Concentration (Dixon Plot) at a Ca^{2+} Concentration of 5 mM.

Legend:

Reaction conditions same as for Figure 10 and at Ca^{2+} concentration of 5 mM.

Figure 16

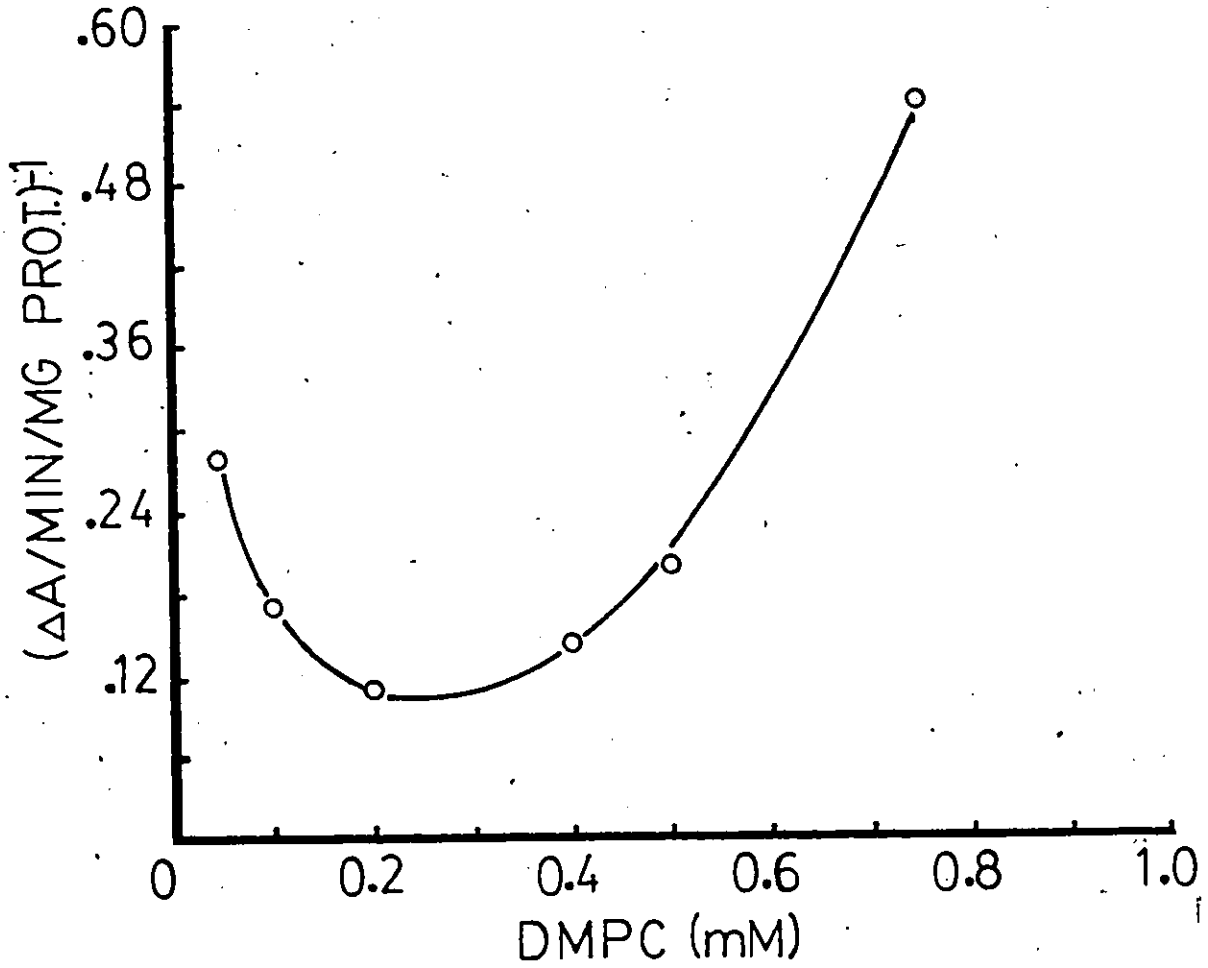


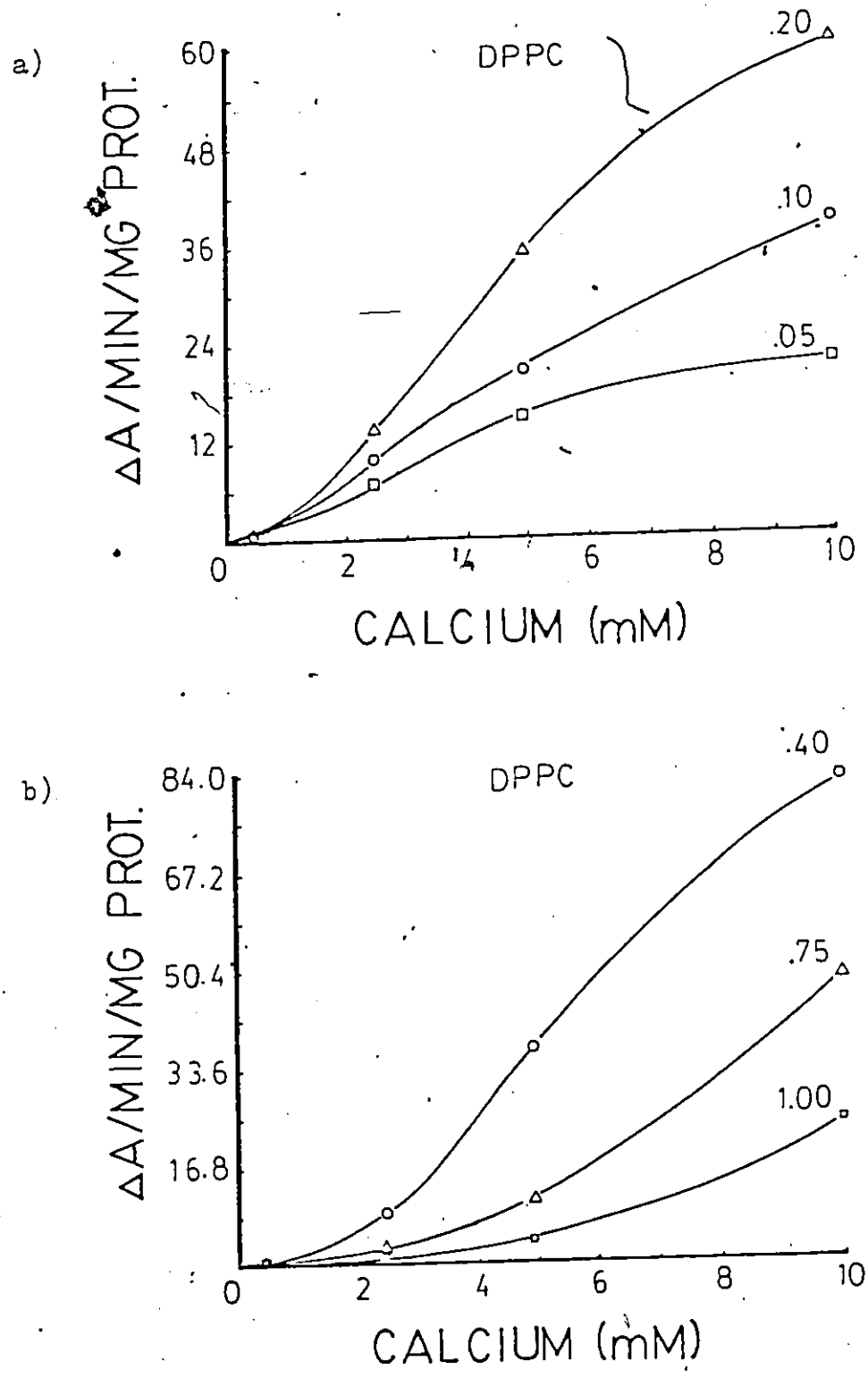
Figure 17

Initial Rates as a Function of Ca^{2+} Concentration at Various High Concentrations of DPPC.

Legend:

Reaction conditions same as for Figure 8 and at DPPC concentrations of (a): 0.05 mM (□), 0.10 mM (○) and 0.20 mM (△); (b): 0.40 mM (○), 0.75 mM (△) and 1.00 mM (□).

Figure 17


a)
DPPC
.20
.10
.05
60
48
36
24
12
0
2
4
6
8
10
ΔA/MIN/MG PROT.
CALCIUM (mM)
b)
DPPC
.40
.75
1.00
84.0
67.2
50.4
33.6
16.8
0
2
4
6
8
10
ΔA/MIN/MG PROT.
CALCIUM (mM)

tration. Figures 17a, b show a sigmoidal dependency of velocity on Ca^{2+} concentration. This is a strong indication that the reaction involves at least two types of calcium binding, consistent with the multiple role of Ca^{2+} suggested by Heller [51] and Rakhimov [77]. According to Gutfreund [127], an illustrative procedure obtained by plotting a family of curves of velocity against metal ion concentration would help in distinguishing between the mechanisms with compulsory order or random order of addition of substrate. Such plots, according to Gutfreund [127] give one of two results in clear-cut cases. (1) If the substrate has to be associated with a metal ion before it will combine with the enzyme (compulsory order) then at low metal-ion concentration the family of curves are superimposed, and the velocity is dependent on substrate-metal complex and independent of free metal or free substrate concentration. (2) If, however, the free substrate will combine with the enzyme, either with the same or a slightly different affinity as the metal-substrate complex, but form an unreactive enzyme-substrate complex, then the family of curves would depict this fact. At a constant total metal-ion concentration, increasing total substrate concentration will cause and increase in the reaction velocity until the substrate concentration becomes as large and larger than the metal concentration, then the reaction velocity will decrease. The free substrate can behave as a competitive inhibitor for the metal ion-substrate complex [127]. Our data so plotted (Figures 17a, b) is indicative of a random order mechanism whereby free substrate as well as metal-substrate complex both bind to the enzyme and in addition an inactive

metal-substrate-enzyme complex is formed.

Hydrolysis as a Function of Intermediate Range Phosphatidylcholine Concentrations

Following the observation of the diminshed enzyme activity at high substrate concentration, we increased the total detergent to substrate ratio to about 8:1. In other words we reduced the initial molar fraction of substrate though the total detergent concentration at hydrolysis remained unaltered. Under these conditions the velocity-substrate relationships were hyperbolic and showed saturating kinetics for all the phosphatidylcholines studied. These velocity-substrate curves for the various phosphatidylcholines are shown in Figures 18 through 22 and data are given in Tables VII a-e. There are some apparent inconsistencies in the Tables VII when comparison is made with corresponding entries in Tables VI. For example, with 0.1 mM DPPC in the presence of 2.5 mM Ca^{2+} different velocities are reported in Table VIa and VIIa. This simply reflects the fact that different enzyme preparations were used and illustrates the need to avoid comparison of absolute velocities from Table to Table. However, comparison of velocities within a Table is valid. Double reciprocal plots of the above data are plotted as shown in Figures 23 through 27, and Figure 28 is a similar plot of $1/v$ against $1/X_s$ (mole fraction at $[Ca^{2+}] = 10$ mM) for the phospholipids. One striking feature common to all the phosphatidylcholines is the linearity of these plots at high Ca^{2+} concentration. These plots become progressively

Figure 18

Intial Rates as a Function of Intermediate Concentration of DPPC at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for intermediate DPPC concentration (D_o:PC $\geq$ 8:1) at Ca^{2+} concentrations of 2.5 mM (△), 5 mM (O) and 10 mM (□).

Figure 18

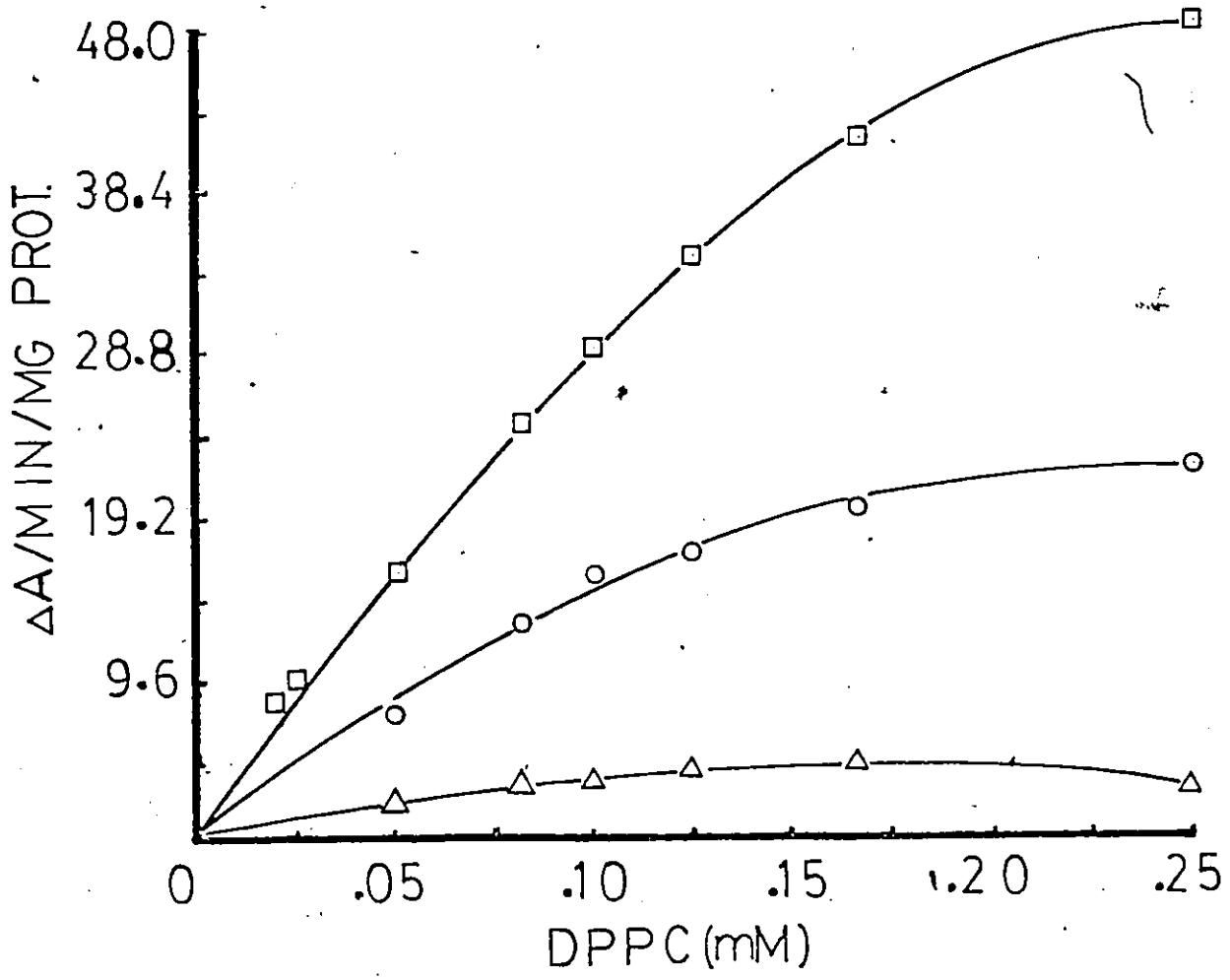


Figure 19

Intial Rates as a Function of Intermediate Concentration of DPPC with the Peanut PLD at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for intermediate DPPC concentration (D_o:PC $\geq$ 8:1) at various Ca^{2+} concentrations of 0.5 mM (□), 2.5 mM (Δ) and 5 mM (○) using the peanut PLD.

Figure 19

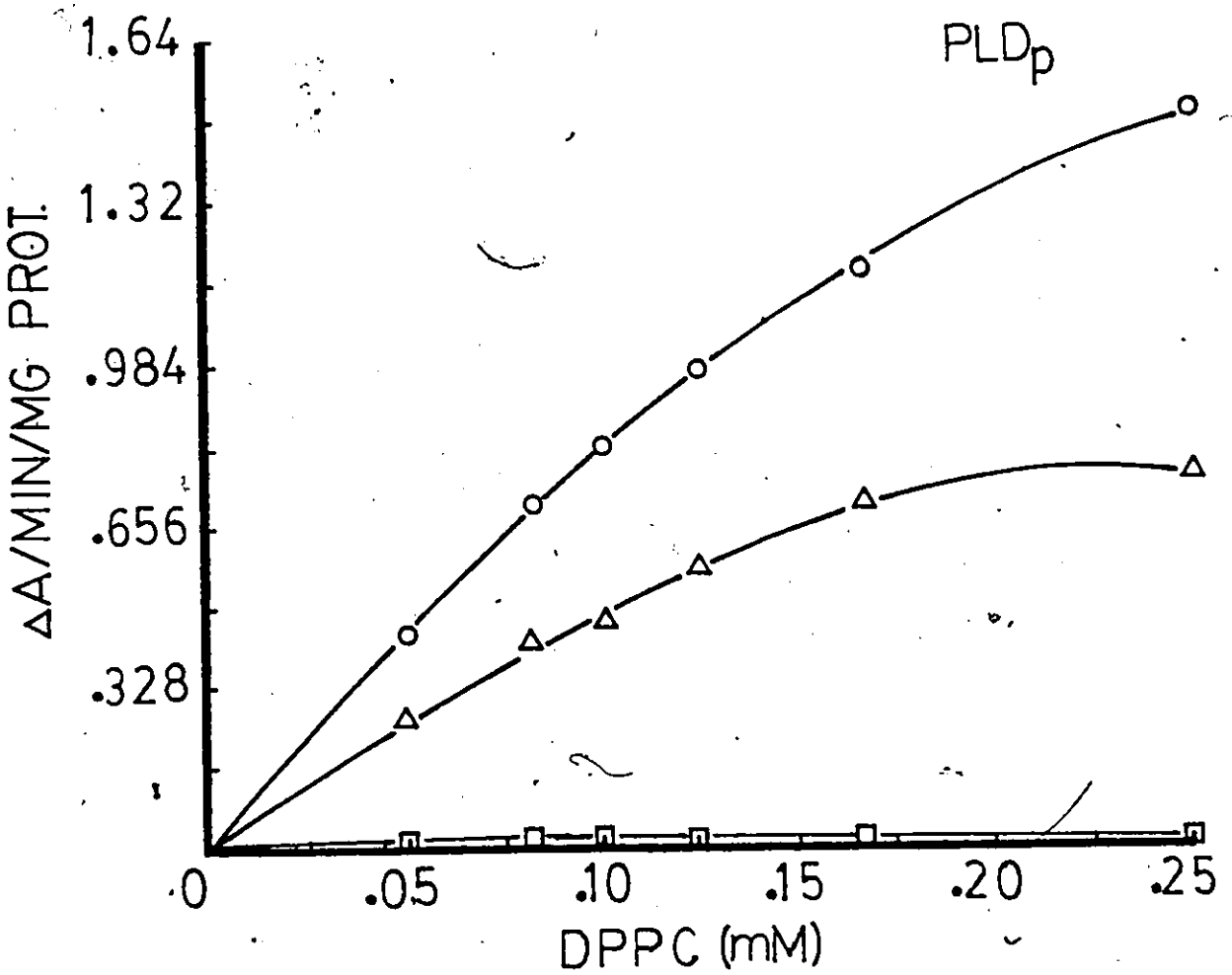


Figure 20

Intial Rates as a Function of Intermediate DDPC Concentration at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for intermediate DDPC concentration (D_o:PC ≥ 8:1) at various Ca^{2+} concentrations of 0.5 mM (Δ), 2.5 mM (□) and 10 mM (O)

