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PURIFICATION AND ENZYMATIC PROPERTIES OF HEAT STABLE PHOSPHOLIPASE A

by

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ABBREVIATIONS

APS - aqueous phase solvents.

OPS - organic phase solvents

DEAE - diethylaminoethyl

DOC - deoxycholate

EDTA - ethylenediaminetetraacetate

mμ - equiv. -millimicroequivalent

poly-Lys - poly-lysine

poly-Lys-Leu - lysine-leucine copolymer

poly-Orn-Leu - ornithine-leucine copolymer

poly-Orn-Leu-Ala - ornithine-leucine-alanine copolymer

SE - sulphoxyethyl

Tris - tris (hydroxymethyl) aminomethane

ABSTRACT

Phospholipase A was prepared from three different sources; bovine pancreas, human pancreas and Crotalus adamanteus venom. The enzymes were prepared by heat-treatment and chromatography in the case of the bovine and snake preparations and by heat-treatment and precipitation in the case of the human enzyme. Electrophoretic analysis indicated that the snake and the bovine preparations were relatively homogenous with regard to their protein content. The human enzyme was still quite impure. Further attempts to purify the human enzyme were unsuccessful.

The ability of each of these preparations to hydrolyze purified phospholipids was determined for phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol both in the presence and the absence of sodium deoxycholate. The effect of various metal ions on the hydrolysis also was determined. The rate of hydrolysis of lecithin in the presence of sodium deoxycholate was shown to be inhibited by sodium and potassium ions in the case of the mammalian enzymes but not in the case of the reptilian one.

Each enzyme was shown to attack certain lipoprotein preparations. The beef and the snake enzyme hydrolyzed

phosphatidylcholine and phosphatidylethanolamine in egg yolk suspensions. The addition of sodium chloride (0. 154 M) to the suspensions resulted in a partial inhibition of the two enzymes. The addition of sucrose (0. 25 M) did not affect the reaction. The human enzyme did not attack egg yolk lipoproteins.

All three enzymes caused the release of free fatty acids from water or isotonic sucrose homogenates of rat brain. In contrast, homogenates of rat brain prepared in isotonic saline were not hydrolyzed by any of the phospholipase A preparations.

The snake enzyme readily attacked beef serum lipoproteins both in the presence and the absence of sodium deoxycholate. The beef enzyme acted on this preparation only in the presence of sodium deoxycholate, while the human enzyme was without effect, even when deoxycholate had been added. Intact bovine erythrocytes were completely unaffected by these phospholipase A preparations.

The formation of lysophosphatidylcholine and lysophosphatidylethanolamine was measured at different time periods during the hydrolysis of egg yolk lipoprotein. The beef enzyme preferentially hydrolyzed phosphatidylethanolamine while the snake enzyme demonstrated no substrate preference.

The formation of lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine and the lyso-derivative

of the ethanolamine plasmalogen were determined at two different times during the hydrolysis of rat brain homogenates. All four phosphatides were attacked by the three enzyme preparations. The beef enzyme appeared to attack phosphatidylethanolamine and phosphatidylserine preferentially. The snake preparation, although hydrolyzing all four components, ceased action after only a small amount of the total hydrolyzable phospholipid had been broken down. The human enzyme, on the other hand, showed a marked preference for phosphatidylethanolamine as a substrate.

I INTRODUCTION

Phospholipase A (lecithinase, phosphatidyl acyl-hydrolase, EC 3. 1. 1. 4) is an enzyme which catalyzes the removal of a single ester-linked fatty acid from most glycerophosphatides. The enzyme acts specifically on the acyl ester linkage at the 2-position of a substrate molecule. It is an enzyme of wide distribution (Kates, 1960; Slotta, 1960; Zeller, 1951), being particularly abundant in many snake venoms. The richest known mammalian source of phospholipase A is the pancreas. The enzyme has a number of unusual characteristics. For example, its heat stability, particularly in acidic media, is noteworthy. It demonstrates resistance to a large variety of organic solvents. The enzyme is very active against substrates such as lecithin suspended in moist diethyl ether.

When phospholipase A attacks a molecule such as lecithin, lysolecithin is produced. Lysolecithin is an extremely surface-active, cytolytic substance. Its production, for example, has been shown by many investigators to be responsible for the hemolysis produced by snake venoms. The addition of lysolecithin to brain homogenates or egg yolk suspensions results in a rapid clearing of these preparations, presumably by dissociating the

large molecular aggregates they contain into particles too small to demonstrate a Tyndall effect. Also, lysolecithin and other lysophosphatides can cause a rapid destruction of cell membranes, producing as a secondary consequence the release from many tissues (e. g. brain) of other enzymes capable of causing additional cellular damage.

Phospholipase A has been shown to be present in brain tissue (Gallai-Hatchard, Magee, Thompson and Webster, 1962) although at a very low level. Therefore, it is possible that lysolecithin can be produced in brain. However, two pathways have been demonstrated which could dispose of this potentially harmful agent under normal circumstances. Marples and Thompson (1960) reported an appreciable level of lysolecithinase in nervous tissue. This enzyme removes the remaining fatty acid from lysolecithin, converting it to glycerylphosphorylcholine, a harmless, non-cytolytic compound. As well, lysolecithin can be reacylated in nervous tissue to form lecithin (Webster, 1962). Thus the accumulation of lysolecithin in brain could result from either an excessive rate of production or a deficient rate of removal. Alternatively, an increase in exogenous lysolecithin, perhaps in the blood, could affect nervous tissue. As yet, elevated lysolecithin levels have not been demonstrable in brains from individuals suffering

from multiple sclerosis. A level sufficient to cause local damage might be difficult to detect, however. Also, all of the phospholipase A preparations studied thus far hydrolyze other phospholipids present in nervous tissues, including particularly phosphatidylethanolamine and phosphatidylserine. The concentrations of these other lysophosphatides in tissues from multiple sclerotic patients have not been determined. In this regard, McClure (1966) found that during Wallerian degeneration of cat sciatic nerve, lysophosphatidylethanolamine was produced, a substance which is approximately twice as cytolytic as lysolecithin (Matsumoto, 1961).

The above observations suggest that phospholipase A, either directly or indirectly, could be involved in producing cell destruction or demyelination in the nervous system. More than sixty years ago, Marburg (1906) postulated that the demyelination which occurs in multiple sclerosis was due to the action of a lipolytic enzyme on myelin. Similar suggestions have been made by other workers. For example, Morrison and Zamecnik (1950) found that treatment of excised portions of spinal cord with either phospholipase A or lysolecithin produced areas of demyelination resembling the plaques seen in tissue samples taken from the central nervous system of patients suffering from multiple sclerosis.

In general, phospholipase A preparations from both reptilian and mammalian sources attack the phospholipids in tissues more readily than they attack purified phospholipids. This is particularly true of lecithin. Magee, Gallai-Hatchard, Sanders and Thompson (1962) found that a phospholipase A preparation isolated from human pancreas required the addition of sodium deoxycholate to the reaction system before lecithin hydrolysis would occur. The enhancement of the rate of hydrolysis by the addition of various activators to the reaction system has led to the development of many unusual reaction systems, ranging from that of Hanahan, Rodbell and Turner (1954) in which the reaction was carried out in moist diethyl ether to the system used by Doizaki and Zieve (1961) where a product of the reaction (lysolecithin) was added to the assay system. In general the problem appears to be one of solubilizing the substrate in such a way as to render it susceptible to the action of the enzyme. The problem of the susceptibility of the phospholipid to attack by phospholipase A is also apparent when lipoprotein substrates are used. For example, the phospholipase A present in Vipera palestinae readily attacks the lecithin present in egg yolk lipoproteins but is unable to attack the lecithin present in red blood cells. Klibansky, Shiloah and Vries (1964) showed that cobra venom phospholipase A could

attack the phospholipids present in brain homogenates, but not in brain slices.

Most of the studies on phospholipase A have been carried out using the enzymes present in snake venoms. Only a few investigations have been performed using mammalian enzymes, generally those present in pancreas. Unfortunately, it is very difficult to compare the enzymes from various sources since widely different substrate preparations and reaction systems have been used.

In this investigation phospholipase A has been isolated and purified from three different sources; two mammalian pancreatic phospholipase A preparations were obtained from bovine and human pancreas, as well as one from the venom of eastern diamondback rattlesnake (Crotalus adamanteus). These three enzymes have been compared with regard to their action on purified phospholipids and intact lipoprotein preparations under carefully controlled and consistent experimental conditions.

II REVIEW OF THE LITERATURE

This review covers the literature available up to the completion of this work (March, 1968). The review will be divided into two parts: the first part will deal with research carried out on the purification of phospholipase A from various sources and the second part will deal with research that has been carried out on the properties of the enzyme.

A. Preparation and Purification of Phospholipase A

The actions of venoms of poisonous snakes has been chronicled for many years. The gross effects of venom on blood and the nervous system were reported as far back as the American Civil War by Mitchell (1860 and 1886). Since then, many studies have been carried out on the specific actions of venom phospholipase A, but most of these were secondary to studies on the toxicity of the venom. It was many years before the purification of phospholipase A from any source was attempted with reported success. The first partially purified preparation was obtained by Gronchi (1936a) using pancreatin as the enzyme source. He showed that pancreatin phospholipase A was remarkably heat stable, as was the enzyme present in bee and snake venoms. The enzyme from pancreatin

precipitate was extracted with glycine-NaOH buffer. Increasing the pH of the extraction medium increased the amount of the enzyme extracted into the buffer. The precipitate from the heat treatment step was extracted at pH 9. 8 with buffer containing 32 per cent ethanol (at this basic pH the enzyme was denatured by higher concentrations of ethanol). The supernatant from this extraction was adjusted to pH 6. 5-7. 0 and the precipitate which formed was collected. This precipitate was the most active phospholipase A fraction isolated. This fraction was about forty times as active as the starting material and hydrolyzed phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine. No activity towards either lysolecithin or triglyceride was observed.

Phospholipase A has been partially purified from human pancreas by Magee, Gallai-Hatchard, Sanders and Thompson (1962). Acetone powders of pancreas were prepared by sequential extraction of the pancreas with ice cold acetone, n-butanol and acetone. The dried powder was extracted with 25 per cent glycerol in 1 per cent sodium bicarbonate. The enzyme was precipitated by the addition of ice-cold acetone. Following precipitation this fraction was redissolved and the pH of the solution was adjusted to 4. 5 and the solution heat-treated at 75° C for five minutes. The active enzyme was again precipitated with acetone. A more active

was soluble in 50 per cent ethanol. Use was made of the above-mentioned properties to prepare extracts which were relatively free from other enzymes such as proteases, amylases and lipases (Gronchi, 1936b). Unfortunately, this work was published in Italian and appears to have been unavailable to some of the later workers in the field. There have been only a few papers published on the purification of the enzyme from mammalian pancreas. Hanahan (1952) showed that basic glycine buffer extracts of pancreatin could be heated at pH 4 so that 90 per cent of the protein was precipitated but less than 10 per cent of the phospholipase A activity was lost from the supernatant. Rimon and Shapiro (1959) achieved a partial purification of the enzyme from aged beef pancreas by selective extraction, precipitation and heat treatment of the material. The soluble proteins were extracted with water from a pancreas that had been stored in a frozen condition for at least two weeks. The extract was centrifuged for one hour at 20,000 x g. The precipitate was washed with 0.05 M sodium ethylenediaminetetraacetate at pH 6, then with water. The precipitate was then extracted by homogenization with 0.067 M Sørensen phosphate buffer pH 6.5. The total homogenate from this homogenization was treated at 70-75^{o} for ten minutes and cooled. This treatment was reported to eliminate phospholipase B (lysolecithinase) activity. The

preparation could be isolated from this fraction, but the results were variable and the enzyme yields were generally poor (W. L. Magee, personal communication). Most authors that have studied this enzyme have used the precipitated heat-treated fraction (Ibrahim, Sanders and Thompson, 1964; Ibrahim and Thompson, 1965; Haas, Heemskerk, Deenen, Baker, Gallai-Hatchard, Magee and Thompson, 1963). This preparation did not hydrolyze lysolecithin or tributyrin.

Much more work has been done on the purification of the enzymes present in snake venoms. With the exception of the purification of the phospholipase A present in C. adamanteus venom, only the briefest outlines of the purification methods used prior to this work will be given. Slotta and Fraenkel-Conrat in 1938 isolated a crystalline protein from the venom of Crotalus terrificus which was claimed to be electrophoretically pure and had both neurotoxic and hemolytic activity, but which has been shown in recent years to be impure (Slotta, 1960). De (1944) isolated from hooded cobra (Naja naja) venom by precipitation and absorption a crystalline substance which contained only three per cent of the protein but fifty per cent of the phospholipase A activity. The enzyme from bee venom has been purified by chromatography on cellulose and IRC- 50 columns (Slotta, 1960). The venom from

spitting cobra (Naja nigricolis) has been fractionated by electrophoresis (Habermann, 1954). Bowan and Kaletta (1957) separated the venom of C. adamanteus on a DEAE-cellulose column. The venom was applied to the column in 0. 02 M THAM (tris (hydroxymethyl) aminomethane) buffer at pH 8. 9. Elution was carried out with this buffer initially, followed by 0. 33 M THAM buffer in a stepwise gradient. Two poorly separated phospholipase A peaks were found. Bjork (1959) separated two active phospholipase A preparations from the venom of Ringhals cobra (Hemaculatus hemaculatus) by electrophoresis of the phospholipase A peak obtained by chromatography of the venom on DEAE-cellulose at pH 8. 6. Further work (Bjork, 1959a) showed that the phospholipase A could readily pass through the walls of dialysis tubing. Gradient elution with phosphate buffer on DEAE-cellulose has been used to give a phospholipase A fraction from Vipera palestinae that was contaminated with a protease which was readily removed by boiling the solution (Klibansky, Shiloah and Vries, 1964). Sasaki (1960) separated the phospholipase A from the neurotoxin in Formosan cobra (Naja naja atra) venom by zone electrophoresis. Doery and Pearson (1961) isolated two phospholipase A peaks from the venom of the common black snake (Pseudechis porphyriacus) by chromatography on IRC-50 columns using an ammonium acetate buffer for elution. A peak which contained

phospholipase A activity was obtained when western diamondback rattlesnake (Crotalus atrox) venom was chromatographed on DEAE-cellulose (Fleiderer and Sumyk, 1961). CM-cellulose has been employed in the separation of Habu venom (Maeno, Mitsuhashi, Okonogi, Hoshi and Homma, 1962); in this study three overlapping phospholipase A peaks were found. Condrea, Vries and Mager (1964) used paper electrophoresis to isolate phospholipase A fractions from the venoms of V. palestinae, H. hemaculatus and V. russelli. Bicher, Klibansky, Shiloah, Gitte and Vries (1965) found the phospholipase A of N. naja venom was obtained in a single band on paper electrophoresis. Ibrahim, Sanders and Thompson (1964) routinely obtained a purified phospholipase A preparation from N. naja venom by heat treatment and acetone precipitation.

Saito and Hanahan (1962) carried out an extensive study on the purification of phospholipase A from C. adamanteus venom. The venom was dissolved in water and the pH adjusted to 9. 0. The precipitate was removed and the pH of the supernatant was adjusted to 7. 0. This was dialyzed against 0. 1 mM ethylenediaminetetra-acetate for forty hours. The dialyzed solution was adjusted to pH 3. 0 and heated at 90° C. for five minutes. After readjusting to pH 7 the precipitate was removed and the solvent replaced with 0. 005M phosphate buffer pH 7. 0. This was applied to a DEAE-cellulose

column and a linear gradient up to 0. 1 M phosphate buffer was started once the breakthrough point had been reached. All of the column buffers contained 1 mM ethylenediaminetetraacetate. Two distinct phospholipase A peaks were obtained. Each of these peaks was present in the starting material and each peak could not be converted into the other by any of the treatments used in the purification procedure.

In the last few years molecular sieving has been used in separating venom components. Haberman and Reiz (1965) used Sephadex G-50 to purify the phospholipase A from bee venom by a factor of nine. Marinetti (1965) fractionated nine different venoms on Sephadex G-75. All of the venoms gave one phospholipase A peak with the exception of V. russelli, which gave two active peaks.

B. Properties of Phospholipase A

Although much research has been carried out on the enzymatic actions of phospholipase A, both *in vivo* and *in vitro*, the majority of these studies have been concerned primarily with an examination of the toxic principles present in various snake venoms. In many of these, specific phospholipase A effects are difficult to interpret, since crude venoms contain many other enzymes and enzyme factors. Representative reviews on the subject include those of Zeller (1948), Hanahan (1957), Condrea and Vries (1965), Meldrum (1965) and Deenen (1966). In this section only papers relevant to the enzymatic properties of the enzymes found in *C. adamanteus* venom and in beef and human pancreas will be discussed in depth.

Both reptilian and heat-stable phospholipase A preparations act specifically on acyl ester linkages present in the 2-position of appropriate glycerol-containing substrates. Deenen and Haas (1963) showed that the enzyme from *C. adamanteus* venom hydrolyzed the 2-acyl ester linkage of L-α-glycerophosphatides, whereas D-α-glycerophosphatides were not attacked. They further showed that ester-linked choline, ethanolamine or serine did not have to be present in phospholipase A substrates since phosphatidic acid was readily attacked. Ethylene glycol analogues with the same

stereochemical configuration as the L-α-glycerophosphatides were also attacked, although at a much slower rate than the corresponding glycerol derivatives. Short chain fatty acid derivatives were shown to react at a much slower rate than derivatives containing fatty acids normally found in natural lecithins. The specificity of phospholipase A for the 2-position of the phosphatides has been extended by Heemskerk, Deenen, Baker, Gallai-Hatchard, Magee and Thompson (1963) to phospholipase A from human pancreas. This enzyme was shown to remove the fatty acid from the 2-position of phosphatidylcholine and phosphatidylethanolamine. Studies by Rimon and Shapiro (1959) indicated that the phospholipase A present in beef pancreas also attacked the 2-position of glycerophosphatides.

Prior to the late 1950's phospholipase A was thought to remove the fatty acid from the 1-position of phospholipids (Hanahan, 1957). This error arose as a result of chemical degradation of the lysolecithin product. Marinetti, Erbland and Stotz (1959) obtained evidence that was interpreted to mean that the enzyme hydrolyzed the ester bond at either position. Plasmalogens also were found to be susceptible to deacylation by phospholipase A by these authors. This was regarded as further proof for the lack of specificity of the enzyme. Tattrie (1959) showed that the unsaturated fatty acids of egg lecithin occupied mainly the 2-position of the lecithin. He

further showed that C. adamanteus venom removed mainly unsaturated fatty acids from lecithin. As stated earlier the positional specificity of phospholipase A is now well documented.

More recent work has indicated that mammalian tissues also contain a phospholipase A which catalyzes the removal of the fatty acid from the 1-position of lecithin (Lloveras, Douste-Blazy and Valiquie, 1963). Bosch and Deenen (1964) have shown that various tissues contain phospholipases which yield 1-acyl- and 2-acyl-glycerol-3-phosphorylcholine. Bosch, Postema, Haas and Deenen (1965) have shown that the phospholipase A removing the fatty acid from the 1-position is unstable to heat. The phospholipase A which removes the fatty acid from the 2-position of lecithin, on the other hand, has been shown to be very heat-stable. Saito and Hanahan (1962) showed that a phospholipase A preparation isolated from C. adamanteus venom could be heated at pH 3.0 for five minutes at 90° C without appreciable loss of activity. Magee et al. (1962) showed that the phospholipase A present in human pancreas was quite stable to heating at 75° C for five minutes at pH 4.5. Bovine pancreatic phospholipase A was shown to be heat resistant by Rimon and Shapiro (1959). They reported that heating the enzyme preparation at 70-75° C did not affect its activity.

The interpretation of the action of phospholipase A on purified

phospholipids is complicated by the difficulty in preparing these substances in a state suitable for enzymatic action. Generally these compounds are sparingly soluble in water and are also amphipathic, forming various types of micelles in solution as well as tending to accumulate at interphases (Dawson, 1966). Many of the early studies were carried out in aqueous phosphate solutions (Chargaff and Cohen, 1939; Fairbairn, 1945; Levene and Rolf, 1923). Generally the reaction was, at best, slow and rather incomplete. Many studies were, therefore, carried out in the presence of certain reagents that were found to cause large increases in the reaction rate. Most of these substances are thought to have their effect through action on the substrate rather than on the enzyme, since most of them can be classified as lipid solubilizing agents (Condrea and Vries, 1965).

Hanahan (1952) found that diethyl ether could be used as the main phase of the reaction system and this led to a series of studies on phospholipid hydrolysis in this medium (Hanahan, Rodbell and Turner, 1954; Long and Penny, 1957). Hanahan showed that during the reaction the enzyme was present in the ether phase. The substrate, lecithin, was essential for this solubilization. He demonstrated the transfer of enzymatic activity using portions of the ether phase. Later in the reaction the enzyme was precipitated

along with the residual substrate and products but this did not appear to affect the rate of the reaction. The use of diethyl ether as an activator was extended to aqueous systems by Magee and Thompson (1960), using ether-saturated 2, 4, 6-collidine buffer. Vogel and Zieve (1960), in a study of the phospholipase A present in human duodenal contents, showed that sodium deoxycholate had a marked activating effect in the reaction. It was added by these authors to an assay system used to assess the activity of phospholipase A in various tissues (Zieve and Vogel, 1961). Magee et al. (1962) showed that the optimal concentration of sodium deoxycholate used depended on the concentration of the substrate. It was also demonstrated that the presence of deoxycholate effectively inhibited the breakdown of lysolecithin by phospholipase B. The latter enzyme has been shown to occur in pancreas at a high level by Shapiro (1952) and could readily interfere with the simpler methods used to determine the rate of the reaction (Magee and Uthe, 1968).

