Phospholipase A Activity of E. coli

by

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Thesis

Submitted to the School of Graduate Studies in partial fulfilment of the requirements for a Master's degree.

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December, 1969

ACKNOWLEDGMENTS

I wish to express my appreciation and gratitude to Dr. P. Proulx for his constant guidance and advice throughout the course of this work.

I would also like to extend my thanks to Miss G. Benns and Mrs. Gaertner for their valuable assistance in checking the purity of E. coli cultures.

To Mrs. J. Shadwell for her excellent assistance by typing this thesis my deepest appreciation.

RESUME

The hydrolysis of labelled phosphatidyl ethanolamine and lecithin by E. coli O15 was studied in vitro. Phospholipase A, as detected by ^{32}P-labelled lysophosphatidyl ethanolamine formation, had two apparent pH optima, one at 5 and the other at 8.4. On the other hand lysophospholipase, also present, had a pH optimum of 10, and was inhibited by high concentrations of either sodium lauryl sulfate or sodium deoxycholate. Phospholipase A required Ca^{++} addition for maximal activity at both pH optima. Mg^{++} also stimulated the activity but other divalent ions tested were slightly inhibitory. Sodium lauryl sulfate completely inhibited at pH 5, but stimulated at pH 8.4. Experiments with singly and doubly labelled phosphatidylethanolamine indicated that phospholipase A1 activity was predominant at both acid and alkaline pH. Lower levels of phospholipase A2 were detectable only at alkaline pH. Whereas the acid phospholipase activity was slightly stimulated by heating cell sonicates at 60° for 30 minutes, the combined phospholipase A activity at alkaline pH was not appreciably effected by this treatment in the absence of detergent. Addition of deoxycholate during this treatment caused a marked fall in the activity at pH 8.4. The activity at alkaline pH was found to reside mainly in the particulate fraction.

The significance of these results is discussed in terms of the lipid-turnover pathways of E. coli and their lypolytic

enzymes, the properties of which have been compared to those of animal and snake venom phospholipase A and lysophospholipase.

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INTRODUCTION

General Comments

Although lipid metabolism has been the subject of great interest in the last two decades, the field still lags behind many other areas of metabolism. Lack of rapid and efficient methods for separation of lipids was the main curtailing factor. With the advent of chromatographic techniques, lipid chemistry and metabolism has progressed immensely. Nevertheless, important aspects still dealing with the basics of lipid metabolism such as fatty acid synthesis have been only very recently elucidated and this only for a few organisms (1,2).

An analogous situation exists in the case of phosphoglyceride biosynthesis. The basic pathways dealing with synthesis of glycerolipids are well known mainly because of Kennedy's group however recent alternative pathways such as the dihydroxyacetone pathway (3) have been put forward and by no means can one consider phosphoglyceride anabolism a closed issue. The enigma of ether lipid biosynthesis and the discovery of several new lipids every year each requiring elucidation of new metabolic pathways all serve to illustrate this point further. Even in the better known pathway leading to phosphatidic acid formation, there is incomplete knowledge as to which precursor is the acyl donor for

indeed, besides acyl CoA, acyl ACP may also be used (4) as well as perhaps other high energy acyl esters yet uncharacterized (5), depending on the biological material studied. The situation with lipid metabolism contrasts clearly with that of carbohydrate metabolism in which case the operating of pathways has been studied in much greater detail.

Although more definite information has been available regarding the catabolism of phosphoglycerides, here again new knowledge pertaining to the specificity and mode of action of lypolytic enzymes has emerged in the last few years, thus bringing new concepts. Phospholipase A activity, for example, is now known to result from either of two enzymes acting at different positions of glycerol (6), phospholipase D favors transphosphatidylation as well as hydrolysis (7), etc. Although the role of these enzymes in partial or complete turnover is often implied ipso facto, very few detailed studies on a complete catabolic pathway or on the factors controlling metabolic turnover of lipids have been made.

One major problem in further understanding the functioning of lipid metabolism, aside from the complexity of the pathways themselves, is the complexity of most of the biological systems studied. Plant and animal tissues not only contain a large variety of lipids which require further elucidation of pathways, but they also represent

an heterogenous composition of cells each equipped with specialized subcellular organelles. Discussions pertaining to the operation of lipid pathways in higher organisms cannot rightly exclude consideration of these morphological factors.

E. coli could potentially offer clear advantages for the detailed study of lipid metabolism. Cultures of this organism represent a relatively homogeneous population of cells devoid of complex intracellular organization. The main anabolic pathways of the relatively few phosphoglycerides present in this bacterium (8) are established and much is known about its intermediary metabolism including closely allied pathways such as those responsible for fatty acid synthesis (1). Growth conditions can be easily varied and therefore the operation of the pathways in vivo can be studied with greater ease. Infact, the general features of the turnover of phosphoglycerides in E. coli have already been studied (9) however further understanding of the lipid metabolism and its regulating factors has not yet been possible. For, despite the impressive data available on the lipid anabolism of this organism, its catabolic pathways have been almost completely overlooked.

Our studies have therefore centered on the

characterization of some of the lypolytic enzymes in this organism. A natural course to follow has been to also study some of the properties of these catabolic enzymes and to compare our data with known properties of analogous phospholipases present in other organisms.

It is hoped that this and other such studies will have served to completely elucidate the catabolism of E. coli lipids and that eventually one organism will be available in which all of the salient features of its phosphoglyceride metabolism are known. It should then be fully possible to study factors regulating the turnover and metabolism of the lipids of this organism.

Lipid composition of E. coli

Early studies by Law (10) and by Kanfer and Kennedy (9) revealed that the lipids of E. coli B consist of over 90% phosphoglyceride. Although phosphatidylethanolamine (PE) is the main phosphatide fraction accounting for over 75% of the total lipids, phosphatidyl glycerol (PG) is also present in amounts comprising most of the remainder. The lipid composition was found to depend on the age of the culture. Whereas the PE content increases to over 90% in the stationary phase, the PG levels decrease proportionally (9). Cardiolipin (DPG)

has also been detected in amounts comprising 5-12% of the total phosphatide fraction, whereas, phosphatidic acid (PA) and phosphatidylserine (PS) are present in only trace amounts (8). Recently Ames (11,12) corroborated these findings with E. coli K-12 in which PE, PG, and DPG are again the main phosphoglycerides present and PA, PS, as well as two partially characterized lipids which occur in only minor quantities. It is important to note that lyso PE has also been detected in one strain of E. coli (13), although this finding has not yet been extended to other strains. Lecithin which is widely occuring in animals, plants and in algae yeast and fungi (14,15) is totally absent in E. coli. Kennedy (9) suggested that the conversion PG to water-soluble amino-acyl derivatives might account for the loss of P^{32} label in this fraction following chase experiments. The amino acid derivatives of PG extracted from other bacteria (16) or chemically synthesized (17), even those containing basic amino acid residues are lipid-soluble under usual extraction conditions and in any case none of these derivatives have yet been found in E. coli. On the other hand the loss of label can be explained in terms of a conversion of PG to DPG (18) and probably, of a degradation

to inorganic phosphate and other water-soluble products.

A passing reference to the presence of highly complex water-soluble liposaccharides in E. coli and other gram-negative bacteria is appropriate. Upon mineral acid hydrolysis of this fraction, Lipid A is obtained which Burton (19) characterized as consisting of two fractions. The basic structural unit of these two fractions appears to be the N-acylated derivative of glycosaminyl glucosamine 4-phosphate.

The fatty acid composition of E. coli lipids was reviewed by Kates (14) and by Lennarz (15). Characteristic of E. coli and other bacteria, there is an absence of polyenoic acids and a predominence of monoenoic and cyclopropane fatty acids as well as palmitic acid which is the main saturated species. The cyclopropane fatty acids, cis-9, 10-methylene hexadecanoic acid and cis-11-12-methylene octadecanoic acid are formed by a methyl transfer from s-adenosyl methionine to phospholipid-bound monoenoic acid present at the 2 position of glycerol (20). The various species of phosphatidyl ethanolamine have been analyzed by Van Golde and Van Deenen using a strain of E. coli which excretes this phosphoglyceride in the culture medium (21).

The fatty acid and lipid composition of E. coli

have been shown to vary with the type of culture medium used, the age of the culture and the temperature. Accordingly it was shown that young cultures of E. coli contain high proportions of C_{16} and C_{18} monoenoic acid and low amounts of cyclopropane fatty acid (14). Older cultures are typified by an increased proportion of cyclopropane fatty acids. The composition of the medium likewise affects not only the actual amounts of lipids in a culture but also its fatty acid composition. With a low glucose medium the amount of palmitic acid is much greater than that of unsaturated fatty acids whereas supplementation with casamino acids increases the proportion of unsaturated fatty acids. Studies on the effect of temperature showed that with low temperatures, unsaturated fatty acids such as hexadecenoic and octadecenoic acid increased whereas palmitic acid decreased (22). The results of Okuyama (23) also showed that after a downward shift in temperature, the proportion of vaccenic acid increased two-fold during a five hour lag period; however, palmitic acid did not decrease substantially. The mechanism whereby the cell adapts to cold by increasing unsaturation is not clearly known however from a teleological point of view, this mechanism might serve to prevent increased rigidity of the biomembrane as a result of cold. This is accomplished by introducing fatty acids which have lower melting points.

Biosynthesis of phosphoglycerides

The classical pathway elucidated by Kennedy and coworkers (24) is the major biosynthetic route for E. coli phosphoglycerides. In this pathway, there is a successive acylation of sn-glycerol-3-phosphate (G-3-P) to form phosphatidic acid as a key intermediate. Glycerol is not incorporated into phospholipid unless it is first phosphorylated by glycerol kinase, a condition which also prevails in animal tissues (25). The acyltransferases involved make use of either long chain acylCoA (26) or fatty acyl derivatives of acyl carrier protein (ACP) (4). The evidence indicates that there is a direct transfer of the acyl groups from ACP to G-3-P rather than a prior transfer to CoA. This facultative acylation process is also present in Cl. butyricus (27,28). As would be expected, with acyl CoA as substrate, the intermediate products are lysoPA and PA but with acyl ACP as substrate both lyso PA and monoglyceride (MG) are formed (4).

The formation of MG via acyl ACP is an interesting finding since it implies ipso facto the presence of a lyso PA phosphatase; however such a phosphohydrolase or a phosphatase acting on PA to produce glycerides has not been clearly demonstrated in vitro, assumably because their levels are very low and rapid conversion of intermediates to phospholipid make the assay of these enzymes difficult. The very small glyceride pool in E. coli is no doubt a consequence of limited conversion of lyso PA or PA to neutral

lipid as the in vivo, pulse-labelling experiments of Kennedy and Chang have shown (29). Their results indicated a very small incorporation of glycerol ^{14}C into the neutral lipid fraction, which under optimal growth conditions for E. coli, was metabolically stable. On the other hand, the results of Okuyama showed that when growth conditions are modified by a downward shift in temperature, the neutral lipid fraction does turnover and becomes an important supplier of acyl groups for phospholipid synthesis (23). In such a case diversion of the anabolic pathway towards the synthesis of glycerides followed by breakdown of their acyl ester bonds and resynthesis of acyl esters as phospholipid all of which is accompanied by increased ATP expenditure, might be an efficient way of producing extra heat. Further investigations dealing with formation of neutral lipid under various growth condition are clearly needed and may serve to elucidate the enigmatic role of the very active diglyceride kinase (30) present in E. coli.

Once formed, PA is converted to CDP-diglyceride via a cytidolysis reaction yielding pyrophosphate as by-product (31). Formation of CDP-diglyceride appears to be an obligatory step in phospholipid biosynthesis, since reactions analogous to those directly utilizing diglyceride and CDP-ethanolamine, which are known to occur in animal tissue, do not occur in E. coli (32). The cytidine triphosphate; phosphatidic acid cytidyltransferase involved is a particulate enzyme depending on Mg for activation and its

properties have recently been studied in detail by Carter (33).

CDP-diglyceride can then be converted by a particulate, Mg-dependent enzyme, serine: CMP phosphatidyltransferase, to PS and CMP (34). PS is in turn decarboxylated by a decarboxylase to yield PE. The enzyme involved does not require the cofactors necessary for mammalian decarboxylases (35).

CDP-diglyceride can be converted to PG and DPG. The first step involves the enzyme, sn-glycero-3-phosphate: CMP phosphatidyltransferase (36), catalyzing the synthesis of phosphatidyl glycerophosphate (PGP). This particulate enzyme is dependent on Mg or Mn for activation. PGP does not accumulate but is rapidly converted to PG by the action PG phosphatase, a particulate, Mg-dependent enzyme (37). PG may be converted to DPG by the action of phosphatidyl-glycerol: CMP phosphatidyltransferase also a particulate enzyme which is dependent of Mg (18). A similar series of reactions effect the synthesis of PG and DPG in animal tissues (38).

Catabolism and turnover of phosphoglycerides

The catabolism of phosphoglycerides by E. coli homogenates was first studied by Proulx and Van Deenen (39) who characterized lysophospholipase and phospholipase C and gave evidence for the presence of phospholipase A in this organism. The presence of phospholipase C and lysophospholipase has been confirmed by Nojima and Okuyama

(8) who also showed concurrently with our own results (40) the definite occurrence of phospholipase A in this organism.

The phosphodiester produced by the action of lysophospholipase can be degraded further (39, 8). ^{32}P-labelled glycerophosphoryl ethanolamine for example, is converted to labelled glycerophosphate and inorganic phosphate (Pi)(8). Thus enzymes effecting the complete breakdown of phospholipids are present in E. coli.

A partial turnover of the acyl moiety is likely since a lysophosphoglyceride: CoA acyltransferase acting on either lyso PE or lyso PC has been characterized *in vitro* (41). It is not yet certain to what extent the phospholipase A; acyltransferase cycle, first described by Lands (42) is operating *in vivo*. For E. coli it has been suggested that phosphoglycerides turnover mainly *de novo* under certain conditions (23). In *Haemophilus parainfluenzae* it seems that the Lands cycle is operating *in vivo* since the rate of turnover at each ester position of phosphoglycerides is different (43). Comparable studies designed to show such partial turnover have not yet been performed with E. coli.

A striking feature brought out by pulse labelling and chase experiments is that whereas PS and PA are very transient intermediates (9) and PG and DPG turnover at slower rates (11,23), PE, under normal growth conditions

for E. coli, is a metabolically stable pool (9). If however the growth temperature is decreased there is turnover of PE fraction as well as the other phosphatide fractions during the induced lag period (23). Thus the environmental conditions have a very strong influence not only on fatty acids or phosphoglyceride composition but also on the turnover of E. coli lipids. As Ames further points out, in many of the culture mediums used for ^{32}P-labelling, inorganic phosphate is kept low to favor incorporation into phospholipid. This pattern of incorporation however may reflect the deficiency of phosphate in the medium as his own results with E. coli showed (12).

Our presentation so far has indicated the presence and possible role of phospholipase A and lysophospholipase in E. coli. Since our ensuing experimental approach will be centered on the characterization and mode of action of these enzymes in this organism, we felt it a propos to point out our present knowledge of the properties of both pholpholipases from the various sources studied.