Figure 20

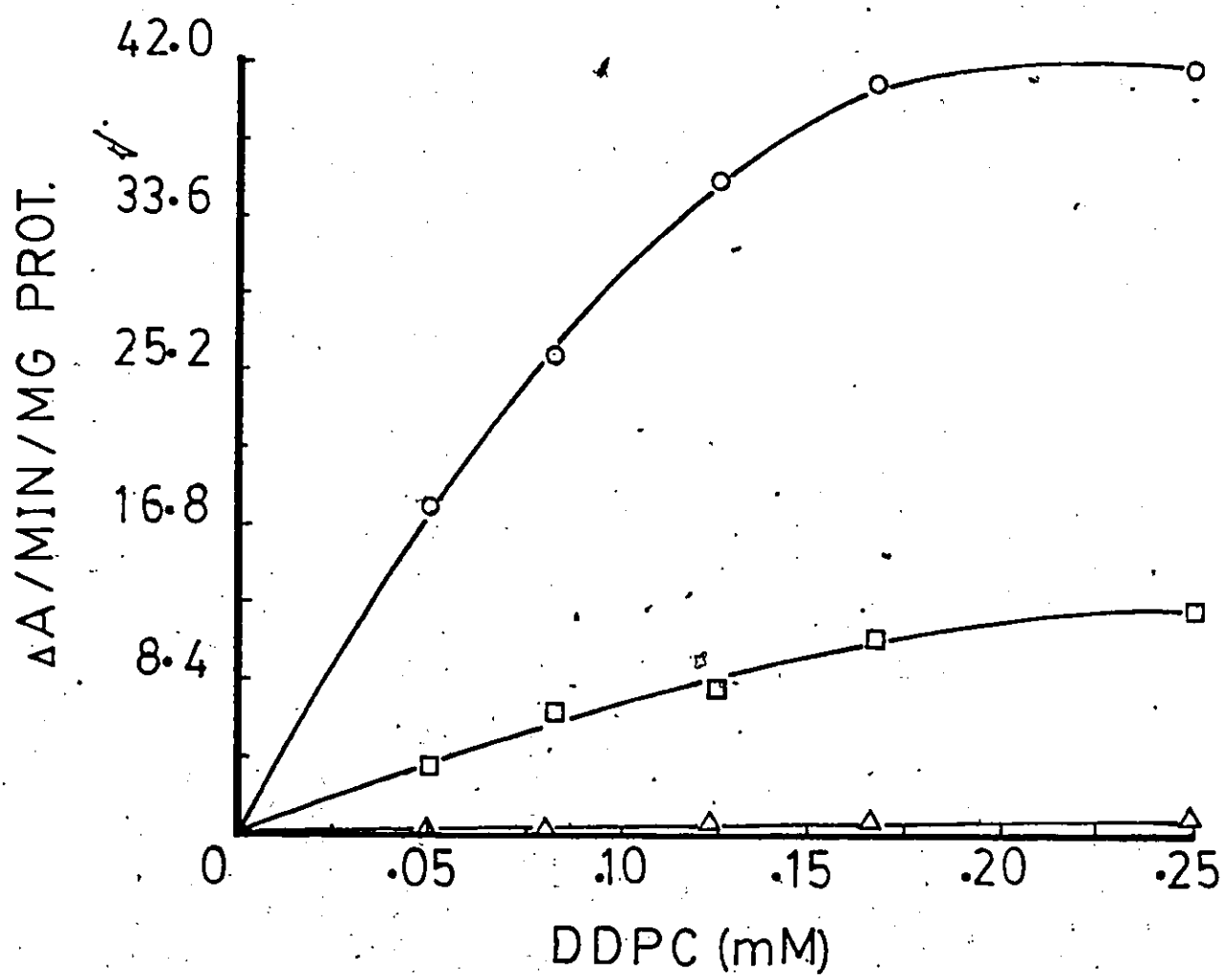


Figure 21

Intial Rates as a Function of Intermediate DMPC Concentration at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for intermediate DMPC concentration (D_o:PC $\geq$ 8:1) at various Ca^{2+} concentrations of 2.5 mM (Δ), 5 mM (□) and 10 mM (O).

Figure 21

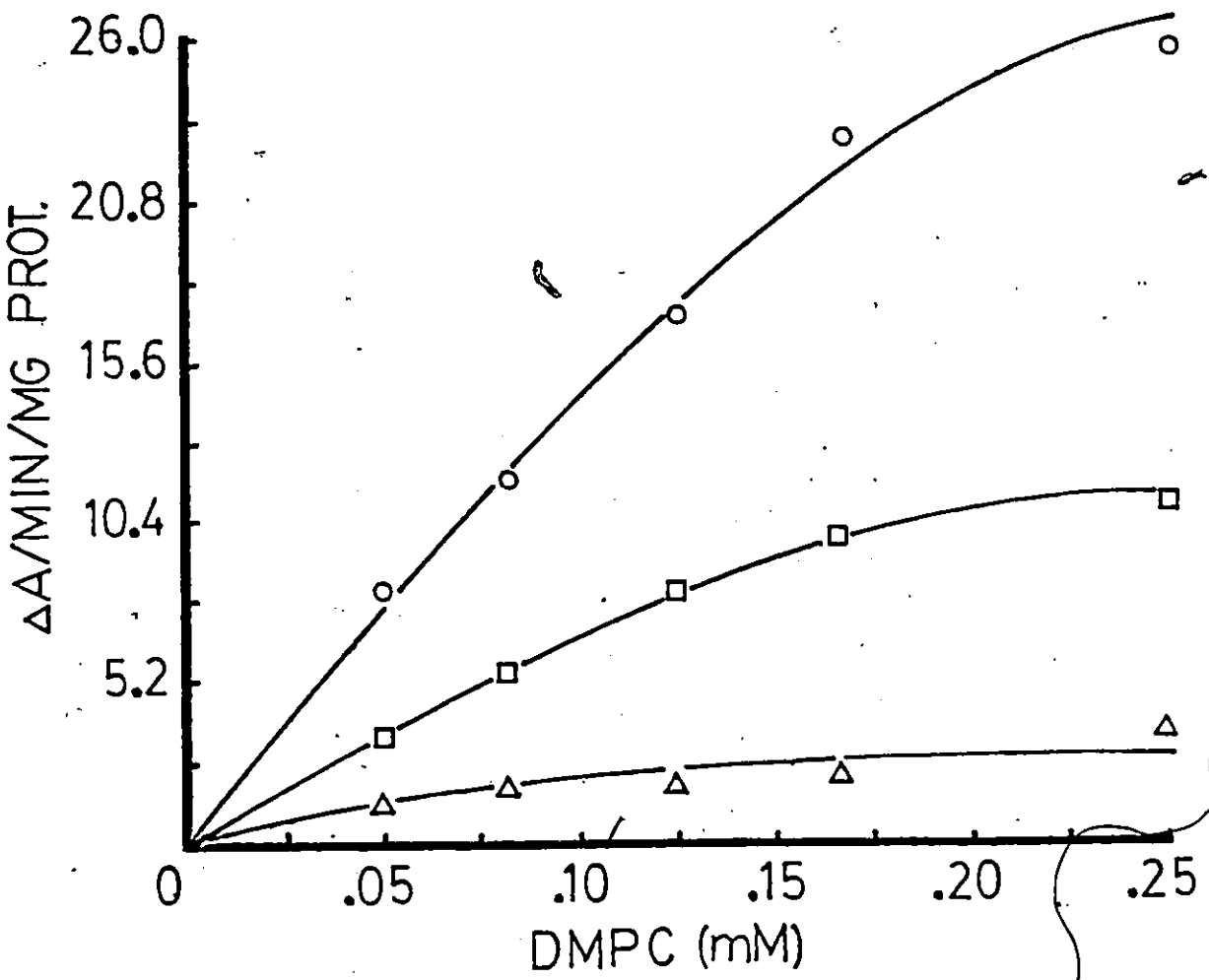


Figure 22

Intial Rates as a Function of Intermediate DSPC Concentration at Various Ca^{2+} Concentrations.

Legend:

Initial rates were determined by standard rate assay for intermediate DSPC concentration (D_o:PC $\geq$ 8:1) at various Ca^{2+} concentrations of 2.5 mM (O), 5 mM (Δ) and 10 mM (□).

Figure 22

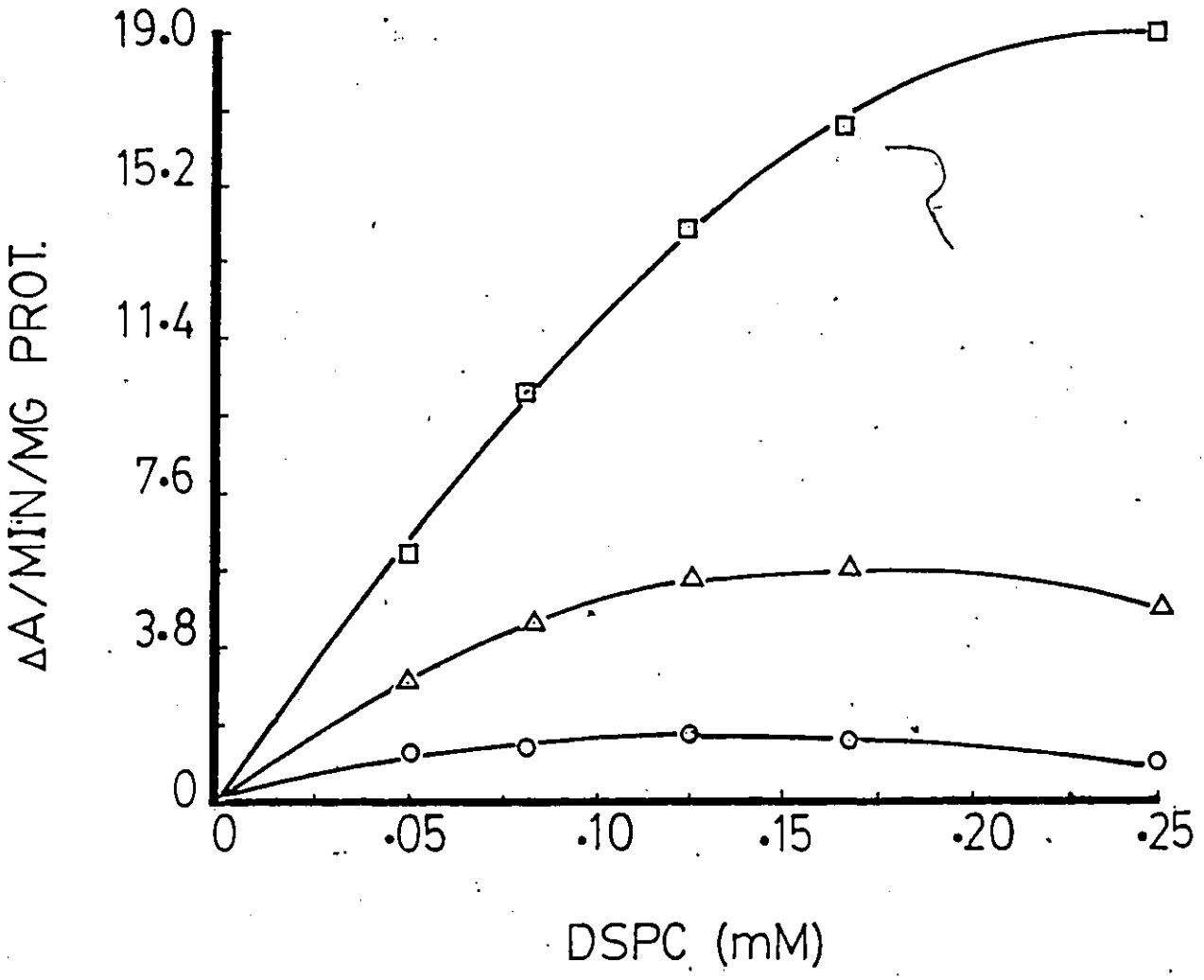


Table VII(a)

PLD_c Hydrolysis of Intermediate Concentration of DPPC

(D_o:PC ≥ 8:1)[a]

[DPPC]	ΔA/min/mg protein			
mM	Ca = 2.5 mM	Ca = 5 mM	Ca = 10 mM	Ca = 20 mM
0.050	2.094	7.199	15.506±0.188	18.519
0.083	3.139	12.712	24.164±0.161	32.329
0.100	3.453	15.542	28.798±0.000	35.782
0.125	4.002	16.871	34.173±0.353	49.592
0.167	4.316	19.460	41.039±0.079	65.286
0.250	3.672	21.883	47.866±0.784	40.176

[a]Standard rate assay conditions. DPPC and Ca^{2+} concentrations are final cuvette concentrations. Reactions at [Ca] = 10 mM are duplicate runs. Data for Figures 18, 23 and 29.

Table VII(b)

PLD_p Hydrolysis of Intermediate Concentration of DPPC (D_o:PC ≥ 8:1)[a]

[DPPC]	ΔA/min/mg protein			
mM	Ca = 0.5 mM	Ca = 2.5 mM	Ca = 5 mM	Ca = 10 mM
0.050	0.0115	0.2517	0.4371	0.5035
0.083	0.0150	0.4056	0.6993	0.8378
0.100	0.0154	0.4545	0.8091	0.8916
0.125	0.0157	0.5594	0.9650	1.1189
0.167	0.0182	0.6923	1.1629	1.1608
0.250	0.0182	0.7517	1.4909	1.6224

[a]Standard rate assay conditions. DPPC and Ca^{2+} concentrations are final cuvette concentrations. Data for Figures 19 and 24.

Table VII(c)

PLD Hydrolysis of Intermediate Concentration of DDPC

(D_o:PC ≥ 8:1)[a]

DPPC	ΔA/min/mg protein			
mM	Ca = 0.5 mM	Ca = 2.5 mM	Ca = 5 mM	Ca = 10 mM
0.050	0.078±0.026	3.860±0.088	10.593±0.258	17.363±2.480
0.083	0.191±0.010	6.645±0.548	18.065±0.207	25.630±0.827
0.125	0.315±0.005	8.006±0.822	21.393±0.103	35.242±1.137
0.167	0.465±0.010	10.547±0.305	25.217±0.000	40.513±0.207
0.250	0.685±0.013	11.975±0.266	34.002±0.517	41.236±0.930

[a]Standard rate assay conditions. DPPC and Ca^{2+} concentration are final cuvette concentrations. All entries are duplicate runs. Data for Figures 20, 25, and 31.

Table VII(d)

PLD Hydrolysis of Intermediate Concentration of DMPC

(D_o:PC $\geqslant$ 8:1)[a]

DMPC	ΔA/min/mg protein		
mM	Ca = 2.5 mM	Ca = 5 mM	Ca = 10 mM
0.050	1.205±0.100	3.425±0.013	8.080±0.703
0.083	1.594±0.088	5.496±0.075	11.744±0.301
0.125	1.822 -	8.043±0.063	17.064±0.251
0.167	2.233 -	9.787±0.427	22.836±0.502
0.250	3.714 -	10.816±0.075	25.571±0.025

[a]Standard rate conditions with concentration expressed as final cuvette concentration. Values with uncertainties were duplicate runs. Data for Figures 21, 26 and 32.

Table VII(e)

PLD Hydrolysis of Intermediate Concentration of DSPC

(D_o:PC $\geq$ 8:1)[a]

DSPC	ΔA/min/mg protein		
mM	Ca = 2.5 mM	Ca = 5.0 mM	Ca = 10 mM
0.050	1.017±0.002	2.792±0.000	6.042±0.000
0.083	1.208±0.042	4.406±0.136	10.000±0.000
0.125	1.427±0.010	5.354±0.146	13.958±0.208
0.167	1.354±0.000	5.625±0.083	16.529±0.154
0.250	0.813±0.104	4.652±0.123	18.833±0.333

[a]Standard rate conditions with concentrations expressed as final cuvette concentration. All values were duplicate runs. Data for Figures 22, 27 and 33.

Figure 23

Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of DPPC at Various Ca^{2+} Concentrations.

Legend:

Reaction conditions were same as in Figure 18. The symbols stand for 2.5 mM (Δ) 5 mM (O) and 10 mM (□) of Ca^{2+} concentration.

Figure 23

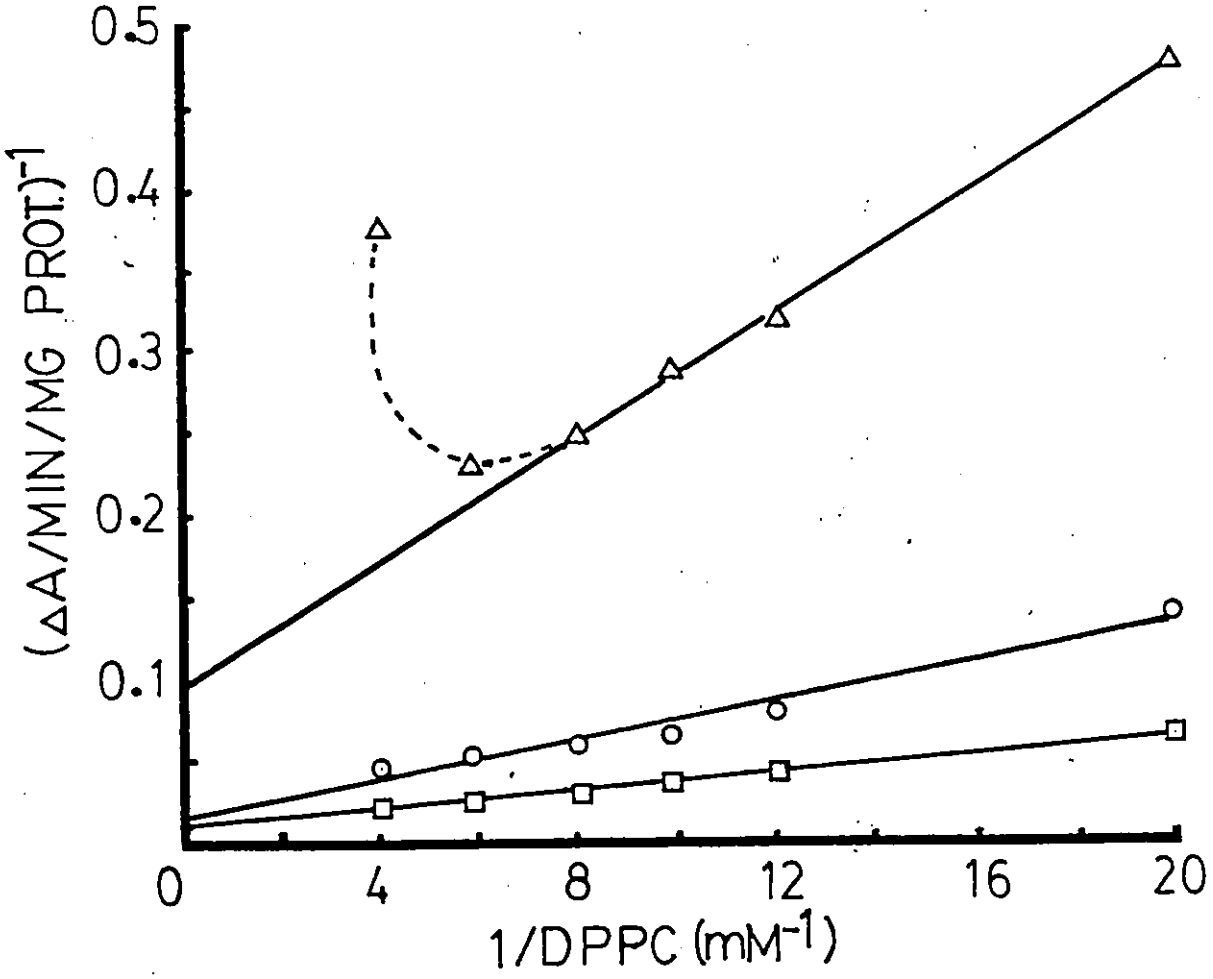


Figure 24

Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of DPPC at Various Ca^{2+} Concentrations for the Peanut PLD.

Legend:

Reaction conditions are same as in Figure 19. The symbols stand for 2.5 mM (O) and 5 mM (□) of Ca^{2+} concentration. The plot for Ca^{2+} concentration of 0.5 mM not shown due to wide scale disparity.