Another method of approach to the study of phospholipase A is that of Roholt and Schlamowitz (1961). These authors were fully aware of the difficulties involved in the use of naturally occurring lecithins and made a study of the use of dihexanoyl-L-α-glyceryl-phosphorylcholine as a substrate. They concluded that this compound was a good substrate for phospholipase A and had the added

advantage of being water soluble and having a known molecular composition with regard to the fatty acid constituents. They also showed that this compound existed in solution as single molecules rather than as aggregates. Deenen and Haas (1963) studied the effect of fatty acid constituents on the reaction and concluded that the dihexanoyl derivative was as good a substrate as dioleyl lecithin. Interestingly, the distearoyl derivative was a relatively poor substrate. Derivatives of acetic and butyric acids were very poor substrates.

Early work on the activity of the enzyme towards purified phospholipids indicated that lecithin was the preferred substrate (Hanahan, 1959), although Levene and Rolf had shown in 1923 that the attack of the enzyme on egg yolk resulted in the formation of both lysolecithin and lysophosphatidylethanolamine and Fairbairn (1945) concluded that cephalins were attacked in crude brain preparations. Chargaff and Cohen (1939), however, found that purified cephalins were not attacked. Later work by Long and Penny (1957), using Hanahan's etherial system (Hanahan et al., 1954) showed that purified egg phosphatidylethanolamine was attacked by the enzyme present in water moccasin (Agkistrodon piscivorous piscivorous) venom. Some evidence was also obtained for the breakdown of phosphatidylserine and ethanolamine plasmalogen.

Deenen and co-workers have carried out an extensive study on the effect of the polar group on the susceptibility of the phospholipid to attack by phospholipase A preparations from various sources. Synthetic stereoisomers of known fatty acid composition were used (Deenen and Haas, 1963; Haas et al., 1963). The enzyme from human pancreas was shown to hydrolyze phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. Magee et al. (1962) stated that this enzyme could slowly hydrolyze ethanolamine plasmalogen. The enzyme present in C. adamanteus venom was found to hydrolyze phosphatidylcholine, phosphatidylethanolamine, phosphatidyl serine, phosphatidic acid and cardiolipin. Choline plasmalogen was also hydrolyzed (Gottfried and Rapport, 1962). Studies by Rimon and Shapiro (1959) showed that the enzyme from beef pancreas could attack phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidic acid.

Dawson (1963) carried out a study on the effect of the addition of various substances on the hydrolysis of phospholipids by phospholipase A isolated from N. naja venom by electrophoresis. The ether-containing 2, 4, 6-collidine system of Magee and Thompson (1962) was used. He concluded that unlike the phospholipases present in Clostridium perfringens and Penicillum notatum, the activity of the venom phospholipase A was not dependent on the

zeta potential of the phospholipid micelle. He suggested the role of the activator was one of orientation of the substrate molecule in such a manner as to enable the enzyme to attack it. This orientation may simply involve an "unpacking" of the closely stacked lecithin molecules. Substances such as ether may also serve to prevent the released fatty acid from gathering at the interphase and inhibiting the hydrolysis. No such studies have been carried out with the water-soluble forms of lecithin such as dihexanoyl-L-α-glycerylphosphorylcholine where the lecithin would exist in monomeric form in aqueous solution.

Haas et al. (1963) made some interesting observations on the effect of the addition of various acidic phospholipids on the hydrolysis of lecithin. The addition of either phosphatidylserine or phosphatidic acid to the reaction system markedly accelerated the breakdown of lecithin. Phosphatidylethanolamine was not effective in bringing about this activation. It was shown that, in the case of the addition of phosphatidylserine, the added phosphatidylserine also was hydrolyzed. It was suggested that this was due to the increase in the negative potential of the substrate molecules, but this appears doubtful in light of Dawson's work (Dawson, 1963) which showed that the activating effect of these substances was inherent in the substance itself since other negatively charged

material which had the same effect on the potential of the micelle did not activate the reaction.

Much work has been done on the effect of snake venoms on tissue preparations, both in vivo and in vitro. Less work has been done on the purified phospholipase A present in the venom, although the first well-documented effect of venom preparations was their ability to cause hemolysis in blood (Mitchell, 1860; Flexner and Noguchi, 1902). Flexner and Noguchi (1902) also noted that the washed red blood cells of various animals were not lysed by snake venoms unless the plasma was added back to the cells. They also showed that the hemolytic power of the venoms was unaffected by heating at 75-80° C for ten minutes. Later Roy (1945) showed that washed red blood cells of all species tested were lysed by venom if the suspending medium contained lecithin. This confirmed the work of Delezenne and Ledebt (Meldrum, 1965) who had shown that lecithin was the substrate and a fatty acid a product of the action of venom on horse serum or egg yolk. Much species variation was noted regarding the susceptibility of the washed cells to the direct action of the venom (Turner, 1957), and it was postulated that the susceptibility of the erythrocyte to the venom depended on the presence of lecithin in the membrane of the cell as well as its exposure or availability. This concept did not,

however, explain the lack of hemolysis occurring with certain venoms which contained large amounts of phospholipase A. It soon became apparent that there was in the venom another factor which was involved in the direct hemolysis of the erythrocyte which was distinct from the phospholipase A of the venom. This component of the venom is called the "direct lytic factor." It has been separated from the phospholipase A of a number of venoms (Habermann and Neuman, 1954; Doery and Pearson, 1961; Condrea, Vries and Mager, 1964). Condrea et al. (1964) showed that the addition of the direct lytic factor isolated from N. naja venom enabled the phospholipase A isolated from Vipera palestinae and Vipera russelli to attack washed erythrocytes, thus indicating a role for the direct lytic factor in rendering the phospholipids of the erythrocyte susceptible to the action of phospholipase A. However, there are still erythrocytes of certain species (e. g. camel and sheep) that are resistant to the actions of venoms such as N. naja even though these venoms contain the direct lytic factor (Condrea, Mammon, Aloof and Vries, 1964). These authors showed that the most probable explanation was that the direct lytic factor did not affect the membrane of the resistant cells to render the phospholipids present therein accessible to the action of the venom phospholipase A. It should be pointed out again that all erythrocytes are hemolyzed

if lecithin is present in large enough quantities in the incubation medium. The relationship between the breakdown of plasma lipids, the hydrolysis of red cell lipids and the amount of hemolysis produced is complicated. Ibrahim and Thompson (1965) have shown that the amount of hemolysis is more directly related to the hydrolysis of red cell phospholipids than to the total hydrolysis occurring in treated whole blood. They also noted that phospholipase A from human pancreas could not hydrolyze the phospholipids of the intact human red blood cell, but that the phospholipase A present in N. naja could attack the intact erythrocyte. These authors also showed that these two enzymes, as well as crude sea snake (Enhydrina schistosa) venom rapidly hydrolyzed the phospholipids of red cell ghosts. Similar differences between intact erythrocytes and red cell ghosts were found by other investigators. Heemskerk and Deenen (1964) showed that C. adamanteus venom readily hydrolyzed the phospholipids in lysed, but not intact rabbit erythrocytes. Condrea, Vries and Mager (1964) studied the hydrolysis of phospholipids in human erythrocyte ghosts and intact erythrocytes by purified phospholipase A preparations isolated from H. hemaculatus, N. naja and V. palestinae. The phospholipase from the first two sources could attack the erythrocyte ghosts in the absence of the direct lytic factor which was required for attack by these enzymes on the intact erythrocyte.

The phospholipase A from V. palestinae could only attack the erythrocyte ghosts if the direct lytic factor isolated from N. naja venom was present. This work was extended to other erythrocyte ghosts by Condrea, Mammon, Aloof and Vries (1964). The phospholipase present in H. hemaculatus attacked phospholipids present in red cell ghosts obtained from human, sheep, rabbit, guinea pig and camel blood. V. palestinae phospholipase A could not attack any of these ghosts unless ringhals direct lytic factor was added. Under these conditions the ghosts of camel and sheep erythrocytes still were unaffected.

The action of phospholipase A from N. naja and V. palestinae venoms on blood platelets is similar to that found for erythrocyte ghosts (Kirschmann, Condrea, Moav, Aloof and Vries, 1964). The phospholipids of the platelet were readily hydrolyzed by N. naja but not by V. palestinae phospholipase A unless the direct lytic factor was present. It should be emphasized that intact platelets were used.

Condrea, Avi-Dor and Mager (1965) also studied the swelling of isolated mitochondria as accentuated by treatment with snake venom phospholipase A preparations isolated from V. palestinae and H. hemaculatus venoms. The mitochondrial swelling was associated with the breakdown of the phospholipids of the mitochondria.

Again the presence of the direct lytic factor was a prerequisite for the action of the V. palestinae phospholipase A. The role of the direct lytic factor was postulated to be one of reaction with the mitochondrial membrane in such a manner as to allow the phospholipase to react with the phospholipids. The direct lytic factor is highly cationic at neutral pH (Condrea et al., 1965). Presumably this reacts with electronegative sites on the surface of the mitochondrion. The role of the direct lytic factor is specific since the action of the direct lytic factor cannot be duplicated by protamine. Various other basic polypeptides could replace the direct lytic factor in making the red cell ghosts susceptible to the action of V. palestinae phospholipase A. It appeared to be necessary that the polypeptide be both basic in nature and possess lipophilic groups (Klibansky, London, Fraenkel and Vries, 1968). Gramicidin S and the synthetic copolymers poly-Orn-Leu, poly-Lys-Leu and poly-Orn-Leu-Ala were effective in replacing the direct lytic factor. The synthetic polymers composed completely of basic amino acids (poly-Lys and poly-Orn) were inactive. It is suggested that the basicity of the factors is involved in binding the polypeptide to the cell membrane and the lipophilicity of the polypeptide is involved in penetration of the lipid portion of the membrane in such a way as to enable the phospholipase to reach the phospholipid substrate.

The reaction is a reversible one, since treatment of the red blood cell with direct lytic factor followed by its removal with heparin blocks the action of the enzyme.

The action of phospholipase A on serum is not simply one of lecithin breakdown. Marinetti (1961) showed that C. adamanteus venom converted serum phosphatidylcholine and phosphatidylethanolamine to their corresponding lyso-derivatives, but no free fatty acids were released into the medium. It was suggested that the released fatty acids were complexed with the serum albumin. The reaction required the presence of calcium ions and was inhibited by the presence of ethylenediaminetetraacetate (EDTA), oxalate and citrate. The EDTA inhibition was readily reversed by the addition of excess calcium ions to the medium. In a later paper Marinetti (1965) showed that the products resulting from the hydrolysis of phospholipids of egg yolk lipoprotein remain bound to the lipoprotein. Ibrahim et al. (1964) showed that serum phosphatidylcholine and phosphatidylethanolamine were susceptible to the action of N. naja venom and the phospholipase A purified from human pancreas. The effectiveness of the human enzyme was greatly increased by the addition of sodium deoxycholate.

Sodium deoxycholate or bile salt addition produces a variety of effects. As stated previously, the action of phospholipase A

on purified lecithin in aqueous media is markedly accelerated by the addition of these agents, particularly sodium deoxycholate, to the medium (Vogel and Zieve, 1960; Magee et al., 1962). Vogel and Zieve (1960) showed that the optimal activity of human duodenal phospholipase A was obtained when the weight ratio of sodium deoxycholate to lecithin was four to five. The reaction was carried out at pH 8.4. Later Doizaki and Zieve (1961) published a turbidimetric method for phospholipase A assay in which the above ratio was about one to three in a system buffered at pH 8.8. Magee et al. (1962) found that the optimal ratio was two to one at pH 7.3. The activating effect of sodium deoxycholate depends markedly on the nature of the substrate and the source of the enzyme. Magee et al. (1962) showed that the addition of sodium deoxycholate was obligatory for the action of human pancreatic phospholipase A on lecithin. In contrast Deenen, Haas and Heemskerk (1963) found that phosphatidylethanolamine was attacked by the human enzyme in the absence of deoxycholate. Ibrahim, Sanders and Thompson (1964) showed that the addition of sodium deoxycholate to the phosphatidylethanolamine-human phospholipase A system resulted in marked inhibition. This inhibition of the breakdown of phosphatidylethanolamine in the presence of sodium deoxycholate also was observed with N. naja venom. Studies with

purified phosphatidylserine under the same conditions showed that the addition of sodium deoxycholate gave a slight activation of both the human and reptilian enzymes. The latter authors also studied the effects of the addition of sodium deoxycholate on the hydrolysis of plasma phospholipids. The addition was without effect in the case of the reptilian enzyme, but showed a marked stimulatory effect in the case of the human enzyme. The addition of sodium deoxycholate did not result in any inhibition of the phosphatidylethanolamine breakdown. In a later paper, Ibrahim and Thompson (1965) reported the effect of the addition of sodium deoxycholate on the breakdown of the phospholipids present in red cell ghosts. Sodium deoxycholate slightly activated the breakdown caused by the human enzyme, had no effect on the action of N. naja venom, and slightly inhibited the activity of sea snake (E. schistosa) venom. There was no decrease in the hydrolysis of phosphatidylethanolamine upon addition of the sodium deoxycholate. The increase in activity noted in the experiments where sodium deoxycholate was added to lipoprotein preparations were, generally, of small magnitude when compared to the increase caused by its addition to purified lecithin. This is not surprising, since Condrea, De Vries and Mager (1962) have shown that the rate of breakdown of phospholipids in lipoproteins was much faster than the rate of breakdown of

isolated, emulsified phospholipids. This was true even if there was a fairly large percentage of phosphatidylethanolamine in the sample.

N. naja and human pancreatic phospholipase A rapidly attacked the phospholipids present in rat diaphragm homogenates. The addition of sodium deoxycholate to the system was not essential, in fact its addition caused a slight but consistent inhibition of both enzymes (Ibrahim et al., 1964). The action of the two enzymes was different towards intact diaphragms. The cobra enzyme readily attacked the intact diaphragm phospholipids while the human pancreatic enzyme was essentially inert. The small amount of activity obtained for the human enzyme was ascribed to action of the enzyme on damaged cells.

Gallai-Hatchard and Grey (1968) studied the action of various enzymes on isolated rat liver cells. N. naja phospholipase A attacked the phosphatidylethanolamine and phosphatidylcholine of the cell without the need for any activator. Phosphatidylethanolamine was the preferred substrate for the N. naja enzyme, but C. atrox and H. hemaculatus phospholipase A preferentially attacked lecithin. The release of glutamic-oxaloacetic transaminase was used as an index of cell membrane damage. About 25 per cent of the total phosphatidylethanolamine was hydrolyzed before a sig-

nificant release of this enzyme occurred. No difference in the morphology of the cells was detected by phase-contrast microscopy, even after complete release of the transaminase.

Various studies have indicated that metal ions, especially calcium, are involved in the reaction between phospholipids and phospholipase A (Zeller, 1950; Hayashi and Kornberg, 1954). The activating effect of calcium ions on the reaction was pointed out by Neuman and Habermann (1954). They also showed that barium and zinc ions inhibited the reaction. Magnesium ions had a slight inhibitory effect.

Long and Penny (1957) carried out a study on the action of calcium ions on the activity of the phospholipase A present in the venoms of A. piscivorous piscivorous and C. adamanteus. Calcium ions were found to activate optimally at a certain concentration. This optimal concentration was not dependent on the concentration of the enzyme present in the reaction system, but rather on the concentration of the substrate. Hanahan's etherial system (1954) was employed by the above investigators. Sodium, potassium, barium, strontium, magnesium and cadmium ions could not replace calcium ions. Copper and zinc ions inhibited the reaction. EDTA also inhibited the reaction. These authors suggest that the calcium plays a role in the binding of the enzyme to the substrate.

In his earlier work, Hanahan (1957) did not find a requirement for calcium ions in his etherial system. He suggested that the role of calcium ions was one of fatty acid removal from the reaction site; a role that would be filled by the ether in his reaction system. Later Saito and Hanahan (1962) found that calcium ions activated purified C. adamanteus phospholipase A in the same manner as described by Long and Penny (1957) in that there was a certain optimal calcium ion concentration. Concentrations greater than this optimal concentration resulted in inhibition of the reaction. Saito and Hanahan also noted that there was an optimal sodium chloride concentration. Roholt and Schlamowitz (1961) concluded that calcium ions were not involved in fatty acid removal since no other heavy metal ion could replace them. They showed that the fatty acid released from the water-soluble dihexanoyllecithin used, did not inhibit the reaction even at ten times the concentration of fatty acid found in the reaction system. These authors also showed that barium and zinc ions inhibited the hydrolysis of the water-soluble lecithin.

Dawson (1963) determined the effect of the addition of calcium ions on the activity of the phospholipase A isolated from N. naja venom, using the ether-activated system of Magee and Thompson (1960), who had found that calcium ions activated the enzyme in A. piscivorous

piscivorous venom. He showed that the hydrolysis of lecithin was markedly dependent on the addition of calcium ions. EDTA inhibited the reaction. Phosphatidylethanolamine hydrolysis was not so dependent on the addition of calcium ions, presumably due to the presence of calcium ions in the lipid preparation. He also noted an enhanced activity in the presence of EDTA when excess calcium ions had been added to saturate the chelating agent. This was attributed to the removal of contaminating, inhibiting metal ions from the enzyme. The role of the calcium was found to be an obligatory one, and the inhibition by other compounds was dependent upon the nature of the compound and was not related to the zeta potential, as is the case with bacterial phospholipase C. Magee and Thompson (1960) studied the effect of metal ions on the activity of the phospholipase A present in A. piscivorous piscivorous. As stated earlier these authors showed that this enzyme was strongly activated by the addition of calcium ions. An optimal concentration of calcium was noted, but additions in excess of this were not particularly inhibitory. Magnesium ions did not replace calcium ions in this system. Both copper and zinc ions were inhibitory. They also noted that the reaction was much more sensitive to the addition of calcium ions if the phosphatidylcholine was washed according to the method of Folch et al. (1957)

Marinetti (1965) studied the effect of various metal ions

on the rate of clearing of an egg yolk suspension by A. piscivorous piscivorous venom. Calcium ions were required, but could be partially replaced by magnesium, manganese, ferrous and zinc ions. Cupric and aluminum ions could not replace the calcium, although the aluminum ions caused a clearing of the suspension even in the absence of the venom. Cuprous citrate also caused a clearing of the suspension without hydrolysis of the phosphatides. EDTA inhibited the reaction, and this inhibition was not reversed by the addition of excess calcium ions. It should be pointed out that in an earlier paper Marinetti (1961) showed that the EDTA inhibition of plasma phospholipid breakdown by C. adamanteus venom was reversed readily by the addition of excess calcium. Since Long and Penny (1957) also showed that the enzyme from A. piscivorous piscivorous was inhibited by EDTA and that this inhibition was not reversed by the addition of calcium ions, there may be an enzyme-EDTA complex of some sort which is specific for the enzyme from this venom.

Rimon and Shapiro (1959) studied the effect of adding calcium ions on the activity of phospholipase A isolated from bovine pancreas. The addition of calcium was necessary for activity towards phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine. The hydrolysis of phosphatidic acid was inhibited by the addition of

calcium ions. Vogel and Zieve (1960) showed that the addition of calcium resulted in a slight inhibition of the enzyme present in human duodenal fluid. They also observed that EDTA inhibition was not reversed by the addition of excessive amounts of calcium. The lack of calcium stimulation of the intestinal enzyme is similar to that found by Epstein and Shapiro (1959) for rat intestinal mucosa lecithinase. In a later paper Doizaki and Zieve (1961) found that low concentrations of calcium ions slightly stimulated the activity of the duodenal enzyme. In this latter paper the assay was based on the clearing effect of lyso-compounds on a lecithin-lysolecithin-sodium deoxycholate suspension while, in the former case, the assay was based on titration of the released fatty acids. Doizaki and Zieve also found that EDTA inhibition of the reaction was relieved by the addition of calcium ions. They further showed that sodium, zinc, cobalt, magnesium and copper ions all inhibited the reaction. Aluminum ions once again were found to accelerate clearing of the suspensions by the enzyme.

Magee et al. (1962) studied the properties of the phospholipase A from human pancreas. In this work calcium ions were found to be inhibitory, as were lead, silver, zinc, ferric and mercuric ions. Aluminum ions stimulated the reaction. In this case the aluminum ion effect represents a true stimulation of hydrolysis since the

assay was based on the decrease in the acyl ester content of the reaction mixture. EDTA was found to inhibit at a high (1. 0 mM) concentration, but activated at low (e. g. 0. 01 mM) concentrations. It was suggested that the inhibition of the reaction by the calcium at high concentrations was due to the effect of the ion on the lecithin-deoxycholate reaction system.

It has long been known that snake venoms can have a marked action on the nervous system (Meldrum, 1965). Flexner and Noguchi (1902) showed that treatment of venom with brain tissue lowered the toxicity of the venom and suggested that this was due to a combination of certain factors present in nervous tissue with the neurotoxic factor of the venom. Weil (1930) showed that treatment of excised portions of spinal cord with N. nigricolis venom resulted in breakdown of the myelin sheath of myelinated nerve fibres. Morrison and Zamecnik (1950) showed that these demyelinating changes could be brought about by treatment of excised cord with cobra and rattlesnake venom but not by the venom of the south american pit viper (Bothrops jararaca). Sanders, Akin and Soret (1954) and Hudson, Quastel and Scholefield (1960) showed that injection of cobra venom, which had been heated to inactive enzymes other than phospholipase A, caused paralysis. Petrushka, Quastel and Scholefield (1959a) studied the effect of heated A. piscivorous

piscivorous venom on respiring mitochondria. Liver and kidney mitochondria showed an initial increase in respiration followed by a sudden decline. The larger the amount of venom used, the shorter was the period of increased respiration. Brain mitochondria, on the other hand, showed a similar stimulation of respiration upon addition of the venom which was followed by a gradual decrease. The final rate of respiration attained was approximately 50 per cent of the maximum, in contrast to the complete loss of respiratory activity observed with liver and kidney mitochondria. The formation of lysolecithin in these preparations was demonstrated. Glutathione exercised a protective action against the inhibitory effects of phospholipase A on the oxygen uptake of rat liver and rat kidney mitochondria. This prolongation of the initial stimulated respiratory phase of respiration was not apparent with brain mitochondria. The effects found with brain mitochondria could be demonstrated using brain slices, but not with either liver or kidney slices. In a later paper (Petruska, Quastel and Scholefield 1959b) it was shown that the venom caused a rapid uncoupling of oxidative phosphorylation during the initial phase of the reaction. This was reversed by either serum albumin or egg lecithin.