Phospholipase A

Occurrence:

Phospholipase A is very widely distributed throughout nature (44). It is present in snake and insect venoms and

a number of animal tissues including testes, spleen, lung and liver which display moderate activities whereas pancreas and small intestine are very rich sources of this enzyme (45). At the subcellular level phospholipase appears to be distributed among the microsomal (46,47), mitochrondria (46,47, 48) and lysosomal fractions (49,50,51).

Mode of Action:

Initially it was not clear whether phospholipase A acted on a specific ester position or an ester containing a particular type of fatty acid. The subsequent work of Tattrie (52), Hanahan (53) and Van Deenen (54) and their coworkers working with snake venoms revealed that the 2-acyl position was attacked rather than the 1-acyl position and thus phospholipase A was accepted by most as being exclusively a 2-acyl ester hydrolase.

Recently however, the studies of Lloveras et al (55) and of Van den Bosch and Van Deenen (56) have revealed that in rat lung, liver and kidney, not only the 2-acyl ester is hydrolysed but the 1-acyl ester as well: Evidence pointed to the existence of two enzymes, phospholipase A1 and A2 designated according to their positional specificity and which differed in properties. Phospholipase A1 is heat labile and usually has an acid pH optimum (50,57,58) whereas phospholipase A2 has a pH optimum of 7-7.5 and is heat

resistant (44).

It has been suggested that complete breakdown of phosphoglycerides may be accomplished by the combined action of phospholipase A1 and A2. This implies that lysophospholipase activity might be attributable to one or the other phospholipase A. In other words hydrolysis by A1 would be followed by A2 activity and vice versa; however, there is yet no clear evidence for such a supposition. On the other hand Mellors and Tapper (49) found that a lysosomal enzyme of liver cleaves both acyl positions of PE and PC without lysophosphatide formation. They attributed this activity to a phospholipase of the type formerly designated B (). Perhaps in this case phospholipase A1 and A2 might be strongly coupled and prevent lysophosphatide accumulation although the other explanation that there is here a combined action of phospholipase A and lysophospholipase might be just as likely. The recent work of Waite et al strongly favors the latter possibility (51).

Specificity and general properties of phospholipase A2

a) Snake venoms

Most of the relevant studies dealing with purification have been performed on phospholipase A2 from snake venom or pancreas. The snake venom enzyme from Crotalus adamanteus

was first purified by Hanahan (59) who found that it was labile to heat treatment in the alkaline range but quite stable in the acid range till about pH 4. In fact this property has been extensively utilized to inactivate other enzymes present when crude preparations of phospholipase A were used. Snake venom phospholipase A required Ca at a concentration of about 10 mM for optimal activity. Long and Penny (60) concluded that Ca may serve as a bridging ion between the enzyme and substrate since a Ca complex of enzyme, substrate and lysolecithin precipitates during the lypolytic reaction. As in the case of phospholipase C or D, ether activates phospholipase A (59) and Dawson (61) explains this effect by concluding that ether penetration into the lipid miscelles causes wider spacing of the substrate molecules. This improves the access of the enzyme to the ester linkages and prevents the inhibition of the enzymatic reaction by removing the fatty acid formed.

Further purification of phospholipase A by Saito and Hanahan showed the presence of two enzymes in Crotalus adamanteus venom which differed in electrophoretic mobility and isolectric point but which each exhibited the same A2 type of activity (62). A very recent study by Wells and Hanahan have confirmed the existence of these two phospholipase A enzymes and revealed their properties further (63). Both had a MW of about 30,000 and both had similar

sedimentation and diffusion coefficients. Their pH optima were identical and so were their general assay requirements, however they were clearly separable by disc-electrophoresis. On the other hand Wu and Tinker (64) in their purification of phospholipase A from Crotalus atrox showed the presence of only one enzyme displaying A2 activity, having a MW of 30,000, a pH optimum of 6.8-7.6 and an optimal temperature of 46°. It was activated by Ca and other divalent cations and was quite resistant to heat treatment at acid pH. Thus it is apparent that the phospholipases A2 of various snake venoms may differ appreciably in their properties however they all seem to have the same positional specificity.

The substrate specificity of a crude phospholipase A preparation from C. adamanteus was studied in detail by Van Deenen and De Haas (65). They found the enzyme specific for the L-isomers of 3-phosphoglycerides (sn-3-phosphoglycerides). Except for the water-soluble short-chain phosphatides which were hydrolysed very slowly, the enzyme has no preference for the type of the constituent fatty acids. The nature of the polar head-group in the phosphoryl moiety turned out not to form a prerequisite since its presence appeared dispensable. As far as prerequisites for substrate activity are concerned, it appears that the compound needs only one acylester bond vicinal to the phosphoryl ester bond.

b) Pancreas

Phospholipase A from pancreas was first partially purified by Magee et al (66) and appeared to have properties similar to the snake enzymes. Conflicting results by Rimon and Shapiro (67) working with porcine pancreatic enzyme indicated however that phosphatidic acid was degraded to 2-monoacyl-sn-glycerol-3-phosphate. Thus in this case phospholipase A1 activity seemed to be present. The difference in results obtained by Rimon and Shapiro was explained on the basis that their method for identifying the lyso product yielded erroneous results (68). On the other hand as mentioned previously, it is now known that both A1 and A2 activities are present in this tissue and that a lack of A1 activity might be attributable to the heat treatment which is often a routine step in the purification of phospholipase A.

Recently De Haas et al have purified two proteins from porcine pancreas, one which they characterized as the zymogen of phospholipase A2 (69) and the other, phospholipase A2 (70). The zymogen has a MW of about 15,000 appears to consist of a single polypeptide chain terminating at the NH_2 region in the amino acid sequence: Glu-Gly-Glu-Ile-Ser-Arg-Ala. The N-terminal glutamic acid residue has no free $-NH_2$ group. Phospholipase A is a protein of about 13,800 $\pm$ 500 , appears to consist of a single polypeptide

chain terminating in alanine (NH_2) and cystine (COOH), and crossed-linked intramolecularly by disulfide bridges. The active enzyme can be released from the zymogen by peptidase action.

Pancreatic phospholipase A acts sterospecifically on all common types of sn-3-phosphoglycerides by hydrolysing exclusively the fatty acid ester bonds at the glycerol C_2-position regardless of chain length or degree of unsaturation. In contrast to the snake venom phospholipase A, the pancreatic enzyme shows a marked preference for anionic phospholipid such as phosphatidic acid, cardiolipin and phosphatidyl glycerol. It requires Ca for activation (70).

Properties of phospholipase A1:

It is only recently that phospholipase A1, has been recognized (6) and thus rather little is known about its properties. Besides its occurrence in the several tissues described (55,56) this enzyme was also found in brain by Gatt (71) who showed that it has a pH optimum of 4.0 and a Km of 8×10^{-4}. A particulate enzyme, it requires triton X-100 for activation but is inhibited by the addition of sodium cholate. It is active on lecithin but not on 1-acyl-lyso-lecithin.

Phospholipase A1 is also present in the lysosomal fraction of adrenal medulla. It has a low pH optimum of

4.2 and does not seem to require Ca for its activation (50).

There appears to be a relation between phospholipase A1 activity and lipase activity of pancreas since highly purified pancreatic lipase was found to act on the 1-position of phosphoglyceride (72). Vogel and Bierman (73) also found with post-haparin plasma that lipase and phospholipase activity could not be separated. This phospholipase was again characterized as acting on 1-position of PE (74). Further studies are required to establish whether phospholipase A1 is a true phospholipase or just a lipase with broad substrate specificity. The evidence to date reveals however that the action of purified lipase on phosphoglycerides is quite slow and quite possibly phospholipase A1 is a distinct enzyme. (75)

Lysophospholipase

Occurrence:

Lysophospholipase was first recognized in rice and bran by Contradi and Ercoli (76). It was also found to occur in Penicillium notatum (77), Serratia plymuthicum (78) snake venom (79), and E. coli (39); and a number of mammalian tissues (80). Noguchi (81) found that the richest sources of lysophospholipase are pancreas and lung. Marples and Thompson (80) studied the distribution of this enzyme in a variety of rat tissues and extended its occurrence in some

areas of human brain. They indicated that intestine, lung and spleen were the most active sources in rat while nervous tissue and heart had little activity. It has also been found in red blood cells and Ferber and Munder (82) indicated that the aged human erythrocytes were 40-50% lower in activity than the young cells.

Mode of action, specificity and properties:

Lysophospholipase catalyzes the hydrolysis of a single acyl ester linkage of lysophosphoglycerides yielding a water-soluble phosphodiester and fatty acid (44). It is not known whether the enzyme shows any positional specificity. Migration of the acyl group from the 2 to the 1 position of glycerol during incubation and separation procedures has so far curtailed a clarification of this point.

The action of lysophospholipase from Penicillium notatum does not hydrolyze purified ovalecithin but is active against brain or soybean lysolecithin. In this organism, the action of lysophospholipase was not limited to monoacyl phosphoglycerides since further studies by Dawson, indicated that when phosphatidylinositol or diphospholinositide are added as activators, the P. notatum enzyme will also attack pure lecithin (83). The activators are required to impart a net negative charge to the lecithin miscelles. Kates et al demonstrated that the P. notatum

enzyme, phospholipase B, catalyzed rapid deacylation of purified egg lecithin in the absence of any activator when the substrate was ultrasonically dispersed. The rate of enzyme hydrolysis depends on the degree of unsaturation (84). In this case the possibility of a combined action of phospholipase A2 and lysophospholipase, accounting for B activity, was not precluded. On the other hand, the pancreas enzyme will not attack lecithin even in the presence of similar activating lipids (44). Thus the specificity depends largely on the physicochemical state of the substrate and on the source of the enzyme.

The general properties of lysophospholipase also vary with the source of enzyme. Accordingly, the enzyme from pancreas is similar to the enzyme isolated from liver, having a pH optimum of 6.0 whereas the enzyme from P. notatum is most active between pH 3-4. The results of Marples and Thompson (80) indicated that the lysophospholipases from mammalian tissue are heat labile. Heating at 6.0 for 15 minutes completely abolished the enzyme activity. Ca and EDTA had no significant effect and again unlike the case for phospholipase A, Dawson (85) showed that ethylether inhibits the lysophospholipase from rat liver. Shapiro (86) was able to purify the pancreas enzyme 40-fold and crystallize it in low yields. The purified enzyme required no Ca ion and ether inhibited the activity. In contrast the lysophospholipase identified in snake and bee venoms

have pH optima between 8.0 and 10, are stable to heat treatment and are activated by both Ca and Mg ion (79).

STATEMENT OF PROBLEM

In preliminary studies by Proulx and Van Deenen, incubation of PE with E. coli sonicates led to the accumulation of GPE and fatty acid. It was proposed that these products arose by the combined action of phospholipase and lysophospholipase, however no direct evidence for the presence of the first enzyme was given.

In the present studies we have first attempted to study the conditions which might favor phospholipase A activity *in vitro* and the accumulation of lyso phosphoglyceride, a key product of this reaction.

Using several labelled substrates we have attempted to further characterize this enzyme with respect to its pH optimum, its action requirements, its substrate and positional specificity, its subcellular distribution and its susceptibility to heat treatment.

We have also studied the pH requirement for lysophospholipase activity.

MATERIALS AND METHODS

Extraction of lipids

Total lipids were extracted quantitatively by the method of Bligh and Dyer (87) modified for our purposes. To 1 volume of aqueous suspension or incubation mixture, 2.5 volumes of methanol and 1 volume of chloroform were added. The monophasic mixture was then stirred 20-30 minutes at room temperature and 1 volume of each, chloroform and water were added. A biphasic system formed which was stirred for an additional 20 minutes. After centrifugation, the bottom phase containing the lipids was removed with a pasteur pipette and the top aqueous phase was re-extracted with 1 volume of chloroform by stirring 15-20 minutes. After centrifugation, the second bottom chloroform phase was removed and pooled with the first. The pooled extract was then evaporated to dryness and kept under nitrogen or dissolved in a known volume of either benzene or a mixture of chloroform methanol 1:9 (v/v). Samples were stored at -20^{o}.

When ^{32}P-labelled phosphoglycerides were extracted, water was replaced by an 0.1M Na-phosphate buffer pH 7.0 to remove all traces of ^{32}P-labelled ortho-phosphate in the lipids.

Preparation of labelled substrates

The procedures followed were essentially those of Van Deenen et al (58,88,89).

(a) ^{32}P-labelled phosphatidylethanolamine and lecithin

About 2-3 grams of liver from a male rat 3-4 weeks old, was rinsed in ice-cold Krebs-Ringer bicarbonate buffer pH 7.4. It was then cut into small pieces and the mince was introduced into a 40 ml centrifuge tube containing 2 ml of ice-cold Ringer buffer. 2.5 mC of ^{32}P-orthophosphate (purchased from Radiochemical Center, Amersham, England) was neutralized with dilute NaOH and transferred to the liver suspension. The mixture was incubated for 3-4 hours at 37° with shaking and throughout the incubation period a gentle stream of oxygen was bubbled through the suspension. The mixture was diluted to 10 ml with distilled water and homogenized with a hand glass tissue grinder. The homogenate was then transferred to a large centrifuge bottle and the lipids were extracted as described previously.

The lipid extract was evaporated to dryness in vacuo at 37° and dissolved in a small volume of chloroform. An incorporation of 15-20 uC was usually obtained for the total lipid fraction.

The labelled phosphatides were then separated by thin layer chromatography on activated silica-gel-G with a

mixture of chloroform-methanol-water 65:25:4 (v/v) as solvent. The nitrogen containing lipids were stained reversibly by spraying with a solution of Ponceau red prepared as follows: 2 gms of uranyl nitrate and 0.05g Ponceau red were dissolved in 1 liter of 0.01N HCl, PE and PC showed up as major red coloured bands and were identified by cochromatography with highly purified PE and PC from egg yolk or with synthetic phosphatides obtained from General Biochemicals Corp., Ohio. The stained components were scrapped off, suspended in 10 ml of water, and each substance was extracted by the modified Bligh and Dyer procedure described previously. In this procedure following the centrifugation step, the water-soluble products as well as the Ponceau red dye remain in the aqueous phase whereas the silica gel separates out as well packed interphase layer. The lipids are recovered in the bottom chloroform phase. The chloroform phase for each fraction was transferred with a pasteur pipette to a conical flask and dried in vacuo. Each lipid was dissolved in 1 ml of redistilled chloroform and then further diluted to 10 ml with methanol. Samples were stored at -20°C. The purity of each labelled substrate was checked by re-chromatography on silica gel G using chloroform-methanol-water (65:25:4 v/v) as solvent. Scanning each chromatoplate with a Chicago Actigraph II Radiochromatogram Scanner revealed that the radiopurity of each compound was

usually over 97%. A typical purity check is shown in plate I.

(b) ^{14}C-labelled Phosphatidylethanolamine and Phosphatidylcholine

(i) Substrates labelled in the 1-acyl position

2.5 gms of liver from a young rat (2-3 weeks old) was homogenized in 10 ml of 0.2 Tris-HCl buffer pH 7.4 containing 0.125M KCl and 0.002M $MgCl_2$. To 3 ml of this suspension was added 1 ml of a 0.2M Tris-HCl solution containing 75 mg of ATP (Nutritional Biochemicals Corp.) adjusted to pH 7.4. Finally, 10 uC of stearic acid 1-^{14}C (New England Nuclear Corp.) was added as a sonicated suspension in 2 ml of 0.2M Tris-HCl buffer. The mixture was incubated with shaking for one hour at 37°. The lipids were extracted and separated as described previously.