Figure 24

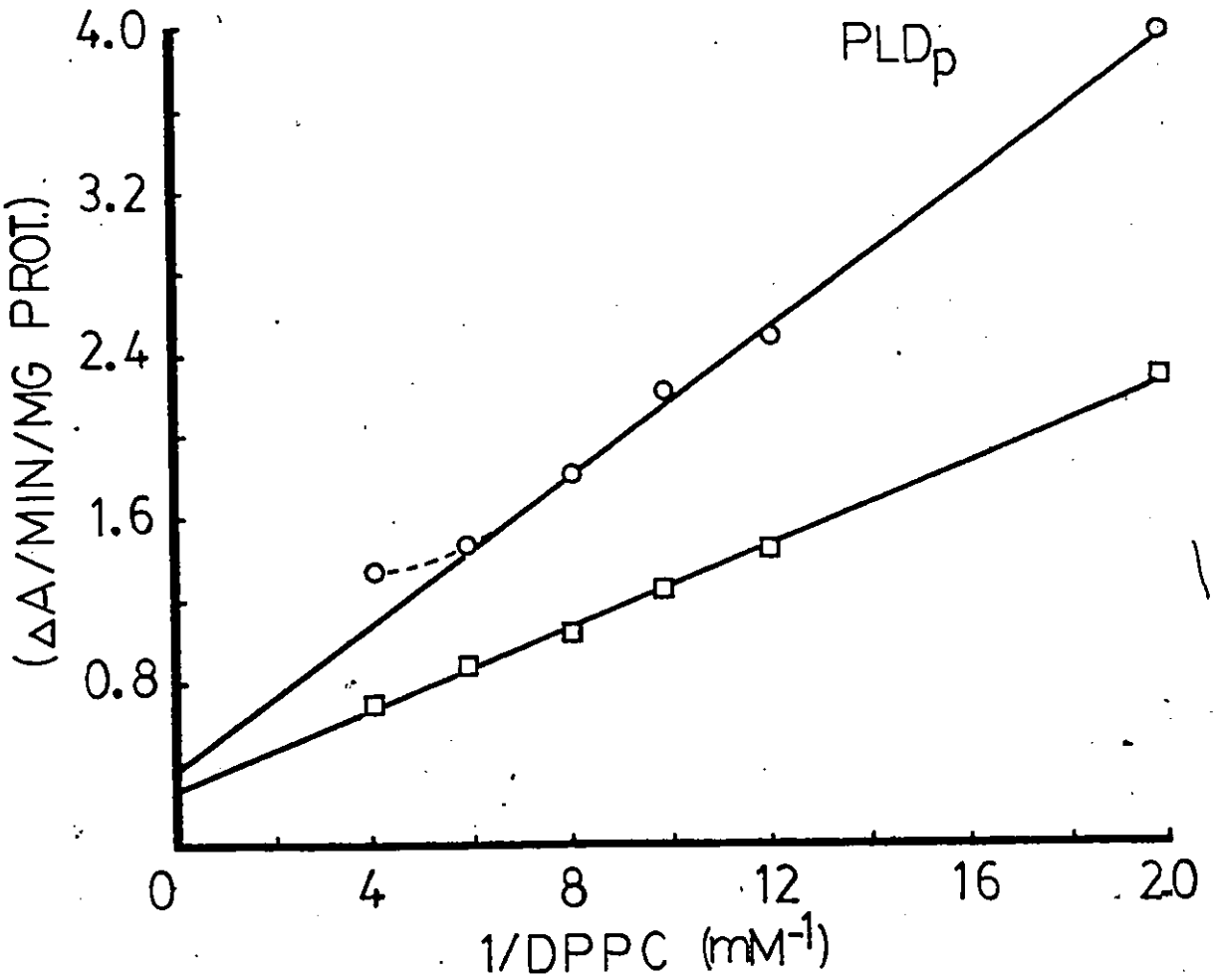


Figure 25

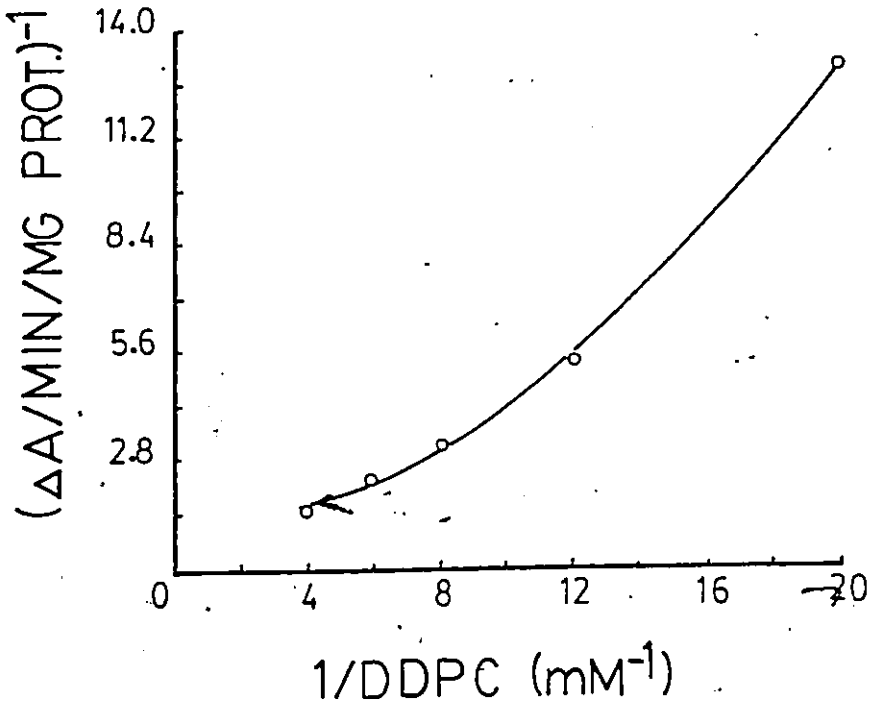
Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of DDPC at Various Ca^{2+} Concentrations.

Legend:

Reaction conditions were same as in Figure 20. (a) Ca^{2+} concentration is 0.5 mM. (b) The symbols stand for 2.5 mM (□) 5 mM (△) and 10 mM (O) of Ca^{2+} concentration.

Figure 25

a)


(ΔA/MIN/MG PROT.)-1
14.0
11.2
8.4
5.6
2.8
0
4
8
12
16
20
1/DDPC (mM-1)

b)

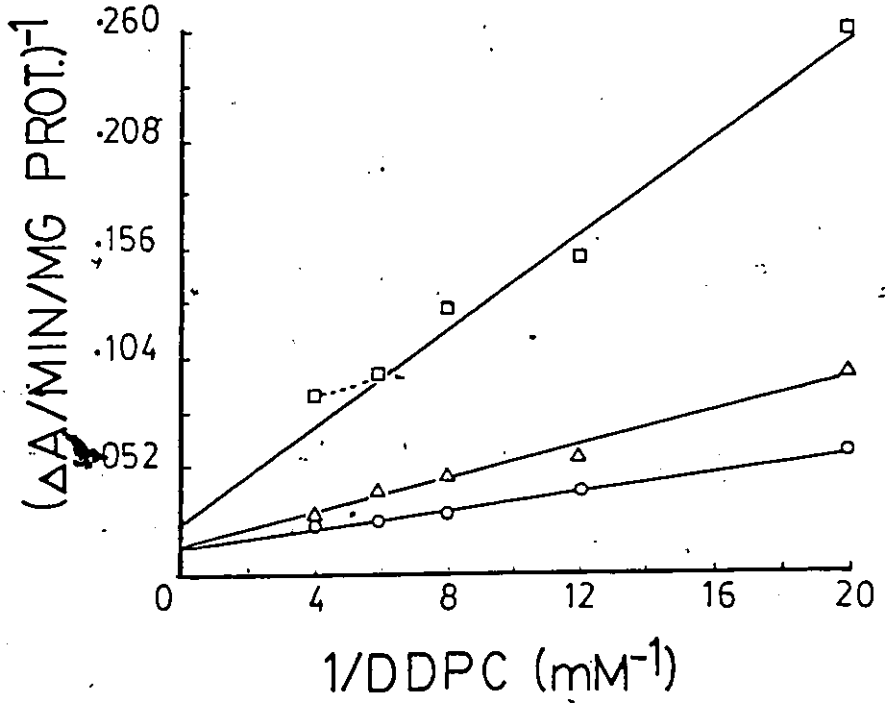

(ΔA/MIN/MG PROT.)-1
·260
·208
·156
·104
·052
0
4
8
12
16
20
1/DDPC (mM-1)

Figure 26

Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of DMPC at Two Ca^{2+} Concentrations.

Legend:

Reaction conditions were same as in Figure 21. Symbols stand for 5 mM (□) and 10 mM (○) of Ca^{2+} concentrations.

Figure 26

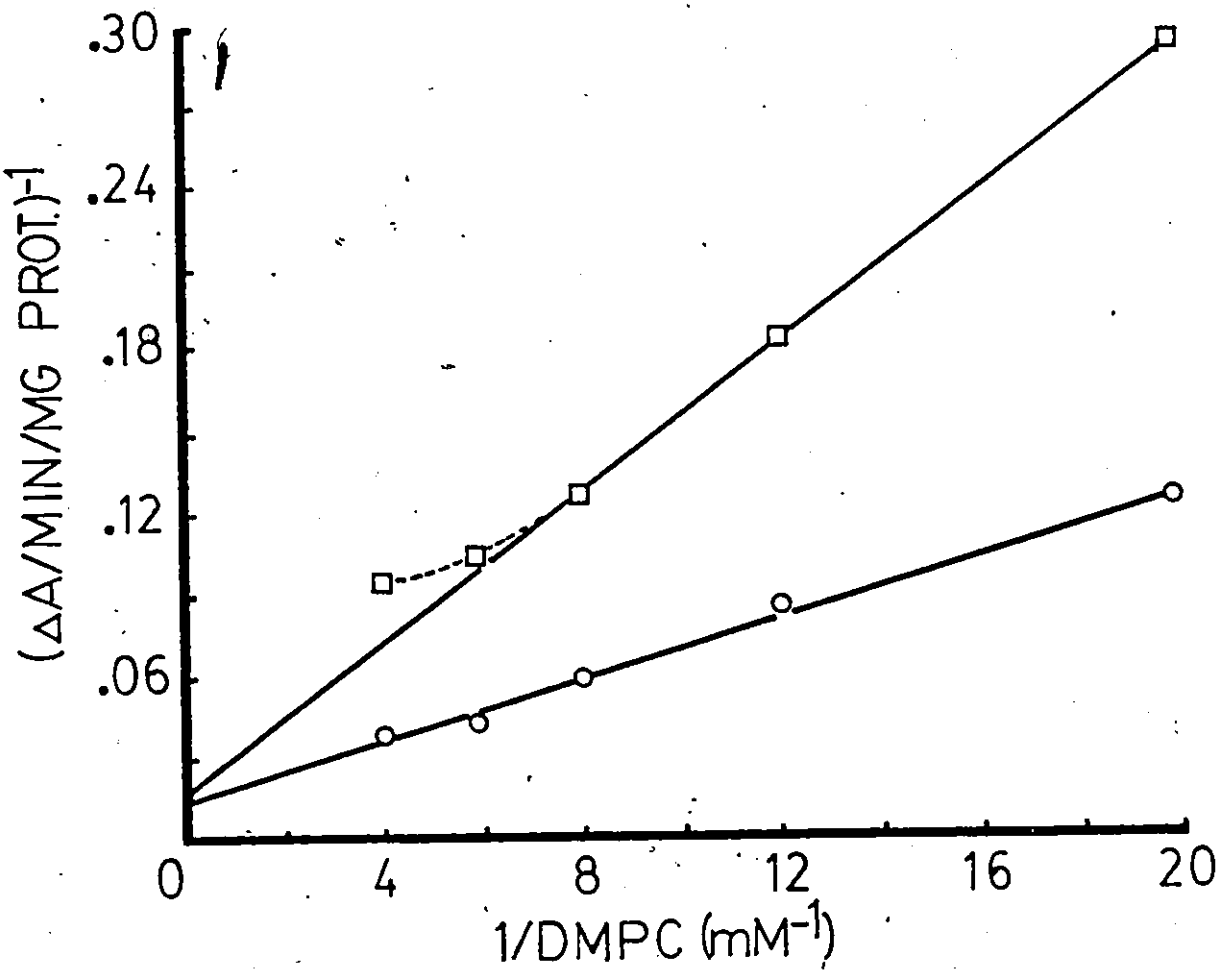


Figure 27

Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of DSPC at Various Ca^{2+} Concentrations.

Legend:

Reaction conditions were same as in Figure 22. The symbols stand for 2.5 mM (O) 5 mM (Δ) and 10 mM (□) of Ca^{2+} concentration.

Figure 27

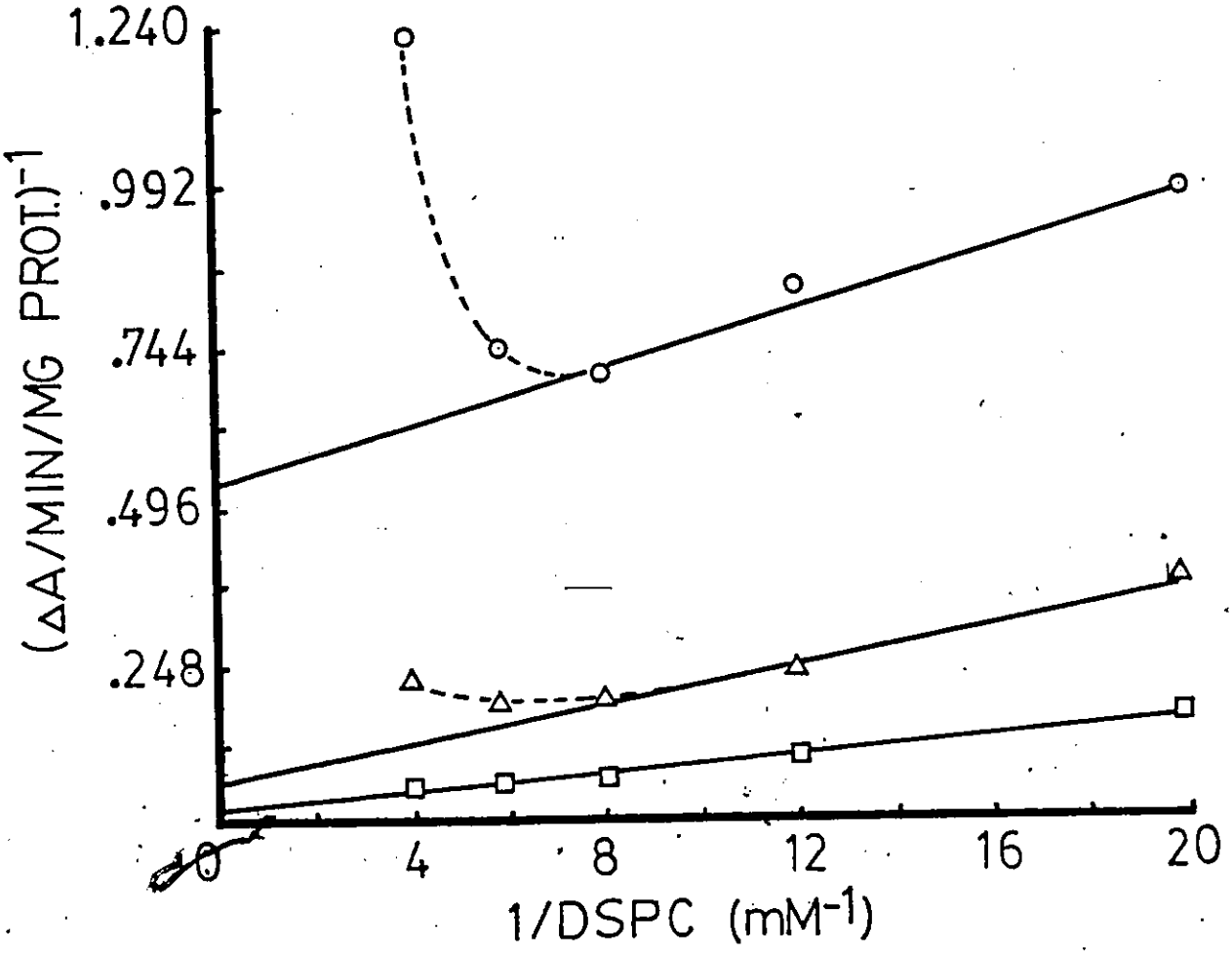


Figure 28

Double Reciprocal Plots of Initial Rates Against Intermediate Concentration of Substrate Expressed as Mole Fractions at 10 mM Ca^{2+} Concentration.

Legend:

Initial rates were determined by the standard rate assay for the various phospholipids. Mole fractions were expressed as the ratio of the phospholipid concentration to phospholipid plus total detergent concentration per reaction mixture. The symbols stand for DSPC (△), DMPC (□), DDPC (○) and DPPC (●) concentration.

Figure 28

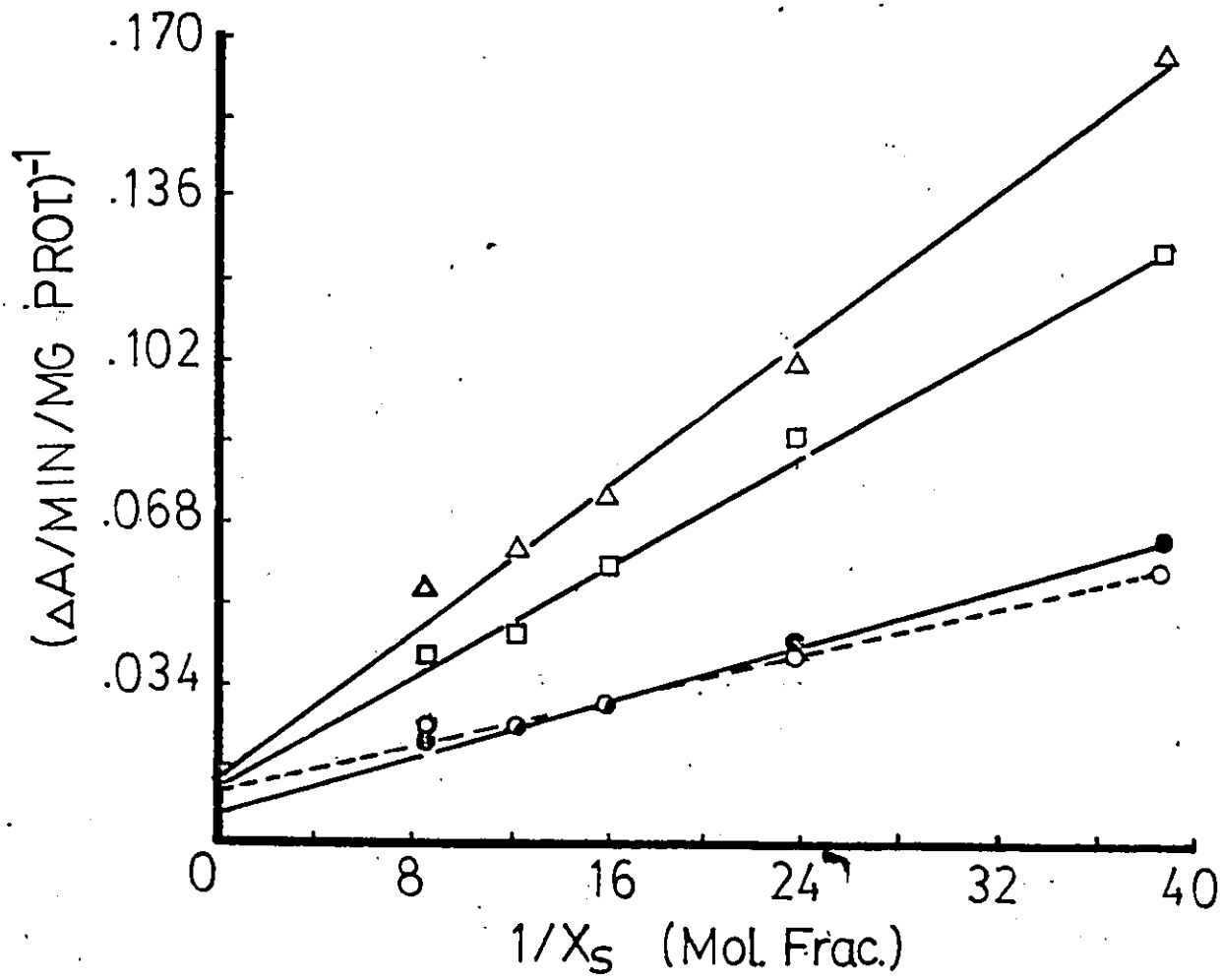


Table VIII

Kinetic Parameters For PLD Catalyzed Hydrolysis Of Phosphatidylcholines[a]

Substrate	Ca^{2+}	Km^{app}	Vmax	r^2
DDPC	2.5	0.45±0.04	39.2±0.4	0.9918
	5.0	0.28±0.03	71.4±4.1	0.9909
	10.0	0.17±0.02	78.7±2.3	0.9914
DMPC	5.0	0.44±0.05	34.3±2.3	0.9954
	10.0	0.37±0.04	66.7±3.6	0.9961
DPPC[b]	2.5	0.51±0.03	2.82±0.1	0.9992
	5.0	0.42±0.02	4.1±0.1	0.9992
DPPC	2.5	0.20±0.01	10.4±0.2	0.9990
	5.0	0.43±0.19	72.5±18.5	0.9795
	10.0	0.35±0.02	125.0±4.4	0.9976
DSPC	2.5	0.04±0.01	1.9±0.03	0.9921
	5.0	0.23±0.03	16.0±1.0	0.9937
	10.0	0.40±0.05	55.0±3.5	0.9946

[a]Data in Tables VII (a-e) were treated by linear regression analysis for the Michaelis-Menten model. Correlation coefficients, r^2, are noted. Except as noted the cabbage enzyme was used. Kinetic runs were individual or duplicate measurements each substrate concentration as noted in Tables VII (a-e).