Hudson, Quastel and Scholefield (1960) studied the effect of heated N. naja venom on the metabolism of spinal cord slices. There

was no initial stimulation of respiration as had been observed with mitochondria and eventually respiration ceased. Incorporation of phosphate into ATP and ADP was much less than that expected if the mitochondria had remained tightly coupled. It should be pointed out that the rate of these biochemical changes was considerably slower than the rate at which paralysis developed. McArdle, Thompson and Webster (1960) studied the release of glutamic-oxaloacetic transaminase from brain slices treated with lysolecithin or A. piscivorous piscivorous venom. Both caused a rapid release of the intracellular glutamic-oxaloacetic transaminase, indicating breakdown of cell membranes. In a later paper, Ibrahim et et., (1964) showed that the release of the glutamic-oxaloacetic transaminase paralleled the release of fatty acid from brain slices which had been treated with heated N. naja venom. The phospholipase A purified from human pancreas also caused the release of both the intracellular enzyme and free fatty acids, but the major portion of the activity appeared to be directed against damaged cells. Homogenates of rat brain were attacked even more rapidly. Neither enzyme required the presence of sodium deoxycholate for activity towards rat brain homogenates or slices. An extensive breakdown of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine by these two enzymes was demonstrated. No quantitative

determinations of phospholipid hydrolysis were reported other than the total amount of fatty acid released.

Klibansky, Shiloah and Vries (1964) studied the reaction of N. naja and V. palestinae on cat brain slices, homogenates and mitochondria. Neither phospholipase A had phospholipid splitting ability when applied to brain slices. N. naja phospholipase A rapidly attacked phosphatidylcholine, phosphatidylethanolamine, ethanolamine plasmalogen and phosphatidylserine in brain homogenates. V. palestinae phospholipase A did not attack homogenates. The action of the N. naja enzyme was enhanced by the presence of calcium ions, and did not appear to be affected by the presence of the direct lytic factor. The addition of blood or plasma which had been pretreated with phospholipase A to brain slices did not enable the N. naja phospholipase A to attack the phospholipids of the slices. The hydrolysis of the above-mentioned phospholipids was documented chromatographically but only the disappearance of lecithin was quantitatively measured.

Studies by Rosenberg and others (Rosenberg and Podleski, 1962; Rosenberg and Podleski, 1963; Rosenberg and Hoskin, 1965) showed that A. piscivorous piscivorous and N. naja venoms were highly potent when used on squid axons, whereas V. russelli and C. adamanteus venoms were relatively inactive, both in their direct effects on conduction and in their ability to render the axon sensitive

to curare and acetylcholine. Condrea, Rosenberg and Dettbarn (1967) related the block of conduction to the breakdown of phospholipid upon treatment of lobster axons with various venoms and H. hemaculatus phospholipase A. A. piscivorous piscivorous and H. hemaculatus venoms and their isolated phospholipase A blocked conduction at high concentrations and rendered the axon sensitive to abnormally low levels of curare and acetylcholine. This action was associated with breakdown of phospholipids, namely lecithin, phosphatidic acid, phosphatidylserine and phosphatidylethanolamine. C. adamanteus and V. palestinae venoms neither attacked the phospholipids of the axon nor prevented conduction nor increased sensitivity of the axon to curare or acetylcholine. The addition of the direct lytic factor to the last two venoms did not change the results although the direct lytic factor when added at high concentration blocked conduction by itself. All four venoms readily attacked the phospholipids present in nerve homogenates. This work was extended to squid axon in a later paper (Condrea and Rosenberg, 1968). Essentially similar results were obtained, only with this tissue all four venoms could attack the intact axon, although the C. adamanteus and the V. palestinae venoms had to be used at high concentrations. The addition of the direct lytic factor had no effect on the activity, although large amounts could block conduction without affecting phospholipid breakdown.

III. METHODS

A. Purification Procedures

1. Phospholipid Substrates

i. Phosphatidylcholine and Phosphatidylethanolamine

One egg yolk was added to 500 ml of chloroform-methanol (2:1, v/v) with stirring. The resulting mixture was sonicated for five minutes using the highest power setting available on the instrument (Bronson Sonifier; Model S125; Heat Systems Incorporated, Melville, N. Y.) to give a fine suspension. The solution temperature was kept below 40° C. This solution was filtered through Whatman No. 1 filter paper on a large Buchner funnel. The filtrate was transferred to 250 ml. glass centrifuge bottles in measured amounts. An appropriate volume of 0.9% NaCl was added to each of the bottles to give a Folch biphasic distribution (Folch, Lees and Sloane-Stanley, 1957). The solutions were mixed by sonication. The two phases were well separated by centrifugation and the upper phase was removed by aspiration. The lower phase was washed with Folch upper phase solvent (chloroform-methanol-water,

3:48:47, v/v). The lower phase was then taken to dryness in a rotary evaporator at a bath temperature of 35-40° C. Chloroform was used to remove the last traces of solvent from the lipid. The lipid was taken up in chloroform for application to the silicic acid column. For the column preparation 300g of silicic acid was slurried with a liter of chloroform-methanol (2:1, v/v). The slurry was poured into a 8. 5 cm I. D. glass column fitted with a sintered glass disc at the bottom. After the silicic acid had settled, a filter paper disc was fitted into place on the surface of the silicic acid. Glass beads (3 mm diameter) were layered on top of the filter paper. The original solvent was displaced by pure chloroform and the lipid applied to the column with a loading limit of 0. 5 mg of lipid phosphorous per g of silicic acid. One liter of chloroform was run through the column to elute the neutral lipids and the major portion of the yolk pigments. Two hundred milliliters of chloroform-methanol (10:1, v/v) was then used to elute the residual pigments. Chloroform-methanol (6:1, v/v) was then used to elute the phosphatidylethanolamine. The elution of the phosphatidylethanolamine was monitored by applying 100 µl of the effluent to a piece of filter paper and spraying the spot with ninhydrin (0. 5% in ethanol). The colour was developed by heating the spot with a hair drier. The fractions which were positive with ninhydrin were taken to dryness

in a rotary evaporator and the lipid was made up in 25 ml of chloroform. This was stored at -20° C. Although no changes could be detected by thin-layer chromatography after storage for about a month, the effectiveness of the lipid as a substrate for phospholipase A was greatly reduced by prolonged storage.

Following the elution of the phosphatidylethanolamine, the solvent was changed to chloroform-methanol (1:4, v/v) which eluted the phosphatidylcholine. The elution of this lipid was monitored by plating 100 µl of the eluant on a silica gel G thin-layer plate. The plate was sprayed with 18 N sulphuric acid and heated at 250° C for ten minutes to char the lipid. Only the most concentrated fractions were taken as phosphatidylcholine.

The purity of the lipids was checked by thin-layer chromatography. Silica gel G (E. Merck, Darmstadt) plates were prepared using a commercial spreader (Shandon, London, England). The plates averaged 0. 5 mm in thickness. The plates were dried at 110° C and stored in a dessicating cabinet. Prior to use the plates were activated for 30 minutes at 110° C. Each fraction was applied to each of two plates at a load of about 10 µg. One of the plates was developed in a basic solvent and the other in an acidic solvent. The solvent systems used were:

a) Chloroform-methanol-ammonia-water (65:25:2:2, v/v)

b) Chloroform-methanol-formic acid-water

(70:28:7.5:2.5, v/v).

After development the plates were air-dried in a warm place. Each plate was sprayed with ninhydrin (Kates, 1960) and warmed gently. The ninhydrin-positive areas were outlined, after which the plates were sprayed with 18 N sulphuric acid and then charred by heating for ten minutes at 250° C. Appropriate lipid standards were included in each run. The lecithin and phosphatidylethanolamine preparations both ran as single spots in the two solvent systems employed.

ii. Phosphatidylserine

Folch "Fraction C" was prepared essentially as described by Folch (1942). However, during the acetone precipitation, the crude cephalin was taken up in petroleum ether and the phospholipids precipitated by adding the ethereal solution to ten volumes of acetone. After storage at 2-4° C overnight the phospholipids were collected by filtration. "Fraction C" was dissolved in 400 ml of chloroform-methanol (2:1, v/v) and the solution was washed with 0.25 volumes of 0.1 N HCl. After separation of the two layers, the upper phase was removed and the lower phase shaken

with the usual Folch upper phase solvents to remove excess acid. The lower phase was taken to dryness and the lipid was made up in chloroform. A 70 g silicic acid column was prepared as described previously. The crude phosphatidylserine was applied to the column and the column was washed with 400 ml of chloroform. Chloroform-methanol (4:1, v/v) was used to elute the phosphatidylserine. The bulk of the lipid was eluted in the first 300 ml. These fractions were taken to dryness in a rotary evaporator and the lipid was made up in chloroform. The solution was stored at -20° C. Thin-layer chromatography was carried out on the lipid as described under the purification of phosphatidylcholine and phosphatidylethanolamine. Only one spot was found in each case. The purification method is similar to that described by Rathbone, Magee and Thompson (1962).

iii. Phosphatidylinositol

The method used throughout was that of Colacicco and Rapport (1967). Five grams of Asolectin (Associated Concentrates Inc., Woodside, N. Y.) was dissolved in 50 ml of ice-cold chloroform and 120 ml of ice-cold methanol was added rapidly

with stirring. The white precipitate was collected by centrifugation. The residue was redissolved in 25 ml of cold chloroform and reprecipitated with 60 ml of cold methanol. The precipitate was collected and dissolved in 1500 ml of cold chloroform-methanol (2:1, v/v). This solution was shaken with 300 ml of cold water and the mixture was allowed to stand in the cold overnight. The clear lower layer was taken to dryness with chloroform-methanol (2:1, v/v) and the lipid was made up in chloroform. A 100 g silicic acid column was prepared as described previously. The chloroform was displaced by chloroform-methanol-ammonia (80:20:2, v/v). One gram of the lipid was applied to the column and the column was washed with three liters of the same solvent. The eluting solvent was switched to ethanol and 50 ml fractions were collected. Two distinct peaks were obtained. The major fractions of each of these peaks were pooled. Each of the peaks was analyzed by thin-layer chromatography and the peak containing the phosphatidylinositol was taken to dryness and made up in chloroform. The lipid was stored at -20° C. Thin-layer chromatography indicated that this preparation was contaminated with a slight amount of phosphatidylserine.

2. Enzymes

i. Preparation of Acetone Powders

Beef pancreas was obtained from a local slaughter house. Human pancreas was obtained at autopsy from individuals without any gross signs of pancreatic defects. Canine pancreas was obtained from research animals. The pancreas was freed from blood vessels and fat and cut into small pieces. These were wrapped in plastic and frozen at -20° C. A sample of the frozen pancreas was homogenized in a Waring blender in 10 volumes of acetone. At all stages of the extraction procedure, except as otherwise noted, the temperature was kept at less than 0° C by the addition of small amounts of powdered dry ice to the mixture. Homogenization of the pancreas was carried out for 10 minutes. The homogenate was quickly filtered using a large Buchner funnel. The precipitate was resuspended in 10 volumes of n-butanol and homogenized for a further 10 minutes in the blender. The mixture was filtered and resuspended in 10 volumes of cold fresh acetone. Filtration was again carried out and the recovered precipitate was mixed with five volumes of reagent grade diethyl ether and allowed to sit at room temperature for 15 minutes with

intermittent stirring. The mixture was filtered and the precipitate broken up and dried in a vacuum dessicator. This is the method of Magee, Gallai-Hatchard, Sanders and Thompson (1962). The resulting acetone powders are referred to as Fractions A. Assays over a period of a few months indicated that the phospholipase A of the beef pancreas is stable in the dry state, as is the phospholipase A isolated from human pancreas (Magee et al, 1962).

ii Bovine Phospholipase A

One gram of the acetone powder was homogenized in a homogenizer fitted with a Teflon pestle (A. H. Thomas, Philadelphia) with 25 ml of glycerol-water (1:1, v/v) until all of the powder appeared to be well suspended in the medium. Following this 25 ml of 1 per cent sodium bicarbonate was added and the suspension homogenized for a further ten minutes. The suspension was then centrifuged at 10, 000 x g for 15 minutes at 4^{o} C. The resulting supernatant is referred to as the glycerinated extract. This was transferred to inch-wide dialysis tubing of a length approximately twice that filled by the liquid. Dialysis was carried out against 500 ml of 0. 005 M calcium acetate. The dialysing solution was changed hourly four times and finally

dialysis was carried out for sixteen hours against four liters of the calcium acetate solution. The dialyzing solution was stirred slowly with the aid of a magnetic stirrer. The resulting cloudy solution was clarified by centrifugation and is referred to as Fraction B. The pH of the solution was adjusted to 4. 5 with 1 N HCl and the solution was heated for five minutes in a hot water bath at 75° C. A large amount of precipitate formed which did not settle well during centrifugation, but could be removed by filtering the mixture through a fine sintered glass filter. The resulting filtrate was clear and colourless. This solution is referred to as Fraction C. It was quickly frozen and lyophilized. The resulting white powder was mixed thoroughly and then stored in a dessicator over phosphorous pentoxide. Optical density measurements at 260 and 280 mμ indicated that this powder was still contaminated with something that absorbed strongly at 260 mμ. These measurements were carried out with a Beckman DU spectrophotometer. Further purification of the phospholipase A was carried out by gradient elution on sulfoxyethyl Sephadex (SE-Sephadex; C-50 medium, Pharmacia, Uppsala, Sweden). The ion exchanger was swelled in distilled water. After filtration it was washed with 0. 5 N NaOH and then with distilled water and then allowed to sit for a short time in 0. 5 N HCl. After filtration

the resin was washed with many portions of 0. 02 M Tris (tris (hydroxymethyl) aminomethane) buffer pH 7. 4. Following these washings the resin was suspended in about three volumes of the above buffer. This was packed into a 1 inch I. D. column (Pharmacia) to a bed height of 40-45 cm. The column was flushed overnight in the cold room with cold buffer flowing through it. The next morning 25 mg of freeze-dried Fraction C was dissolved in 5 ml of 0. 02 M Tris buffer pH 7. 4 and was applied to the column. The column was eluted from a constant volume reservoir containing 150 ml of 0. 02 M Tris buffer pH 7. 4. This reservoir was connected to a second reservoir containing 2. 0 M NaCl in the above buffer to obtain the necessary gradient. A flow rate of 12-15 ml per hour was maintained and 5 ml fractions were collected. The elution pattern of the column was obtained by measuring the absorbance of each fraction at 280 and 260 mμ. Four distinct peaks were obtained. The chromatogram is illustrated in Fig. 2. The first two peaks, although large, did not contain any protein by the Lowry method. The last two peaks, especially the first of them, contained the protein. The last peak contained all of the phospholipase A activity. This peak was approximately 0. 15 M with respect to NaCl. The fractions composing this last peak were quick-frozen and the water removed

by lyophilization. The NaCl was allowed to remain to act as a carrier for the enzyme, since in many of the assay experiments a total of less than 1 mg of the enzyme was required and the total yield of protein from this peak was not large, although the recovery of activity was good.

iii. Canine Phospholipase A

The extraction and purification of the acetone powder of dog pancreas was carried out in a manner identical to that employed in the procedure for the isolation of the freeze-dried Fraction C of the beef pancreas.

iv. Rattlesnake Phospholipase A

Lyophilized eastern diamondback rattlesnake (C. adamanteus) venom was obtained from a commercial source (Ross Allen's Reptile Institute Inc., Silver Springs, Florida). A 10 mg per ml solution of this crude venom was prepared in glass distilled water. The pH of this solution was adjusted to 3.0. The solution was then heated at 90° C for five minutes. There was no visible precipitation and no detectable loss of activity. Adjustment to pH 7 produced

a precipitate which was removed by filtration. The solution was applied to a molecular sieve made up as follows: Polyacrylamide molecular sieve (Bio-Rad P-60) was allowed to swell overnight in 0.9 per cent NaCl. This was then packed into a 1 inch I. D. column (Pharmacia) to a bed height of 90-95 cm. The resulting column was allowed to equilibrate overnight in the cold room (0-4°C). The next morning the heated enzyme solution was applied and the column developed using 0.9 per cent NaCl at a flow rate of 20 ml per hour. Three distinct peaks were obtained when the absorbance at 280 mμ was determined for each of the 5 ml fractions. The middle peak contained the major portion of phospholipase A activity. This peak was frozen and lyophilized, using the contained salt as a carrier for the protein for the same reasons as outlined under the purification of the phospholipase A from beef pancreas acetone powders.

v. Human Phospholipase A

One gram of the acetone powder was extracted and centrifuged in the same manner as was described previously for the beef pancreas acetone powder. The supernatant obtained was adjusted to pH 4 with 0.1 N HCl and 3 volumes of acetone at -15° C were

added. After a short time in the cold, the precipitate was collected by centrifugation. The supernatant was discarded and the precipitate kneaded with fresh cold acetone to convert the rather gummy precipitate into a granular one. After centrifugation the acetone was discarded and the kneading repeated with diethyl ether. The recovered, dried precipitate is referred to as Fraction B. A one per cent solution of this fraction was prepared in dilute NaOH (pH 7. 5-8. 0), after which the pH was adjusted to 4. 5. The preparation was heated at 75° C for five minutes and rapidly cooled. After centrifugation the pH of the supernatant was checked, readjusted to pH 4. 5, if necessary, and the enzyme precipitated as before using cold (-15° C) acetone. The dried powder is referred to as Fraction C and was stored in a dessicator after drying in vacuo.

B. Cellulose Acetate Electrophoresis

Electrophoretic determinations were carried out using the Gelman Rapid Electrophoresis apparatus and the Gelman Sepraphore III cellulose acetate strips (Gelman Instrument Co., Ann Arbor, Mich.). Electrophoretic runs were performed at pH 8. 6 using the Gelman High resolution buffer diluted to 2200 ml,

and at pH 5.01 using 0.025 M sodium acetate buffer. In both cases the electrophoresis was carried out for 45 minutes at an amperage of 1 milliampere per cellulose acetate strip. The strips were removed and stained for 10 minutes in 0.1% Amido Black B in 7% acetic acid. The excess stain was removed by washing the strips in 0.5% acetic acid in 50% methanol.

C. Molecular Weight Determinations by Molecular Sieving

A 1 cm I.D. column was packed to a height of 65 cm with Bio-Gel P 60 (Bio-Rad) that had been swollen in 0.9%NaCl. The elution volumes were determined for human serum albumin, human hemoglobin, carboxypeptidase B, trypsin and beef pancreatic ribonuclease A. The elution volume was also determined for the three phospholipase A preparations. The elution volume was determined as follows: 5-10 mg of protein was applied to the column. A 10 and a 5 ml fraction were collected then 1 ml fractions were collected until the peak had been eluted. The optical density of each of the 1 ml fractions was determined at 280 mμ. The logarithm of the molecular weight of the known proteins was plotted against elution volume. A straight line was obtained from which the apparent molecular weight of the

phospholipases could be calculated. This is essentially the method of Andrews (1964).

D. Assay Procedures

1. Phosphorous

Quantitative phosphorous analysis was carried out by ashing a portion of the lipid (containing from 1-10 µg of phosphorous) with 1 ml of 60 per cent perchloric acid for twenty-five minutes in a 10 ml Kjeldahl flask. After cooling, 7 ml of water, 1 ml of 2.5 per cent ammonium molybdate and 1 ml of 10 per cent ascorbic acid were added to each flask. The tubes were shaken and then heated in a boiling water bath for five minutes, then cooled. In the determinations where silicic acid was present in the samples it was removed by centrifugation at this stage. The optical density of each of the tubes was determined at 820 mµ using a Beckman B spectrophotometer. This is essentially the method of Rouser, Siakotos and Fleischer (1966).

2. Protein

Each sample containing from 50-400 µg of protein in a 0. 3 ml volume was put into a standard test tube. Three milliliters of Lowry's alkaline reagent was added (1 ml of 0. 5 per cent copper sulfate in 1 per cent sodium tartrate mixed with 50 ml of 0. 4 per cent sodium hydroxide in 2 per cent sodium carbonate), and the tube shaken and allowed to stand for ten minutes. Following this, 0. 3 ml of commercial Folin-Ciocalteu reagent (British Drug Houses) was added and the tube was mixed as rapidly as possible. After standing for thirty minutes, the optical density at 700 mu was determined using a Beckman B spectrophotometer. Human serum albumin (Sigma) was used as a standard. This is essentially the method of Lowry, Rosebrough, Farr and Randall (1951).

3. NaCl Gradient for Column Chromatography

One milliliter of each fraction was mixed with 3 ml of water and one drop of 10 per cent potassium chromate. This was titrated with approximately 0. 1 M silver nitrate which had been standardized against 1 ml portions of the 2 M sodium chloride 0. 02 M Tris buffer used to develop the gradient.