(ii) Substrates labelled in the 2-acyl position

4 gms of liver were removed from a young rat (2-3 weeks old) starved overnight. The tissue was rinsed and chilled in several volumes of ice-cold 0.25M sucrose and then homogenized in 18 ml of 0.25M sucrose. Nuclei, mitochondria and cell fragments were removed by centrifuging at 15,000 g for 15 minutes at 5°C using a Lourdes refrigerated

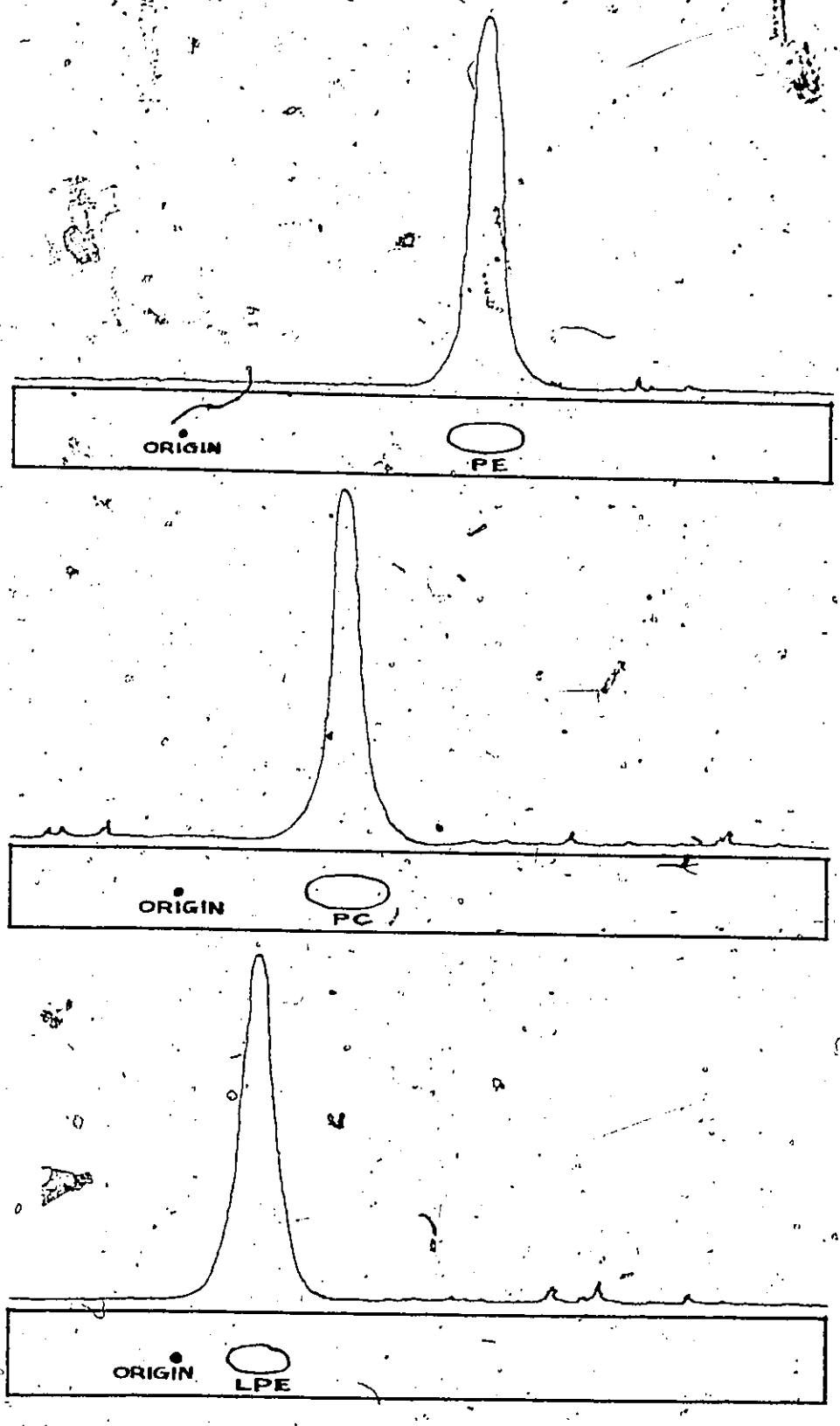
centrifuge. The supernatant was then centrifuged for 1 hour at 100,000 g using a Spinco preparative ultra-centrifuge. The crude microsomal pellet was suspended in 4 ml of KCl-Tris buffer pH 7.4 and served as the source of enzyme for labelling of the phosphatide fractions. 50uC of a benzene solution of 1-^{14}C linoleic acid (New England Nuclear) was evaporated to dryness under a nitrogen stream. 1 ml of solution containing 25 mg ATP, 0.7 mg CoA and 20 um moles of $MgCl_2$, adjusted to pH 7.4 was added over the linoleic acid film and the suspension was sonicated at 65W for 3-5 minutes in a salt-ice mixture. The sonicate was transferred to a 40 ml centrifuge tube and 2 ml of microsomal suspension was added. The mixture was incubated 1 hour at 37° with shaking and was then stopped with the addition of 2.5 volumes of methanol. Lipids were extracted and isolated as previously described. The radio-purity of each compound was checked by thin layer chromatography (plate I).

Distribution of radioactivity in th ^{14}C-labelled substrates

The distribution of 1-^{14}C stearic acid or linoleic acid within each ester position was estimated by degrading each labelled substrate with phospholipase A (EC 3.1.1.4) from Crotalus adamanteus venom (Sigma Chemicals). 1 uMole

Plate I

Chromatographic of the purity of substrates



substrate ($3-4 \times 10^4$ cpm) was added as a methanol-chloroform solution to a 12 ml conical tube and evaporated to dryness with a stream of nitrogen. The lipids were then redissolved in 1 ml of ether and layered over 1 ml solution of 0.1M borate buffer containing 1 mg snake venom and 5 umoles of $CaCl_2$. The mixture was vigorously agitated at room temperature for 3 hours. A control containing no enzyme was prepared in the same manner. Most of the ether was then removed by evaporation *in vacuo* at 37° and following lipid extraction, the reaction products were separated by thin layer chromatography using activated silica-gel-G plates and a mixture of chloroform-methanol-water (65:25:4 v/v) as solvent. The separated components were identified by co-chromatography with unlabelled, highly purified lysophosphatides, their diacyl analogues and stearic or linoleic acid. The spots were revealed with iodine vapors, marked, and then freed from iodine by a gentle flow of warm air. The components were then scrapped off the plate and eluded by suspending the silica-gel-G in 1-2 ml of water and extracting by the modified method of Bligh and Dyer.

The products as well as any unhydrolyzed substrate were then counted as described in a section following. After correction for 1-2% non-enzymatic hydrolysis any radioactivity in the lysophosphoglyceride was taken as the amount of label in the 1 position and likewise any

radioactivity in the fatty acid fraction was interpreted as labelling in the 2 position. Assayed in this manner, the designated 1-(stearoyl-1-^{14}C) phosphoglycerides contained approximately 88-90% of the label in the 1 position and 2-(linoleoyl-1-^{14}C) phosphoglycerides contained 90-92% of the label in the 2 position.

Doubly-labelled substrates

These were prepared by mixing various proportions of ^{32}P-labelled phosphoglyceride with either 1-(stearoyl-1-^{14}C)-phosphatide or 2-(linoleoyl-1-^{14}C)-phosphatide.

Preparation of lysophosphoglycerides

PE and PC, either cold, or labelled in the 1-acyl ester position with stearic acid-1-^{14}C or with ^{32}P-ortho-phosphate were converted to their 1-monoacyl analogues by the action of phospholipase A2 (EC 3.1.1.4) from Crotalus adamandeus venom. The reaction mixture was carried out as described in the preceeding section. The lysophosphoglyceride formed was extracted by the modified method of Bligh and Dyer and isolated by preparative thin layer chromatography using silica-gel-G and system A as solvent. The product revealed by the Ponceau red stain was eluded by the extraction method previously described for the preparation of other labelled phosphatides. The purity was checked by re-

running in the same chromatographic system (plate I).

Preparation of sn-glycero-3-phosphoryl ethanolamine and sn-glycero-3-phosphoryl choline

The water-soluble phosphodiester derivatives of PE and PC were prepared by the mild alkaline hydrolysis method of Dawson (90) as modified by Benson and Ferrari (91). 0.5 ml of chloroform solvent containing 10 umole of highly purified, cold or ^{32}P-labelled phosphoglyceride was transferred to a flask containing 5 ml of 1% toluene in methanol and 5 ml of 0.2N methanolic KOH. The mixture was incubated exactly 15 minutes at 37°C, then plunged in ice and 5 ml of ice-cold water were added together with 2 drops of 1% phenolphthalein in ethanol. A 30% (w/v) suspension of Dowex 50 was added drop by drop while stirring vigorously the mixture under ice until the pink color disappeared. The mixture was then centrifuged and the supernatant together with a 3 ml water and a 3 ml methanol rinse of the resin were pooled and evaporated to dryness at 50°C in vacuo. A mixture of 100 ml chloroform, 50 ml isobutanol and 75 ml of distilled water was shaken and allowed to separate into two phases. The residue was then taken up with 1 ml of upper phase and 2 ml of lower phase and after vigorous shaking the mixture was centrifuged to separate both phases. The upper layer containing the phosphodiester

was used as chromatographic standard.

Preparation of ^{32}P-labelled phosphoryl choline

5 umole of ^{32}P-labelled lecithin obtained biosynthethically were dissolved in 5 ml of ether contained in glass stoppered flask. 1 ml of 0.1M borate buffer pH 7.0 containing 5 mM $CaCl_2$ and 1 mg of phospholipase C from Cl. welchii was added to the flask and the mixture was shaken vigorously for 4 hours at room temperature. The ether was then distilled off in vacuo and the water phase was extracted by the modified method of Bligh and Dyer. The upper phase thus obtained was concentrated to 0.5 ml and used as chromatographic standard. Cold synthetic o-phosphorylcholine and o-phosphorylethanolamine obtained from Sigma Chemicals were also used as chromatographic reference compounds.

Chromatographic Procedures

(a) Chromatography of water-soluble compounds

Water-soluble products were separated and identified by paper chromatography using as solvents systems (A) phenol-water 5:2 w/w and (B) propanol-ammonia-water 6:3:1 v/v.

Tanks were saturated 24 hours before use, each with the appropriate solvent. An aliquot of 25-50 ul of extract

containing labelled product was applied with a Hamilton syringe as a small spot 4 inches from one end of a strip of Whatman No.3 mm paper. Several other aliquots each containing a comparable amount of non-labelled E. coli extract were applied as spots alined with the radioactive sample. Over each of these unlabelled spots $3-5X10^3$ cpm of a different labelled reference compound was also applied. The solvent was allowed to descend 18 hours, the paper was then removed, the front was marked and the solvent was allowed to evaporate off completely in a fume hood. Labelled components were identified by scanning the chromatograms with a Nuclear Chicago Actigraph III Radioscanner equipped with 4 pi detector. Spots were also revealed by spraying with ninhydrin (92) periodate-Schiff reagents or ammonium molybdate-perchoric acid reagent (93) however these did not always give conclusive results because of interfering non-labelled substances present in the extract. The R_f values of a number of reference compounds are given in Table(i)

Table (i)

Compound	R_f A	R_f B
Pi [1]	0.08	0.07
GP [1]	0.20	0.10
GPE-P^{32} [2]	0.60	0.44
GPE [3]	0.62	0.44
GPC [1]	0.96	0.54
GPC [1]	0.96	-
GPC-P^{32} [2]	0.96	0.54
PhE [1]	0.35	0.30
PhC [1]	0.75	0.35
PhC-P^{32} [4]	0.78	0.35

(1) obtained commercially (2) prepared from ^{32}P-phosphoglyceride isolated from rat liver (3) prepared from the parent phosphatide isolated from egg yolk. (4) prepared from PE-^{32}P (liver) by the action of phospholipase C. Abbreviations: Pi, inorganic phosphate; GP, DL-glycero-3-phosphate; GPE, glycerophosphorylethanolamine; GPC, glycerophosphorycholine; PhE, phosphorylethanolamine; PhC, phosphorylcholine.

(b) Chromatography of Lipids

Lipids were separated by thin layer chromatography. A slurry of silica gel G (25 gms/50 ml water) was spread as a layer 0.25 mm thick on glass plates (20X20 cm or 5X20 cm) using Desaga equipment. Thicker layers of up to 1.5 mm were also used for preparative chromatography. The plates were allowed to stand 15 minutes at room temperature and then activated 2 hours at 110°. They were then used immediately or stored in a desicator-cabinet.

(i) Phospholipids

Phospholipids were separated by first running the plates in a mixture of chloroform-petroleum ether-acetic acid (system C) and, after brief drying in air, the plates were transferred to a second solvent (D) composed of chloroform-methanol-water (65:25:4 v/v). A first run in solvent C was important only when highly labelled fatty acid fractions were present along with the phospholipids. Such was the case for experiments dealing with the biosynthesis of ^{14}C-labelled lipids. Solvent C+D permitted a distinct separation of fatty acids from labelled phospholipids.

(ii) Neutral lipids

Neutral lipids were separated, using as solvent, a mixture of petroleum ether 60/90-ether-formic acid in the proportion 75:25:1.5 v/v system (E) or in the proportion (55:35:1.5 v/v) system (F). System E separates all neutral lipids except monoglyceride which remains close to the phospholipids at the origin. System F separates monoglycerides from phospholipids however fatty acids and triglycerides move closer together. Typical separation are illustrated in plate II.