[b]Peanut phospholipase D was used.

parabolic as the metal ion concentration is lowered. In the region where $[Ca^{2+}] >> [PC]$ the reciprocal plots are reasonably linear and extrapolates to $1/Vmax$ and $-1/Km^{app}$. Note that the reciprocal plots for $[Ca^{2+}] < 5$ mM approach linearity only at high values of $1/[PC]$ and cannot reliably be extrapolated to obtain $-1/Km^{app}$. Table VIII shows the kinetic parameters estimated from the double reciprocal plots of the data in Tables VIIa-e. The Km^{app} decrease for both DDPC and DMPC as Ca^{2+} concentration is increased. However, with DSPC the trend is reversed with increasing Ca^{2+} concentration. With DPPC, the Km^{app} increased from 0.197 mM at $[Ca^{2+}] = 2.5$ mM to 0.428 mM at $[Ca^{2+}] = 5.0$ mM then decreased to 0.350 mM at $[Ca^{2+}] = 10$ mM for the cabbage enzyme. As noted earlier, the extrapolation of the double reciprocal plots for $[Ca^{2+}] < 10$ mM which allows estimation of Km^{app} may not be completely valid and these values actually change depending on the number of data points considered for linear regression analysis. On the other hand, Km^{app} values obtained at $[Ca^{2+}] = 10$ mM which took all the data points into consideration may be a more reliable estimate of this parameter (Table IX). The Vmax values are well-behaved as this quantity increases proportionally with increasing Ca^{2+} concentration for all the phosphatidylcholines (Table VIII).

By virtue of our experimental approach, calculation of the apparent surface Michaelis constant (Km^{app}, mole fraction) and Vmax was possible. These values are also shown in Table IX as mole fractions. These parameters expressed in terms of the molar fraction of substrate may be a common

Table IX

Kinetic Parameters For PLD Catalyzed Hydrolysis Of Phosphatidylcholines in Terms of Surface Concentration[a]

Substrate	Km^{app}	Km^{appb}	Vmax	r^2
	Mole fraction	mM, Bulk conc.		
DDPC	0.10±0.01	0.17±0.02	86.2±2.5	0.9915
DMPC	0.24±0.02	0.37±0.04	82.6±4.0	0.9961
DPPC	0.23±0.02	0.35±0.02	153.9±5.0	0.9976
DSPC	0.26±0.03	0.40±0.05	69.4±4.3	0.9945

[a]Kinetic data from Table VII (a-e) were used only for runs in the presence of 10 mM Ca^{2+}. Substrate concentration were converted to mole fractions (of PC:total of PC + detergents) and subjected to linear regression analysis for the Michaelis-Menten model. The Km^{app} values given in column 2 are mole fractions whereas the Km^{app} values given in column 3 are the values in mM taken from Table VIII.

[b]The values arise from the regression with mole fractions.

Figure 29

Initial Rates as a Function of Ca^{2+} Concentration at Various Intermediate Concentrations of DPPC.

Legend:

Reaction conditions were same as in Figure 18. Symbols stand for 0.050 mM (△), 0.083 mM (○) and 0.125 mM (□) DPPC. Other DPPC concentrations not shown for clarity.

Figure 29

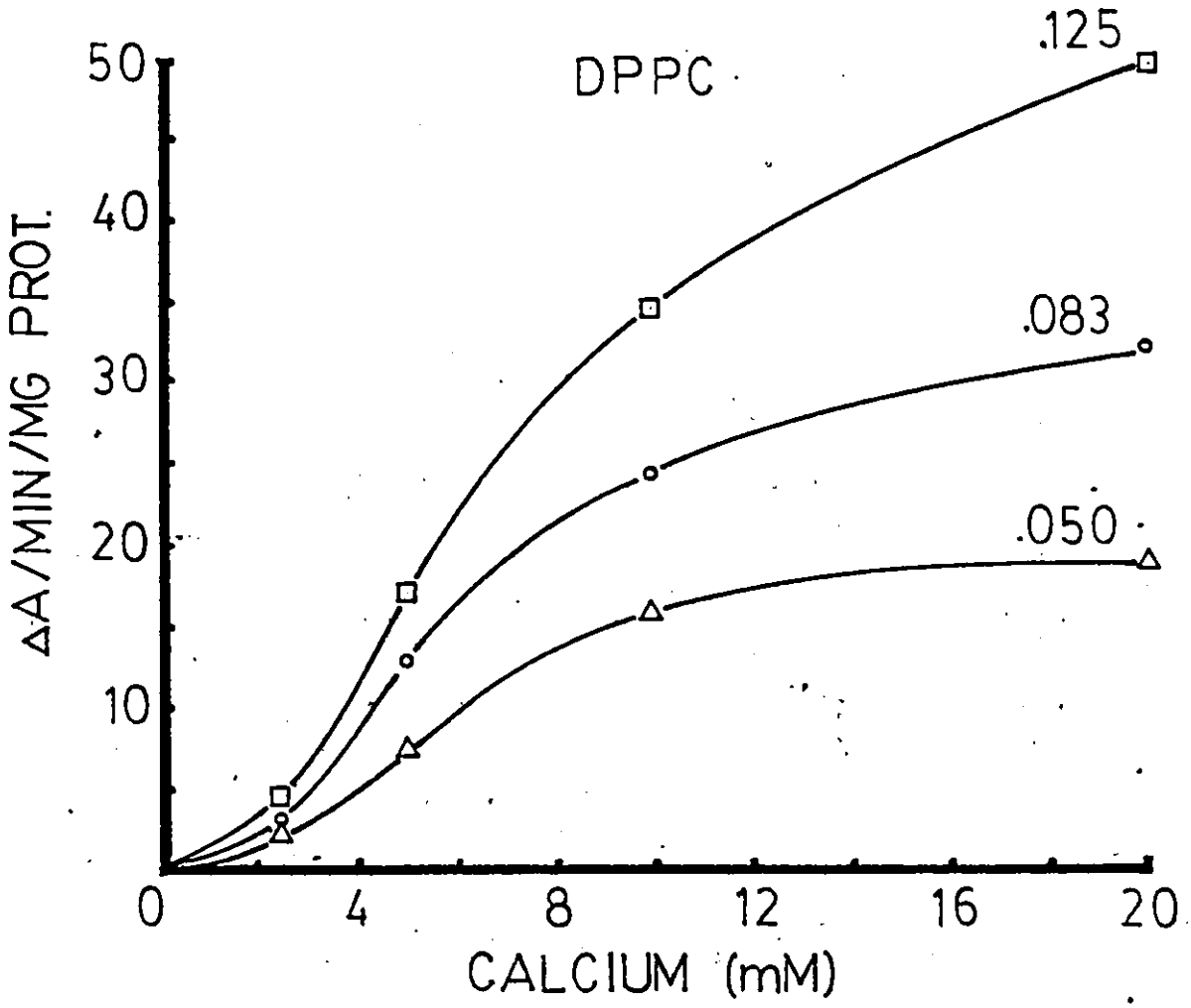

DPPC
.125
.083
.050
ΔA/MIN/MG PROT.
50
40
30
20
10
0
4
8
12
16
20
CALCIUM (mM)

Figure 30

Initial Rates as a Function of Ca^{2+} Concentration at a Fixed Concentration of DPPC for Both Cabbage and Peanut PLD.

Legend:

Initial rates were determined by the standard rate assay using 0.125 mM DPPC (D_o:PC ≥ 8:1) for both the peanut and cabbage PLD. See data in Table VII(f).

Figure 30

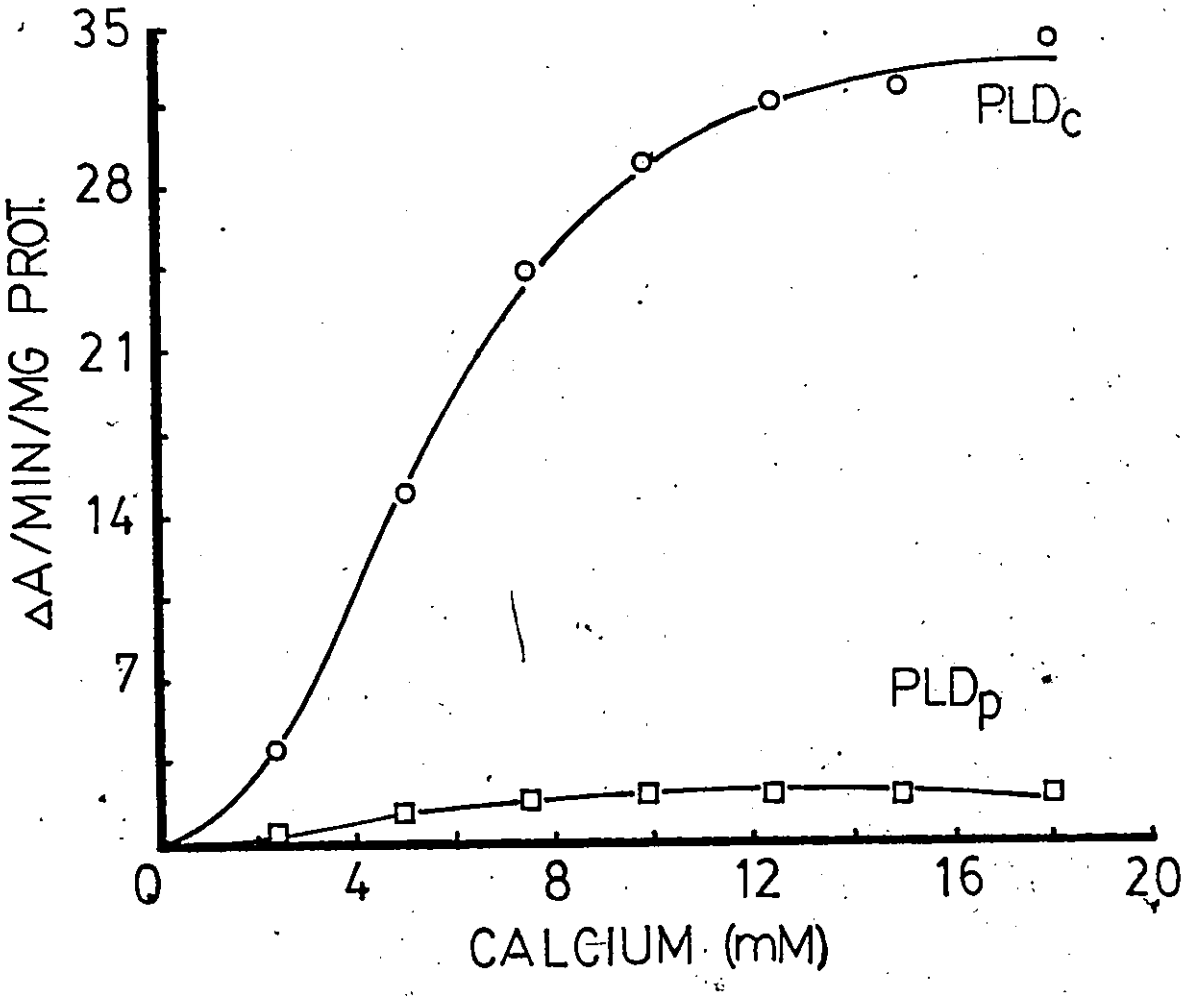


Figure 31

Initial Rates as a Function of Ca^{2+} Concentration at Various Intermediate Concentrations of DDPC.

Legend:

Reaction conditions were same as in Figure 20. The symbols stand for 0.050 mM (△), 0.125 mM (□) and 0.250 mM (○) of DDPC. Other DDPC concentrations not shown for clarity.

Figure 31

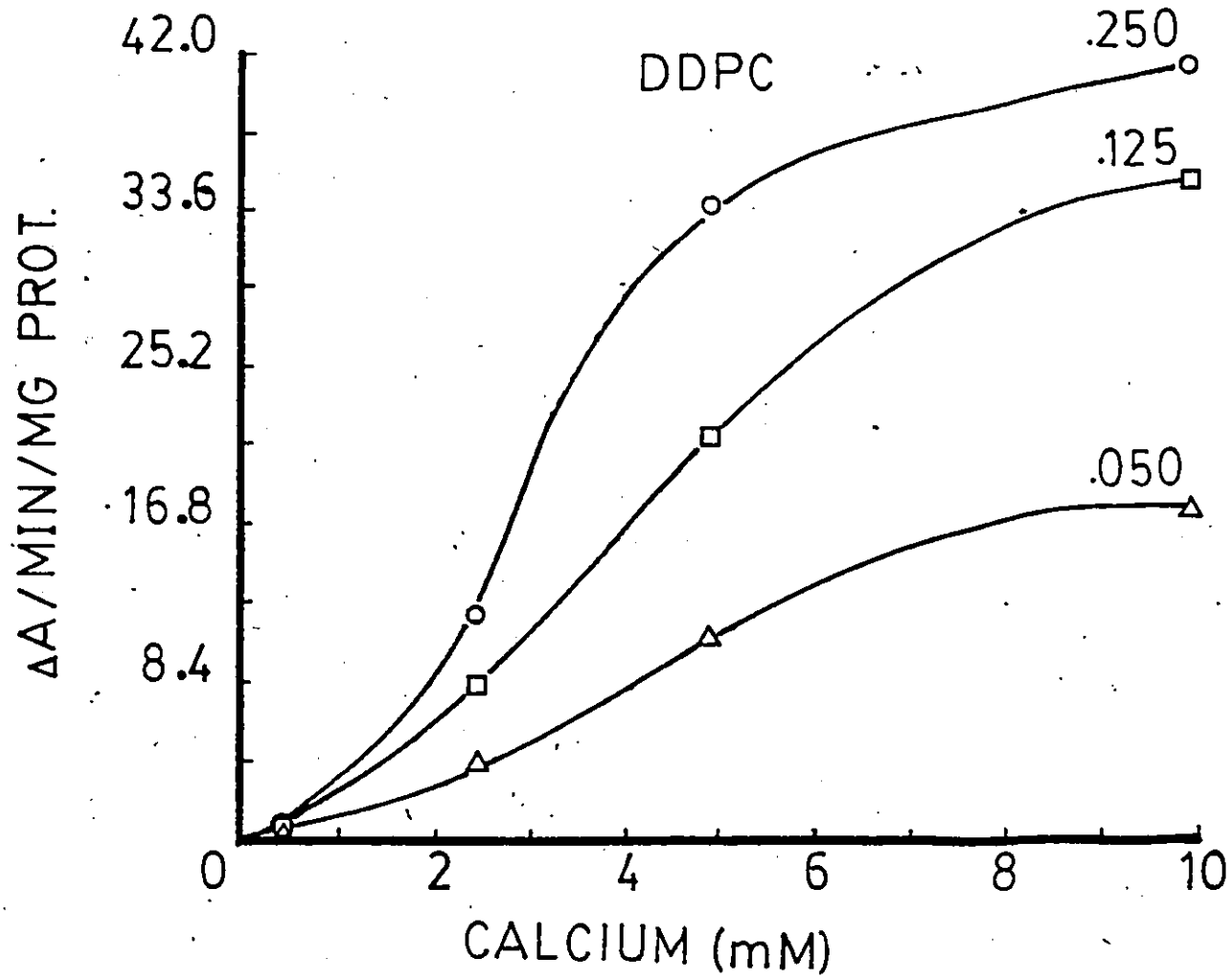

42.0
33.6
25.2
16.8
8.4
0
ΔA/MIN/MG PROT.
DDPC
.250
.125
.050
2
4
6
8
10
CALCIUM (mM)

Figure 32

Initial Rates as a Function of Ca^{2+} Concentration at Various Intermediate Concentrations of DMPC.

Legend:

Reaction conditions were same as in Figure 21. The symbols stand for 0.050 mM (□), 0.125 mM (○) and 0.167mM (△) DMPC. Other DMPC concentrations were not shown for clarity.

Figure 32

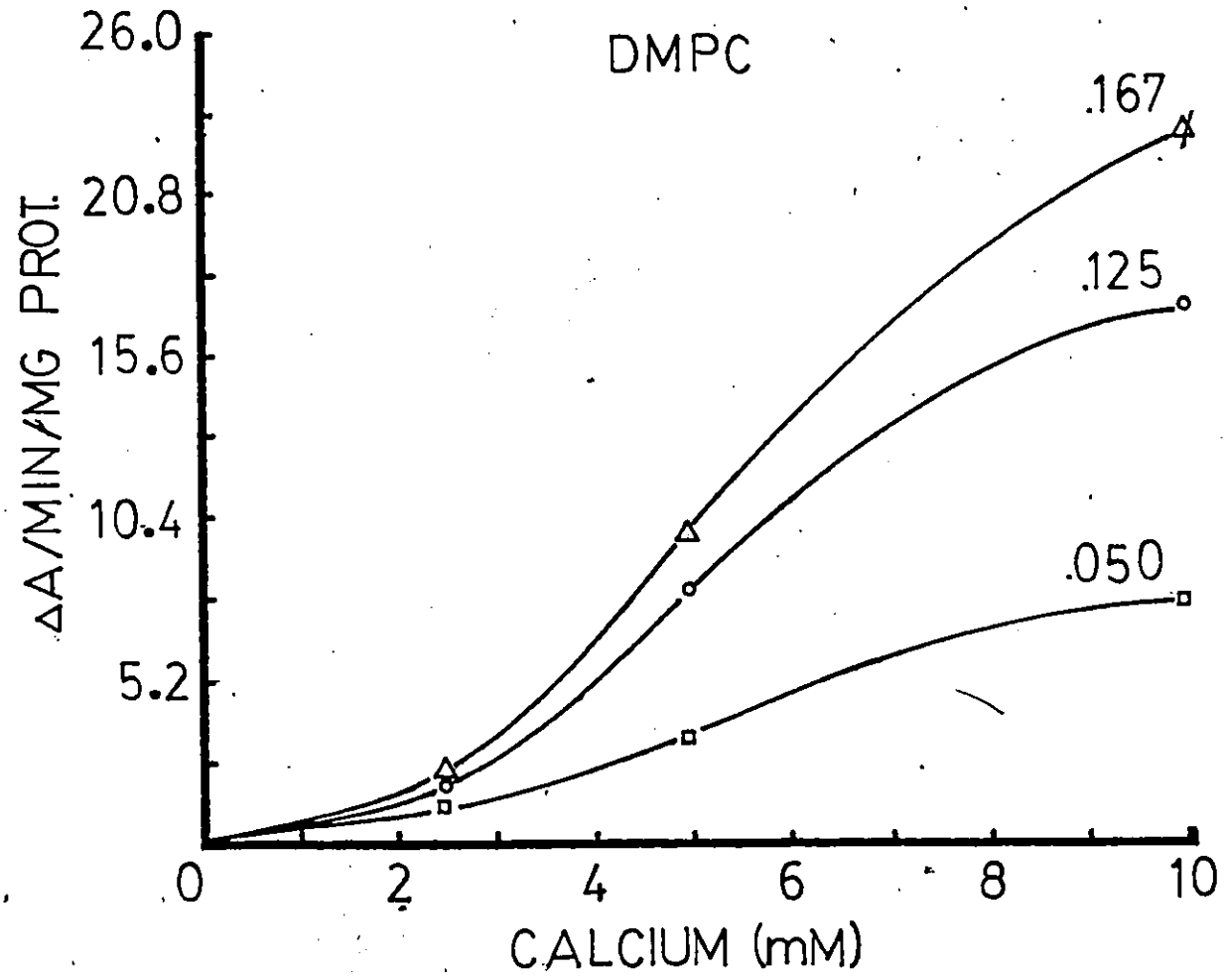

DMPC
26.0
20.8
15.6
10.4
5.2
ΔA/MIN/MG PROT.
.167
.125
.050
0
2
4
6
8
10
CALCIUM (mM)

Figure 33

Initial Rates as a Function of Ca^{2+} Concentration at Various Intermediate Concentrations of DSPC.

Legend:

Reaction conditions were same as in Figure 22. Symbols stand for 0.050 mM (□), 0.083 mM (○) and 0.167 mM (△). Other DSPC concentrations not shown for clarity.