4. Phospholipase A Assay Procedures

i. The Basic System Used During the Purification Procedure

Lecithin (40 µg of lipid phosphorous) was emulsified with 0. 5 ml of 0. 1 M glycyl-glycine buffer pH 7. 4, 0. 2 ml of a sodium deoxycholate solution (containing 10 mg of sodium deoxycholate per ml) and 0. 1 ml of 10 mM calcium acetate. The mixture was sonicated for one minute using the lowest setting available on the instrument. The resulting clear substrate was brought to temperature (38° C) in a shaking bath. The enzyme test solution (also at 38° C) was added in a volume of 0. 2 ml. Generally the above amounts were increased by a factor of ten and 1 ml samples were taken at appropriate times to determine the initial reaction velocity. In the case of the beef enzyme, there was an initial burst of activity followed by a linear period of hydrolysis until at least 80 per cent of the substrate had been utilized. Since the reaction rate became linear after this initial burst of activity, the specific activity was calculated from the slope of the linear portion of the reaction. In the experiments with purified enzyme initial reaction rates were calculated from the start of the reaction. This assay method is essentially the same as that developed by Magee et al (1962).

In the purification of the enzyme, calcium ions were not added routinely to the assay system since Magee et al. (1962) found a certain amount of evidence suggesting that calcium ions were not required by this enzyme. Calcium salts were added routinely to the reaction system for the snake and the beef enzyme assays. The reaction was stopped by adding 1 ml portions of the reaction system to 5 ml of Dole's reagent (propan-2-ol-heptane-1 N sulphuric acid, 40:10:1, v/v) in a glass stoppered shaking tube (15 x 150 mm) and mixing vigorously. Three milliliters of water and 2 ml of heptane were added to each tube. The tubes were again shaken. After the separation of the two phases, 3 ml of the upper phase was taken and titrated against methanolic NaOH, which was prepared by combining 150-200 ml of methanol (previously gassed out with N_2) and 10 µl of saturated NaOH. The methanolic NaOH container was stoppered and shaken vigorously. Vigorous shaking was necessary to redissolve the NaOH which precipitated during the addition of the saturated NaOH to the methanol. The methanolic NaOH was added to the reservoir of a microburet (Fisher Scientific Co.) which was then fitted with a drying tube filled with lithium hydroxide. The top of the calibrated portion of the buret was also fitted with a small tube of the carbon dioxide trapping agent. With these precautions it was necessary to make up fresh NaOH only

every three to four days. Cresol red in ethanol (1 ml) was added to each sample titrated. The cresol red was made up at a concentration which would give a sharp, readily discernible end point. Standard mixtures containing 2000 mμ-equiv. of either stearic or palmitic acid were titrated along with the samples. The specific activity was calculated from the initial reaction velocity and is expressed in terms of mμ-equiv. of fatty acid released per minute per mg protein. This assay can only be used as it stands when lecithin is employed as the substrate.

ii. Purified Phospholipid Substrates

The incubation system was much the same as that described for the measurement of the specific activity during the purification of the enzymes. In a typical experiment a phospholipid substrate containing 400 μg of lipid phosphorous was suspended in 5 ml of 0.1 M glycyl-glycine buffer at pH 7.4. Three milliliters of water was added. In the cases where sodium deoxycholate was added to the system, 3 ml of a solution containing 20 mg of sodium deoxycholate was added in place of the water. The resulting solution was sonicated for one minute at the lowest power setting available. The enzyme was dissolved in water at a concentration ten times

that required in the final reaction system. One volume of this solution was mixed with one volume of either water or the ion being used, which was also made up at a concentration ten times that required in the final reaction mixture. This solution was incubated for thirty minutes at 37° C. At the end of this period 2 ml of the enzyme solution was added to the substrate solution. At appropriate times 1 ml samples were taken and added to 5 ml of Dole's reagent in a glass stoppered shaking tube (15 x 150 mm). After stopping the reaction with Dole's reagent, 3 ml of water and 2 ml of heptane were added to each tube. The tubes were shaken well and 3 ml of the upper phase was transferred to a 15 ml glass centrifuge tube. Chloroform (1 ml) was added and the contents mixed on a Vortex mixer. A small scoop of silicic acid (approximately 300 mg) was added and the tube again agitated on the Vortex mixer. The tubes were stoppered with a hollow polyethylene stopper and centrifuged to completely sediment the silicic acid. Titration of 3 ml of the supernatant was carried out as described under the assay system used during the purification of the enzymes. The silicic acid was added to ensure complete removal of the phospholipid from the heptane phase. Ibrahim (Ibrahim, Sanders and Thompson, 1964; Ibrahim, 1967) has shown that acidic phosphatides are extracted into the heptane phase in varying amounts.

Since they are titratable under the conditions of this assay they must be removed prior to titration. Ibrahim removed the contaminating phospholipids by evaporating the upper phase to dryness, redissolving the lipid in chloroform and absorbing the phospholipid on silicic acid. Since this method is not easily applied to a large number of determinations the above method was developed. Model experiments with various phospholipids and free fatty acids indicated that, at a level of 40 µg of lipid phosphorous per sample the new procedure would completely absorb phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. Phosphatidic acid, on the other hand, was not picked up by the silicic acid under these conditions. This is not surprising, since Privett, Aurmont and Böttcher (1966) have shown that phosphatidic acid is readily eluted from silicic acid columns with relatively non-polar solvents, whereas the above-mentioned phosphatides require relatively polar solvents for elution from silicic acid. In the experiments where lecithin was used as a substrate it was not necessary to extract the heptane layer with silicic acid since it does not interfere under the titration conditions used (Ibrahim, Sanders and Thompson, 1964). The specific activity was determined as described previously.

iii. Lipoprotein Substrates - Fatty Acid Release

Stock solutions of tissue preparations were prepared in solutions at such a concentration that could be readily diluted with stock enzyme solutions to give the following concentrations of materials in the incubation mixture:

1. Lipoprotein - 40 µg of lipid phosphorous per ml.
2. Glycyl-glycine buffer, pH 7.4 - 0.05 M.
3. Calcium ions - 1.0 mM.
4. Enzyme protein
   - 1 µg per ml for snake.
   - 15 µg per ml for beef.
   - 25 µg per ml for human.
5. Isotonic solutions
   - sucrose - 0.25 M.
   - NaCl - 0.154 M.
   - KCl - 0.159 M.

In each case the enzyme was pre-incubated with the activating metal ion for 30 minutes at 37° C. The reaction was sampled by adding 1 ml portions of the reaction mixture to 5 ml of Dole's reagent. The samples were well shaken and then sonicated to give a fine suspension of the material in the extracting medium.

Model studies and comparative studies indicated that this method extracted all of the free fatty acid out of the tissue. Following the addition of 3 ml of water and 2 ml of heptane, each of the tubes was briskly shaken for thirty seconds. The phases were allowed to separate and 3 ml of the upper phase was taken for the estimation of the free fatty acid content. The estimations were carried out in the same manner as described under the procedure for determining the specific activity of phospholipase A towards purified phospholipids. Specific activities were calculated as described previously.

iv. Lipoprotein Substrates - Lysophosphatide Formation

a. Egg Yolk Suspensions

An intact egg yolk was separated from the white, washed with 0.9% NaCl and blotted dry with a tissue. A portion (10 ml) of yolk was made up to 50 ml with 0.9% NaCl and 1 ml of this mixture was taken and the amount of phospholipid determined by determining the amount of phosphorous present in the lower phase of a Folch extract (Folch, Lees and Sloan Stanley, 1957). The reaction between the enzyme and the yolk suspension was carried out in 0.9% NaCl containing 0.05 M glycyl-glycine buffer pH 7.4.

The enzyme was made up in 1 ml of 0.9% NaCl and pre-incubated 30 minutes with 1 ml of 10 mM $CaCl_2$ in 0.9% NaCl at 37° C. Eight milliliters of egg yolk suspension containing 10 mg of lipid phosphorous was added to the enzyme solution and the reaction sampled at appropriate times. Samples (1 ml) were taken and added to 20 ml of chloroform-methanol (2:1, v/v). The mixtures were sonicated to give a fine dispersion and filtered through a medium grade sintered glass filter into a graduated mixing cylinder. The precipitate was washed carefully three times with the extracting solvent. Isotonic saline was added to the combined extract to give a Folch biphasic distribution and the cylinder shaken and allowed to stand in the cold until the two phases had separated. The upper phase was aspirated off and discarded. Care was taken to ensure that none of the lower phase was removed. The lower phase was washed with Folch upper phase solvent and transferred to a round bottom flask and the solvent removed under vacuum. The residue was dissolved in chloroform and the sample was again taken to dryness. The residue was then dissolved in a small volume of chloroform, filtered through a fine sintered glass filter and made up to 10 ml. The amount of phosphorous present in the extracts was then determined. The phospholipids were then separated on a silicic acid column. Six grams of silicic acid was

slurried with about 30 ml of chloroform-methanol (2:1, v/v) and the slurry poured into a 1 cm I. D. glass column. The solvent was changed to chloroform when the liquid level had reached the top of the bed. After the column was well washed with chloroform, about 500 µg of lipid phosphorous was put on the column. The neutral lipids were eluted from the column with 50 ml of chloroform and then the solvent was switched to chloroform-methanol (5:1, v/v). The intact ethanolamine-containing phospholipids were eluted with 50 ml of this solvent. The lysophosphatidylethanolamine was eluted with 25 ml of chloroform-methanol (1:1, v/v). The phosphatidylcholine and the majority of the sphingomyelin were eluted in the first 50 ml of chloroform-methanol (1:4, v/v) and the lysophosphatidylcholine was eluted in the next 100 ml of this solvent. Thin-layer chromatography, as described under the purification of phosphatidylcholine and phosphatidylethanolamine, was used to establish the purity and elution pattern of these fractions. Determination of the amount of phosphorous present in each of the fractions gave results from which the amount of hydrolysis of the phosphatidylethanolamine and the phosphatidylcholine could be calculated.

b. Rat Brain Homogenates

The required amount of phospholipase A was made up in 1 ml of water and pre-incubated with 1 ml of 10 mM $CaCl_2$ 30 minutes at 37° C. Eight milliliters of a brain glycyl-glycine buffer sucrose solution (pH 7.4) was added to give an incubation mixture containing 1 g of brain and having a final glycyl-glycine concentration of 0.05 M and which was isotonic with respect to sucrose. The reaction was stopped by the adding the mixture to approximately 200 ml of chloroform-methanol (2:1, v/v). This solvent was used to rinse the incubation flask. The extraction mixture was sonicated for a few minutes to give a fine dispersion and the mixture was filtered into a round bottom flask through a sintered glass filter. The precipitate was extracted three more times with 50 ml portions of chloroform-methanol (2:1, v/v). The combined extract was taken to dryness under vacuum. Fresh chloroform-methanol (2:1, v/v) containing 4% water was added and the mixture was taken to dryness again using more chloroform-methanol (2:1, v/v). The lipid was taken up in chloroform-methanol (2:1, v/v) and a Folch distribution was carried out using 1.12% $CaCl_2$. After separation, the upper phase was removed and discarded and the lower phase solvents were evaporated. Chloroform was used to

remove the last traces of water. The lipid was taken up in chloroform, filtered through a fine sintered glass filter and made up to 10 ml in a volumetric flask. Phosphorous was determined on small portions of each fraction. The lysophosphatidylserine and lysophosphatidylcholine content of each of the fractions was determined as follows:

Six grams of silicic acid was slurried with chloroform-methanol (2:1, v/v) and the slurry poured into a 1 cm I. D. glass column. The solvent was changed to chloroform when the liquid level reached the top of the bed. After the column was well washed with chloroform, approximately 1 mg of lipid phosphorous was applied to the column. Neutral lipids were eluted with 50 ml of chloroform. The acidic phospholipids, both intact and lyso-derivatives, were eluted with 25 ml of chloroform-methanol (1:1, v/v). Choline-containing phospholipids were eluted with chloroform-methanol (1:4, v/v). The first 50 ml of this solvent contained all of the phosphatidylcholine and some of the sphingomyelin. The next 100 ml contained all of the lysophosphatidylcholine and the rest of the sphingomyelin. Thin-layer chromatography was carried out on the first fraction. Glass plates were prepared by spreading them with a slurry of 40 g of silica gel H (Merck, Darmstadt) and 90 ml of 1 mM sodium carbonate. The plates were activated one

remove the last traces of water. The lipid was taken up in chloroform, filtered through a fine sintered glass filter and made up to 10 ml in a volumetric flask. Phosphorous was determined on small portions of each fraction. The lysophosphatidylserine and lysophosphatidylcholine content of each of the fractions was determined as follows:

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hour at 110° C before use. From 10-15 µg of lipid phosphorous was applied to the plate in a streak 5-10 mm long. The plates were developed in chloroform-methanol-acetic acid-water (25:15:3:3, v/v). This is essentially the system employed by Skipski, Peterson and Barclay (1964). After development the plates were dried and sprayed with ninhydrin. The colour was developed in a warm place and the positive areas outlined. The plates were then sprayed with 50% sulphuric acid and charred in a hot oven. The areas corresponding to lysophosphatidylserine were transferred to 10 ml Kjeldahl flasks and the phosphorous content determined. Spots containing measured amounts of the total extract were also analyzed. The amount of lysophosphatidylserine formed was then calculated.

To determine the lysophosphatidylcholine content of the last fraction of the column, a portion of the lipid containing approximately 50 µg of lipid phosphorous was taken to dryness in a small tube. The lipid was dissolved in 0. 5 ml of benzene-methanol (1:1, v/v). A drop of cresol red indicator (0. 1% in ethanol-water (1:1, v/v)) and 0. 5 ml of 0. 1 N methanolic NaOH were added and the tube gently mixed. The tube was incubated at 37° C for 15 minutes with intermittent mixing. After incubation the excess NaOH was neutralized by the addition of ethyl formate, the hydrolysate was

taken to dryness and appropriate volumes of both the aqueous phase solvent (APS) and the organic phase solvent (OPS) from a biphasic solvent system added. The biphasic solvent system was prepared by combining one volume of water with four volumes of chloroform-methanol (2:1, v/v), as described by Folch et al. (1957). Appropriate volumes of each of these solvents were added so that the concentration of phosphorous did not exceed 200 µg per ml in the APS and 50 µg per ml in the OPS. This is essentially the method of Dawson, Hemington and Davenport (1962) and Magee (unpublished). The phosphorous present in the APS results only from the hydrolysis of lysophosphatidylcholine to give glyceryl-phosphorylcholine. The phosphorous present in the OPS represents that present as sphingomyelin, which is not affected by the mild hydrolytic conditions used in this procedure.

The lysophosphatidylethanolamine and lysophosphatidal ethanolamine content of each sample was determined as follows: A 6 g silicic acid column was prepared as described under the first part of this section. The neutral lipids were eluted with 50 ml of chloroform. Phosphatidylethanolamine, phosphatidylserine, ethanolamine plasmalogen and a portion of the lysophosphatidyl serine were eluted with 50 ml of chloroform-methanol (4:1, v/v). The rest of the lysophosphatidylserine and the lysophosphatidyl- and

lysophosphatidalethanolamine were eluted with 25 ml of chloroform-methanol (1:1, v/v). This second fraction was hydrolyzed as described under Part A of this section. In this case the OPS contains the lysophosphatidalethanolamine and the deacylated alkyl ether ethanolamine-containing phospholipids. The phosphorous present in this phase is reported as lysophosphatidalethanolamine since no attempt was made to determine the amount of the deacylated alkyl ether phospholipid present in this fraction. The APS from this hydrolysis contains glycerylphosphorylethanolamine formed by the hydrolysis of lysophosphatidylethanolamine and glycerylphosphorylserine formed from the hydrolysis of the small amount of lysophosphatidylserine present in the chloroform-methanol (1:1, v/v). These two water-soluble products were separated by paper chromatography as described by Spencer and Dempster (1962). After development the papers were dried and washed in ether to remove the excess phenol. The paper was dried and then sprayed with ninhydrin. The spots corresponding to glycerylphosphoryl-ethanolamine were cut out, along with spots containing a known amount of the APS. The paper spots were minced and transferred to 40 ml Kjeldahl tubes. The paper was charred in a boiling water bath after the addition of 1 ml of 72% perchloric acid and 1 drop of 1% ammonium molybdate. After this preliminary charring the amount of phosphorous was determined as described above.

IV RESULTS

A. Purification of the Enzymes

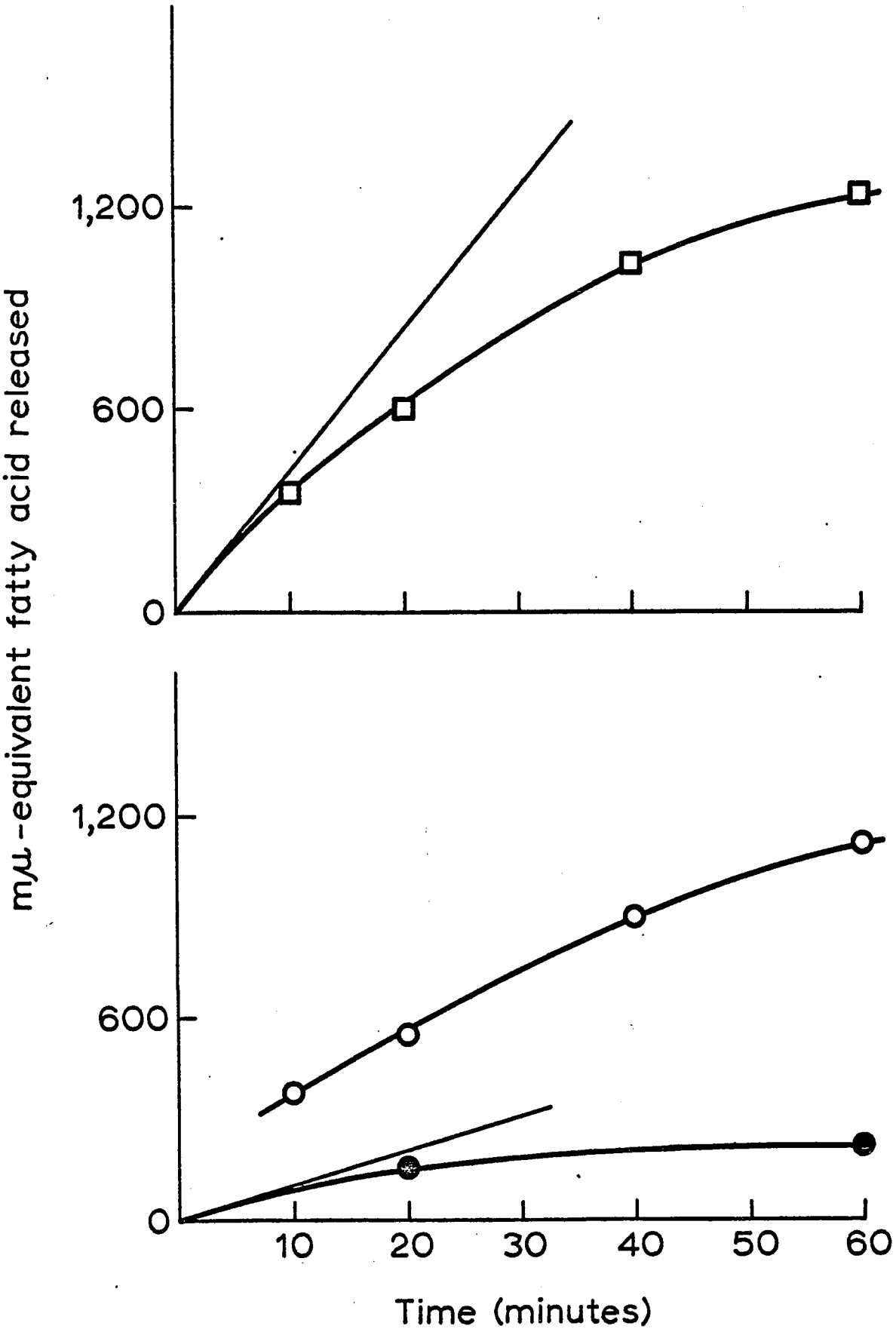
Representative reaction rate curves which were obtained during these investigations are shown in Fig. 1. The upper curve illustrates an average plot used for the determination of an initial reaction rate. The initial reaction rate was calculated from a tangent drawn to the curve at zero time. The lower two curves are typical of the results obtained in certain experiments in which lecithin was the substrate. With high levels of the enzyme, especially the bovine enzyme, there appeared to be an initial burst of activity which was followed by a relatively linear rate of hydrolysis for a considerable time period. The lower curve in this set demonstrates the hydrolysis of lecithin in the absence of deoxycholate. It can be seen that the percentage hydrolysis of lecithin when deoxycholate is omitted from the system is low and usually does not go beyond 20 per cent.

The specific activity and the total activity of the enzymes were determined at various stages during their purifications. Except for the purification of the bovine pancreatic enzyme, the specific activity was calculated from the initial reaction rate and, in all

Figure 1

Representative Hydrolysis Curves Obtained during the Hydrolysis of Purified Phospholipids by Phospholipase A

The upper figure represents the hydrolysis of lecithin in the presence of sodium deoxycholate. The upper curve of the lower figure represents the hydrolysis of lecithin in the presence of sodium deoxycholate and high levels of the bovine pancreatic enzyme. The lower curve represents the hydrolysis of lecithin in the absence of sodium deoxycholate.


mμ-equivalent fatty acid released
1,200
600
0
1,200
600
0
10
20
30
40
50
60
Time (minutes)

cases, is reported in terms of the number of millimicroequivalents of fatty acid released per minute per milligram of protein. Purification of the mammalian enzymes was carried out at least twice. The results agreed reasonably well in each case. The large yield of product obtained in the initial purification of the snake venom rendered duplication of the procedure at this level unnecessary. However, a similar experiment with a small (10 mg) amount of venom gave essentially the same results.