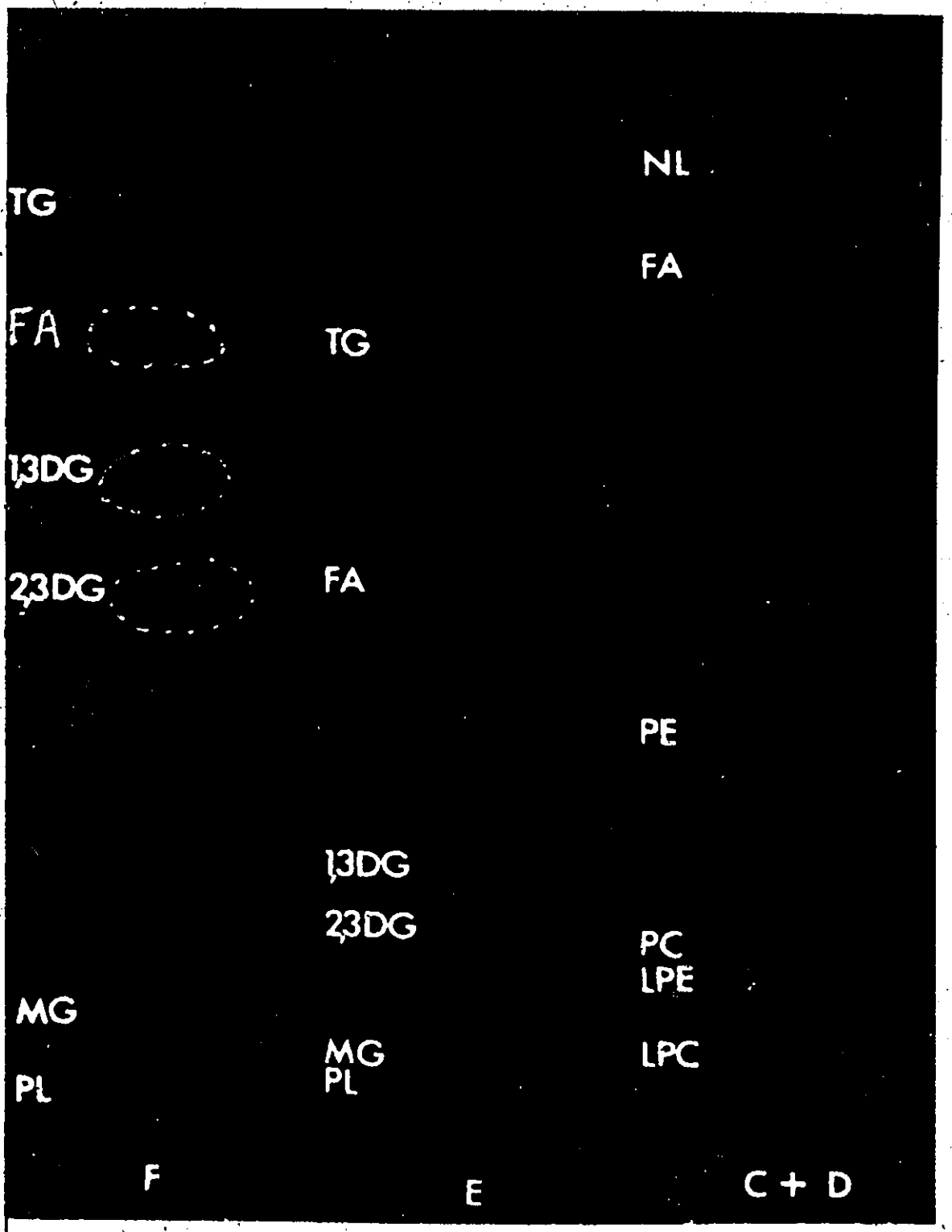
Radioisotope counting procedures

Preparation of counting sample

Lipid components, singly-labelled with ^{32}P were usually counted in one of two manners; (i) after separation of lipids by thin layer chromatography, the plates were scanned using an Actigraph III Radio Activity Scanner equipped with a digital integrator and a gas flow detector. The efficiency of counting was estimated at about 20-25% using a standard for each plate.

(ii) After chromatography the samples were scrapped off and transferred to liquid scintillation vials containing 15 ml of 0.5% PPO (2.5-diphenyloxazole) and 0.05% dimethyl POPOP (1.4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) in

Plate II Separation of lipids



toluene. Counting was performed in a Mark I Nuclear Chicago Spectrometer. A quench curve was prepared with addition of increasing amounts of chloroform to a series of ^{32}P standards and plotting the change in channel ratio as a function of efficiency. Although both methods gave comparable results method (i) was preferred in most of the later experiments.

Singly-labelled ^{14}C lipids method

Procedure (i) was unsatisfactory in this case since the efficiency varied between 1 and 3% and variations due to the thickness of the gel layers could not be corrected satisfactorily by spotting a ^{14}C standard on each plate. Method (ii) was satisfactory provided 4% Cab-o-sil (a thixotropic gel) was added to the scintillation fluid to suspend the silica gel. A separate quench curve was prepared for counting in the presence of Cab-o-sil.

For doubly-labelled substances, method (ii) did not prove satisfactory and elution was performed as follows: the components was scrapped off the plate, suspended in 5-10 ml of water and the lipid was extracted by the method of Bligh and Dyer. The chloroform phase thus obtained was evaporated to dryness and dissolved in a small volume of methanol. Samples were counted in the scintillation mixture described for method (ii). Quench curves for ^{32}P-^{14}C

simultaneous counting were made using chloroform as quench and an external standard (57,94).

Aqueous extracts were counted as 0.1 ml aliquot in the scintillation fluid described for method (ii) to which 2 ml of Triton-X-100 was added per vial.

Water-soluble components on paper chromatograms were counted by method (i).

Culture and preparation of E. coli

E. coli strains 015 and 0118 were supplied by Dr. M. Beaulieu of the Department of Bacteriology, University of Ottawa. The cells were cultured to an early stationary phase (OD_{625} 0.25) in a medium containing per liter: 15g of bactopetone, 1g of yeast extract, 20g glucose and 5 gm sodium chloride. The purity of the culture was routinely checked by the Gram stain and by culture on McKonkey plates or citrate slants. Typing of the strains was kindly performed by Mr. D. Peters, Laboratory of Hygiene, Department of National Health and Welfare.

The cells were collected by centrifugation at 8000 rpm in a Lourdes refrigerated centrifuge kept at 5°C. The sediment was washed once with fresh medium and finally suspended on an appropriate buffer described subsequently for each experiment. The suspension was then sonicated 15 minutes in ice at 125W using a Biosonik II

ultrasonicator. In preliminary trials unbroken cells were removed by centrifuging the sonicate 10 min. at 3000 rpm (26); however this step proved unnecessary since no significant difference in the results obtained when subsequently the whole cell sonicate was used. Protein, assayed by the Lowry method (95) was maintained at a concentration about 20 mg/ml by diluting the sonicate with an appropriate amount of buffer.

Phospholipase A activity

The incubation mixture contained 1 ml of whole cell sonicate (20 mg protein), 0.1 ml of 0.2M $CaCl_2$ 0.1 ml sodium lauryl sulfate or sodium deoxycholate and 0.8 ml of 0.1M appropriate buffer. The substrate, 1-2 umole of labelled phosphoglyceride was dissolved in 1 ml of ether and layered over the aqueous extract. Incubation was carried out at room temperature for up to 4 hours with vigorous shaking.

On some occasions the substrate was sonicated at 100w for 3-5 minutes in buffer and 0.8 ml of this suspension containing 1-2 umole of substrate was added to the incubation mixture. In this case no ether was present in the system. For pH studies the following buffers were used:

pH 4.0-5.7	0.1M acetate buffer
pH 5.8-7.4	0.1M phosphate
pH 5.0-7.4	0.1M histidine
pH 7.4-9.0	0.1M Tris-HCl or borate
pH 9.0-11.0	0.1M carbonate or borate

Lysophospholipase activity

Identical conditions as for phospholipase A were used except that labelled lysophosphoglyceride served as substrate and Ca and detergent were omitted.

EXPERIMENTAL RESULTS

The presence of phospholipase A and lysophospholipase E. coli 015 and 0118

In a preliminary investigation we tested our E. coli sonicates for the presence of lypolytic enzymes using as substrates, ^{32}P-labelled PE and 1-(stearoyl-1-^{14}C)-PC. The incubation was carried out at pH 7.4 in borate buffer and in the presence of DOC and ether. These conditions were arbitrarily chosen from known conditions favoring animal phospholipase A. As can be seem from Fig. 1, E. coli 015 degraded ^{32}P to lyso PE and water-soluble products. At a 5 mM concentration of DOC, lysophospholipase was not completely inhibited since water-soluble products also accumulated. However phospholipase A activity was clearly demonstrated under these conditions.

The aqueous phase, obtained following the Bligh-Dyer extraction of a 4 hours incubation mixture containing no detergent, were concentrated to a small volume and subjected to paper chromatography. Identification of the products was performed by cochromatography with reference compounds as described in the Methods. As can be seen in Table I inorganic phosphate was the main product but GPE and GPC were also present in significant amounts, together with glycerophosphate and phosphorylethanolamine and phosphorylcholine which were present in only trace quantities.

As can be seem from Fig. 2 E. coli 0118 degraded 1-(stearoyl-1-^{14}C)PC to labelled fatty acid mainly although

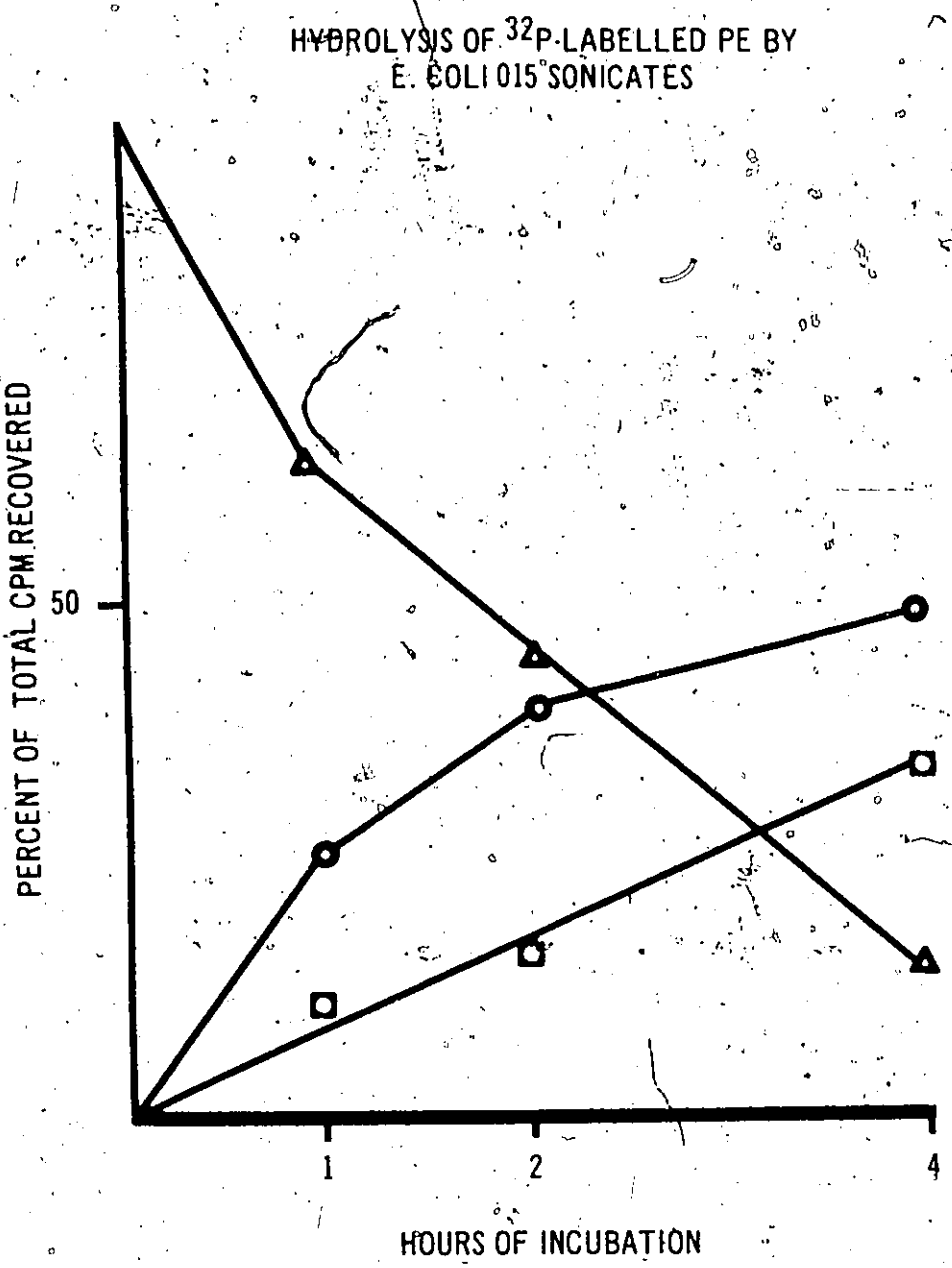


Fig. 1. ^{32}P-labelled phosphatidylethanolamine (2umoles) dissolved in 2 ml of ether was incubated at room temperature for various times with 1 ml of broken E. coli 015 cells. The cell suspension was sonicated 15 min at 100 W using a Biosonik II ultrasonic probe and contained per milliliter: 15-20 mg of protein, 4 mg sodium deoxycholate, 100 umoles of borate buffer, pH 7.4, and 10 umole $CaCl_2$. Labelled products were separated with chloroform-methanol-water, 65:25:4, v/v/v on activated silica gel G. (▲) PE; (o) lyso-PE; (□) water-soluble products.

Table I. Recovery of Water-Soluble Products by Paper Chromatography

Substrate	Labelled Product	% of total cpm recovered as water-soluble product	
		A	B
^{32}P-PE			
	Pi	22.5	35.0
	GP	6.3	10.0
	GPE	9.7	9.0
	PhE	1.8	-
^{32}P-PC			
	Pi	18.0	27.6
	GP	8.9	11.0
	GPC	6.5	7.8
	PhC	1.2	0.8

The mixture was incubated for 4 hours in the absence of detergent and other conditions were the same as stated for Fig. 1. The aqueous phases obtained after the Bligh-Dyer extraction were concentrated to a small volume. Water-soluble fractions were spotted on strips of Whatman No. 3 mm filter paper and chromatographed in a descending system with (A) phenol-water 5:2 w/w (B) propanol-ammonia-water 6:3:1 v/v as solvent. Labelled components were identified by scanning the chromatograms with a Nuclear Chicago Actigraph III Radioscanner. For abbreviations see Table (i), Methods.