Figure 33

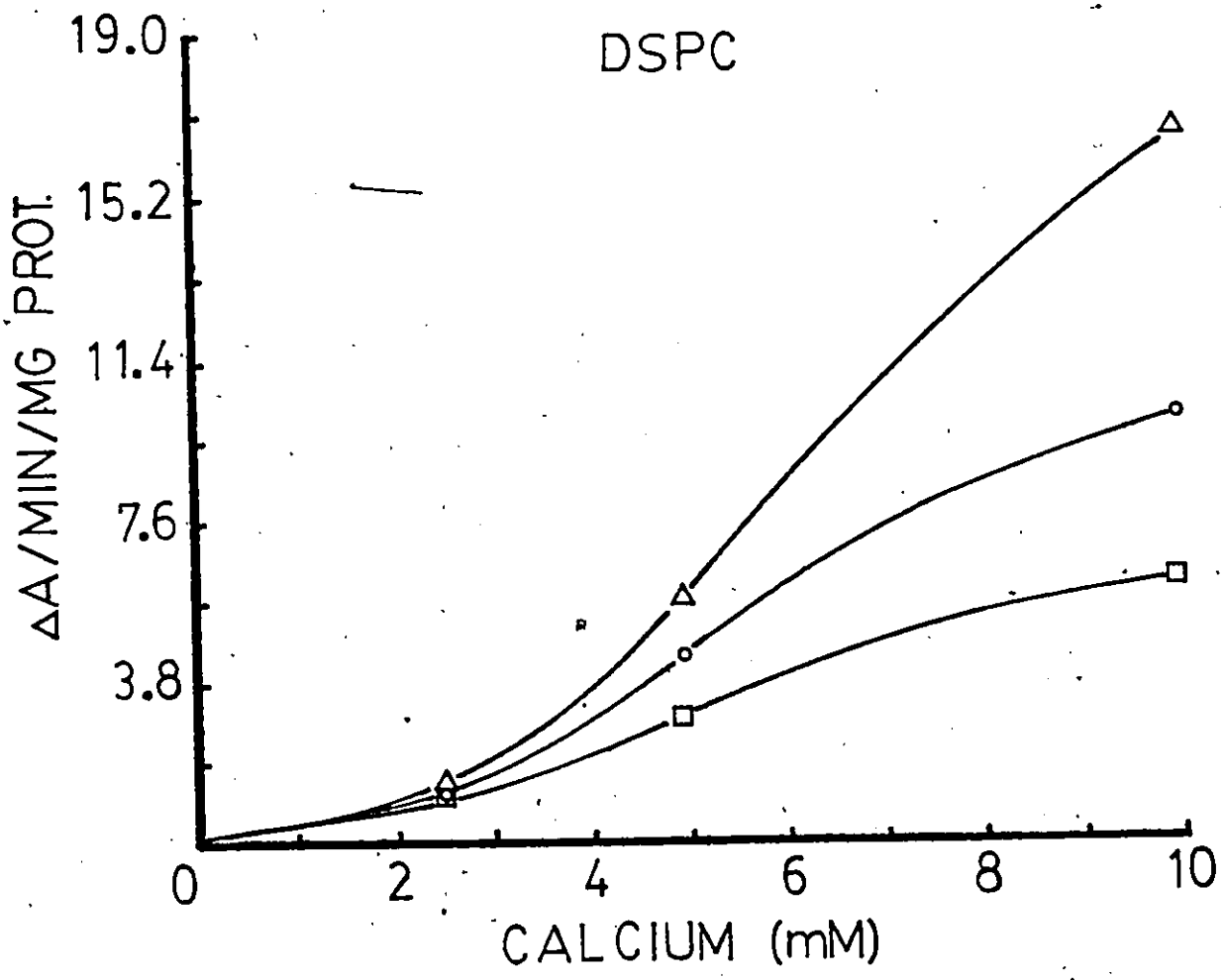


TABLE VII(f)

Rate of DPPC Hydrolysis as a Function of Calcium Concentration For PLD_c and PLD_p[a]

[Calcium]	ΔA/min/mg protein	
	PLD_c	PLD_p
0.0	0.000±0.000	0.000±0.000
2.5	3.815±0.000	0.446±0.001
5.0	14.900±0.232	1.144±0.030
7.5	24.036±0.127	1.525±0.058
10.0	28.741±0.000	1.642±0.000
12.5	31.412±0.382	1.654±0.014
15.0	32.141±0.076	1.724±0.000
18.0	34.209±0.201	1.833±0.043

[a]DPPC concentration was 0.125 mM (D_o:PC ≥ 8:1).
Standard rate concentration and calcium concentrations are cuvette concentrations.
Data for Figures 30, 44 and 45

denominator for comparing the velocity dependency on substrate concentration for PLD catalyzed hydrolysis in reaction carried out at different detergent to PC ratios for phosphatidylcholines of varying chain length.

The rate dependence on Ca^{2+} concentration for the phosphatidylcholines at various fixed concentrations of PC are shown in Figures 29 through 33 (data taken from Tables VIIa-e or f). Similar to comparable profiles obtained using the high substrate solutions (D_o:PC $\geq$ 1:1) (Figures 17a,b) these curves were also sigmoidal and the v increased with increasing PC concentration at a fixed Ca^{2+} concentration. In order to determine whether the systematic curvature of the v against Ca^{2+} plots are indicative of cooperative binding of Ca^{2+} to the components of the reaction system, and if so, estimate the extent of cooperative binding, the data for DPPC (D_o:PC $\geq$ 8:1, Table VIIa,b) were fitted to a Hill equation. These plots were linear. Figure 34 and Figure 35 show some representative plots. Data for these plots are shown in Table X. A Hill coefficient, n_H of 2.5$\pm$0.1 and $[Ca^{2+}]_{0.5}$ value at one-half maximal velocity of 6.2$\pm$0.2 mM were estimated (See Table X for the various n_H and $[Ca^{2+}]_{0.5}$ values).

It is not possible to compare Vmax values for the various substrates since, as noted earlier, comparison of individual measured rates from enzyme preparation to preparation is not valid. However for a given substrate comparison of the Vmax's obtained for various Ca^{2+} concentrations is valid. Some trends are apparent but discussion of Ca^{2+} effects is postponed until some of the physical data concerning interactions amongst various components is considered.

Figure 34

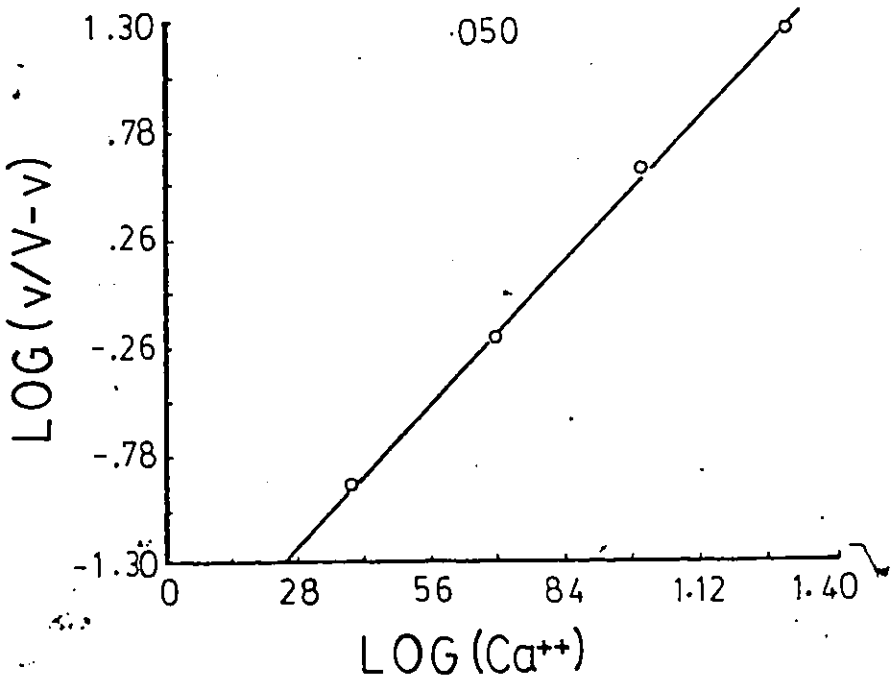
Hill Plots for Ca^{2+} Binding for Various Intermediate Concentrations of DPPC.

Legend:

Reaction conditions were standard rate assay conditions. Data shown in Table X. Original data taken from Table VII(a). A Hill coefficient of 2.4 was estimated in both (a) 0.050 mM and (b) 0.100 mM DPPC and coefficient of linear correlation of 0.9992 (a) and 0.9987 (b) were estimated.

Figure 34

a)


1.30
.78
.26
-.26
-.78
-1.30
0
28
56
84
1.12
1.40
LOG (v/V-v)
LOG (Ca++)
·050

b)

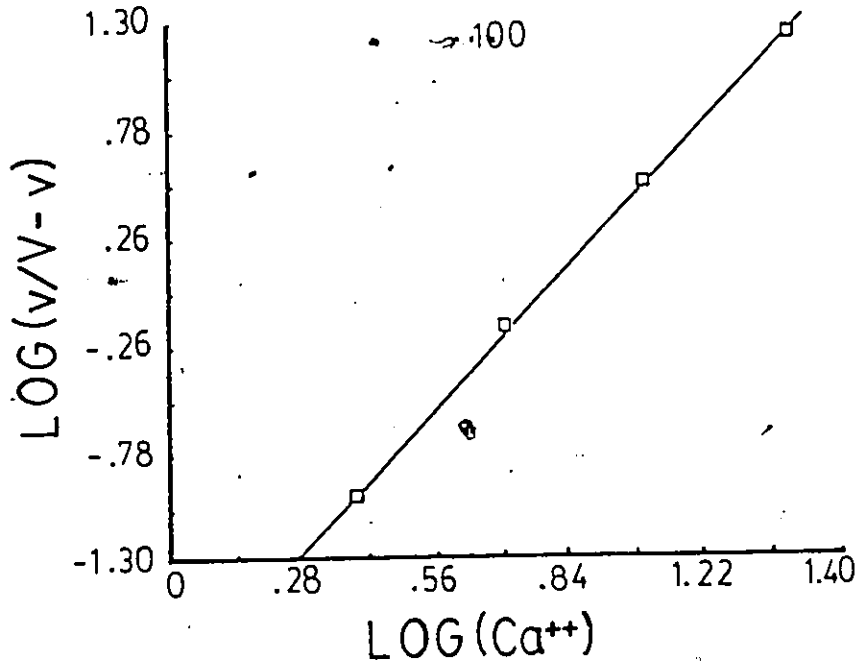

1.30
.78
.26
-.26
-.78
-1.30
0
.28
.56
.84
1.22
1.40
LOG (v/V-v)
LOG (Ca++)
·100

Figure 35

Hill Plots for Ca^{2+} Binding at a Fixed Intermediate Concentration of DPPC for Both Cabbage and Peanut PLD.

Legend:

Reaction conditions were standard rate assay conditions at 0.125 mM DPPC using both the cabbage and peanut PLD. Data shown in Table X. Original data taken from Table VII(f). A Hill coefficient of 2.5 (□) and 2.6 (○) and correlation coefficients of 0.9993 and 0.9999 were estimated, respectively.

Figure 35

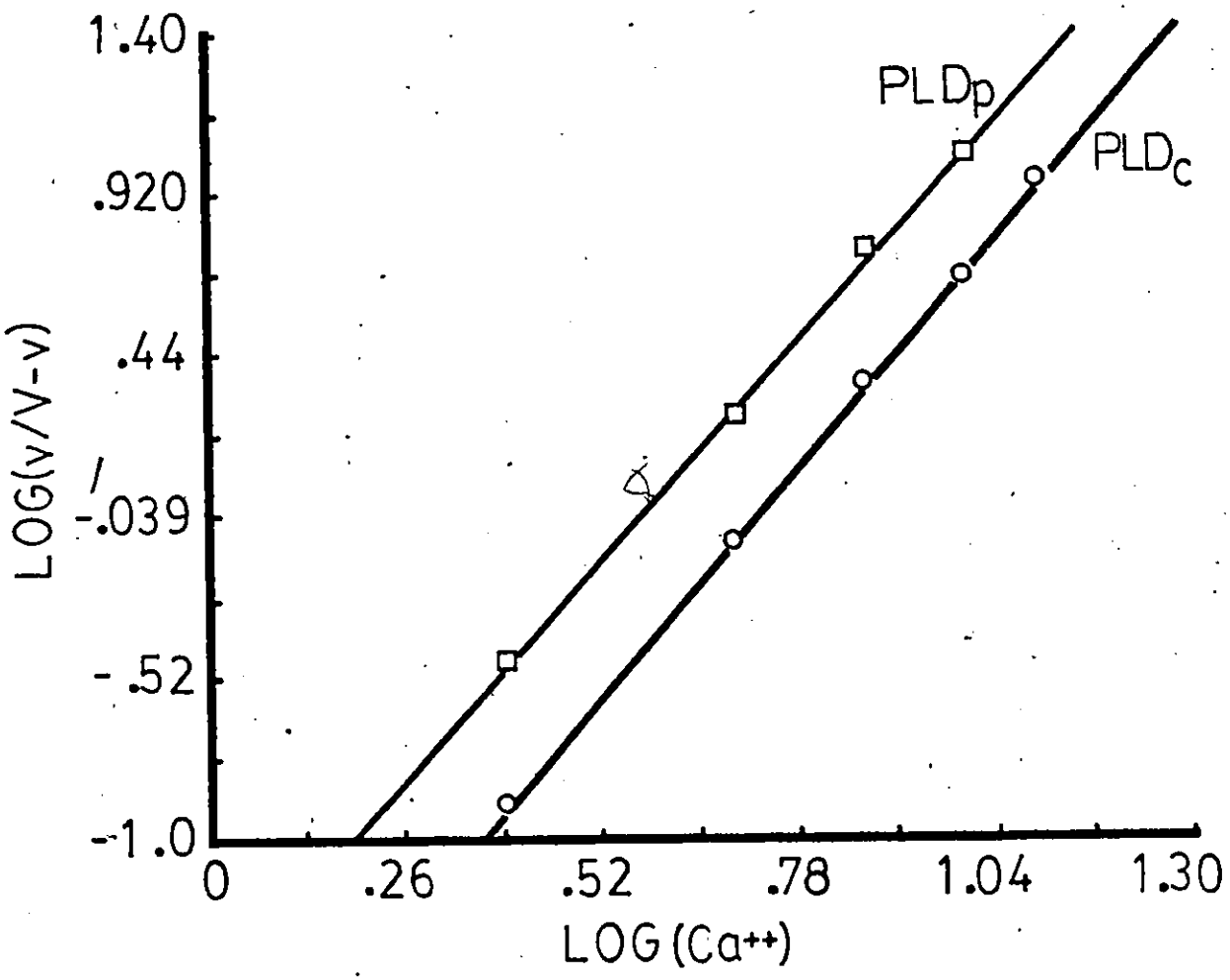


Table X

Data for Hill Plots

Log[Ca^{2+}]	Log v/(Vmax - v)			
	(a)	(b)	(c)	(d)
0.398	-0.920	-1.000	-0.482	-0.912
0.699	-0.233	-0.160	0.242	-0.130
0.875			0.744	0.341
1.000	0.589	0.495	1.017	0.661
1.100	1.259	1.208		0.942

[a]Conditions: PLD_c, [DPPC] = 0.050 mM, standard rate assay n_H = 2.4 $[Ca]_{0.5}$ = 6.0 mM

[b]Conditions: PLD_c, [DPPC] = 0.100 mM, standard rate assay n_H = 2.4 $[Ca]_{0.5}$ = 6.2 mM

[c]Conditions: PLD_p, [DPPC] = 0.125 mM, standard rate assay n_H = 2.5 $[Ca]_{0.5}$ = 4.0 mM

[d]Conditions: PLD_c, [DPPC] = 0.125 mM, standard rate assay n_H = 2.6 $[Ca]_{0.5}$ = 5.9 mM

Data for Figures 34 and 35.

Physical Measurements

Determination of CMC as a Probe of the Physical State of PC-SDS-Triton Mixture

CMC determinations are usually made on the basis of a sharp change in the colligative properties, surface tension, solubilization of a hydrophobic dye or conductivity of a detergent solution with respect to concentration [128].

The absorption spectrum of Coomassie Brilliant Blue G-250 (CBBG) show two major peaks at 655 nm and 465 nm. Binding of monomeric nonionic detergent e.g., Triton X-100, cause no change in the CBBG spectrum, however, at the onset of micelle formation, a shift and concominant proportional increase at 620 nm and decrease at 470 nm are observed in absorption with increasing micelle formation (see Figure 36). This pattern of absorption results in a sharp break in the concentration-dependent absorbance of the detergent-CBBG solution at the critical micelle concentration of the detergent [101]. Determination of the CMC for Triton X-100 and various mixtures of Triton X-100 and SDS and PC were made from plots of the A_{620} and A_{470} with respect to concentration as shown in Figure 37a, b. We observed a peak at A_{640} instead of A_{620} when SDS was mixed with Triton X-100. CMC for SDS alone could not be determined by this method as no sharp break in absorption is caused by this anionic detergent as also reported by Rosenthal and Koussalle [101]. The CMC reported in Table XI are in terms

Figure 36

Absorption Spectra of Coomassie Brilliant Blue G-250 and its Mixture with Triton X-100.

Legend:

(1) Absorption spectrum of pure Coomassie Brilliant Blue G-250 (CBBG); (2) absorption spectrum of CBBG plus 0.006% Triton X-100; (3) absorption spectrum of CBBG plus 0.03% Triton X-100. See text for details.