The specific activity and the total activity of the various fractions obtained during the purification of the phospholipase A isolated from beef pancreas are shown in Table 1. The specific activity was not calculated from the initial reaction rate but from the linear portion of the curve. With Fraction D the specific activity was calculated from both the initial reaction rate and the linear portion of the curve. The specific activity shown in brackets in Table 1 refers to that calculated from the initial reaction rate. During dialysis of the glycerinated extract, an appreciable amount of precipitate formed in the dialysis tubing. The supernatant, referred to as Fraction B, had an increased activity over that of the glycerinated extract. The total activity of Fraction B was about double that of the glycerinated extract. Heat treatment of Fraction B increased the specific activity of the preparation

TABLE 1

Phospholipase A Activity[a] of Various Preparations from Bovine Pancreas

Enzyme Source	Fraction Weight (mg)[b]		Specific Activity[c]		Total Activity	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Glycerinated Extract	623	580	7.8	8.9	4,870	5,150
Fraction B	364	378	25	27.5	9,100	10,400
Fraction C	177	192	34	35	6,200	6,750
Fraction D	19	18	300 (2,000)[d]	315	5,650	5,700

a - Enzyme estimations were carried out in the lecithin-deoxycholate reaction system.

b - Milligrams of protein from one gram of acetone powder.

c - Specific activities are expressed as millimicroequivalents of fatty acid released per minute per milligram of protein and were calculated from the linear portion of the hydrolysis curve.

d - Specific activity calculated from the initial reaction rate.

but in this case about one-third of the total activity was lost. The column purification of the enzyme (Fig. 2) resulted in a marked increase in specific activity; at the same time there was no significant change in the total activity of the preparation. The active peak was lyophilized. The resulting powder had a protein concentration of about 50 mg per g dry weight. This fraction was analyzed by cellulose acetate electrophoresis at two different pH's. Only one band was found in this preparation in each of the runs. The electrophorograms are shown in Fig. 3.

The specific activity and the total activity of the various fractions obtained during the attempted purification of the phospholipase A present in acetone powders of dog pancreas are shown in Table 2. It is apparent that the activities are much less than those found in any of the other materials used. For example, the heated extract had a specific activity of less than 15. This small amount of activity was lost when the fraction was lyophilized.

The specific activity and the total activity of fractions obtained during the purification of the phospholipase A present in C. adamanteus venom are shown in Table 3. It can be seen that there was no loss of either protein or activity during the heat treatment. The elution pattern for the separation of the enzyme on the molecular sieve, Bio-gel P-60, is shown in Fig. 4. The initial large peak represents

Figure 2

Column Chromatography of the Heated Beef Pancreatic Fraction on SE-Sephadex

Five ml of 0. 02 M Tris buffer pH 7. 4 containing 25 mg of lyophilized Fraction C was applied to the column and the gradient developed with 2 M NaCl in 0. 02M Tris buffer pH 7. 4. Three ml fractions were collected and the chromatogram determined by measuring the optical density at 280 mμ and measuring the protein concentration of each tube. The hatched bars indicate the fractions that contained phospholipase A activity. These were lyophilized as Fraction D.

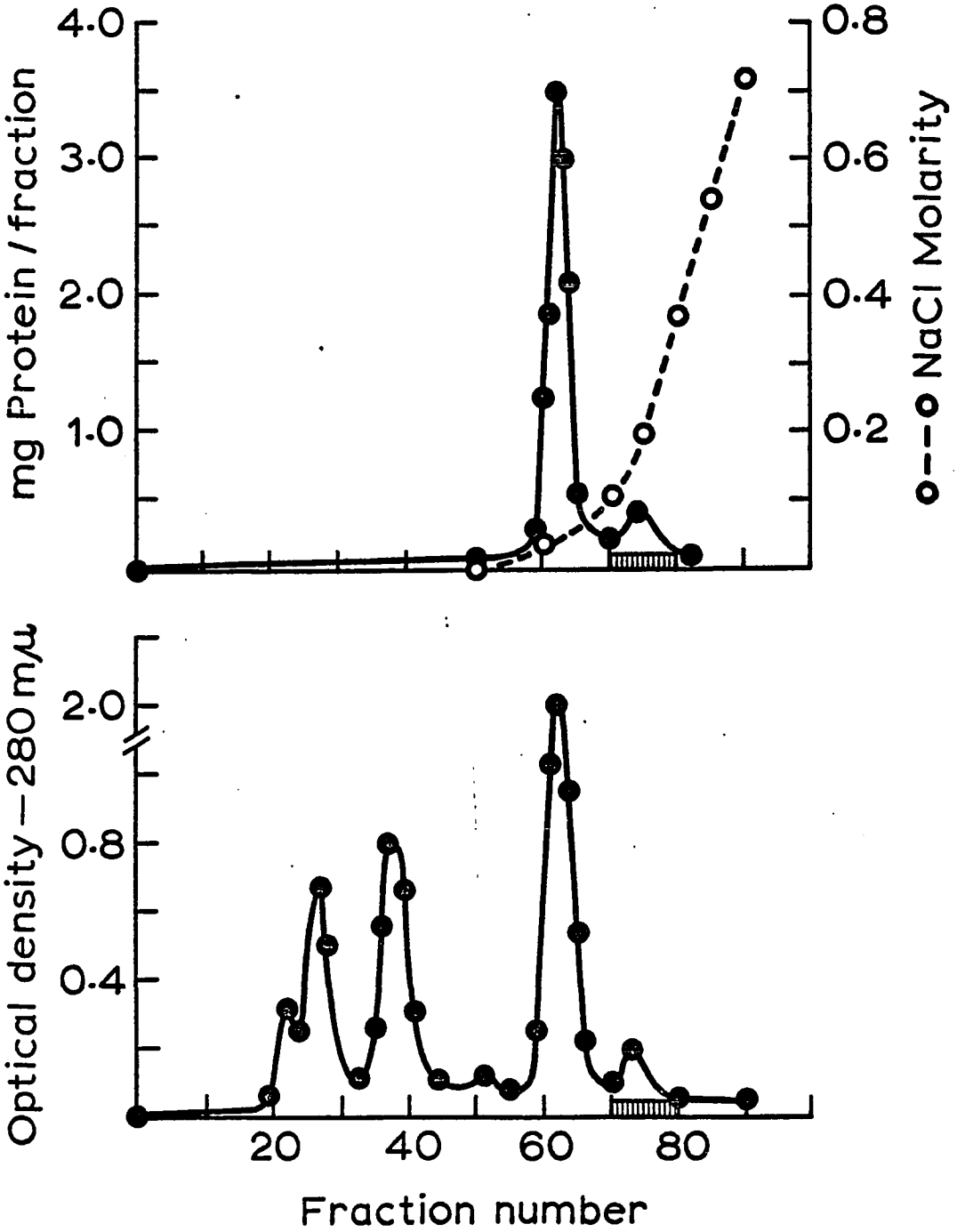

4·0
3·0
2·0
1·0
mg Protein / fraction
0·8
0·6
0·4
0·2
NaCl Molarity
Optical density — 280 mμ
2·0
0·8
0·4
20
40
60
80
Fraction number

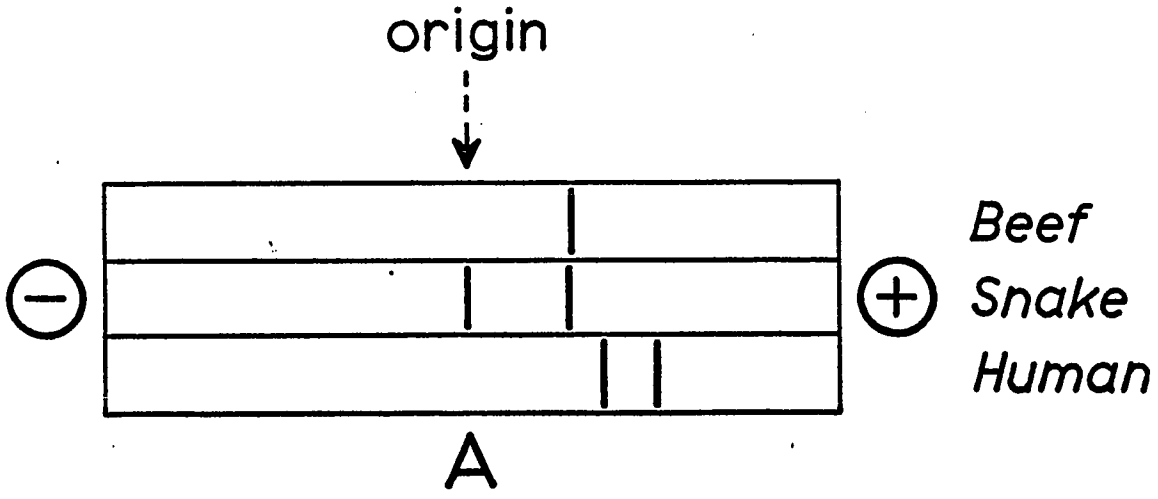
Figure 3

Cellulose Acetate Electrophoresis of Beef, Snake and Human Phospholipase A Preparations

A - Gelman H. R. buffer; pH 8. 6.

B - 0. 025 M sodium acetate buffer; pH 5. 0.

Other conditions as described in the text.


origin
Beef
Snake
Human
A

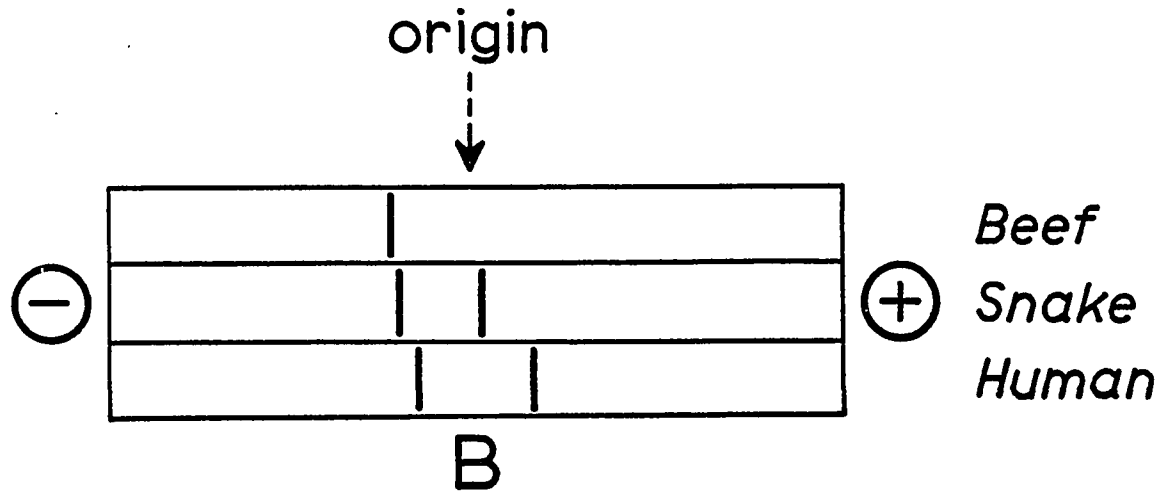

origin
Beef
Snake
Human
B

TABLE 2

Phospholipase A Activity[a] of Various Preparations from Canine Pancreas

Enzyme Source	Fraction Weight (mg)[b]		Specific Activity[c]		Total Activity	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Glycerinated Extract	650	615	5	8	3, 320	4, 850
Fraction B	434	465	10	8	4, 340	3, 720
Fraction C	163	127	15	12	2, 500	1, 490
Fraction D	-	-	0	0	-	-

a - Enzyme estimations were carried out in the lecithin-deoxycholate reaction system.

b - Milligrams of protein from one gram of acetone powder.

c - Specific activities are expressed as millimicroequivalents of fatty acid released per minute per milligram of protein and were calculated from the initial reaction rate.

TABLE 3

Phospholipase A Activity[a] of Various Preparations of C. adamanteus Venom

Enzyme Source	Fraction Weight (mg)[b]	Specific Activity[c]	Total Activity
Lyophilized Venom	95	8,750	840,000
Heated Venom	95	8,750	840,000
Column Fraction	18	20,000	356,000

a - Enzyme estimations were carried out in the lecithin-deoxycholate reaction system.

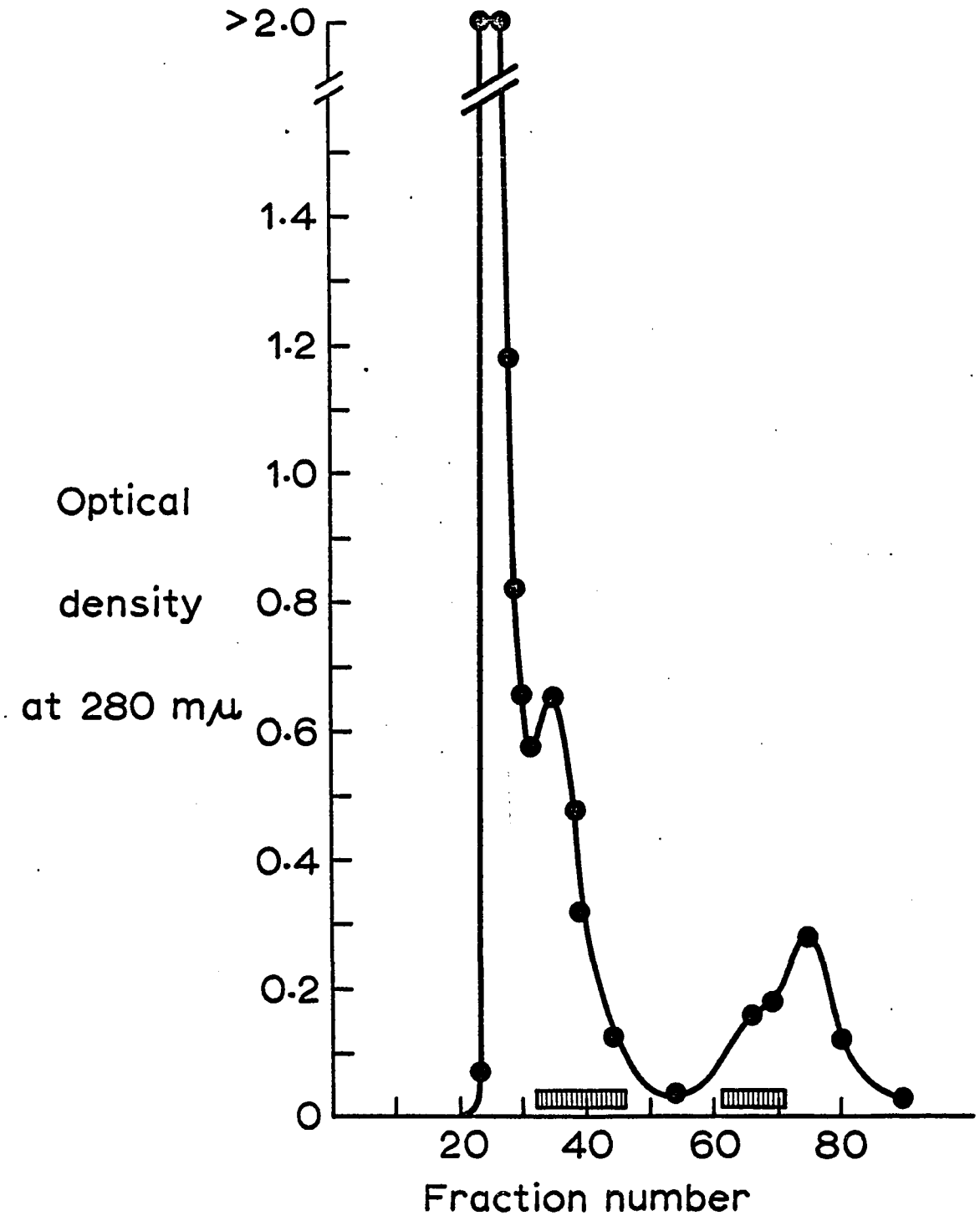
b - Milligrams of protein from 100 mg of lyophilized venom.

c - Specific activities are expressed as millimicroequivalents of fatty acid released per minute per milligram of protein and were calculated from the initial reaction rate.

Figure 4

Separation of Heat-treated C. adamanteus Venom on the Molecular Sieve; Bio-gel P-60

An amount of heated snake venom equivalent to 100 mg of crude venom in 0.9% NaCl was applied to the column and the protein eluted with 0.9% NaCl. The hatched bars indicate the fractions which contained phospholipase A activity.


>2·0
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
Optical
density
at 280 mμ
20
40
60
80
Fraction number

inert aggregated protein and appears at the void volume of the column (as determined by the use of Blue Dextran 2000; Pharmacia). Two active peaks were obtained, but the activity of the first peak was much greater than the second. The second peak was discarded. The first peak was lyophilized. The resulting powder had a protein concentration of less than 50 mg per g dry weight. This fraction was analyzed electrophoretically at pH 5. 1 and 8. 6. The electropherograms are shown in Fig. 3. Only one band was found away from the origin. Some Amido Black B-stainable material was found at the origin in both of the runs. Presumably this represents inert protein from the first peak.

The specific activity and the total activity of various fractions obtained during the purification of the phospholipase A present in human pancreas are shown in Table 4. The specific activity was increased by a factor of four over that of the glycerinated extract, but the increase over that found in the acetone powder is greater (approximately tenfold). Electrophoretic analysis of Fraction C gave two distinct bands at both pH's (Fig. 3). Column chromatography of this fraction on a molecular sieve (Bio-gel P-60) gave two protein peaks, neither of which corresponded well to the location of the activity eluted from the column. The total amount of activity eluted was low, however. Ultraviolet absorption measurements at 260 and

TABLE 4

Phospholipase A Activity[a] of Various Preparations from Human Pancreas

Enzyme Source	Fraction Weight (mg)[b]		Specific Activity[c]		Total Activity	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Glycerinated Extract	375	435	148	115	55, 000	50, 000
Fraction B	165	163	332	298	54, 800	48, 600
Fraction C	56	23	675	530	37, 800	12, 200

a - Enzyme estimations were carried out in the lecithin-deoxycholate reaction system.

b - Milligrams of protein from one gram of acetone powder.

c - Specific activities are expressed as millimicroequivalents of fatty acid released per minute per milligram of protein and were calculated from the initial reaction rate.

280 mμ indicated that this fraction was still badly contaminated since a 260/280 ratio of approximately three was found. Attempts were made to purify the final fraction (Fraction C) by ion exchange chromatography. These were not successful as little, if any, activity could be detected in the eluant.

The apparent molecular weights of the three enzymes were estimated by determining their position of elution on a molecular sieve from which they were partially excluded. Various proteins of known molecular weight were used to calibrate the column. The results are plotted in Fig. 5. The molecular weights for the various phospholipases A preparations were:

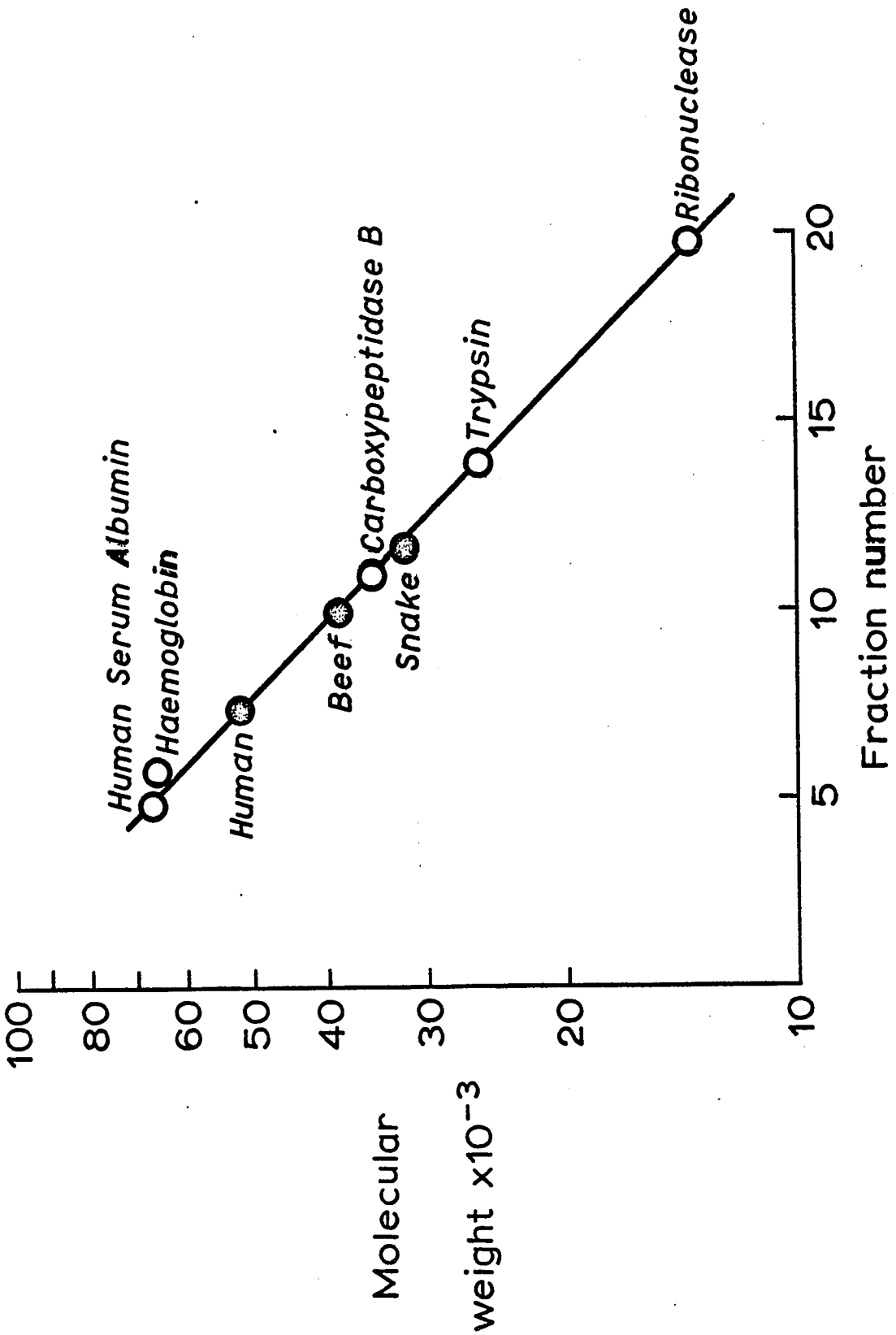
- Beef enzyme - 39,000
- Snake enzyme - 31,000
- Human enzyme - 51,000.