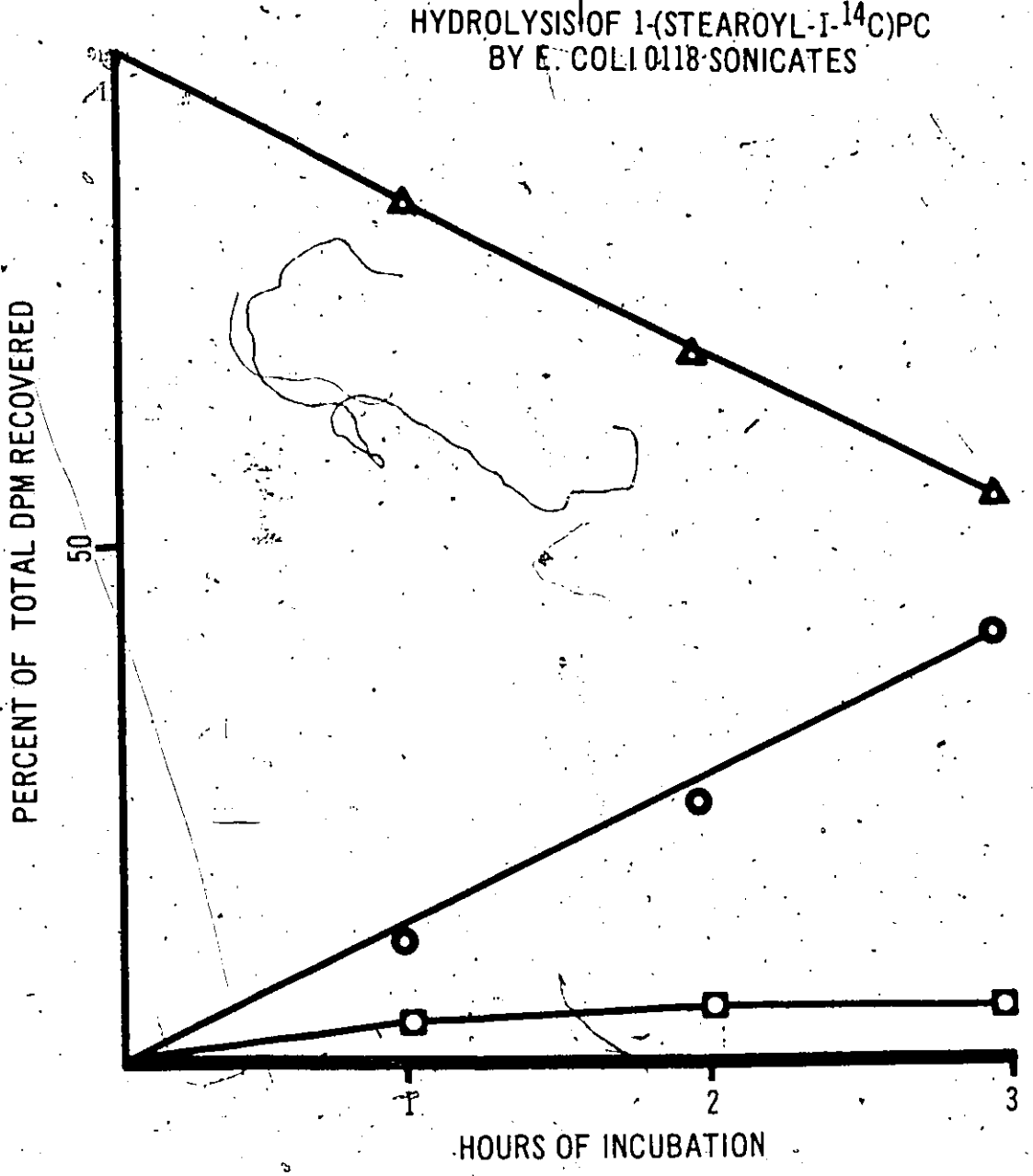


Fig. 2. 1-(stearoyl-1-^{14}C)-phosphatidylcholine (1 umole) was incubated for various times with E. coli 0118 under the conditions stated for Fig. 1. The products were separated on activated silica gel G with petroleum ether-ether-formic acid, 75:25:1.5 v/v/v. (o) Fatty acid; (▲) PC; (□) diglyceride.

some labelled DG was also produced. Identical results were obtained with E. coli 015 acting on either this same substrate or the PE anologue.

It appears from these results that phospholipase A is a predominant enzyme in the two E. coli studied. Lysophospholipase activity was also indicated and was not completely inhibited with 5 mM DOC.

Furthermore since with ^{14}C-labelled substrates the label was located mainly in the 1-acyl ester position and since the hydrolytic product in this case was mainly labelled fatty acid, it seems that the predominant lypolytic enzyme operating for both strains of E. coli is phospholipase A1 despite the rather high pH conditions used.

The effect of detergents on lysophospholipase and phospholipase A

Since lysophospholipase was also present in our E. coli sonicates we studied conditions which would inhibit this enzyme and at the same time favour phospholipase A activity. Since detergents have often been used to inhibit mammalian lysophospholipase (66) we tested the effect of SDS and DOC in our system.

Fig. 3 shows the effect of increasing concentrations of SDS and DOC on the hydrolysis of 1-(stearoyl-1-^{14}C)-lyso PE. At concentrations higher than 20 mM, SDS was as effective as DOC in inhibiting lysophospholipase activity. The

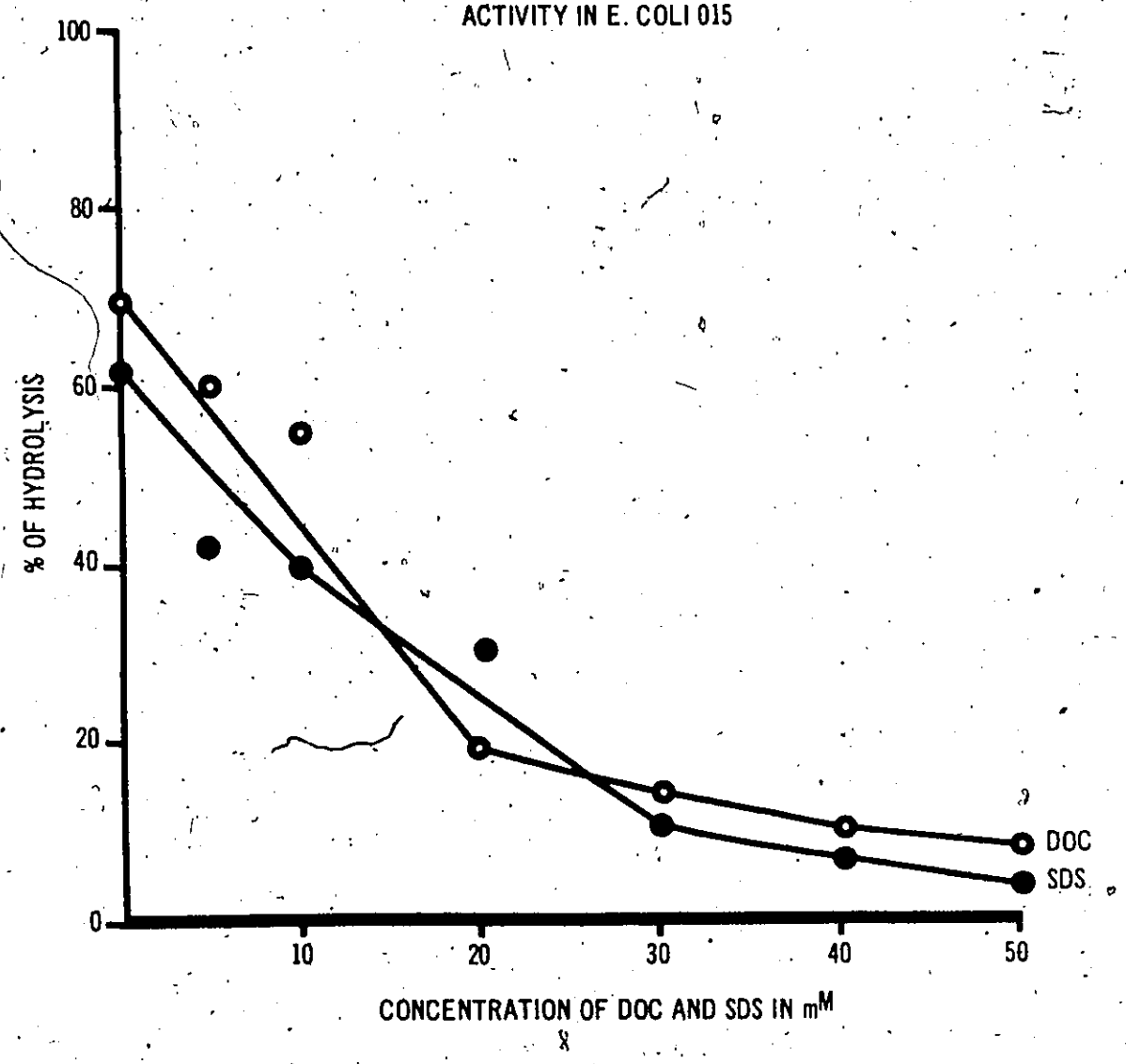


Fig. 3. 1-(stearoyl-1-^{14}C) lysoPE (1-2umoles) dissolved in 2 ml of ether was incubated at room temperature for 4 hours with 1 ml of sonicated E. coli cells, various concentrations of DOC and SDS and 0.1M of Tris-HCl buffer, pH 8.4. The products were separated as stated for Fig. 2. (○) with DOC (●) with SDS.

inhibition was not complete however even at a 50 mM concentration. The enzyme from E. coli appears to be more resistant to detergents than mammalian lysophospholipase which is completely inhibited by 5 mM DOC (97). As can be seem from Table II, addition of DOC greatly stimulated the accumulation of lysophosphoglyceride when ^{32}P-labelled PC or PE were used as substrate. This could be explained by inhibition of lysophospholipase. However when, 1-(stearoyl-1-^{14}C)PE was used as substrate, labelled fatty acid production was also enhanced by DOC addition and it seems therefore that detergents exert their action at least partly by stimulating phospholipase A acitivity.

The effect of pH on phospholipase A activity

When E. coli sonicate was incubated with ^{32}P-labelled PE in the presence of ether at various pH, lysophosphoglyceride production exhibited 2 peaks, one at pH 5 and other at pH 8.4(Fig. 4). Whereas the activity at acid pH could be detected without addition of detergent, the activity at alkaline pH required SDS or DOC. The same two peaks were obtained when the buffer combinations were changed as described in the Methods or when the substrate was added as a sonicated suspension rather than an ethereal solution. When DOC was omitted, lysophosphoglyceride production was maintained at acid pH but greatly reduced at alkaline pH. At the latter pH there was instead, a slightly increased production of water-soluble products(Fig. 5). The increase

Table II. Effect of Deoxycholate on the Breakdown of Phosphoglyceride in E. coli 015

Substrate	Condition	% of total cpm recovered as product		
		LysoPE	LysoPC	---
^{32}P-PE	-DOC	2.0	-	
	DOC	26.0	-	
^{32}P-PC	-DOC		1.4	
	DOC		25.0	
		Fatty Acid	LysoPE	DG
^{14}C-PE	DOC	26.0	1.0	3.4
	DOC	43.6	2.3	4.8

^{32}P-labelled or 1-(acyl-1-^{14}C)-phosphoglycerides were incubated for 4 hours in the presence or absence of 30 mM DOC. Incubation conditions and separation procedures were as stated for Fig. 1 and 2.

THE EFFECT OF pH ON PHOSPOLIPASE A ACTIVITY ON HYDROLYSIS E. COLI

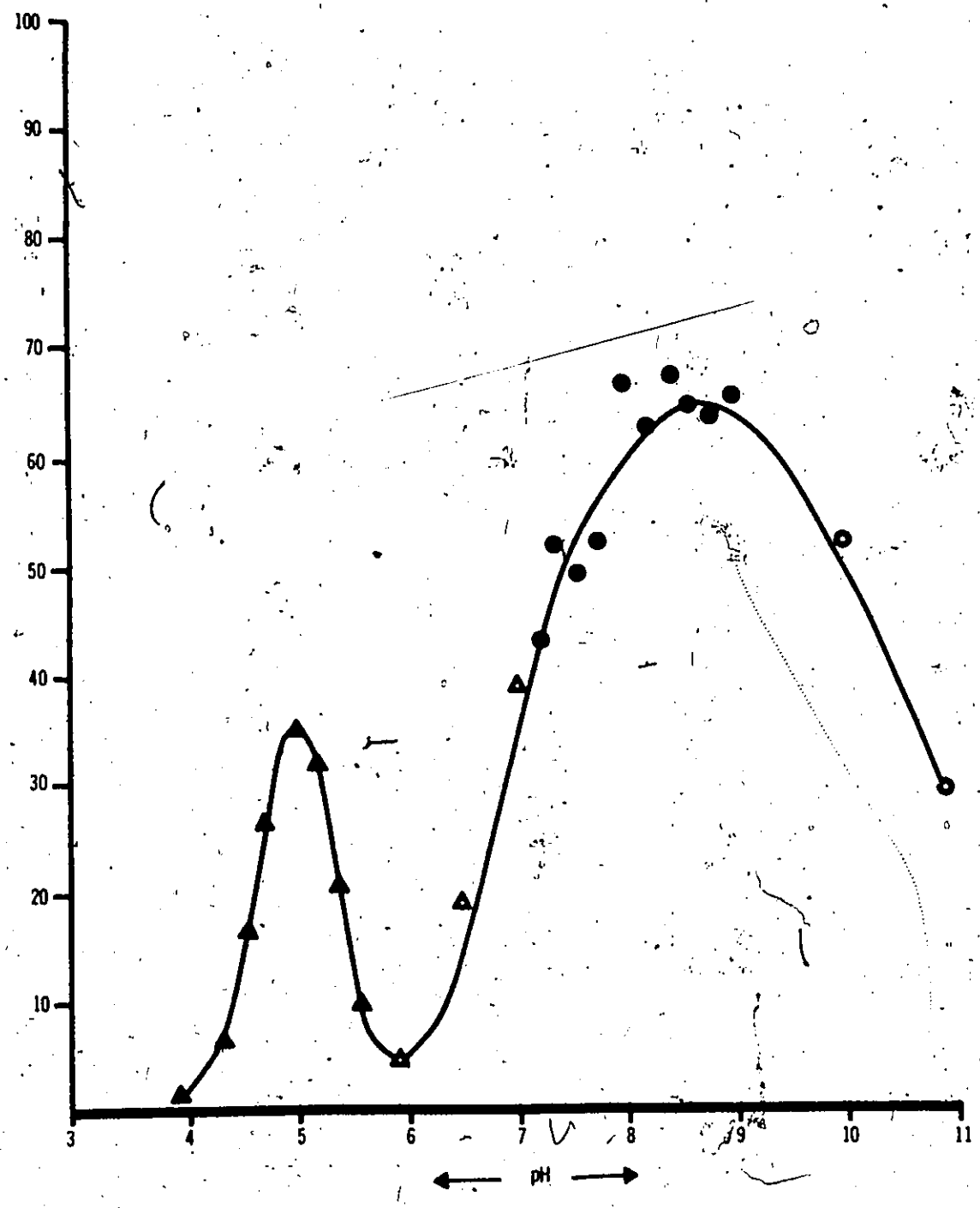


Fig. 4. The incubation mixture contained 1 umole of ^{32}P-labelled PE dissolved in 2 ml of ether, 1 ml of whole cell sonicate, 20 umoles $CaCl_2$; the pH was varied with the following Buffer; (▲) 0.1M acetate buffer, (▲) 0.1M phosphate buffer, (●) 0.1M Tris-HCl buffer with 30m^M DOC, (●) 0.1M bicarbonate buffer with 30m^M DOC, all final concentration. It was incubated for 4 hours at room temperature and the separation of labelled components was performed under the conditions stated for Fig. 1.