Figure 36

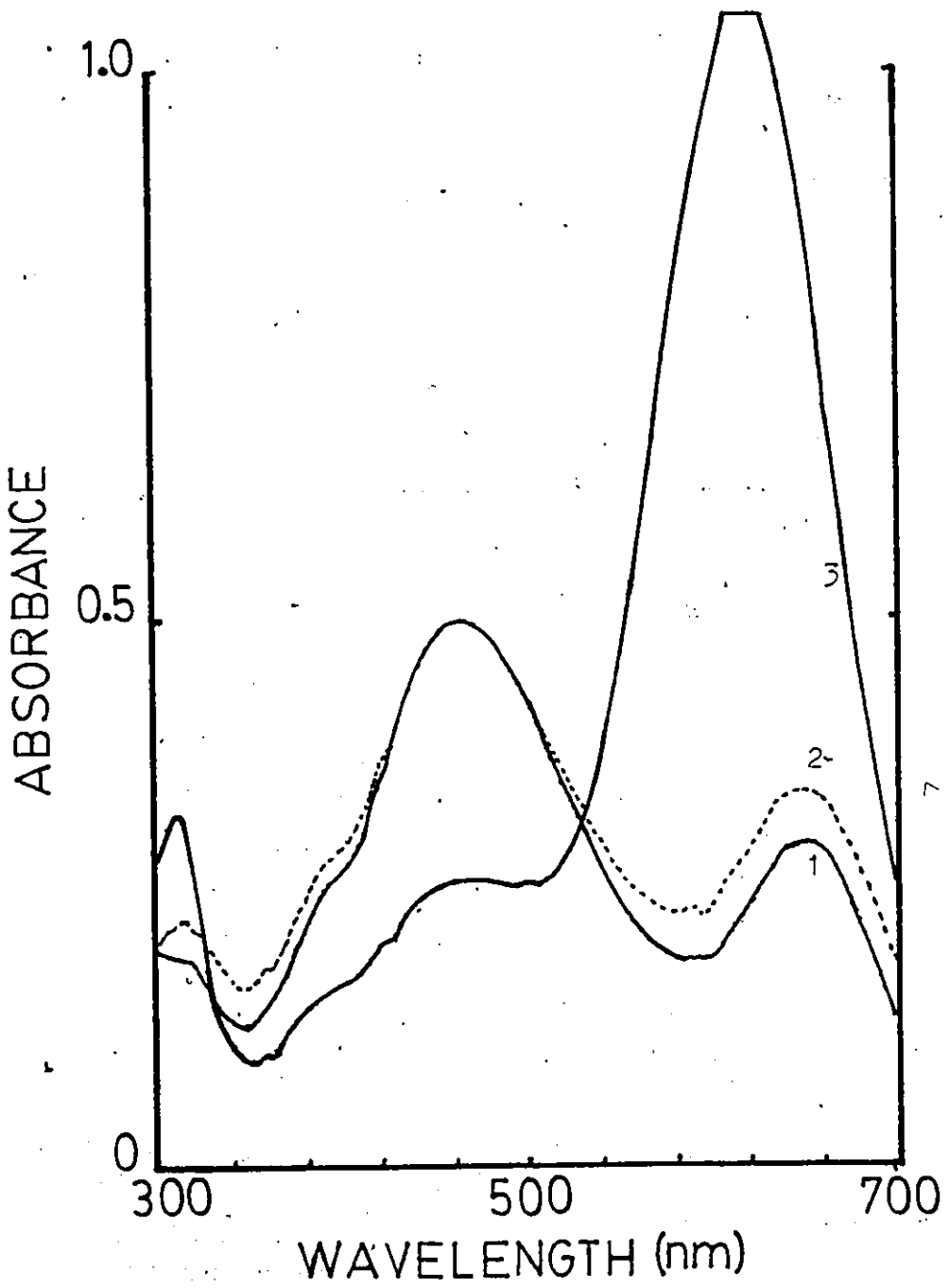


Figure 37

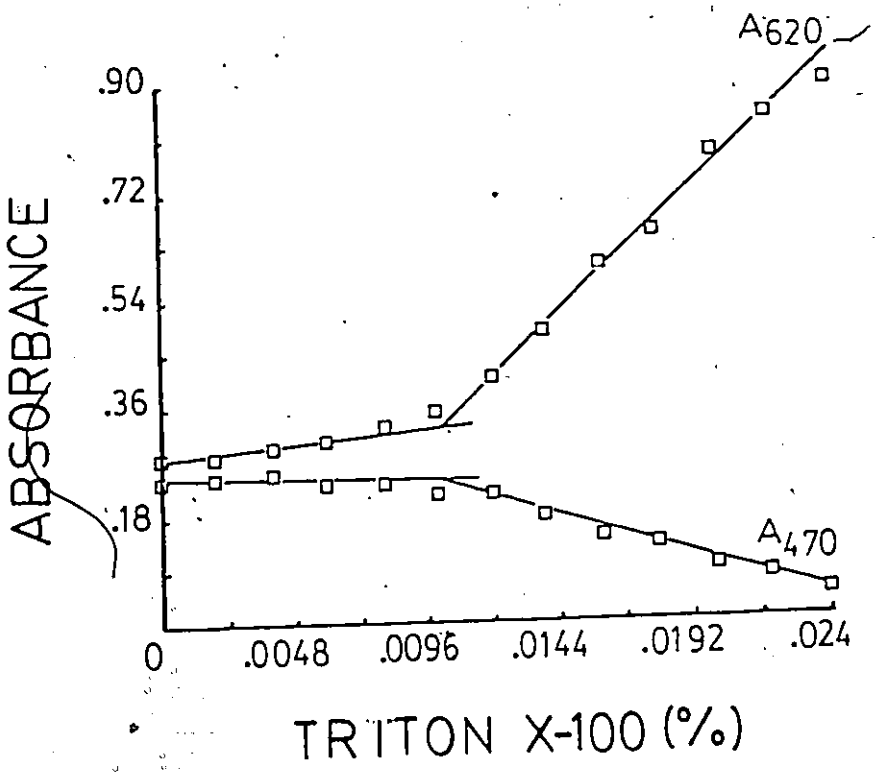
CMC Determination of Triton X-100 and SDS-Triton X-100 Mixture

Legend:

CMC's were determined by the method of Rosanthal and Koussalle [101]. (a) Triton X-100 alone. (b) Mixture of Triton-SDS-PC (70 μM). See text for details.

Figure 37

a)


A620
.90
.72
.54
.36
.18
ABSORBANCE
A470
0
.0048
.0096
.0144
.0192
.024
TRITON X-100 (%)

b)

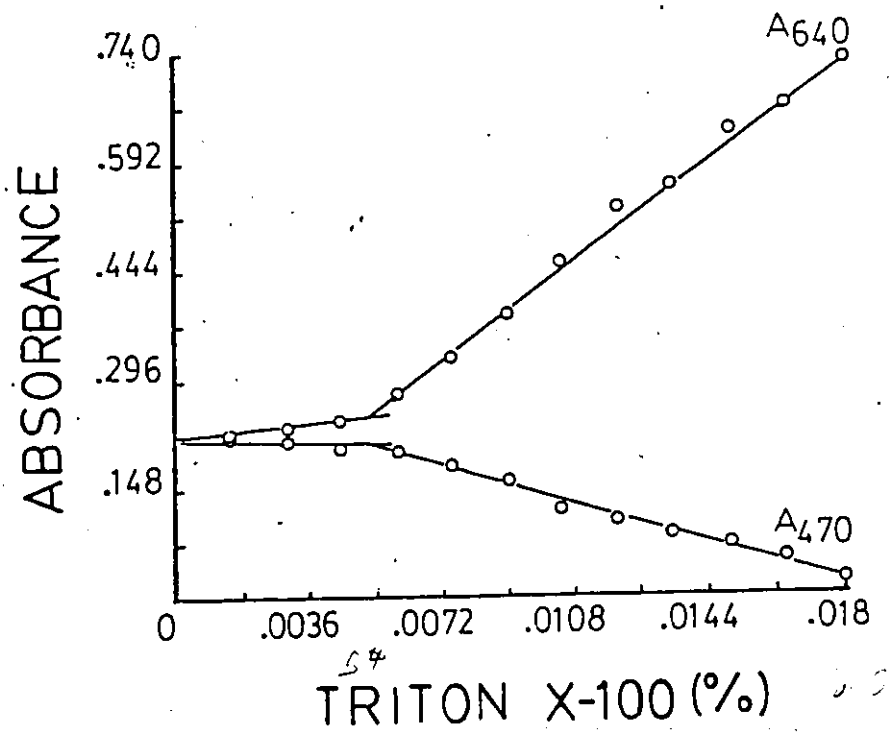

A640
.740
.592
.444
.296
.148
ABSORBANCE
A470
0
.0036
.0072
.0108
.0144
.018
TRITON X-100 (%)

Table XI

CMC Values for Various Mixtures of PC-SDS-Triton-Ca^{2+} [a]

Line	Detergent Mixture	(b) 10^{-3} x CMC	(c) 10^{-3} x CMC
1	TX	10.0	-
2	TX-PC (70 µM)	10.6	9.6
3	TX-SDS	3.0	4.5
4	TX-SDS-PC	1.2	5.1
5	TX-SDS-PC	-	6.9
6	TX-SDS-PC-Ca (70 µM)[3]	1.6	-

[a]TX is abbreviation for Triton X100, concentrations are expressed as % Triton X-100 in the detergent mixture. All entries are accurate to ±0.0003.

[b]Line 1: 0.2% Triton X-100.
Line 2: 0.2% Triton X-100 containing 70 µM DPPC.
Line 3: 0.09% SDS-0.2% Triton X-100.
Line 4: 0.09% SDS-0.2% Triton X-100 containing 70 µM DPPC.
Line 5: 0.09% SDS-0.2% Triton X-100 containing 0.83 mM DPPC
Line 6: 0.09% SDS-0.2% Triton X-100 containing 70 µM DPPC and 9 mM Ca^{2+}.

[c]Line 2: 0.3% Triton X-100 containing 70 µM DPPC.
Line 3: 0.03% Triton X-100.
Line 4: 0.03% SDS-0.3% Triton containing 70 µM DPPC.
Line 5: 0.03% SDS-0.3% Triton containing 0.83 mM DPPC.

of percent Triton X-100 in the various mixtures.

Consistent with thermodynamic prediction, the CMC for Triton X-100 decreased as SDS and PC were introduced into this detergent. Obviously the ratio of Triton X-100 to SDS affects the CMC for Triton X-100. The variation in CMC for Triton X-100 with adducts is indicative of mixed micelle formation [59]. By the dynamics of detergent-CBBG interaction, a change in absorption occurs because at this point the dye is transferred completely from a hydrophilic to a hydrophobic environment assumed to be the micelle interior [101]. The variation of the CMC for Triton in the presence of adducts provide evidence that the microscopic organization of the components in any one mixture is unique.

UV Difference Spectroscopic Studies

In order to understand the interaction of PLD with PC mixed micelles and Ca^{2+} observed from our kinetic studies we studied the binding of PLD_c to Ca^{2+} and to its substrate micelles by UV difference spectroscopy. Also, since the phospholipases D obtained from commercial sources were used without further purification, SDS-PAGE was conducted to check whether or not these enzymes show any unusual electrophoretic mobility patterns. Results of the SDS-PAGE indicate clearly that both PLD_c and PLD_p are not homogeneous.

From our UV difference spectroscopy studies Figure 39b shows the Ca^{2+}-induced difference spectrum of PLD_c. Two

Figure 38

Enzyme Proteins Stained Via Coomassie Blue on SDS-PAGE

Legend:

Gel was stained for proteins with Coomassie Blue according to the procedure of Laemmli [88].

To lanes 2, 4 and 5 was added 10 µg, 10 µg and 5 µg of PLD_c, PLD_p and PLD_p, respectively, solubilized in the Laemmli [88] sample buffer. To lanes 1 and 3 was added 20 µg of the standard protein mixture containing myosin, β-galactosidase, phosphorylase b, bovine albumin, ovalbumin and carbonic anhydrase with molecular weights of 205,000, 116,000, 97,400, 66,000 45,000 and 29,000 daltons, respectively, solubilized in the Laemmli [88] sample buffer. Lanes 1 and 2 were run on one slab and lanes 3-5 on another slab alongside each other in one electrophorectic cell. The two slabs were cut and joined together for the photograph.

Figure 38

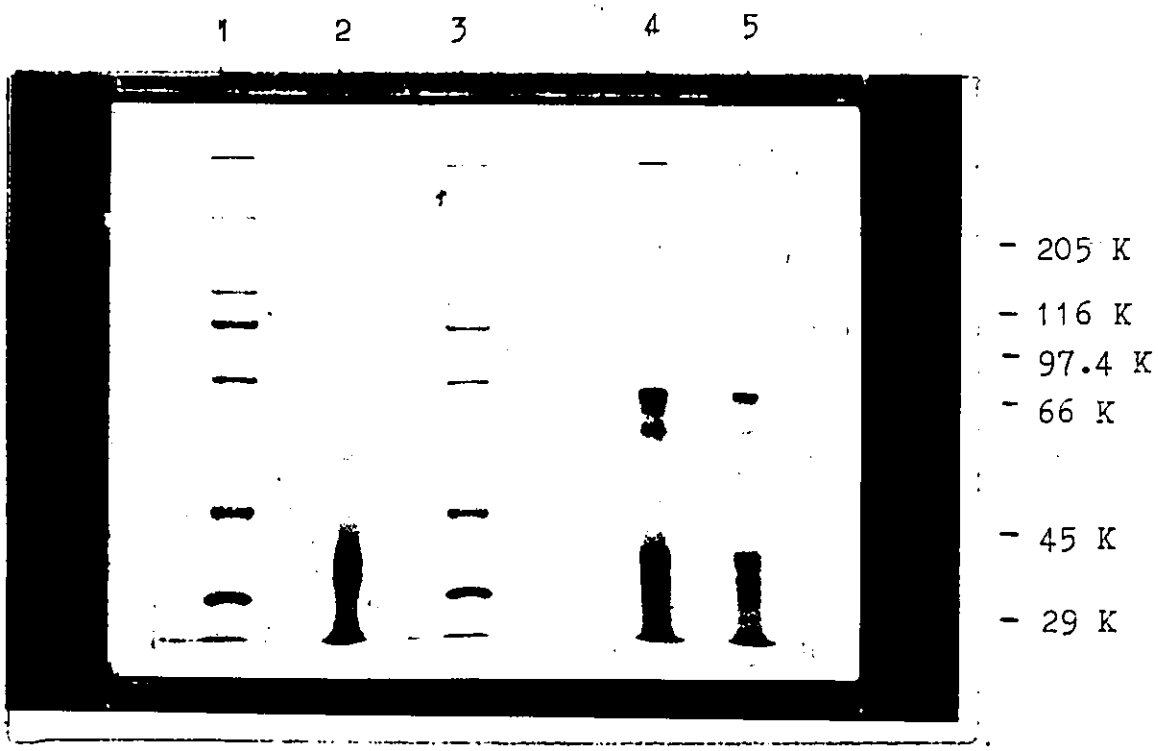


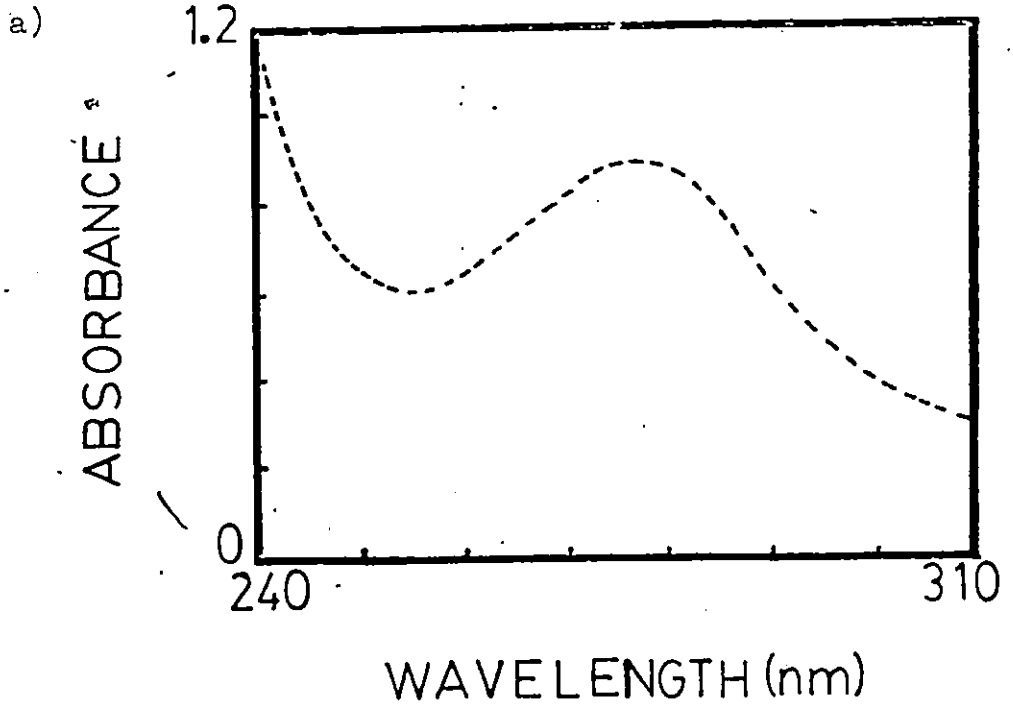
Figure 39

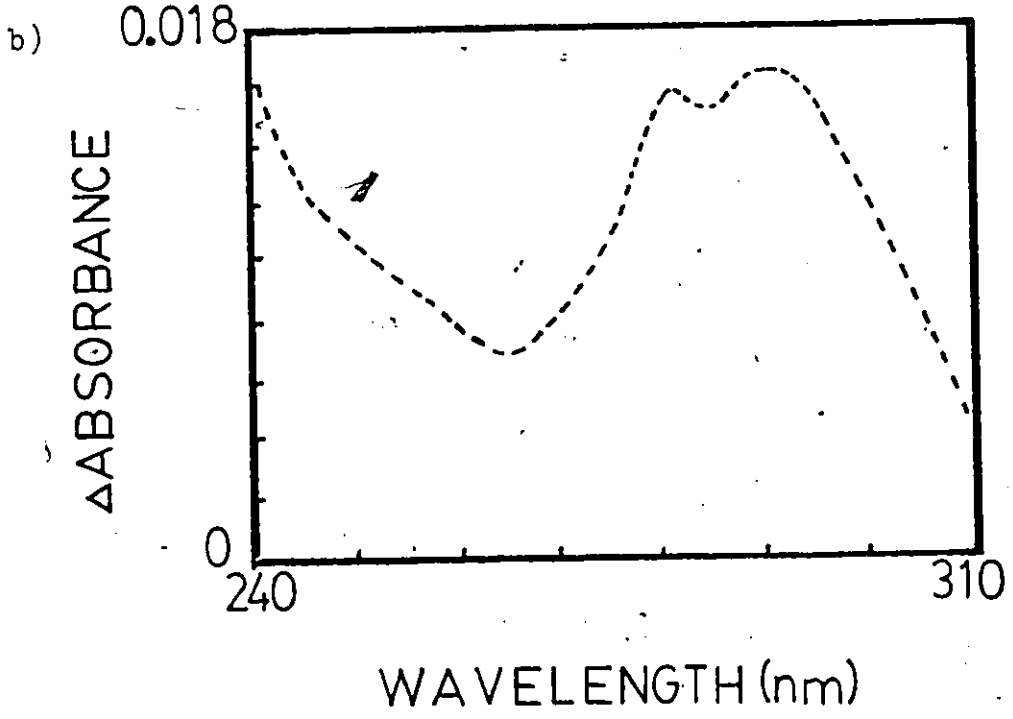
UV Difference Absorption Spectra of Ca^{2+} Interaction with Cabbage PLD.

Legend:

The reference spectrum contained 0.5 mg/mL PLD (950 µL) and 50 µL of water (top figure); the sample contained 0.5 mg/ml PLD (950 µL) and 50 µL (2 M) $CaCl_2$ and the difference spectrum obtained by automatic subtraction of reference from sample spectrum is shown in bottom figure.

Figure 39


a)
1.2
ABSORBANCE
0
240
310
WAVELENGTH (nm)


b)
0.018
ΔABSORBANCE
0
240
310
WAVELENGTH (nm)

distinct peaks appear at 280 nm and 292 nm with a minimum between 260 and 270 nm. Figure 39a shows the reference spectrum due to PLD absorption alone, showing the normal protein peak at 278 nm. Protein concentrations between 0.5-1.0 mg protein/mL which give absorbances up to 1.2 at 240 nm, produce well-defined difference spectra in the presence of 0.1 M Ca^{2+}. Quantitative measurements for the interaction of enzyme and Ca^{2+} were not made since the maximal amplitudes of the difference peaks were relatively small. Furthermore, the purity of the enzyme was not ascertained. Despite the high concentration of Ca^{2+} used to induce the difference spectra, only about 5 mM EDTA was sufficient to nullify the difference spectrum.

Binding of PLD to PC mixed micelles by UV difference spectra could not be demonstrated conveniently possibly because any spectral changes produced as a result of interaction were being masked by the strong and broad absorbances at 278 nm by both PLD and Triton.

Ca^{2+}-induced difference spectra of PLD-PC solutions were unstable to about 10 min. followed by a constant straight line indicating that the spectroscopic signal then corresponded to a stable mixture: the saturation of binding sites. Of course, the inital disproportionate change in the difference spectra might have been due to the complication introduced by the enzymic reaction. Though no quantitative information can be obtained under these circumstances the appearance of a difference signal is indicative of complex

formation with the PLD-PC mixtures.

In order to gain more insight into Ca^{2+}-micelle interaction, Ca^{2+} perturbations of Triton-SDS detergent micelles were studied. Results of this study indicate that Ca^{2+} readily binds to these detergent micelles. Figure 40 shows the Ca^{2+}-induced difference spectra of Triton-SDS micelles. A sharp peak at 288 nm was characteristic of Ca^{2+} titrations of the Triton-SDS micelles. As would be expected if the pertubations were Ca^{2+}-induced, addition of EDTA totally abolished this peak (see Figure 40). When varying amounts of Ca^{2+} were added to the detergent mixed micelles, increasing differences in the spectrum of the micelles were observed until the micelles became saturated. A plot of the change in absorbance against the negative log of the concentration of metal ion permits an estimation of the dissociation constant [129]. In Figure 41 the change in absorbance at 288 nm divided by the maximal absorbance change is plotted against the negative log of the metal chloride concentration for a typical titration as demonstrated by Suelter and Melander [130]. The experimental data are fitted with a theoretical curve of the form of equation

$$pK = pM^{2+} + \log[\alpha/(1-\alpha)]$$

where $\alpha = \Delta A/\Delta Amax$ [129] ($\Delta A/\Delta Amax$ represents the fraction of micelle-metal complex). The midpoint of the curve shown by the small vertical line gives the dissociation constant [130]. In this study 5.69 µmoles of total detergent micelles were titrated with Ca^{2+} concentrations up to a concentration of 20 mM. A mean dissociation constant for

Figure 40

UV Difference Absorption Spectra of Ca^{2+} Interaction with SDS-Triton X-100 Detergent Mixed Micelles.