The three purified preparations were analyzed for activity towards trilaurin and lysolecithin in the purification assay system. Equivalent amounts of either substrate were used instead of the lecithin normally added. In all cases there was no activity towards either of these substrates, indicating that the preparations contained no lipase (glycerol ester hydrolase) or phospholipase B (lysolecithinase) activity.

Figure 5

Determination of the Molecular Weights of the Phospholipase A Preparations

The elution volume of each protein was determined for Bio-gel P-60 in 0.9% NaCl.


Human Serum Albumin
Haemoglobin
Human
Beef
Carboxypeptidase B
Snake
Trypsin
Ribonuclease
100
80
60
50
40
30
20
10
5
10
15
20
Molecular weight x10⁻³
Fraction number

B. Action of the Enzymes on Purified Phospholipids and Lipoprotein

The specific activities of each of the phospholipase A preparations as determined with purified phospholipid substrates in the presence and the absence of sodium deoxycholate are shown in Table 5. In each case the enzyme was pre-incubated with calcium ions for 30 minutes at 37° before use. The rate of lecithin hydrolysis by all of the enzymes was markedly accelerated in the presence of sodium deoxycholate. The reaction tended to go to completion in the presence of the activator, but in its absence the reaction rate leveled off with at least 75 per cent of the substrate remaining unhydrolyzed (Fig. 1). The presence of deoxycholate increased the specific activity of all three enzymes by a factor of approximately ten.

The effect of the activator on the hydrolysis of phosphatidylethanolamine is much more complex. The addition of sodium deoxycholate to the substrate in the case of the snake enzyme resulted in a marked activation of the reaction. Its addition in the case of the mammalian enzymes caused an inhibition which was greater with the bovine than with the human enzyme.

The results obtained with phosphatidylserine as the substrate were similar to those obtained using lecithin. The reptilian enzyme

TABLE 5

Hydrolysis of Purified Phospholipids
by Phospholipase A Preparations
in the Presence or Absence of Sodium Deoxycholate

Values shown are specific activities expressed as millimicro-equivalents of fatty acid released per minute per milligram of protein. All reaction systems contained 1.0 mM calcium ion.

Enzyme source	With DOC	Without DOC
	Phosphatidylcholine	
Snake venom	20,000	3,250
Human pancreas	680	50
Beef pancreas	1,930	133
	Phosphatidylethanolamine	
Snake venom	20,800	11,800
Human pancreas	1,770	2,100
Beef pancreas	1,970	3,800
	Phosphatidylserine	
Snake venom	14,500	0
Human pancreas	670	325
Beef pancreas	2,670	670
	Phosphatidylinositol	
Snake venom	8,000	0
Human pancreas	405	125
Beef pancreas	1,170	510

Values shown are mean values from duplicate determinations.

was completely inactive in the absence of deoxycholate. The mammalian enzymes did not show this obligatory requirement for the activator. Their activities were increased about two times for the human enzyme and about four times for the beef enzyme by its addition.

The effect of sodium deoxycholate on the hydrolysis of phosphatidylinositol resembles its effect with phosphatidylserine. Once again the snake enzyme showed an obligatory requirement for the presence of the activator in the reaction system. The mammalian enzymes could hydrolyze phosphatidylinositol in the absence of sodium deoxycholate but, in each case, the reaction rate was increased at least twofold by its addition.

The effect of pre-incubation of the enzymes with various metal ions on their specific activity towards lecithin in the presence of sodium deoxycholate in shown in Table 6. All three phospholipase A preparations were activated by the presence of calcium ions. None of the other metal ions tested could replace calcium. Added magnesium ions appeared to have little effect on the reaction, while manganese ions were inhibitory. The effect of the metal ions in the absence of sodium deoxycholate was not tested since the activities of the enzymes were very low in this system, even in the presence of calcium ions. The addition of 1 mM EDTA completely

TABLE 6

The Effect of Various Ions on the Specific Activity of Phospholipase A Preparations towards Phosphatidylcholine in the Presence of Sodium Deoxycholate

Specific activity: millimicroequivalents of fatty acid released per minute per milligram of protein.
Final concentration of all ions added to the reaction: 1 mM.

Ion added	Snake enzyme	Human enzyme	Beef enzyme
None	11,000	540	1,330
Ca	20,000	680	1,930
Mg	9,500	412	1,330
Mn	3,000	115	770
EDTA	0	0	0

Values shown are mean values from duplicate determinations.

inhibited the hydrolysis of lecithin by all three phospholipase A preparations.

Pre-incubation of the enzymes with metal ions also affected the hydrolysis of phosphatidylethanolamine (Table 7). Once again calcium ions increased the reaction rate. Magnesium ions were again inert, causing neither stimulation nor inhibition of the reaction. Zinc ions inhibited the reaction whether sodium deoxycholate was present or not. The effect of added sodium deoxycholate on the snake enzyme was one of marked stimulation. In the absence of the activator, activity was found only if the enzyme had been pretreated with calcium.

The results are quite different for the human enzyme. The addition of sodium deoxycholate to the reaction system was not only not necessary for activity in the absence of calcium ions but, in fact, resulted in some inhibition of the human enzyme. Once again stimulation of the activity was found with calcium ions and magnesium ions did not affect the reaction to an appreciable extent. Zinc ions inhibited the hydrolysis of phosphatidylethanolamine both in the presence and the absence of sodium deoxycholate.

The results (Table 7) with the phospholipase A isolated from beef pancreas were very similar to those found with the human pancreatic enzyme. The presence of sodium deoxycholate resulted

TABLE 7

The Effect of Various Ions on the Specific Activity of Phospholipase A Preparations towards Phosphatidylethanolamine in the Presence or Absence of Sodium Deoxycholate

Specific Activity: Millimicroequivalents of fatty acid released per minute per milligram of protein. Final concentration of all ions added to the reaction system: 1 mM. Sodium deoxycholate was added at a level of 2 mg per 40 µg lipid phosphorous.

Ion added	Snake enzyme		Human enzyme		Beef enzyme	
	with DOC	without DOC	with DOC	without DOC	with DOC	without DOC
None	3, 300	0	900	1, 125	865	800
Ca	20, 800	11, 800	1, 800	2, 100	1, 970	3, 800
Mg	3, 950	0	1, 060	1, 235	865	935
Zn	935	0	340	340	100	380
EDTA	0	0	0	0	0	0

Values shown are mean values from duplicate determinations.

in an inhibition of hydrolysis in those experiments where the enzyme was pretreated with metal ions. Calcium ions again activated the reaction while zinc ions inhibited partially and EDTA inhibited completely.

The effects of various metal ions on the hydrolysis of phosphatidylserine by phospholipase A preparations are shown in Table 8. With this substrate the rate of hydrolysis was quite dependent on the presence of sodium deoxycholate in the reaction medium. In the case of the snake enzyme, the reaction was completely dependent on the addition of sodium deoxycholate even when the enzyme had been pretreated with calcium ions. Here too, calcium ions were found to stimulate all three enzymes. Magnesium ions, in contrast to the results obtained in the studies on phosphatidylcholine and phosphatidylethanolamine, partially replaced calcium ions in activating the reaction. Zinc ions partially inhibited the hydrolysis of phosphatidylserine while EDTA completely inhibited the reaction.

The effect of calcium ion concentration on the hydrolysis of phosphatidylethanolamine in the absence of sodium deoxycholate is shown in Table 9. The enzymes were pre-incubated with the ion, but the values shown are those present in the final reaction system. The values are expressed as a percentage of the specific

TABLE 8

The Effect of Various Ions on the Specific Activity of Phospholipase A Preparations towards Phosphatidylserine in the Presence or Absence of Sodium Deoxycholate

Conditions and expression of results as in Table 7.

Ion added	Snake enzyme		Human enzyme		Beef enzyme	
	with DOC	without DOC	with DOC	without DOC	with DOC	without DOC
None	2,750	0	228	40	2,270	312
Ca	14,500	0	670	325	2,670	670
Mg	10,500	0	510	130	2,480	535
Zn	1,000	0	100	40	0	360
EDTA	0	0	0	0	0	0

TABLE 9

The Effect of Calcium Ion Concentration on the Specific Activity of Phospholipase A Preparations towards Phosphatidylethanolamine in the Absence of Sodium Deoxycholate

Values are expressed as a percentage of the specific activity when the calcium ion concentration was 1.0 mM.

Calcium ion concentration (mM)	Snake enzyme	Human enzyme	Beef enzyme
0	0	50	21
0.5	100	86	42
1.0	100	100	100
2.0	100	240	300
5.0	100	100	207
10.0	100	54	207

Values shown are mean values from duplicate determinations.

activities found when calcium ions were present at a concentration of 1.0 mM. The results show that the optimal calcium ion concentration is greater than 1.0 mM for the mammalian enzymes. The minimum concentration of calcium needed for optimal activity of the snake enzyme has not been determined since the maximum activity was obtained with the smallest amount of calcium used. Increasing the calcium ion concentration by a factor of twenty did not affect the specific activity. The optimal calcium ion concentration for the mammalian enzymes was approximately 2 mM. Higher levels resulted in a lessening of the activity, especially in the case of the human enzyme. Optimal calcium ion concentrations could not be determined in reaction systems containing sodium deoxycholate, since concentrations of the ion greater than 1 mM caused precipitation.

Since it has been shown (Magee et al., 1962; Dawson, 1963) that phospholipase A activities could be increased by the addition of low concentrations of EDTA, the effect of varying concentrations of this reagent on the specific activities of the three enzyme preparations was determined. The results for the snake and the beef enzyme are shown in Table 10. It can be seen that concentrations of this chelating agent of one-tenth to one-hundredth that required for the inhibition of the reaction resulted in a stimulation

TABLE 10

The Effect of Ethylenediaminetetraacetate Concentrations on the Specific Activity of C. adamanteus Venom and Beef Pancreatic Phospholipase A Preparations towards Phosphatidylcholine in the Presence of Sodium Deoxycholate

Values shown are mean values from duplicate determinations.

EDTA concentration (mM)	Snake venom enzyme	Beef pancreas enzyme
1.0	0	0
0.1	0	0
0.01	8,000	1,230
0.001	9,500	1,000
0.0001	8,000	750
0	8,000	750
1 mM EDTA + 2 mM $CaCl_2$	24,000	1,670

of the activity of the enzymes, particularly the beef enzyme, when compared to the reaction in the absence of the chelator. Addition of excess calcium ions to the inhibited enzyme, either along with the chelator or after pre-incubation of the chelator with the enzyme resulted in recovery of the activity and, in the case of the snake enzyme a greater recovery of activity than found in the absence of the chelator. Since it has been postulated (Magee et al., 1962; Dawson, 1963) that this activating effect of EDTA is due to the removal of heavy metal ions, a study of the effect of various divalent metal ions and divalent metal ion complexing agents on the activity of the human enzyme in the presence of sodium deoxycholate was carried out. The results are tabulated in Table 11. As found before, of all of the metal ions tested only calcium caused an increase in the reaction rate. All of the other alkali earth metal ions and all of the transition metal ions tested inhibited the reaction. Of the four divalent cation-complexing agents tested only EDTA caused any inhibition of the system. Pre-treatment of the enzyme with N-ethylmaleimide resulted in a partial inhibition of the enzyme.

The effect of carrying out the hydrolysis of lecithin in the presence of sodium deoxycholate in various isotonic solutions as opposed to the use of water as a carrying medium is shown in

TABLE 11

The Effect of Various Divalent Metal Ion Complexing Agents on the Activity of Human Pancreatic Phospholipase A Preparation towards Phosphatidylcholine in the Presence of Sodium Deoxycholate

Values shown are mean values from duplicate determinations.

Agent added	Concentration	Specific Activity
EDTA	1.0 mM	0
EDTA	0.1 mM	0
EDTA	0.01 mM	540
EDTA	0.001 mM	720
EDTA	0.0001 mM	600
None	-	540
$CaCl_2$ + EDTA	2.0 mM + 1.0 mM	600
Oxalate	1 mM	560
Ferrocyanide	1 mM	560
Sulphide	1 mM	520
Ca	1 mM	680
Mg	1 mM	412
Mn	1 mM	115
Ba	1 mM	57
Cd	1 mM	57
Sr	1 mM	57
Zn	1 mM	0
N-ethylmaleimide	1 mM	460

Table 12. It must be emphasized that the compared homogenates also contained the usual buffers and ions at pH 7.4 as described in the Methods. Carrying out the reaction in the presence of isotonic sucrose instead of water did not affect the reaction rate appreciably. Use of either isotonic sodium or potassium chloride as a carrying medium resulted in an inhibition of both mammalian enzyme preparations. These alkali metal ions did not inhibit the phospholipase A from C. adamanteus venom.

The action of the three phospholipase A preparations on various lipoprotein preparations is shown in Table 13. The snake enzyme was capable of hydrolyzing the phospholipids in aqueous egg yolk suspensions (without added deoxycholate) at a rate equivalent to that found with the lecithin-deoxycholate reaction system. Although the beef enzyme attacked egg yolk lipoproteins in water, it was not as active towards this substrate as it was in a comparable substrate system containing lecithin solubilized with sodium deoxycholate (Table 12). With both the snake and the beef enzymes (Table 13) suspension of the egg yolk in isotonic saline caused a marked reduction in specific activity. Egg yolk suspensions in either water or saline were not attacked by the phospholipase A prepared from human pancreas.

Water or isotonic sucrose homogenates of rat brain were

TABLE 12

The Effect of the Medium on the Activity of Phospholipase A Preparations towards Phosphatidylcholine in the Presence of Sodium Deoxycholate

Values shown are mean values from duplicate determinations.

Medium	Specific Activity		
	Snake enzyme	Human enzyme	Beef enzyme
Water	18, 000	525	1, 900
Isotonic sucrose	18, 000	525	1, 900
Isotonic sodium chloride	20, 000	200	900
Isotonic potassium chloride	22, 500	200	900

susceptible to attack by all three enzymes. Preparation of this substrate in isotonic saline, however, rendered the phospholipids completely inert to the action of any of the enzymes.

The snake enzyme was considerably less active in the water or isotonic sucrose homogenates of rat brain than it was in the lecithin-deoxycholate reaction system. The human enzyme was found to have a higher specific activity with water or isotonic sucrose homogenates of rat brain than it had with any other substrate tested in all the experiments reported in this thesis. All three enzymes catalyzed the release of fatty acids from rat brain slices incubated in isotonic sucrose (Table 13), but at reduced rates compared with sucrose homogenates.

Beef serum, either with or without added deoxycholate, appeared to be an ideal substrate for the phospholipase A prepared from snake venom. The addition of deoxycholate to beef serum was necessary for hydrolysis by the phospholipase A from beef pancreas. The human enzyme was completely inactive towards beef serum phospholipids. Intact bovine erythrocytes were not attacked by any of the enzymes under the experimental conditions selected (Table 13), confirming earlier studies, and demonstrating that the refractiveness of the erythrocyte to phospholipase A attack is not simply due to the isotonic saline generally used as an

TABLE 13

Activity of Phospholipase A Preparations
towards Lipoprotein Substrates
Prepared in a Variety of Media as Described under Methods

When present sodium deoxycholate was added at a concentration of 2 mg for every 40 µg of phospholipid phosphorous.

Values shown are mean values from duplicate determinations.

Lipoprotein substrate	Medium	Enzyme Preparations		
		Snake	Human	Beef
Egg yolk	Water	26,400	0	540
Egg yolk	Isotonic saline	5,600	0	145
Rat brain homogenate	Water	14,000	3,900	1,400
Rat brain homogenate	Isotonic sucrose	14,000	3,900	1,400
Rat brain homogenate	Isotonic saline	0	0	0
Rat brain slices	Isotonic sucrose	5,500	1,000	600
Beef serum	Water plus deoxycholate	31,000	0	600
Beef serum	Water without deoxycholate	30,000	0	0
Beef erythrocytes	Isotonic saline	0	0	0
Beef erythrocytes	Isotonic sucrose	0	0	0

incubation medium in red cell studies.

In certain experiments involving egg yolk suspensions in saline the major lysophosphatides produced at various time intervals were determined individually. These were isolated chromatographically. A typical column run is illustrated in Fig. 6. The phospholipids were eluted from these columns in the order: phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine plus sphingomyelin and lysophosphatidylcholine. Only the beef and the snake enzymes were used in these studies since the enzyme prepared from human pancreas did not attack egg yolk suspensions. The results are shown in Tables 14 and 15. The percentage hydrolysis figures shown in brackets were calculated using the original concentration of the parent diacyl glycerophosphatides in the egg yolk suspensions.

The beef enzyme showed a partial preference for phosphatidylethanolamine (Table 14) since the percentage hydrolysis of this phospholipid was higher than that for the phosphatidylcholine at all sampling times.

The snake enzyme (Table 15) appeared to have little or no preference for either phosphatidylcholine or phosphatidylethanolamine.

The total amount of phospholipid phosphorous extracted from equal portions of the suspension did not change during the course of the reaction, showing that any lysophosphatides produced were

Figure 6

The Separation of Phospholipase A-Treated Egg Yolk Phospholipids on Silicic Acid

Five hundred micrograms of phospholipid phosphorous was chromatographed on a 6 g, 1 cm I. D. silicic acid column. The solvent compositions were as follows:

A - chloroform-methanol (5:1, v/v)

B - chloroform-methanol (1:1, v/v)

C - chloroform-methanol (1:4, v/v)

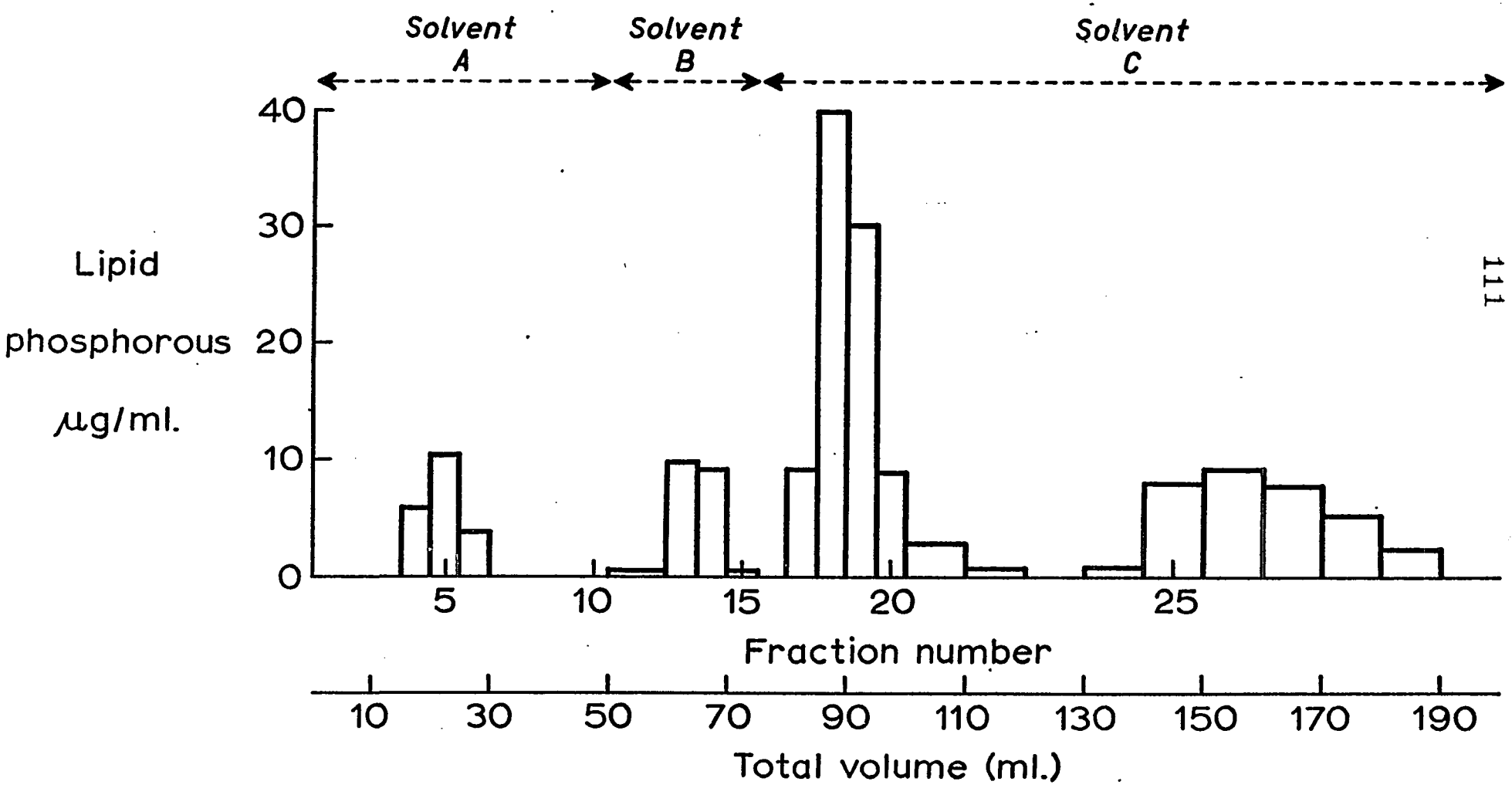

Solvent A
Solvent B
Solvent C
Lipid phosphorous µg/ml.
40
30
20
10
0
5
10
15
20
25
Fraction number
10
30
50
70
90
110
130
150
170
190
Total volume (ml.)