EFFECT OF pH ON HYDROLYSIS OF ^{32}P-LABELLED PE BY E. COLI 015 SONICATES IN THE ABSENCE OF DETERGENT

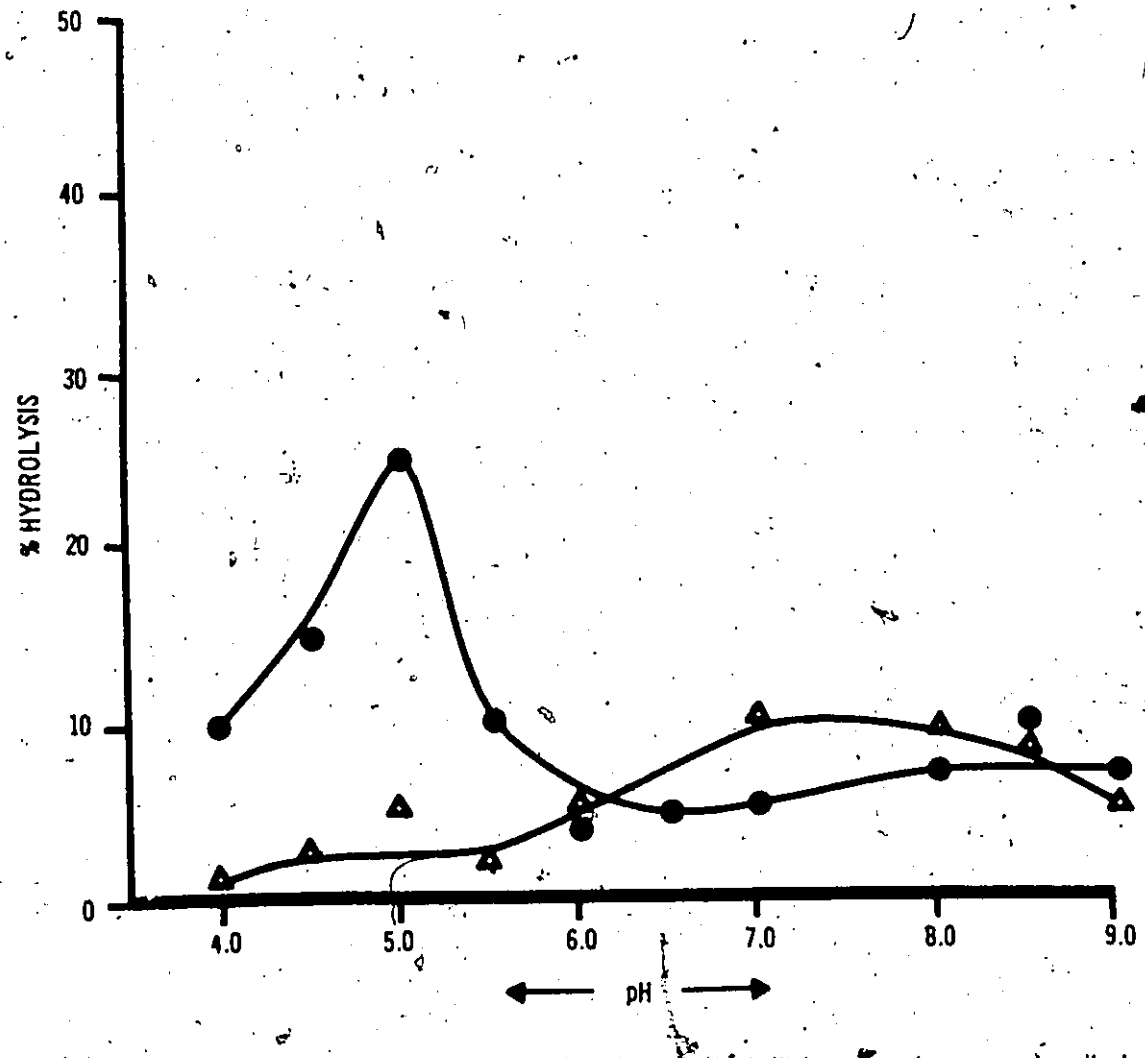


Fig. 5. The incubation conditions and the separation of labelled components were as described for Fig. 4. (●) lysoPE, (▲) water-soluble products.

in water-soluble products at alkaline pH did not match the fall in lysoPE production and this was taken as a further indication that detergent not only inhibits lysophospholipase but actually stimulates phospholipase A activity in the alkaline range.

When 2-(linoleoyl-1-^{14}C)PE was used as substrate in the presence of DOC, hydrolysis at acid pH resulted in lysoPE accumulation although small amounts of fatty acid were also found. At alkaline pH, lyso PE and fatty acid were produced in almost equal amounts, (Fig. 6). These results indicated the possibility that at least two types of phospholipase A with different pH optima are present in E. coli. Furthermore the result with 2-(linoleoyl-1-^{14}C)PE indicated that at pH 5 phospholipase A1 is a predominant enzyme whereas at alkaline pH both A1 and A2 are present in about equal proportions. In this experiment performed as part of our early studies, an arbitrary concentration of DOC (5mM) was used instead of the higher concentration (30mM) needed for the almost complete inhibition of lysophospholipase activity. Consequently, part of the labelled fatty acid production might have resulted from lysophospholipase activity. From a strictly equalitative point of view, the results with ^{32}P and ^{14}C-PE corroborate each other in that irrespective of the products analysed, two pH optima were revealed.

pH EFFECT OF PHOSPHOLIPASE A ACTIVITY ON THE HYDROLYSIS OF 2-(LINOLEOYL-1-^{14}C) PE IN E. COLI 015

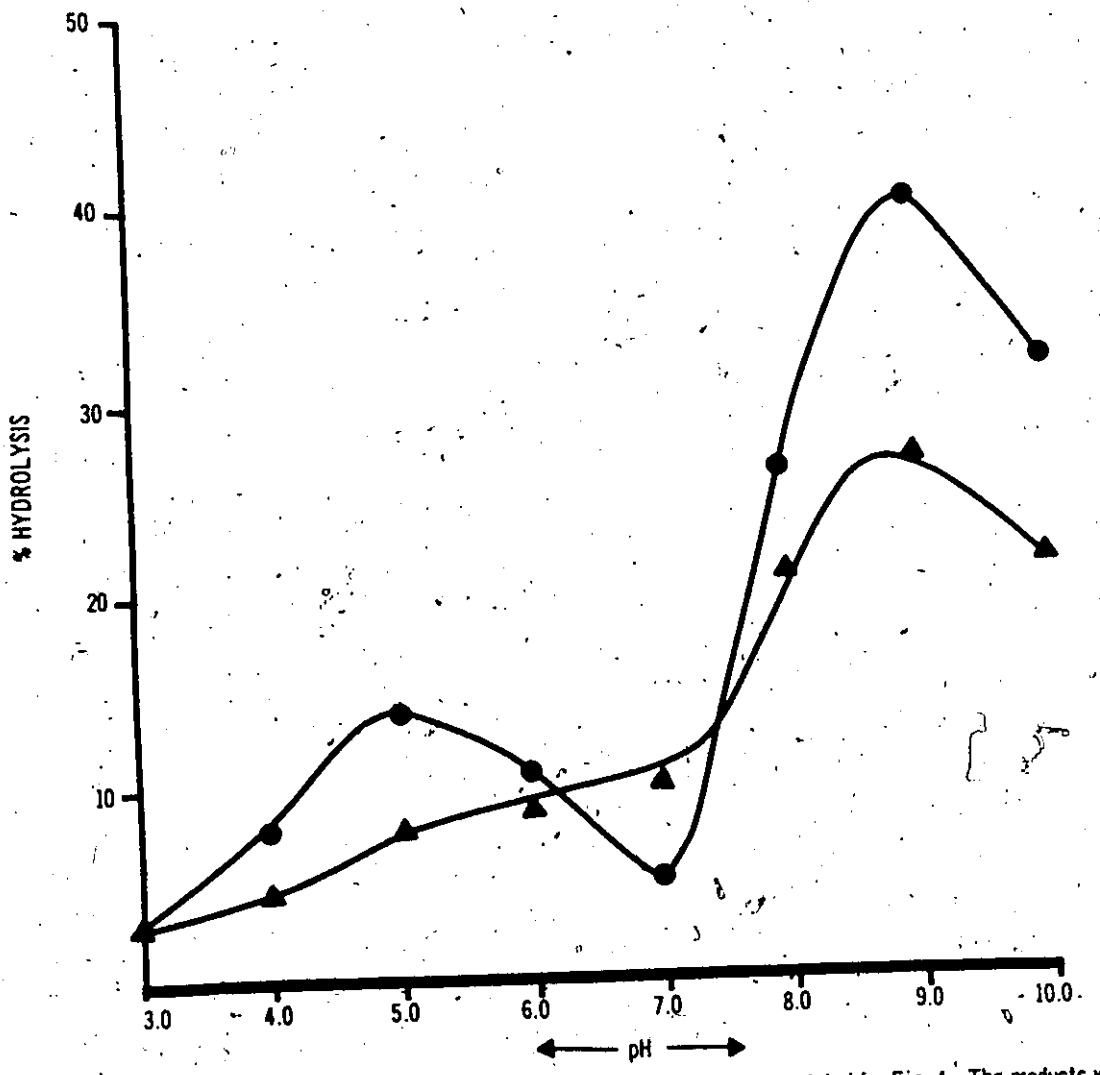


Fig. 6. The incubation conditions and buffers used were the same as stated for Fig. 4. The products were separated as stated for Fig. 1 and 2. (▲) fatty acid (●) lyso PE.

pH EFFECT OF PHOSPHOLIPASE A ACTIVITY ON THE HYDROLYSIS OF 2-(LINOLEOYL-1-^{14}C) PE IN E. COLI 015

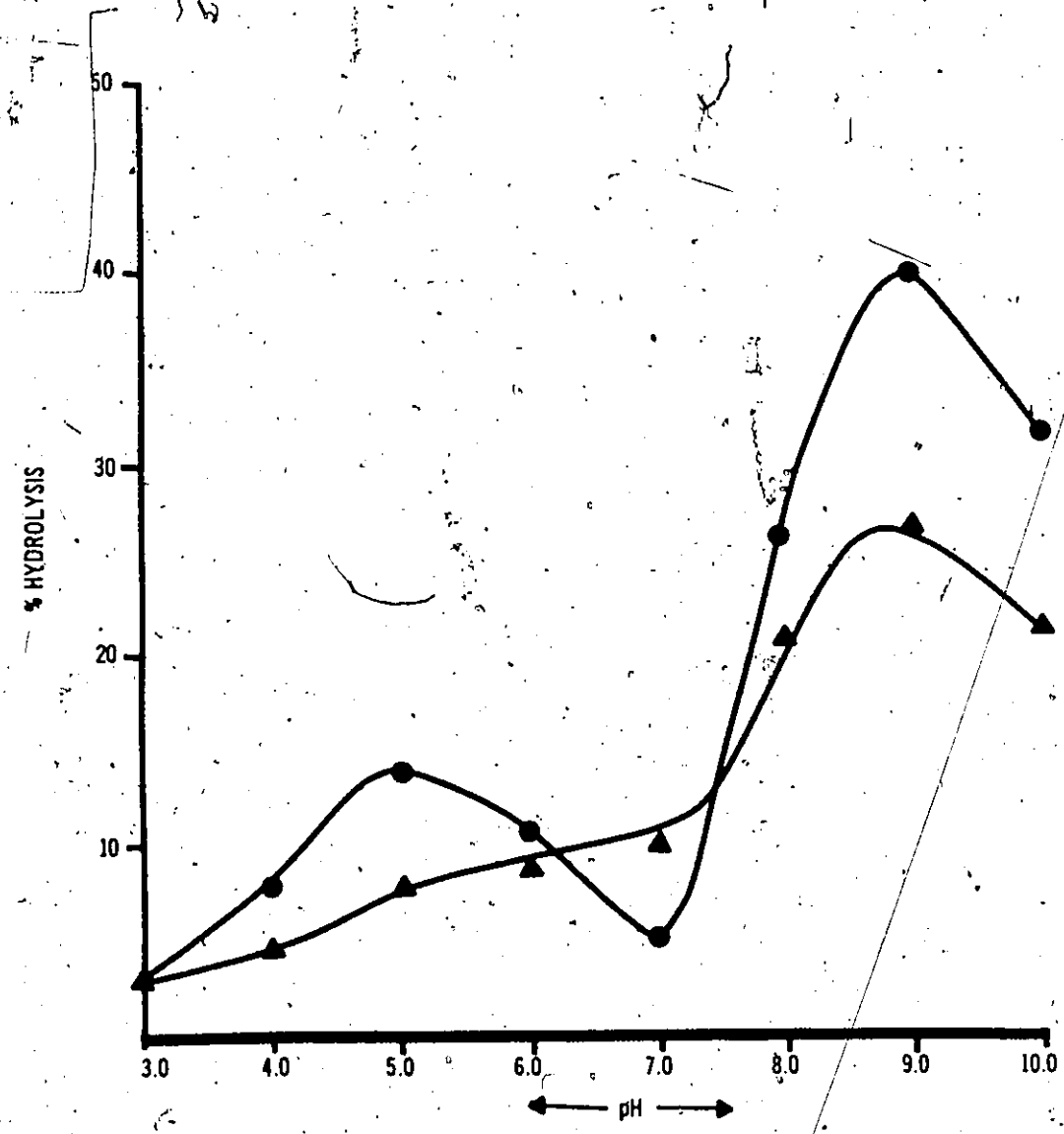


Fig. 6. The incubation conditions and buffers used were the same as stated for Fig. 4. The products were separated as stated for Fig. 1 and 2. (▲) fatty acid (●) lyso PE.

pH optimum of lysophospholipase

When 1-(stearoyl-1-^{14}C)-lysoPE was used as sonicated substrate in the absence of detergent and ether at various pH, lysophospholipase activity was detected mainly in the alkaline range and peak activity was found at pH 10 Fig. 7. Thus from these various experiments dealing with the effect of pH, it can be concluded that most of the lypolytic enzymes of E. coli show optimal activity in the alkaline range. However one of these, which appears to be a phospholipase A1 is active at acid pH.

Positional specificity of phospholipase A

To further test the positional specificity of phospholipase activity at either pH optimum, the degradation of 1-(stearoyl-1-^{14}C)PE and 2-(linoleoyl-1-^{14}C)PE to labelled fatty acid and lyso PE was followed under optimal conditions.

As can be seem from the results in Table III, at pH 5, 1-(stearoyl-1-^{14}C)PE was degraded mainly to labelled fatty acid whereas 2-(linoleoyl-1-^{14}C)PE yielded mainly labelled lyso PE. This indicated that at acid pH, phospholipase A1 was the predominant if not the only lypolytic enzyme operating.