Legend:

The reference medium contained 5.69 umoles of total detergent (0.09% SDS-0.2% Triton X-100) (900 µL) and 100 µL water. Sample volume contained 5.69 umoles of total detergent plus 20 mM $CaCl_2$. (1) Baseline (reference-minus reference spectrum), (2) Ca^{2+}-induced difference spectrum (sample spectrum minus reference) and (3) difference spectrum in (2) plus about 5 mM EDTA (no volume correction made, 20 µL added).

Figure 40

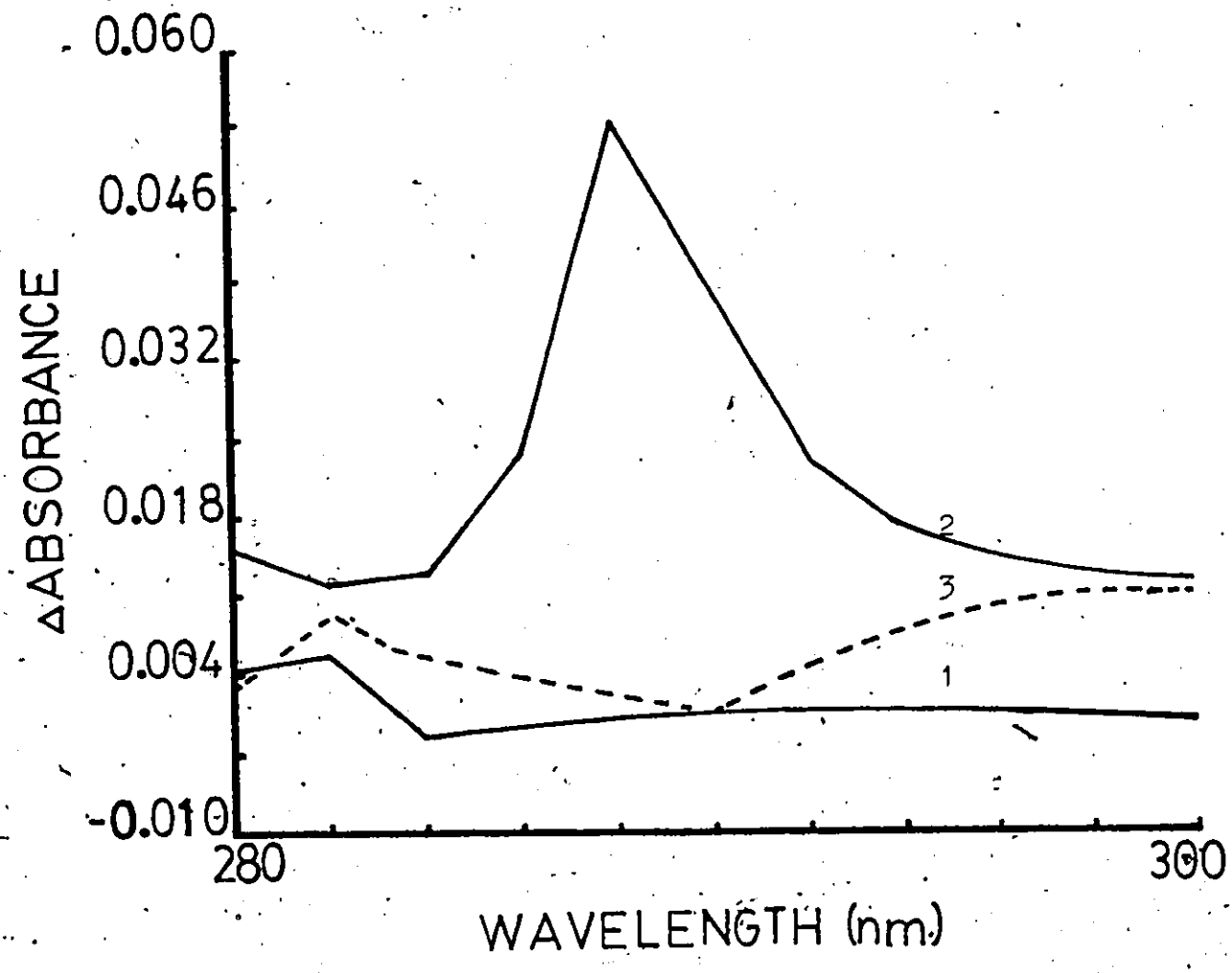

0.060
0.046
0.032
0.018
0.004
-0.010
ΔABSORBANCE
280
300
WAVELENGTH (nm)
1
2
3

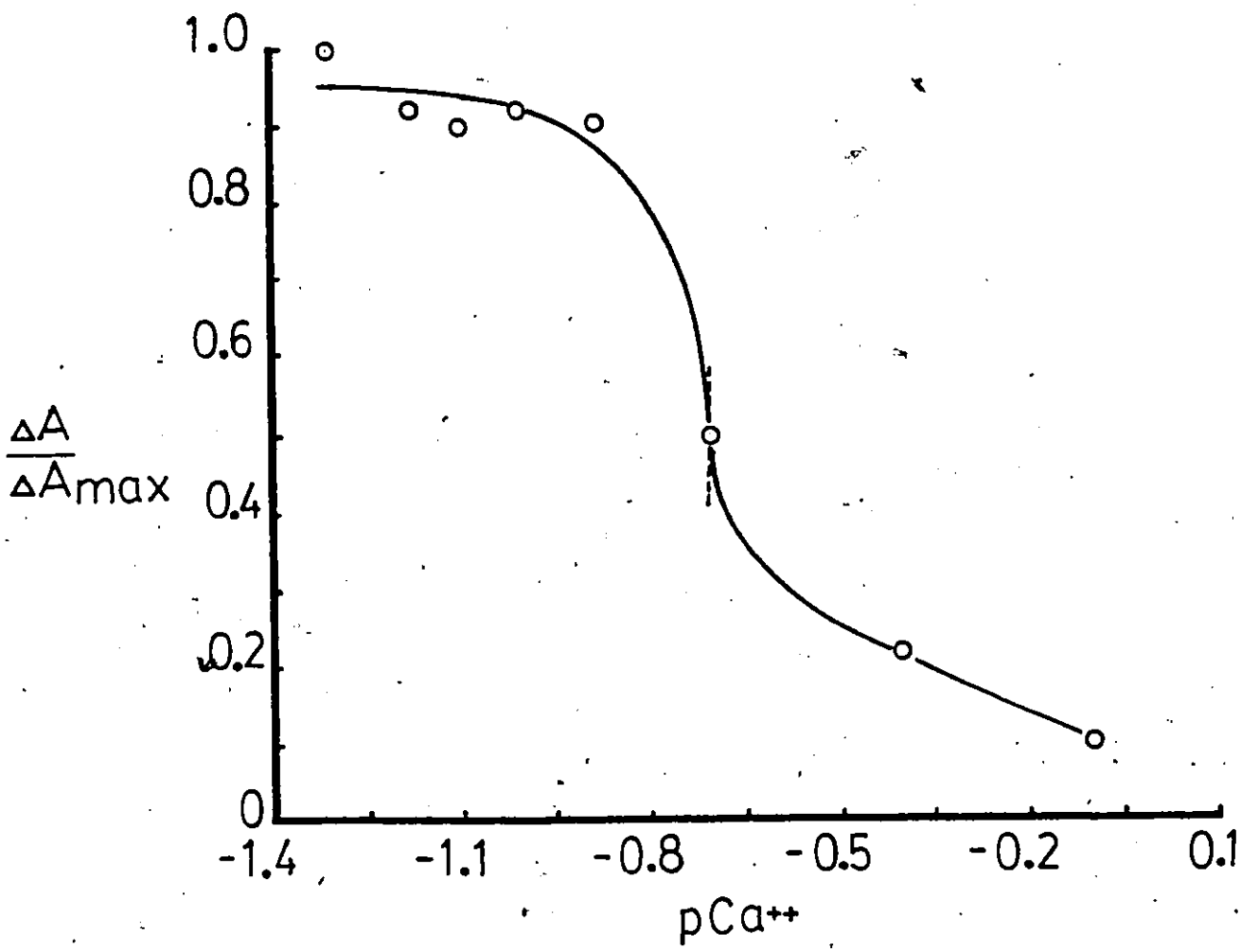
Figure 41

Determination of Ca^{2+} Binding Constant to SDS-Triton X-100 Detergent Mixed Micelles by UV Difference Spectroscopy.

Legend:

Conditions same as in Figure 40. Ca^{2+} titrations were made at constant total detergent concentration (5.69 μmoles). One determintaiton for each $CaCl_2$ concentration. The maximum change in absorbance at 288 nm was taken as the amplitude of the measured signal for each $CaCl_2$ concentration.

Figure 41



Ca^{2+}-detergent micelle complex of 4.8±0.8 mM Ca^{2+} (four determinations) were estimated. As observed earlier with the Ca^{2+}-induced difference spectra, EDTA completely abolished the difference spectra of Triton-SDS mixed micelles.

Ca^{2+}-induced PC mixed micelle difference spectra could not be demonstrated. This may be due to the low threshold of Ca required to initate precipitation of Ca^{2+}-PC aggregates. Fortunately, however, it is known both from the effect of Ca^{2+} on the electrophoretic mobility of lecithin particles [53] and surface potential of lecithin films [131] that the metal is adsorbed by counter ion attraction at the lecithin/water interface but this would only be of significance at much higher concentration of calcium [132]. Hauser and Dawson [132] observed limited adsorption of Ca^{2+} to pure lecithin, however, they noted a considerable amount bound when SDS was introduced into the substrate phase. In fact numerous studies have now shown that Ca^{2+} binds to zwitterionic phosphoplipids [133-140]. Several NMR studies also indicate Ca^{2+} binding to egg lecithin [136-139].

Structural consequences of the metal binding to lecithin indicate that DPPC bilayers immersed in a 1 mM Ca^{2+} solution would separate indefinitely and higher concentrations of $CaCl_2$ caused a progressive decrease in bilayer separation due to the ionic screening of electrostatic repulsion between bilayers charged by the adsorption of divalent cations [135,141]. Further studies by Lis et al.

[133] concluded that the association of Ca^{2+} with lecithin cannot be described either in terms of an "association constant" or a characteristic surface potential as these factors are strongly influenced by the ionic conditions. In another development, Lis et al.[134] showed the preferred order of Ca^{2+} binding to different synthetic phosphatidylcholines in the sequence DOPC < DLPC < DMPC ≈ DPPC ≈ DSPC. They also demonstrated that different density of bound charge are in proportion to the surface density of phosphatidylcholine on the bilayer surface: at room temperature they go as DOPC < DLPC < DMPC < DPPC < DSPC [134]. Therefore as should be expected in the presence of anionic amphipaths the density of bound divalent cation would be augmented correspondingly.

Summarizing the binding data, it is clear that extensive binding between Ca^{2+} and the reaction components does occur consistent with existing data. In effect the presence of Ca^{2+} ions does have a distinct influence in the enzyme-lipid interface interactions though our results do not allow a definite conclusion about the exact location of the group either on the enzyme or the interface responsible for these interactions. The most obvious conclusion therefore is that PLD specifically binds to Ca^{2+} ions and to the residual Ca^{2+}-modified lipid-water interfaces through electrostatic and hydrophobic interactions. The extensive binding prevalent in this reaction system would offer a more plausible explanantion of the nonclassical kinetics observed in this study.

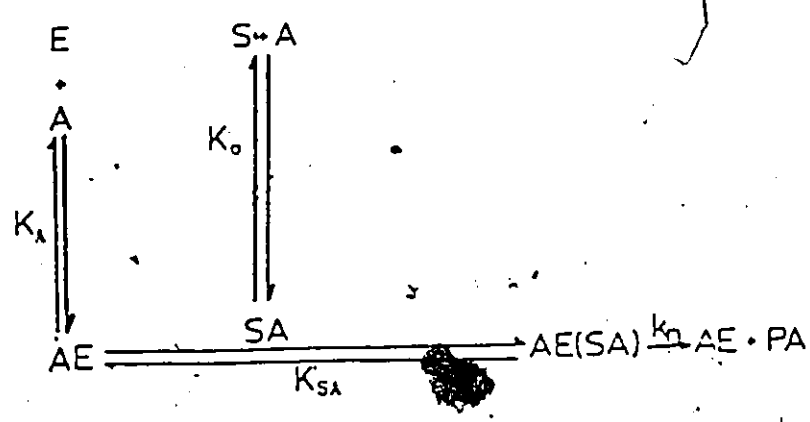
Cooperativity in Ca^{2+} Binding

Despite the linearity of our Hill plots (Figure 34, Figure 35) in the light of our experiments especially considering the multiple role of the metal modifier, it cannot be certainly concluded that the PLD from cabbage and peanut are necessarily allosteric enzymes. Allgyer and Wells [42] observed non-hyperbolic rate dependence on Ca^{2+} at pH < 7 and assumed this as due to the compound effect of Ca^{2+}. Vincent and Thellier [143], from their mathematical analysis of enzymes in structured media and Michaelis-Menten kinetics, concluded that in a structured system the apparent kinetic parameters are generally quite different from the actual molecular parameters of the catalyzing proteins, a biphasic kinetic curve may correspond to a single mechanism, and a sigmoidal kinetic curve may be obtained with a perfectly hyperbolic enzyme. Furthermore, as has been pointed out elsewhere [144] cooperativity may also depend upon the kinetic steps themselves, rather than upon alteration of cooperativity that existed in binding or upon interactions of multiple catalytic or regulatory sites. For example, a monomeric enzyme or an oligomeric enzyme with indepedent sites having a substrate and effector that follow a random, steady-state addition or which undergoes a slow transition in the presence of substrate will give rise to second-order rate equations [144,145].

Analysis of Data

Owing to the complexity and many poorly understood problems in catalysis of phospholipids by lipolytic enzymes, various experimental designs and provisions [15,51,60-62, 103-105] have been made to amend data analysis to the classical Michaelis-Menten model. Recently, the "dual phospholipid model" [1,15,104,105] was advanced for the kinetics of cobra venom phospholipase A_2 with phospholipids where it was suggested that the enzyme binds a phospholipid molecule in the first step, then the interfacial enzyme binds a second phospholipid in the interface. However in these approaches the role of the metal modifier was considered as just essential to enzyme activation.

Our experimental data is consistent with the rapid random equilibrium model [16,17,18,146] in which Ca^{2+}, PC, and PC-Ca^{2+} bind to free enzyme and also to Ca^{2+}-PLD complexes. The Ca^{2+} is obligatory for catalytic activity as well as for the formation of the PC-Ca^{2+} complex but not for ligand binding to the catalytic site. Based on the observed experimental evidence, a kinetic scheme is hereby proposed as summarized below:



Kinetic Scheme: General model describing the equilibria among the various enzyme states.

The following symbols as used stand for: E, free enzyme; S, PC-SDS Triton (micelle); A, free Ca^{2+}; P, products; and AE, AE(SA) and PA are intermediate complexes.

The reaction

$$E + A \rightleftharpoons AE$$

represents an essential activation system where A is obligatory for catalytic activity as well as for formation of SA. This means that all ligands bind in a rapid random equilibrium manner, but only the AE(SA) complex breaks down to yield products.

In general, the diagnostic features of the rapid random equilibrium model are as follows [16].

(a) The ascending portion of the v versus S_o curves are concave or sigmoidal.

(b) The v versus S_o curves have a peak and the v versus A_o curves do not; and

(c) the ascending portion of the v versus A_o curves are always sigmoidal.

According to this model the terminal portion of the v versus S_o profile reflect the unmodified enzyme, and the terminal portion of the v versus A_o curves reflect the modified enzyme [16]. Furthermore, this model does not assume an enzyme with multiple interacting subunits.

Using the equilibria in the kinetic scheme above,

the relevant rate equations can be derived which express the velocity of PC hydrolysis in terms of the ligand concentrations and equilibrium constants. Using the general equilibrium treatment, the general velocity equation is

$$v = k_p[AE(SA)] \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

where

$$E_o = [E] + [AE] + [AE(SA)]$$

$$[AE(SA)] = [AE][SA]/K_{SA}$$

$$[AE] = [A][E]/K_A \text{ and}$$

$$[SA] = [A][S]/K_o$$

Rearranging and substituting concentrations of all the species in the equilibria, the velocity equation becomes

$$v = \frac{Vmax[SA]}{K_{SA}(1 + K_A/[A]) + [SA]} \quad \ldots\ldots\ldots\ldots (2)$$

Equation (2) expresses the fact that [SA], the phospholipid-Ca^{2+} complex, is the substrate for these enzymes. This explains why double reciprocal plots of v versus S_o become

increasingly non-linear at low activator concentration at the high substrate concentration regions (Figures 23-27). As noted by Lis et al. [134] the amount of cationic material bound and eventually desorbed from zwitterion phosphatidylcholines is not small, but is the same magnitude as that which is in solution between bilayers. By analogous reasoning, cation binding behaviour could be similar in micelles of the same phospholipid. Under these circumstances if the Ca^{2+}-phospholipid interaction become comparable or greater than that of the enzyme, the majority of the Ca^{2+} will be bound by the phospholipid and not to the enzyme. Hence little hydrolysis at high substrate concentration should not be unexpected. In a similar way, activation by metal ion at constant substrate concentration is reflected in the competition term $(1 + K_A/[A])$ in equation (2). This term tends to increase the velocity proportionally with increasing A_o (Table VIII). Obviously the term $K_A/[A]$ implies that the rate is zero in the absence of activator [126].

The activating effect is best appreciated when the rate equation is expressed in terms of free S and free A.

$$\frac{v}{Vmax} = \frac{[A]^2[S]}{K_o K_{SA}(K_A + [A]) + [A]^2[S]} \qquad \ldots (3)$$

Obviously the velocity depends on the $[A]^2$. This dependence has been shown to be true when the reciprocal of

Figure 42

Plots of Reciprocal Initial Rates Against Reciprocal of the Square of Ca^{2+} Concentration at Various Intermediate Concentrations of DPPC.

Legend;

Reaction conditions same as for Figure 18. Symbols stand for 0.083 mM (□), 0.100 mM (●), 0.125 mM (△) and 0.167 mM (○) DPPC.