TABLE 14

Formation of Lysophosphatides in Egg Yolk Suspensions by a Beef Pancreatic Phospholipase A Preparation

Incubation as described in Methods, employing an initial phospholipid phosphorous concentration in the reaction system of 1 mg per ml. Values shown are for duplicate experiments expressed as µg of lysophosphatide phosphorous formed per mg of original substrate phosphorous. The mean percentage hydrolysis values are shown in brackets.

	Reaction period in minutes			
Lysophosphatide formed	10	30	60	120
Lysophosphatidyl-ethanolamine	11, 4 (3%)	32, 25 (13%)	74, 63 (33%)	127, 126 (60%)
Lysophosphatidyl-choline	9, - (1%)	52, 62 (8%)	164, 144 (19%)	319, 322 (40%)

NOTE - No decrease in total phospholipid phosphorous was observed during the incubation.

- The enzyme was used at a concentration of 50 µg per ml.

TABLE 15

Formation of Lysophosphatides in Egg Yolk Suspensions by a C. adamanteus Venom Phospholipase A Preparation

Conditions and expression of results as in Table 14.

	Reaction period in minutes			
Lysophosphatide formed	10	30	60	120
Lysophosphatidyl-ethanolamine	22, 28 (11%)	33, 39 (16%)	65, 59 (28%)	86, 90 (40%)
Lysophosphatidyl-choline	62, 61 (8%)	133, 143 (17%)	209, 204 (27%)	305, 301 (40%)

NOTE - No decrease in total phospholipid phosphorous was observed during the incubation.

- The enzyme was used at a concentration of 1 µg per ml.

not hydrolyzed further.

All three phospholipases attacked homogenates of rat brain prepared using isotonic sucrose. The formation of lysophosphatides was measured after either a thirty or a ninety minute reaction period. The separation of the various lysophosphatides was carried out using a variety of chromatographic techniques (see Methods). The separation of phospholipase A- treated brain phospholipids is illustrated in Fig. 7. The upper chromatogram demonstrates the separation of the intact acidic phosphatides and their corresponding lysoderivatives (the first peak) from the choline-containing phospholipids. The second peak contained phosphatidylcholine and the majority of the sphingomyelin and the last peak contained the rest of the sphingomyelin and the lysophosphatidylcholine.

The lower figure shows the separation of the acidic phospholipids into two peaks (the first two). The first of these contained all of the phosphatidylserine, all of the ethanolamine plasmalogen, all of the phosphatidylethanolamine and some of the lysophosphatidylserine. The second peak contained all of the lysophosphatidylethanolamine, all of the lysoethanolamine plasmalogen and the rest of the lysophosphatidylserine. The third peak contained the intact choline-containing phosphatides and the fourth peak the

Figure 7

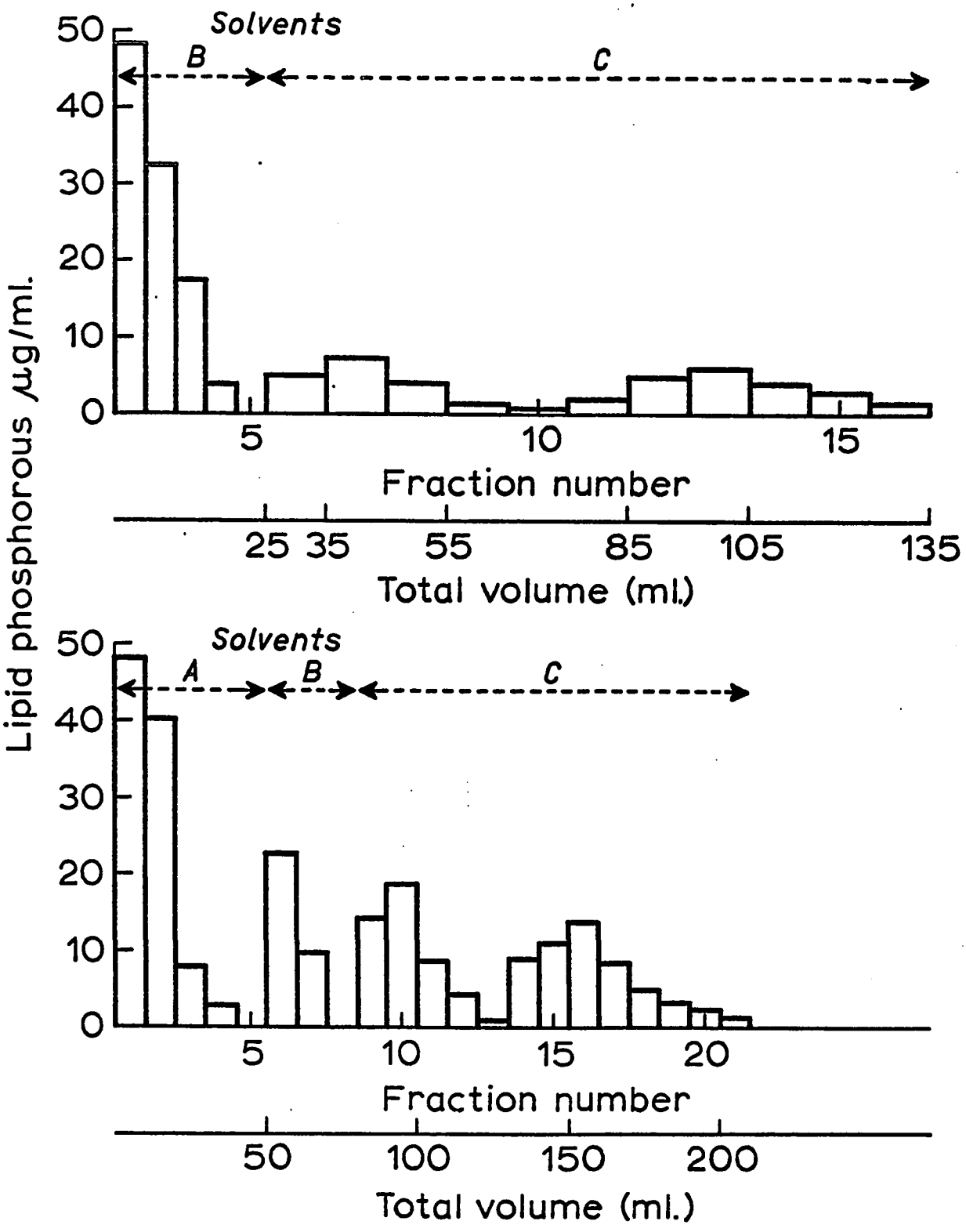
The Separation of Phospholipase A Treated Rat Brain Phospholipids on Silicic Acid Columns

One milligram of phospholipid phosphorous was chromatographed on 6 g, 1 cm I. D. silicic acid columns as described in the text. The solvent compositions were as follows:

A - chloroform-methanol (4:1, v/v)

B - chloroform-methanol (1:1, v/v)

C - chloroform-methanol (1:4, v/v)


Solvents
B
C
50
40
30
20
10
0
5
10
15
Fraction number
25 35
55
85
105
135
Total volume (ml.)
Lipid phosphorous µg/ml.
Solvents
A
B
C
50
40
30
20
10
0
5
10
15
20
Fraction number
50
100
150
200
Total volume (ml.)

lysophosphatidylcholine contaminated with a small amount of sphingomyelin.

As pointed out in the Methods, the first column was used to prepare an acidic phospholipid fraction from which the lysophosphatidylserine could be separated by thin-layer chromatography. The second column was used to isolate fractions from which the individual ethanolamine-containing lysophosphatides could be determined.

The formation of lysophosphatides by the beef pancreatic phospholipase A preparation is shown in Table 16. All four major phospholipids of brain were attacked by the enzyme. The total amount of lipid phosphorous extractable from the homogenates decreased by about 10 per cent during the course of the reaction, probably as a result of phospholipase B activity in the tissue. The actual amount of unhydrolyzed phospholipid remaining in each experiment was not determined and the percentage hydrolysis figures in brackets were calculated from separately determined values for the composition of normal rat brain (Table 16). In the case of the beef enzyme the results suggest that phosphatidylethanolamine and phosphatidylserine are hydrolyzed preferentially.

The enzyme isolated from human pancreas appears to act preferentially on phosphatidylethanolamine (Table 18). Even

TABLE 16

Formation of Lysophosphatides by the Action of a Beef Pancreatic Phospholipase A Preparation on Rat Brain Homogenates

Incubated as described in Methods, employing an initial phospholipid phosphorous concentration in the reaction system of 100 mg of brain tissue per ml. Values shown are for duplicate experiments expressed as µg of lysophosphatide phosphorous formed per g of brain tissue.

	Time (minutes)	
Lysophospholipid	30	90
Lysophosphatidylethanolamine	183, 193 (45%)	292, 294 (70%)
Lysophosphatidylserine	82, 71 (33%)	121, 122 (60%)
Lysophosphatidylcholine	199, 207 (27%)	298, 301 (40%)
Lyso-ethanolamine plasmalogen	45, 42 (10%)	106, 107 (25%)

NOTE - The mean percentage hydrolysis values are shown in brackets and were calculated from the following average values for the phospholipid composition of mature rat brain (W. L. Magee, personal communication): phosphatidylethanolamine 20%, phosphatidylserine 10%, phosphatidylcholine 36% and ethanolamine plasmalogen plus alkyl ether phospholipid 20%. During the course of the reaction approximately 10% of the phospholipid phosphorous disappeared.

- The enzyme was used at a concentration of 75 µg per ml.

TABLE 17

Formation of Lysophospholipids by the Action of C. adamanteus Venom Phospholipase A Preparation on Rat Brain Homogenates

Conditions and expression of results as in Table 16.

	Time (minutes)	
Lysophosphatide	30	90
Lysophosphatidylethanolamine	56, 50 (12%)	57, 53 (13%)
Lysophosphatidylserine	46, 34 (19%)	47, 24 (17%)
Lysophosphatidylcholine	130, 104 (16%)	123, 140 (18%)
Lyso-ethanolamine plasmalogen	6, 8 (2%)	11, 13 (3%)

NOTE - No decrease in total phospholipid phosphorous was observed during the incubation.

- The enzyme was used at a concentration of 5 µg per ml.

TABLE 18

Formation of Lysophospholipids by the Action of a Human Pancreatic Phospholipase A Preparation on Rat Brain Homogenates

Conditions and expression of results as in Table 16.

	Time (Minutes)	
Lysophospholipid	30	90
Lysophosphatidylethanolamine	94, 93 (22%)	190, 183 (45%)
Lysophosphatidylserine	19, 12 (8%)	26, 49 (18%)
Lysophosphatidylcholine	105, 94 (13%)	217, 208 (28%)
Lyso-ethanolamine plasmalogen	26, 24 (6%)	81, 73 (19%)

NOTE - a 2% decrease in total phospholipid phosphorous was observed during the incubation.

- The enzyme was used at a concentration of 62. 5 µg per ml.

though the concentration of phosphatidylethanolamine in the tissue is only one-half that of the phosphatidylcholine the percentage hydrolysis of phosphatidylethanolamine was about twice that of phosphatidylcholine. Phosphatidylserine and the ethanolamine plasmalogen were not attacked as rapidly by the human enzyme as phosphatidylcholine, and phosphatidylserine was attacked at about the same rate as the ethanolamine plasmalogen. A very small decrease (2%) in the amount of extractable lipid phosphorous was noted during the course of the hydrolysis.

The action of the phospholipase A prepared from C. adamanteus venom on rat brain homogenates is shown in Table 17. Again all of the major brain phospholipids were attacked. There was a very definite limit to the amount of phospholipid hydrolysis which could occur (approximately 15 per cent of the theoretical maximum), since no significant production of any lysophosphatide could be detected after the first thirty minutes of incubation.

DISCUSSION

In this study the enzyme phospholipase A has been partially purified from the venom of the eastern diamondback rattlesnake (Crotalus adamanteus), and from bovine, canine and human pancreas. Selection of these sources was based on the relatively high phospholipase A content of many snake venoms and pancreatic tissues from several species, as well as their availability. Dog pancreas proved to be an unsatisfactory source of the enzyme and will not be discussed further. The properties of the other three enzyme preparations were investigated in some detail.

The assay system used to determine the specific activity of the various enzyme preparations is similar to that of Magee et al. (1962). There were two significant differences, however. In the first place the release of fatty acid was used to monitor the reaction rather than the decrease in the acyl-ester linkage content of the substrate. This method was found to be more sensitive and faster than ester determinations. The method of Dole (1956) could be used directly when lecithin was used as the substrate but this procedure had to be modified considerably for use with substrates containing acidic phospholipids. Another variation involved addition of calcium ions to the reaction medium in the determination

of the specific activities of the beef, canine and snake preparations, since it had been shown that calcium ions were required for the optimal activity of both the snake (Saito and Hanahan, 1962) and the beef enzyme (Rimon and Shapiro, 1959). Calcium ions were not routinely added to the human enzyme during the purification procedures since Magee et al. (1962) reported that calcium ions inhibited the action of human phospholipase A in lecithin-deoxycholate reaction systems. During later experiments in this study a small activating effect of calcium ions was noted with the human enzyme.

The activating effect of bile salts on the hydrolysis of phosphatidylcholine was first shown by Zieve and Vogel (1961) using the enzyme present in human duodenal fluid. Magee et al. (1962) have shown, that for optimal activity with the enzyme from human pancreas a weight ratio of 2 mg of sodium deoxycholate to 1 mg of lecithin is required. Experiments not reported in detail in this thesis showed that this ratio was optimal for all three phospholipase A preparations.

Beef pancreas was selected originally as a mammalian source of the enzyme because of its relatively high phospholipase A content, ready availability and because it had been used by other investigators, making certain direct comparisons possible. The phospholipase A present in beef pancreas has been purified to a high degree

by Rimon and Shapiro (1959) although their recovery of total activity was very low, amounting to only 7 per cent of that in the original tissue extract. Because of this an attempt was made to apply the procedure of Magee et al. (1962) to beef pancreas. It was found, however, that excessive activity losses occurred when the glycerinated extract was precipitated with acetone. The glycerinated extract was therefore dialyzed to remove the glycerol and give a more convenient starting material for further purification attempts. As shown in Table 1, the above dialysis step caused a marked increase in the activity of the extract. This may be due in part to the activation of lipases or other phospholipases in the tissue extract. Comparison of the lipase activity of the glycerol-containing and the glycerol-free extracts demonstrated a considerable increase in lipase activity after dialysis. In this regard it has been shown that preparations of pancreatic lipase of very high purity show activity towards lecithin (Deenen, 1966). The increase, however, did not appear to be large enough to account for the total increase in the activity of the preparation towards lecithin. Alternatively, activation of the phospholipase A during this manipulation would not be surprising, since Lesslauer, Slotboom, Postema, Haas and Deenen (1968) have shown that pig pancreas phospholipase A can occur in the form of a zymogen which can be activated by trypsin.

The active form was obtained following the release of a heptapeptide.

Heat treatment of the dialyzed preparation (Fraction B) increased the specific activity of the preparation (Fraction C, Table 1) but decreased its total activity. Much of the decrease in the total activity probably results from lipase inactivation, since the activity of the heated preparation towards trilaurin was markedly reduced, or through the inactivation of the heat-labile phospholipase (phospholipase A') which has been demonstrated by Bosch et al. (1965) in rat pancreas. The heat-treated preparation (Fraction C) was still quite impure. Electrophoretic analysis of this preparation at pH 8.6 gave multiple Amido Black B-staining areas. Measurement of the optical density of a solution of this preparation at 260 and 280 mμ indicated that this preparation was not pure protein, as did protein determinations on the freeze-dried preparation.

Column chromatography of the lyophilized fraction on SE-Sephadex gave a rather pure preparation (Fraction D). Large amounts of non-protein material were eluted from the column which absorbed strongly at 260 mμ suggesting that it was nucleic acid. Electrophoretic analysis of Fraction D at pH 8.6 and 5.0 showed only one band of stainable material indicating that Fraction D was a relatively homogenous protein preparation.

During the preparation of phospholipase A from bovine

pancreas the specific activity was calculated from the linear portion of the hydrolysis curve rather than from the initial reaction rate. Using Fraction D it was shown that the specific activity calculated from the linear portion of the curve was proportional to that calculated from the initial reaction rate with varying amounts of enzyme.

The phospholipase A present in C. adamanteus venom was purified using a heat-treatment step followed by chromatography. The major portion of the activity recovered from the column was found in the second peak. This procedure was used since it was extremely fast and simple, whereas the procedure used by Saito and Hanahan (1962) was quite long. During pH adjustment and chromatography there was a reasonably large loss in the total activity of the preparation. This loss may be due to occlusion of active enzyme in the precipitate and loss due to rejection of the first few fractions in the peak. This was done to eliminate contamination by the protein present in the first peak as much as possible. The preparation appeared to be quite pure on electrophoretic analysis. The small amount of protein remaining at the origin of the electropherograms probably represents carry-over of protein from the first peak.

The phospholipase A preparation (Fraction C) isolated from

human pancreas is by far the most impure of the three enzyme preparations used in these studies. Optical density measurements indicated that this fraction was badly contaminated and protein determinations indicated that it contained less than 40 per cent protein by weight. The most probable contaminant is nucleic acid. Various attempts were made to separate the protein from the contaminating material. Ethanol fractionation, protamine precipitation and column chromatography on DEAE-Sephadex and SE-Sephadex as well as molecular sieving on Bio-gel P-60 gave poor separation of the components and marked losses of activity. For example, chromatography on Bio-gel P-60 gave two partially overlapping protein peaks with the residual activity demonstrable in the fractions between the peaks. Also all fractions containing activity after ion exchange or molecular sieving were still contaminated. Attempts to prepare Fraction D as described by Magee et al. (1962) were largely unsuccessful. Consequently, Fraction C was used in its impure state. For comparative purposes this offers certain advantages since a considerable amount of work has been carried out using this preparation by others (Haas, et al., 1963; Ibrahim, Sanders and Thompson, 1964; Ibrahim and Thompson, 1965).

The apparent molecular weight of the three enzymes was

determined using the method of Andrews (1964). The molecular weight of the snake enzyme was calculated to be 31, 000. This is in good agreement with the value reported by Saito and Hanahan (1962) who reported a value of from 30, 000 to 35, 000 as determined by sedimentation analysis. The molecular weights of both the beef and the human enzymes were higher than this. The molecular weight of the human enzyme is probably lower than the value of 51, 000 found by this method, because of its obvious contamination. The apparent molecular weight of the beef enzyme preparation (Fraction D) was calculated to be 39, 000. This may be quite close to the actual value as the preparation appears to be reasonably pure.

As yet, very few molecular weight determinations have been carried out on mammalian phospholipase A preparations. However, Lesslauer et al. (1968) recently reported that the phospholipase A isolated from pig pancreas had a molecular weight of around 14, 000.

All three enzyme preparations were tested with respect to their action on various phospholipids under a variety of conditions. It was found in these experiments that although the three preparations had a number of common properties, each possessed certain unique characteristics, especially with regard to their action towards the lipoprotein preparations.

All three enzymes were similar in their action on lecithin. They attacked lecithin slowly and incompletely unless sodium deoxycholate was added to the system. Each enzyme was markedly activated in its action on this particular phospholipid by the addition of deoxycholate to the reaction and for all of the enzymes the activation was maximal when the activator was added at a level of 2 mg per mg of lecithin, a molar ratio of about four to one. It has been suggested that activators such as diethyl ether work by removing reaction products such as free fatty acid from the immediate area of the reaction (Dawson, 1963). It was also suggested that the activators could penetrate the micelle and "loosen" the tightly packed molecules in some way to facilitate the attack of the enzyme. Deenen, Haas and Heemskerk (1963) have suggested that deoxycholate functions to modify the zeta potential of the substrate making it sufficiently negative for the enzyme to attack the micelle. Dawson (1963), however, showed that the role of the activator, at least as far as N. naja phospholipase A is concerned, is not directly related to zeta potential. He found that many compounds which affected the zeta potential of the micelle in the same manner as deoxycholate did not activate the reaction.

The zeta potential theory would explain the lack of a requirement for the presence of an activator when phosphatidylethanolamine

is hydrolyzed by either of the mammalian enzymes. With both the addition of deoxycholate resulted in an inhibition of the reaction. This might be explained by the lowering of the zeta potential below some critical level.

This argument is contradicted by the finding that the C. adamanteus phospholipase A preparation did not attack phosphatidylethanolamine rapidly except in the presence of added deoxycholate.

The above observations suggest that there is a fundamental difference in the reaction mechanisms of the reptilian enzymes as compared to the mammalian ones. This conclusion is reinforced by the observation that the reptilian enzyme appears to have an absolute requirement for added deoxycholate in order to hydrolyze either phosphatidylserine or phosphatidylinositol. The mammalian enzymes, on the other hand, although they are more effective in the presence of deoxycholate, are able to hydrolyze either of these phospholipids in its absence.

Ibrahim, Sanders and Thompson (1964) studied the rates of hydrolysis of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in the presence and absence of deoxycholate. A phospholipase A preparation obtained from human pancreas by essentially the same method as used in these studies and a heat-

treated N. naja phospholipase A were used. It was demonstrated that the hydrolysis of lecithin required the presence of deoxycholate, while the hydrolysis of phosphatidylethanolamine was inhibited by the presence of deoxycholate. Phosphatidylserine hydrolysis was much slower than lecithin hydrolysis when both systems contained deoxycholate. The addition of deoxycholate to phosphatidylserine rendered this phospholipid only slightly more susceptible to the action of the enzymes. In this study the addition of deoxycholate to phosphatidylserine increased activity, especially in the presence of certain metal ions.