At pH 8.4 in the presence of 30 mM DOC or SDS, 1-(stearoyl-1-^{14}C)PE was degraded mainly to labelled fatty acid although significant amounts of lyso PE were also produced. Conversely, 2-(linoleoyl-1-^{14}C)PE was degraded mainly to labelled lyso PE although an appreciable

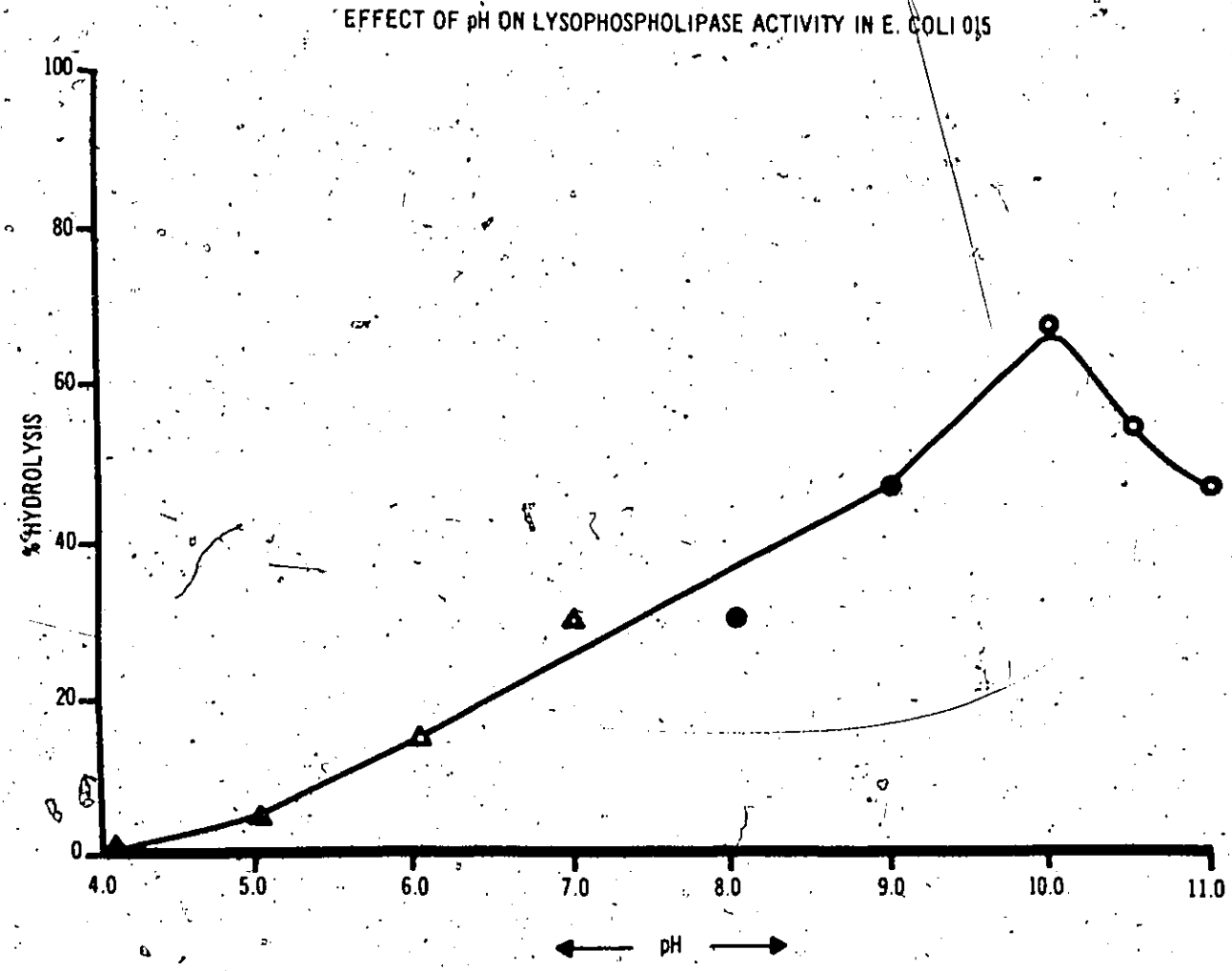


Fig. 7. The incubation conditions were the same as for Fig. 4, except that 1-(stearoyl-1-^{14}C) - lysoPE was used as sonicated substrate in the absence of detergent and ether. The fatty acid product was separated by TLC with petroleum ether-ether-formic acid, 75:25:1.5 v/v/v. (▲) acetate buffer, (△) phosphate buffer, (●) Tris or borate buffer, (○) carbonate or borate buffer, all 0.1M.

Table III. Hydrolysis of ^{14}C-labelled PE Substrates by E. coli C15 Sonicated Cells.

Substrate	pH	% of total cpm recovered as product	
		Lyso PE	Fatty Acid
1-(stearoyl-1-^{14}C)-PE	5.0	4.7	29.3
"	8.4	12.9	40.0
2-(linoleoyl-1-^{14}C)-PE	8.4	40.4	15.4

At pH 5 the incubation mixture usually contained 1 ml of E. coli sonicate, 10 mM Ca, 0.95 ml of 0.1M acetate buffer. 2 umoles of 1-(stearoyl-1-^{14}C)-PE dissolved in 1 ml of ether was then added. At this pH, sodium lauryl sulfate or sodium deoxycholate was omitted. At pH 8.4, similar conditions were used except that the mixture was buffered with 0.1M Tris-HCl and sodium lauryl sulfate was included. The mixture was incubated at room temperature with vigorous shaking for 3 hours.

amount of fatty acid was also formed. Thus it seemed that at alkaline pH both phospholipase A1 and A2 were active.

These conclusions were further supported by results obtained with doubly-labelled substrates. For these experiments 1-(stearoyl-1-^{14}C)PE or the 2-(linoleoyl-1-^{14}C) analogue was admixed in different proportions with ^{32}PE such that varying ^{32}P/^{14}C ratios were obtained in the substrate.

At pH 5 there was a very large increase in the ^{32}P/^{14}C ratio of the lyso product when the ^{14}C-label was initially present in the 1-acyl position of the substrate (Table IV). The large variations in the B/A values can be explained by the fact that only very few ^{14}C counts were recovered in the lyso PE product. It could be estimated from the increase in ^{32}P/^{14}C ratio of the product that no more than 5% to less than 1% of the phsopholipase activity was of the A2 type at acid pH. (The % 1-acyl lsyo PE formed due to phospholipase A2 = ratio in substrate/ratio in product X 100). When 2-(linoleoyl-1-^{14}C)-^{32}P-PE was the substrate at pH 8.4 (Table V) there was again a significant increase in the ^{32}P/^{14}C value of the lyso product indicating the presence of both A1 and A2 activity. From this increase in ratio it was estimated that between 60-70% of the activity at alkaline pH was due to phospholipase A1 and the remainder, to phospholipase A2.

Table IV. Ratio of ^{14}C and ^{32}P Activities of Lysophosphatidyl Ethanolamine from 1-(Stearoyl-1-^{14}C)-^{32}P-Phosphatidyl Ethanolamine by Sonicated E. coli Cells at pH 5.

PE $^{32}P/^{14}C$ (A)	Lyso PE $^{32}P/^{14}C$ (B)	B/A
4.1	1001.0	250
1.1	20.0	20
6.2	4287.0	690

The conditions were essentially those described for Table III except that DOC was not added and the double-labelled substrate used was prepared by mixing for each experiment a different proportion of 1-(stearoyl-1-^{14}C)-PE with ^{32}P-labelled substrate.

Table V. Ratio of ^{14}C and ^{32}P Activities of Lysophosphatidyl Ethanolamine from 2-(Linoleoyl-1-^{14}C)-^{32}P-Phosphatidyl Ethanolamine by Sonicated E. coli Cells at pH 8.4

PE $^{32}P/^{14}C$ (A)	Lyso PE $^{32}P/^{14}C$ (B)	B/A
4.55	7.90	1.53
2.30	3.25	1.40
1.16	1.80	1.55
4.70	7.27	1.45

The conditions were the same as Table III and IV except that the ^{14}C-label was in 2-position and Tris-HCl buffer, pH 8.4 was used in the presence of 30 mM sodium lauryl sulfate.

The effect of Ca on phospholipase A

When ^{32}P-labelled PE was incubated in the presence of increasing concentrations of Ca the activity increased proportionally up to a concentration of about 20 mM at pH 5 (Fig. 8) and 5 mM or less at pH 8.4 (Fig. 9). It is important to note however that addition of DOC, (which is necessary at alkaline pH), to the calcium-containing incubation mixture, resulted in the formation of an insoluble complex. When SDS substituted for DOC, a precipitate also formed but which almost completely dissolved upon addition of ether to the incubation mixture. SDS was therefore used in our later experiments. Nevertheless, the concentration of added Ca can be only tentatively stated and it is likely that only a part of this added Ca is truly accessible to the enzyme. In any event it does appear that phospholipase activity at either pH optima is Ca dependent.

As Table VI illustrates, Mg also stimulates at either pH but to a lesser extent than Ca. Zn, Cd and Cu and EDTA were somewhat inhibitory. However this latter study was complicated by the fact that concurrent addition of Ca was necessary for detection of phospholipase A activity at either pH and counteracted the effect of ionic inhibitors. Furthermore at alkaline pH the divalent cations tended to form insoluble precipitates with the anionic detergents used.

An interesting result appearing in Table VI is the fact that SDS addition completely inhibited the activity at

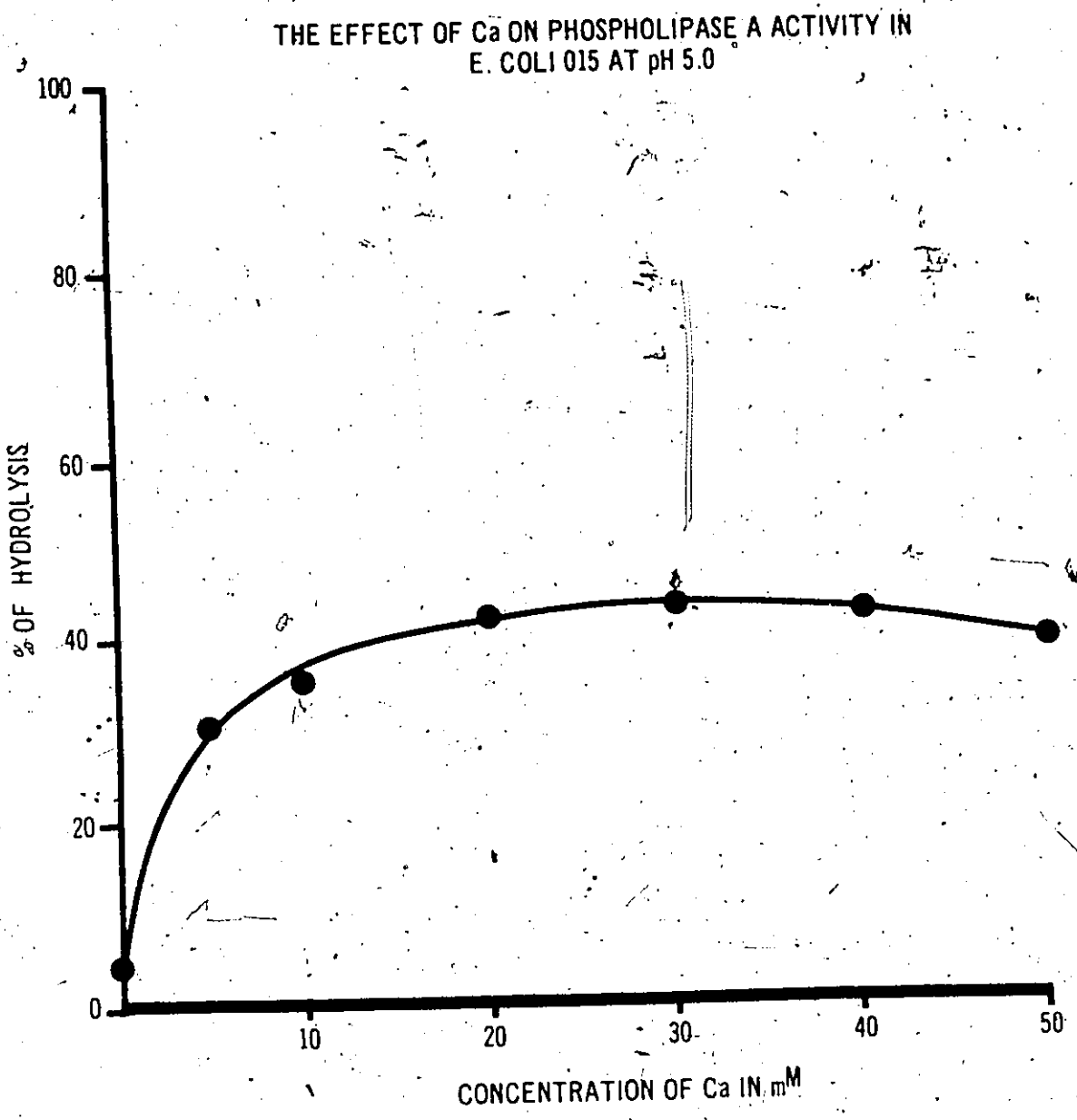


Fig. 8: The incubation mixture contained (0-50m^{M}) ^{32}P-labelled PE (2umoles) dissolved in 2 ml of ether, 1 ml of sonicated E. coli cells 0.8 ml 0.1M acetate buffer, pH 5.0 and 0.2 ml $CaCl_2$ solution final concentration. The mixture was incubated 4 hours at room temperature. The labelled lyso PE product was separated with chloroform-methanol-water, 65:25:4, v/v/v.

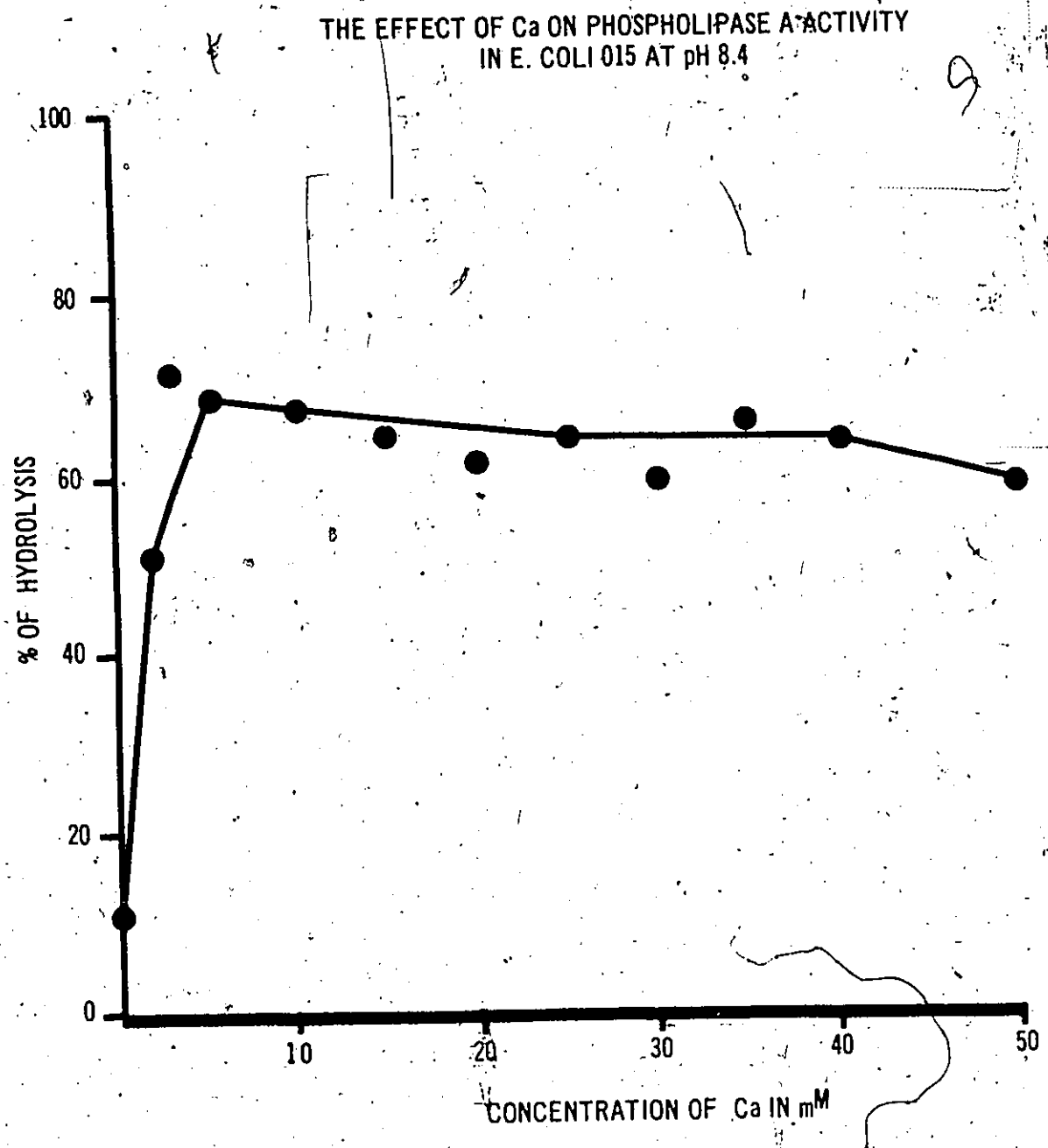


Fig. 9. The incubation conditions were the same as stated for Fig. 8. except that Tris-HCl buffer pH 8.4 was used in the presence of 30mM DOC. The labelled lyso PE product was separated with chloroform-methanol-water, 65:25:4, v/v/v.