Figure 42

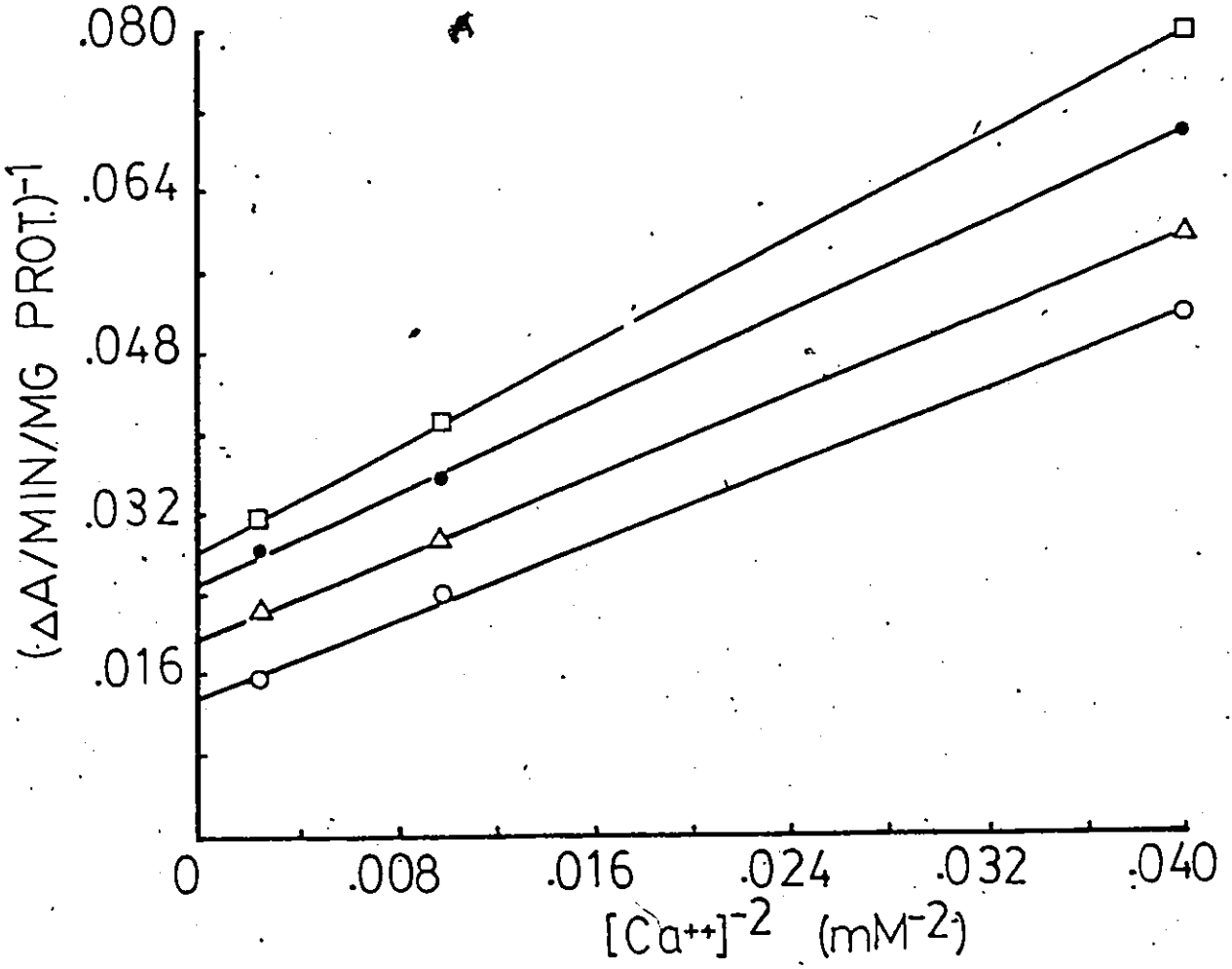


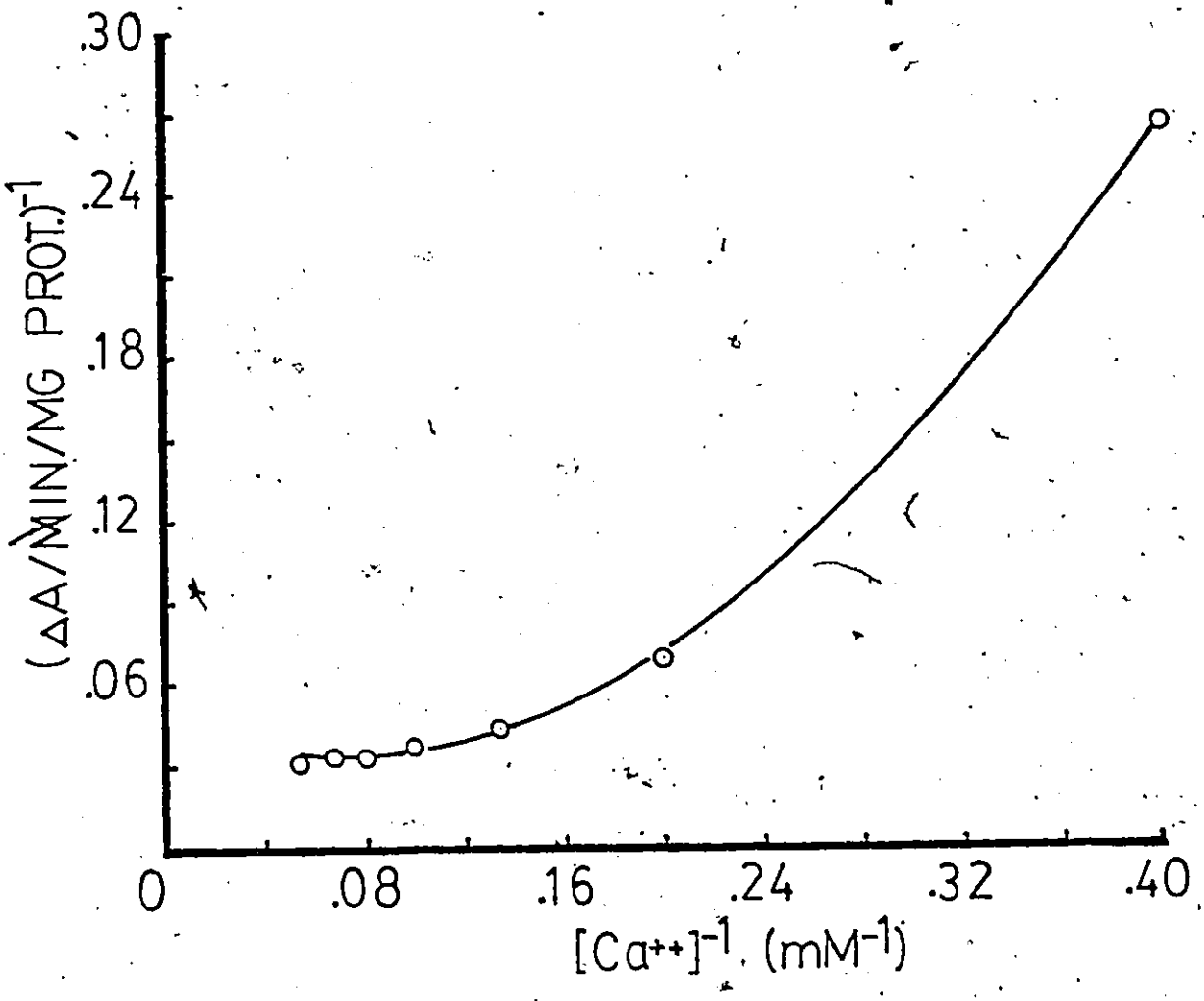
Figure 43

Double Reciprocal Plot of Initial Rates Against Ca^{2+} Concentration at a Given Concentration of Intermediate DPPC.

Legend:

Reaction conditions were as in Figure 30 (cabbage enzyme).

Figure 43



velocity and the reciprocal of the square of metal ion were plotted and found to be linear (Figure 42). On the other hand, Figure 43 shows the double reciprocal plot for velocity versus calcium concentration which is clearly non-linear while the same data plotted as a function of the reciprocal of the square of Ca^{2+} was linear (Figure 44). It must however, be noted that total metal ion concentration, A_o, was plotted instead of free [A] predicted from the equation.

At high concentrations of metal ions, that is when $A_o >> S_o$, the following approximations made by London and Steck [16] apply:

$$[SA] = S_o, \quad [A] = A_o - S_o \quad \text{and} \quad [S] = 0$$

Equation (2) then becomes

$$v = \frac{VmaxS_o}{K_{SA}(1 + K_A/(A_o - S_o) + S_o} \quad \ldots\ldots\ldots (4)$$

where

$$Km^{app} = K_{SA}(1 + K_A/(A_o - S_o))$$

The Km^{app} term reflects the dependence of this constant on Ca^{2+} concentration as shown in Table VIII. With DDPC and DMPC, Km^{app} is inversely proportional to Ca^{2+} concentration and directly proportional for DSPC but this variation is not cut for DPPC.

In conclusion, it is clear from our studies that the

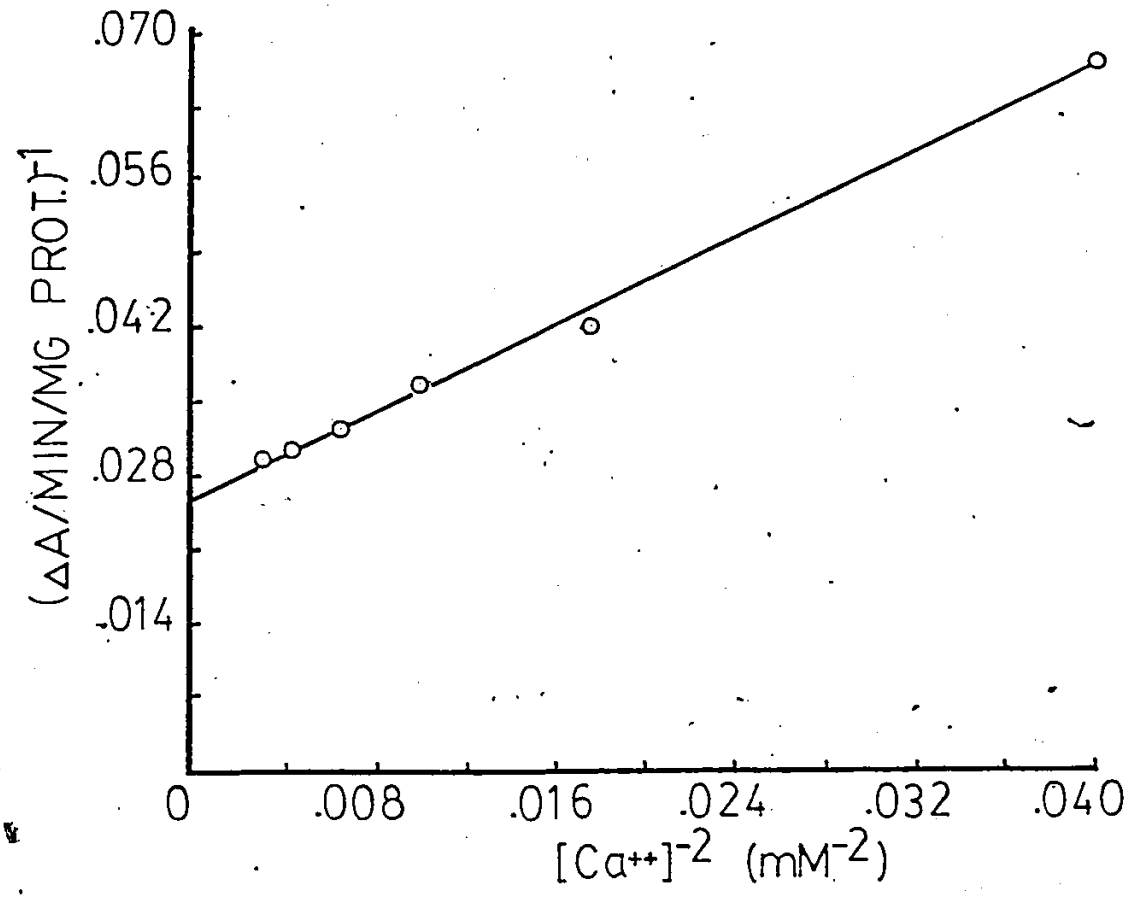
Figure 44

Plot of Reciprocal Initial Rates Against Reciprocal of the Square of Ca^{2+} Concentration at a Fixed Concentration of Intermediate Concentration of DPPC.

Legend:

Reaction conditions were as in Figure 30 (cabbage enzyme)

Figure 44



nonclassical kinetics observed may be due to kinetic cooperativity [144], a phenomenon due to the alteration of catalytic constants as substrate and effector bind with the maintenance of rapid equilibrium binding.

At any rate no meaningful information regarding the complete description of the mechanism of action of an enzyme can be obtained until the following factors are known [146]: (a) the geometric and electronic structure at the active site of the enzyme and of its complexes with substrates and products; (b) the affinity of substrates and specificity of substrates; (c) the kinetic scheme of the reaction; (d) the chemical mechanism of each step; (e) the rate constants of the individual steps; and (f) a rationale for the magnitude of the rate constants in terms of structure.

Despite the lack of any extensive characterization of the phospholipases D, the identification of a general rapid random equilibrium model that fit PC hydrolysis data is one step in the complete elucidation of the mode of action of these lipolytic enzymes. This model has not been previously considered for micellar substrates and lipolytic enzymes but its consistency with data in the foregoing work suggests that it merits consideration.

The complex kinetics of phospholipases D observed *in vitro* might be physiologically important, in that the PLD could allow a more sensitive regulation of phosphatidylcholine content in biological membranes in response to fluctuations in calcium concentration. In fact, there is

sufficient evidence to show that changes in intracellular calcium levels may be associated with the regulation of numerous cellular functions including alterations of membrane permeability and flexibility [147, 148] and that these responses may be mediated by alterations in the state of calcium-responsive membrane proteins. Our observations that enhanced enzyme activity was associated with increases in calcium concentration and alteration in Ca^{2+} associated with the phospholipids and with the enzyme may be indicative of the fact that such interfacial fluctuations could substantially regulate the activity of this enzyme *in vivo*.

Naturally occurring membranes are considered as mixed micellar aggregates [59]; thus our results which indicated that the ratio of the detergents to phosphatidylcholine determined the reactivity of these enzymes. In addition, the equally high efficiency of catalysis of human erythrocyte ghost phosphatidylcholines hydrolysis under our assay conditions is indicative of a good emulation of some of the properties of the natural system. Hence the observed kinetics nontheless gives some insight into the regulating role of Ca^{2+} in the physiological state for these enzymes.

Generally speaking, we can also speculate that PLD together with the base exchange enzymes and phospholipid exchange proteins may be important in intracellular metabolism of phospholipids, regulation of membrane composition and maintenance of membrane asymmetry.

Conclusions

1. A simple, sensitive and rapid spectrophotometric assay for determination of PC has been developed in a continuous end-point and rate-modes, and the latter found suitable for kinetic analysis of phospholipases D.

2. Kinetic curves of initial rates versus substrate concentration were biphasic in the substrate concentration range of 0 to 2 mM, but hyperbolic at concentrations below 0.25 mM.

3. The reactivity of both the cabbage PLD and peanut PLD towards the phosphatidylcholines studied was similar though the cabbage enzyme was several-fold more active.

4. Direct calcium binding to cabbage PLD has been demonstrated by UV difference spectroscopy.

5. Calcium-induced enhancement of PLD activity was correlated with the increase in the concentration of calcium associated with the phospholipids.

6. Initial velocity and binding data fit a rapid random equilibrium mechanism for PLD.

7. The responsiveness of PLD to calcium levels <u>in vitro</u> may be physiologically important as one means for regulation of PC content in membranes.

APPENDIX

The following experiments were conducted on the recommendation of the External Examiner, Dr. J. N. Kanfer.

Kinetics of the PLD Catalyzed Hydrolysis of the Phosphatidylcholines at Constant Total Detergents to PC Ratio.

The hydrolysis reactions were carried out at a constant ratio of D_o:PC = 1:1, using the standard rate assay. Table A shows the data for both DPPC and DDPC at 2.5 mM Ca^{2+} concentration. Comparable data obtained previously at constant total detergents concentration but at varying D_o:PC ratio (D_o:PC $\geqslant$ 1:1) are shown in Tables VI(a) and VI(b) ([Ca^{2+}] = 2.5 mM) (Text) for DPPC and DDPC, respectively.

Figure A.1 shows a plot of the hydrolysis rate versus substrate concentration for both DPPC and DDPC. These curves are biphasic, with one phase distinguishable below 0.4 mM, and the second phase above 0.4 mM PC. This biphasic characteristic may be due to the changing total detergents concentrations when the D_o:PC ratio is held constant. Experiments conducted with 0.8 mM DPPC at a constant D_o:PC = 4:1 also exhibit similar trends but with no measurable rates below 0.2 mM PC. Similarly, experiments

TABLE A

PLD_c Catalyzed Hydrolysis of DPPC and DDPC at Constant Total Detergents to PC Ratio[a]

[Substrate] (mM)	ΔA/min/mg protein, for DPPC	DDPC
0.05	0.000	1.405±0.045
0.10	0.000	1.380±0.035
0.20	0.000	1.354±0.022
0.40	0.115±0.030	1.380±0.025
0.60	0.541±0.050	1.949±0.062
0.80	1.016±0.136	2.924±0.102
1.00	1.131±0.218	3.658±0.165
1.25	1.344±0.278	4.506±0.311
1.50	1.639±0.279	5.646±0.423
1.75	2.098±0.327	6.342±0.374
2.00	2.148±0.389	6.759±0.114

[a]All entries are triplicate runs using the standard rate assay.

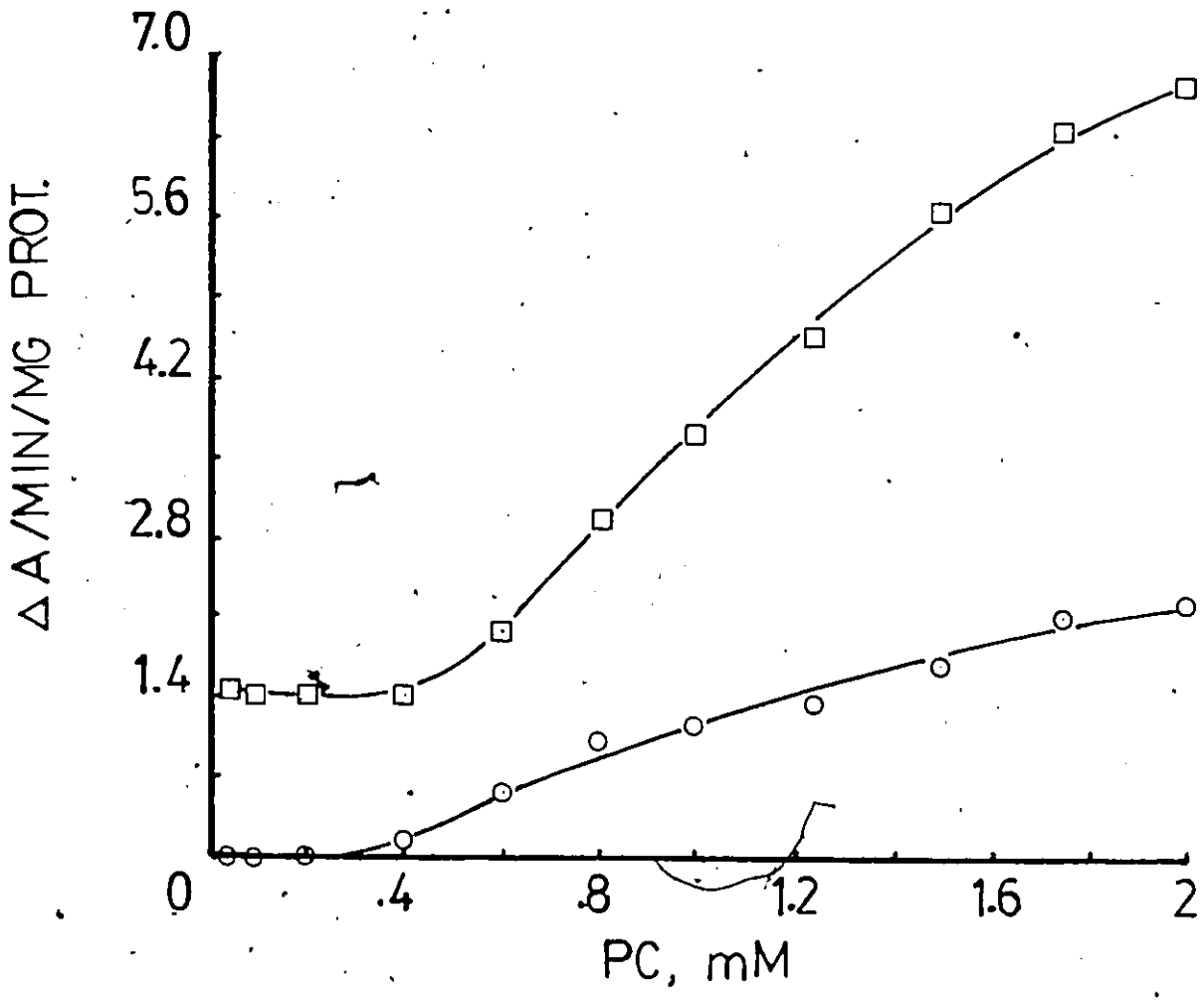
Figure A.1

PLD_c Catalyzed Hydrolysis of DPPC and DDPC at Constant Total Detergents to PC Ratio.

Legend:

Initial rates were measured using the standard rate assay but keeping D_o:PC = 1:1 in the substrate range between 0.05 - 2.0 mM at 2.5 mM Ca^{2+} concentration. Symbols represent (○) DPPC and (□) DDPC.

Figure A.1



with DMPC at constant D_o:PC = 1:1 showed no measurable rates at 2.5 mM Ca^{2+} throughout the entire substrate range (i.e., 0.05 - 2.0 mM). Attempts to use higher Ca^{2+} concentrations resulted in turbidities in the reaction mixtures.

Conclusions from these experiments enlighten us on the kinetic trends observed in Figures 8-11 (Text). Consequently, the drastic fall in enzyme activity observed beyond the peak rates (Figures 8-11) may be indicative of the effect of decreasing D_o:PC ratio along the substrate range studied, so that at a minimum ratio of 1:1, the substrates became least susceptible to PLD attack.

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1981-1984	Teaching Assistant, Department of Chemistry, University of Windsor, Windsor, Ontario.

AWARDS :

1969-1974	Ghana Cocoa Marketing Board Scholarship
1974-1976	Government of Ghana Scholarship
1976-1979	Government of Ghana Scholarship

1984 University of Windsor Postgraduate Research Scholarship (Summer Award).

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