The effect of metal ions on the activity of phospholipase A towards the four above-mentioned phospholipids was determined. All three enzymes were activated by pre-incubation with calcium ions prior to hydrolysis of phosphatidylcholine (Table 6). The activating effect of calcium ions on the C. adamanteus and the beef pancreatic phospholipase A has been reported by Marinetti (1960) and Rimon and Shapiro (1959) respectively. Magee et al. (1962) found that 1 mM calcium produced a 55 per cent inhibition of the human enzyme in a lecithin-deoxycholate system. Under essentially the same assay conditions and pre-treatment of the enzyme with calcium ions a 30 per cent stimulation of activity was found in these studies. The calcium-induced inhibition found by

Magee for the human enzyme is not readily explained in light of these results. Although the assay system used in this study and that used by Magee et al. (1962) were similar , two differences should be noted. In the first case the calcium ion treatment of the enzyme was much longer (30 min.) than the time employed in the earlier study. Also Magee et al. prepared the substrate by vigorous shaking, while substrates used here were prepared by sonication, a process that could conceivably give rise to micellular forms of the substrate which differ considerably from those prepared by mechanical means.

An inhibition of phospholipase A by added calcium ions has been reported by others. Vogel and Zieve (1960) and Vogel and Bierman (1967) inhibited both the duodenal and the post-heparin enzymes. However, these authors worked at a more basic pH and employed levels of lecithin and deoxycholate which were higher than the concentrations used in these studies. Model experiments involving the reaction conditions used here indicated that concentrations of sodium deoxycholate and calcium ions in excess of 2 mg per ml and 1 mM respectively led to the development of precipitates in the reaction system. This could alter drastically the delicate balance between substrate, activator and cofactors, producing an apparent inhibition of the enzyme.

As was first pointed out by Long and Penny (1957), an apparent activating effect of divalent metal ions could be caused by the removal of the released fatty acid as a result of the formation of insoluble soaps. These authors showed, however, that the optimal calcium ion concentration occurred when the calcium to substrate molar ratios were of the order of one to ten, not one to two as would be expected by the above theory. Roholt and Schlamowitz (1961) pointed out that according to the above theory divalent ions which form insoluble soaps should all activate. They found, however, that barium ions inhibited the reaction and compete with calcium ions for the enzyme. The inhibition of the three enzymes by manganese also throws doubt on the above theory. In the case of the human enzyme in the lecithin-deoxycholate system (Table 11), only calcium activated the reaction of all the divalent cations tested.

The effect of metal ions on the activity of the enzymes towards phosphatidylethanolamine (Table 7) is similar to the effects noted in the studies on phosphatidylcholine. Calcium ion activated the hydrolysis both in the presence and the absence of deoxycholate. This suggests that the effect of the metal ion is one of reaction with either enzyme or substrate. The results with zinc, resembling those found in the lecithin system, indicate that this metal ion acts

directly on the enzyme, although substrate and product effects probably play a certain role. Magnesium ions did not cause consistent activation of phosphatidylethanolamine hydrolysis.

Since phosphatidylethanolamine is readily susceptible to the action of phospholipase A in the absence of any activator and can be prepared quickly and easily in a relatively pure state, it is a superior substrate for studying the effects of metal ions on the reaction, since there are no activator-metal ion interactions to complicate interpretation of the results. The effect of varying amounts of calcium ion on the hydrolysis of phosphatidylethanolamine by the three enzymes was determined. The mammalian enzymes had an optimal calcium concentration of 2 mM. The snake venom phospholipase reacted differently; maximal activity was found at a concentration of 0. 5 mM and no change in the specific activity was noted upon increasing the calcium concentration by steps up to 10 mM. This finding is similar to the results reported by Roholt and Schlamowitz (1961) on the effect of increasing concentrations of calcium on the activity of C. durissus terrificus towards lecithin in aqueous reaction systems. Saito and Hanahan (1962) showed that the hydrolysis of lecithin by purified C. adamanteus phospholipase A demonstrated a rather sharp optimal calcium ion concentration. However, it should be pointed out that their studies

were carried out in Hanahan's etherial reaction system.

Unfortunately, phosphatidylethanolamine did not prove to be an ideal substrate for studying this type of reaction. The susceptibility of the lipid to phospholipase A hydrolysis fell off rather sharply during storage. Also preparation of homogenous substrate suspensions was very difficult, requiring long periods of sonication. Also it was noted that the addition of calcium ions at a concentration of 2 mM or greater resulted in precipitation of material from the reaction system, presumably a calcium-phosphatidylethanolamine complex of some sort. This did not appear to hinder the reaction. The precipitate which formed went back into solution as the reaction proceeded. In this regard it should be noted that Hanahan, Rodbell and Turner (1954) observed the precipitation of a lysolecithin-lecithin-enzyme complex during the course of the reaction in the etherial system. The precipitation did not appear to affect the course of the reaction and the reaction went to completion.

Phosphatidylserine was affected by the presence of metal ions in much the same way as phosphatidylethanolamine with the exception that magnesium ions activated hydrolysis. Interpretation of the results is difficult since phosphatidylserine readily binds metal ions. Rathbone (1962) showed that calcium

salt and magnesium salt of phosphatidylserine formed readily and dissociated with difficulty. Hendrickson and Fullington (1965) verified the formation of phosphatidylserine-calcium complexes. Phosphatidylserine appeared to require the addition of deoxycholate to the reaction system before it could act as a good substrate. The requirement for deoxycholate was so strong in the case of the snake enzyme that no activity was found in its absence (Table 8).

The role of magnesium in activating the hydrolysis of phosphatidylserine while having no effect on the hydrolysis of phosphatidylcholine or phosphatidylethanolamine is puzzling. It might be possible that the addition of magnesium ions releases calcium ions from the phosphatidylserine which could activate the enzyme. This is unlikely since the phosphatidylserine was prepared by the method of Rathbone, Magee and Thompson (1962) and should be present as either the free acid or the sodium salt. Another possibility is the formation of a magnesium ion-enzyme complex which, in contrast to the calcium ion-enzyme complex, is active only with phosphatidylserine.

The effect of various metal ions was not studied in detail when phosphatidylinositol was used as a substrate. Preliminary experiments indicated that all three enzymes showed minimal activity with this substrate in the absence of calcium ions. The

reaction rate was accelerated greatly by the addition of sodium deoxycholate and the pre-incubation of the enzymes with calcium. Under these conditions phosphatidylinositol was hydrolyzed at a lower rate than any of the other substrates (Table 6). Long and Penny (1957) found that phosphatidylinositol was not attacked by moccasin venom, whereas Keenan and Hokin (1964) showed that phosphatidylinositol which had been activated by passage through alumina was attacked slowly in the etherial system (1 ug lipid P hydrolyzed per 0.25 mg protein per sixteen hours) by C. adamanteus venom. Carter and Weber (1966) studied the various salt forms of phosphatidylinositol. These authors noted that phosphatidylinositol has a high affinity for calcium ions. It was possible to chromatograph the calcium salt of the phospholipid on silicic acid without cation exchange occurring. They also showed that, using Folch's washing procedure (Folch, Lees and Sloane-Stanley, 1957), it was very difficult to remove bound calcium from the lipid. The differences found between the reactivity of phosphatidylinositol in these studies and those of the above-mentioned investigators may be due to binding of the calcium ion required by the enzyme to the substrate, which would effectively inhibit the reaction. Long and Penny (1957) added calcium ions to their system but at a much lower concentration than was used in these experiments.

They also employed a higher substrate concentration.

The relatively small calcium ion effect generally observed when lecithin was used as the substrate may be related to the findings of Shah and Schulman (1967) who showed that lecithin has a strong tendency to exist in an internal salt form which does not pick up ions from the medium. Dervichian (1956) showed that lecithin will not bind calcium ions.

Ethylenediaminetetraacetate (EDTA) at high concentrations was found to inhibit phospholipase A activity completely when the substrate was phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine. At lower concentrations in a lecithin-deoxycholate reaction system the chelating agent increased the specific activity of all three enzyme preparations (Tables 10 and 11). Dawson (1963) reported that the addition of excess calcium ions to an EDTA-inhibited system containing N. naja venom resulted, not only in the complete recovery of the original activity, but in a stimulation of activity. He ascribed the effect to removal of inhibiting ions from active sites of the enzyme followed by re-activation of the apoenzyme by the added calcium ions. Magee et al. (1962) also noted a stimulating effect upon treatment of human pancreatic phospholipase A with less than inhibitory amounts of EDTA. A role for the chelating agent similar to that mentioned above was postulated, but the source of the calcium ions required

for activation was unclear. Possibly the enzyme preparation itself contained the required calcium.

Using the human enzyme acting on lecithin in the presence of deoxycholate an attempt was made to clarify the action of EDTA. If heavy metal ions were inhibiting the enzyme it should have been possible to remove them selectively without removing the calcium, thus demonstrating an activating effect similar to that found with EDTA. However, no such effect was found (Table 11) with agents which selectively bind heavy metal cations, suggesting that the activating effect of the lower concentrations of EDTA is a highly specific one. At the pH used in this study the EDTA molecule bears a double negative charge. Possibly some interaction occurs with the substrate and facilitates attack by the enzyme.

N-ethylmaleimide is an effective sulphydryl group reagent. A considerable excess of the reagent was found to decrease the specific activity of the human phospholipase A preparation by only 20 per cent (Table 11). Although Magee et al. (1962) showed that cysteine increased the activity of the human enzyme, iodoacetate and mercuric ions were not markedly inhibitory. It appears unlikely, therefore, that the enzyme contains a functionally active sulphydryl group.

Early in these experiments it was found that phospholipase A

preparations were unable to attack rat brain homogenates prepared in isotonic saline. Water homogenates of rat brain, in contrast, were good substrates for the enzymes. It was decided, therefore, to study the effect of the tonicity of the incubation medium on phospholipase A activity using the lecithin-deoxycholate reaction system. In the case of the experiments recorded in Table 12, the medium was isotonic only with respect to the solute being tested. In the presence of added sucrose or salts it was actually hypertonic, since the incubation system routinely contained the buffering agent (glycylglycine, 0.05 M), and sodium deoxycholate. Using this model system the presence of either sodium or potassium ions did not inhibit the action of the reptilian enzyme but inhibited the mammalian enzymes by more than 50 per cent. Isotonic sucrose had no effect on the rate of the reaction for any of the enzymes. This indicates that there is a fundamental difference in the reaction mechanism of the mammalian and the reptilian enzymes. It may be that the phospholipid micelle can absorb cations and effectively repel the mammalian enzymes. This may also explain the inhibition observed with the highest concentrations of calcium ions when the mammalian enzymes were acting on phosphatidylethanolamine (Table 9).

Egg yolk suspensions were selected for study since the

rate at which they could be attacked by certain phospholipase A preparations has been shown to be very fast when compared to the rates found with extracted phospholipids (Condrea, Vries and Mager, 1962). This is certainly the case with the snake enzyme (Table 13) which exhibited a higher specific activity towards the aqueous yolk suspensions (no deoxycholate added) than it did towards any of the purified phospholipids, even in the presence of deoxycholate (Table 5). Egg yolk suspensions were not particularly good substrates for the beef enzyme, and were not attacked at all by the phospholipase A prepared from human pancreas. Water homogenates of rat brain were hydrolyzed less rapidly than egg yolk suspensions by the snake enzyme (Table 13); in fact, the specific activity of this enzyme with brain homogenates was lower than with either phosphatidylcholine or phosphatidylethanolamine plus deoxycholate (Table 5). The mammalian enzymes, particularly the human one, had relatively high specific activities in the water or isotonic sucrose rat brain homogenates. The low or negligible activity of the mammalian enzymes towards either egg yolk suspensions or rat brain homogenates prepared in saline probably reflects in part the inhibitory effect of saline demonstrated with the enzymes in the lecithin-deoxycholate system (Table 12). The effect of saline on the reptilian enzyme

cannot be explained on this basis. Possibly lipoprotein preparations possess negatively charged or acidic groupingswhich can absorb sodium ions to either change the structure of the lipoprotein or prevent the approach of the enzyme. The lower specific activity observed with rat brain slices in sucrose as compared with sucrose homogenates (Table 13) no doubt is related to the greater surface area of the homogenate.

Beef serum in the absence of deoxycholate was attacked by the snake enzyme only. Deoxycholate did not potentiate the action of the snake enzyme but enabled the beef enzyme to attack the serum phospholipids. The human phospholipase A preparation had no effect on this substrate. Presumably the phospholipids of the plasma are orientated in such a manner as to be fully exposed to the action of the snake enzyme but not to the mammalian ones. This again implies that there is a fundamental difference in the mode of attack of the reptilian enzyme as compared to the mammalian ones.

Beef erythrocytes in either the sucrose or saline were not attacked by any of the phospholipase A preparations. Again apparently the phospholipids are protected from the action of the enzymes in some manner, either because of a non-phospholipid outer layer or because of repulsion of the enzyme by surface charges

on the membrane. A combination of these two possibilities seems most likely in light of the studies by Klibansky et al. (1968) who showed that the hydrolysis of the phospholipids by venom phospholipase A was facilitated by the use of basic polypeptides which also possessed lipophilic properties. It was suggested that the basic properties of the polypeptide permitted its absorption onto the surface of the membrane and its lipophilic properties facilitated access of the enzyme to the lipid protein of the membrane.

It was shown that egg yolk phospholipids were acted on in their native state by the snake and the bovine enzymes. The bovine enzyme exhibited a preference for phosphatidylethanolamine as a substrate. This agrees with the studies involving purified substrates where this enzyme readily attacked phosphatidylethanolamine in the absence of deoxycholate. However, phosphatidylcholine also was hydrolyzed readily in egg yolk preparations without the addition of deoxycholate. This is in marked contrast to the results obtained with purified substrates, where the omission of deoxycholate resulted in only a small portion of the total amount of the phosphatidylcholine being broken down and that at a slow rate. The percentage hydrolysis of phosphatidylcholine and phosphatidylethanolamine increased at identical rates in the case of the snake enzyme acting on egg yolk lipoproteins. This probably

indicates that the snake enzyme has no preference for either of these substrates. It should be remembered that the snake enzyme attacks phosphatidylcholine and phosphatidylethanolamine slowly in the absence of deoxycholate.

The human enzyme, however, had no effect on the phospholipids in egg yolk under any of the conditions tested. This is particularly hard to explain in light of the studies by Condrea, Klibansky, Keret and Vries (1963) who showed that human pancreatic phospholipase A prepared by the method of Rimon and Shapiro (1959) readily attacked egg yolk lipoproteins. It should be pointed out that these authors used protein concentrations about a hundred times those used in these studies and also that they found lecithin in the absence of deoxycholate was readily attacked under their experimental conditions, although not as rapidly as the egg yolk. It is possible that some sort of a lipid activator was carried along with the enzyme during the course of their purification which could have been removed during the preparation of the acetone powders used in these studies.

Both mammalian enzymes attacked brain homogenates prepared in either sucrose or water but not saline. The snake enzyme caused an initial burst of hydrolysis which stopped abruptly when less than 20 per cent of the hydrolyzable lipid had been broken down.

Since homogenization was carried out under conditions where the mitochondria and other intracellular particles would be left reasonably intact, this phenomena in the presence of ample substrate suggests that the phospholipids of only certain cellular constituents are accessible to the enzyme. The relatively small breakdown of the ethanolamine plasmalogen suggests that the non-myelin constituents are particularly susceptible since ethanolamine plasmalogen is essentially a myelin lipid (McClure, 1966).

Both of the mammalian enzymes rapidly broke down all four of the major phospholipids present in brain. A marked phosphatidylethanolamine breakdown occurred. This can be contrasted with the breakdown of ethanolamine plasmalogen, which although hydrolyzed to a significant degree was attacked at less than one-half the rate found for phosphatidylethanolamine. It may be that phosphatidylethanolamine in brain homogenates is much more accessible to these enzymes as is suggested in the case of the snake venom enzyme. The bovine pancreatic enzyme appeared to have some preference for phosphatidylserine in contrast to the other two phospholipase A preparations.

From these studies it is apparent that not only the nature of the phospholipid but its association with other materials determines how phospholipids will be acted on by enzymes such as

phospholipase A. This is especially obvious in the case of lipoprotein complexes in in vitro situations. In this context, Wolman (1965) and Wolman and Wiener (1965) have shown that the structural integrity of myelin and other membranes depends on the maintenance of critical ionic ratios in the incubation media employed. The sensitivity of lipoprotein structures to ionic variations and the presence of agents such as deoxycholate complicates the interpretation of the experiments reported here, as does the fact that each of the enzymes investigated was found to possess certain unique characteristics. With regard to this latter point, the differences between the enzyme preparations obtained from snake venom and human pancreas are particularly interesting. In short, the reptilian enzyme exhibited a marked dependence on calcium ions and was not inhibited by sodium or potassium ions when acting on lecithin in the presence of deoxycholate. It attacked the phospholipids in beef serum more avidly than those in any other substrate investigated. In contrast, the phospholipase A prepared from human pancreas was inhibited by sodium and potassium and was only slightly activated by calcium. The human enzyme had no effect on beef serum preparations. It preferentially hydrolyzed phosphatidylethanolamine and its specific activity was higher with water or isotonic sucrose homogenates of rat brain than with any other

substrate. These differences between the enzymes help to explain why the reptilian phospholipases cause hemolysis when injected into the bloodstream, whereas the mammalian enzymes circulate freely without producing any obvious harmful effect. It is also felt that they pertain to certain points made in the introduction to this thesis.

VI SUMMARY

Phospholipase A has been partially purified from three different sources, two mammalian and one reptilian. The enzyme has been purified from beef pancreas acetone powders by extraction with basic aqueous glycerol, dialysis against dilute calcium acetate and heat treatment, followed by ion exchange chromatography on SE-Sephadex using gradient elution. The phospholipase A present in the venom of the eastern diamondback rattlesnake (C. adamanteus) has been purified through a heat treatment and chromatography on a molecular sieve. A phospholipase A preparation from human pancreas has been obtained by acetone precipitation of heat-treated material obtained from glycerinated extracts of human pancreas acetone powders. Although the venom and beef enzymes were reasonably pure, the human phospholipase A preparation was badly contaminated with both extraneous protein and material which is thought to be nucleic acid. Unsuccessful attempts were made to purify this preparation further using a variety of techniques.

All three preparations were very similar in their action towards lecithin in the presence of sodium deoxycholate and various ions. Calcium ions were found to activate, while zinc ions inhibited the reaction. Phosphatidylethanolamine was hydrolyzed by all

three enzymes in the absence of deoxycholate. The addition of deoxycholate caused a marked stimulation of hydrolysis in the case of the snake enzyme but brought about an inhibition of the reaction catalyzed by the two mammalian enzymes. Addition of deoxycholate caused a marked increase in the activity of all three preparations towards phosphatidylserine and phosphatidylinositol. Calcium ions also activated the hydrolysis of the last three phospholipids.

Ethylenediaminetetraacetic acid (1.0 mM) inhibited the hydrolysis of all purified phospholipids tested. Using the lecithin-deoxycholate system it was shown that concentrations of ethylenediaminetetraacetate of about one-hundredth to one-thousandth that required to inhibit the enzymes stimulated their activity. Studies with the lecithin-deoxycholate system and the human enzyme and other divalent cation complexing agents indicated that the activating effect of ethylenediaminetetraacetate was probably not related to the removal of inhibiting heavy metal ions.

High concentrations of either sodium or potassium chloride were found to inhibit the action of the mammalian enzymes towards lecithin in the lecithin-deoxycholate system. There was no inhibition of the snake enzyme. The addition of sucrose at the same

molecular concentration did not affect the action of any of the enzymes. Similar concentrations of sodium ions inhibited the action of the snake and bovine phospholipase A preparations on egg yolk lipoproteins. Once again sucrose did not affect the reaction. Human pancreatic phospholipase A did not attack egg yolk suspensions under any of the conditions used. Rat brain homogenates in saline were unaffected by all three enzymes. Sucrose and water homogenates of rat brain were readily attacked by all three preparations, especially the human one.

Bovine erythrocytes prepared in either saline or sucrose were not attacked by these enzymes. Bovine serum, in the absence of deoxycholate was only attacked by the snake venom phospholipase A preparations and was a particularly good substrate for this enzyme. The addition of deoxycholate to the serum enabled the bovine phospholipase A to attack the serum but did not affect the actions of the snake or the human enzymes to a significant degree.

The action of the beef and the snake preparations on egg yolk lipoproteins were quantitated by measuring the formation of lysophosphatides during the course of the reaction. The snake enzyme hydrolyzed both phosphatidylethanolamine and phosphatidylcholine in the absence of deoxycholate and did not appear to have any preference for either substrate. The beef enzyme, while

hydrolyzing both phospholipids, appeared to have a small preference for phosphatidylethanolamine.

Similar lysophosphatide measurements were carried out in experiments involving rat brain homogenates. The reptilian enzyme, while attacking the four major glycerophosphatides of brain, ceased action when less than 20 per cent of the theoretically available substrate had been hydrolyzed. Both the human and the bovine pancreatic enzymes caused extensive degradation of rat brain phosphatides. The beef enzyme demonstrated a preference for phosphatidylethanolamine and phosphatidylserine as substrates. The human enzyme showed a marked preference for phosphatidylethanolamine.

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ADDENDUM

Reproducibility of Titrations with Fatty Acid Standards

Fatty acid concentration (mμ Equivalents)	Titration value* (μl NaOH)
1000	289 ± 6
2000	575 ± 14

* Mean ± S.E.M. for nine separate determinations.