Table VI. Effect of Various Cations and Sodium Lauryl Sulfate on Phospholipase A Activity.

Addition	% of total cpm recovered as lyso PE	
	pH 5	pH 8.4
no cation, no detergent	4.8	0.
30mM sodium lauryl sulfate	1.0	5.8
$CaCl_2$, no detergent	22.6	4.0
$CaCl_2$, 30mM sodium lauryl sulfate	1.8	34.9
$MgCl_2$	11.3	8.6
$CaCl_2$, EDTA	16.0	6.4
$CaCl_2$, $CuCl_2$	20.6	26.1
$CaCl_2$, $ZnCl_2$	20.7	23.5
$CaCl_2$, $CdCl_2$	10.3	26.5

Except otherwise stated, at pH 5, no detergent was added whereas at pH 8.4, 30 mM DOC was used. All Cation and EDTA final concentration were maintained at 10 mM and 2 mM respectively. Other incubation conditions were as stated for Table III.

pH 5. On the other hand as these and our other results show, DOC or SDS addition at alkaline pH not only permits detection of phospholipase A activity but enhances it (Tables II and VI).

The effect of heat treatment on phospholipase A activity

With ^{32}P-PE as substrate, the effect of heating E. coli sonicate at 65° for various times at pH 5 and 8.4 are shown in Figs 10 and 11.

When the sonicate was heated at pH 8.4 without detergent and the activity was tested at pH 5, there was a small gradual increase of activity with time. When tested at pH 8.4 there was little or no effect on the activity unless detergent was present in the sonicate during the heat treatment. In this latter case there was a marked fall in activity (Fig.10).

When this experiment was repeated by heating at pH 5 and the activity was tested at either pH optimum (Fig. 11), results simlar to those described in Fig. 10 were obtained.

From these latter experiments it seems that several types of phospholipase A are present in E. coli, one phospholipase A1 which is slightly activated by heat treatment has a pH optimum of pH 5. The other phospholipases A1 and A2, have an alkaline pH optimum of 8.4 and at least one of these is labile to heat treatment in the presence of detergent, which at lower temperatures normally enhances the

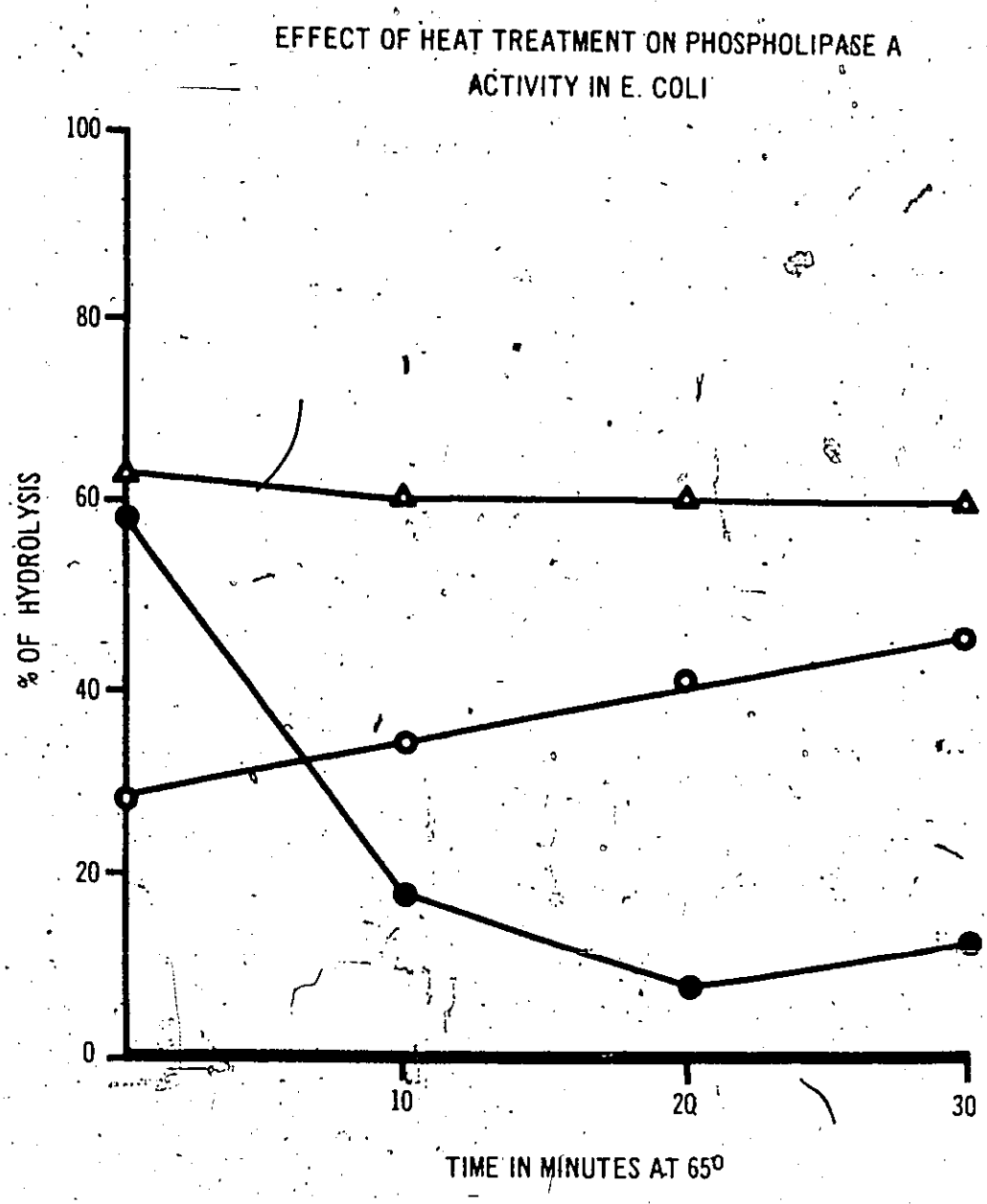


Fig 10. (▲) E. coli sonicate was heated at pH 8.4 without detergent and tested at 8.4. 30m^{M} DOC and 32^{P}-labelled phosphatidylethanolamine (2umoles) dissolved in ether was added after heat treatment. The other incubation conditions were the same as for Fig. 4.

(○) The sonicate was heated at pH 8.4 without detergent and tested at pH 5.0 in the absence of detergent and other conditions were the same as (▲).

(●) The sonicate was heated at pH 8.4 and tested at 8.4 but 30m^{M} DOC was present during the heat treatment and during the assay.

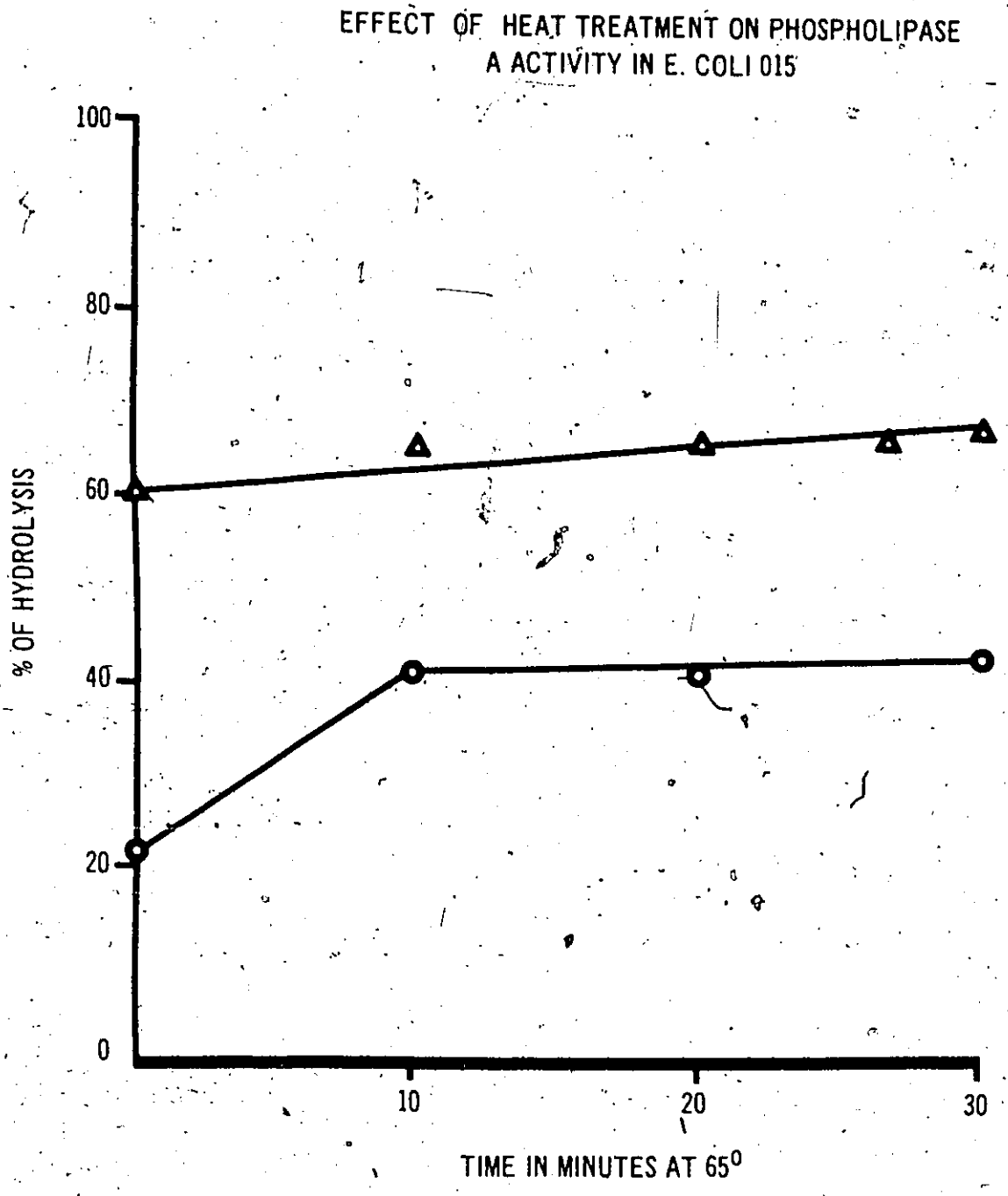


Fig. 11. (▲) The sonicate was heated at pH 5 without detergent and tested at pH 8.4 in the presence of 30mM DOC. The other incubation conditions were as for Fig. 10. (●) The sonicate was heated at pH 5.0 without detergent and tested at pH 5.0 in the absence of DOC.

combined activity at this pH.

The subcellular distribution of phospholipase A

Table VII summarizes preliminary results obtained when the subcellular distribution of phospholipase A was studied at alkaline pH with 1-(stearoyl-1-^{14}C)-PE as substrate. It is evident from these results that both phospholipase A1 and phospholipase A2 are particulate enzymes although small amounts of activity were also recovered in the supernatant and culture medium fraction.

Table VII

The Subcellular Distribution of Phospholipase A in E. coli 015

Subcellular Fraction	Distribution of radioactivity in products as % of substrate cpm added		Total Activity° as umoles of substrate hydrolysed per total volume of each fraction/hr.
	Lyso PE	F.A.	
Particulate*	14.0	54.0	8.0
Supernatant*	2.0	6.4	1.0
Culture Medium◊	1.2	3.4	1.2

* 1 ml of suspension was used for incubation.
◊ 20 ml of medium was used for incubation.
° Calculated from total labelled PE hydrolysed.

Twenty five ml of E. coli cells sonicated in borate buffer 0.1M, pH 8.4 were centrifuged at 100,000 g for 1 hr. The sediment was resuspended in 20 ml of the buffer (protein concentration 20 mg/ml), the supernatant was adjusted to a volume of 24 ml with buffer (2.8 mg protein/ml) and the medium was not buffered but was adjusted to pH 8.4 with NaOH. Other incubation conditions were essentially those described for Table III.

DISCUSSION

Although the main pathways leading to phosphoglyceride synthesis in E. coli have been well established by Kennedy's group (29), the catabolism of these lipids is not yet completely resolved. Earlier studies with E. coli NCTC 2276 (39) did not reveal definitely the presence of phospholipase A. However this strain did degrade PE to GPE and fatty acid at a slow rate and the results indicated a combined action of phospholipase A and lysophospholipase under the incubation conditions used.

The presence of phospholipase A in E. coli

Using high concentrations of DOC or SDS to inhibit lysophospholipase activity we have been successful in clearly demonstrating the accumulation of labelled lysophoglycerides as a key product of ^{32}P-labelled phospholipid degradation in vitro. Both strains of E. coli used in this investigation, strains 015 and 0118, possessed phospholipase A activity which in the alkaline pH range could be detected only in the presence of detergents. Besides inhibiting lysophospholipase the detergents used may have solubilized the phospholipase A, which as our results show is a particulate enzyme, and thus promote exogeneous substrate-enzyme interaction. In earlier studies (39) failure to add sufficient amounts of detergents would explain why phospholipase A was not readily detected, although the possibility that different strains of E. coli might contain different levels of this enzyme could also offer part of the explanation. In a recent paper (8) published

concurrently with ours (40) Nojima and Okuyama also characterized phospholipase A in E. coli B. In addition, they were able to show that phospholipase C was present and accounted for 25% of the water-soluble breakdown products of ^{32}P-labelled PE.

It would seem that a difference exists with respect to the levels and types of phospholipases found in various E. coli strains. In E. coli 015 phospholipase C is not readily detectable under the conditions of incubation used. We have not studied these conditions extensively since our main interest was concerned with further characterization of phospholipase A. An analogous situation exists with other bacteria. For example, in B. cereus the levels of phospholipolytic activity varies extensively between different strains (98).

It is apparent from this discussion that one cannot always generalize about the presence or importance of an enzyme in a particular type of organism since different strains may display different enzyme patterns. Moreover certain of these enzymes might be quite susceptible to induction, or their levels might reflect the culture conditions, the age of the organisms and the conditions of assay used.

Phospholipase A has now been detected in four strains and well characterized in three of these by two independent workers (8,40). It would seem that this lypolytic enzyme has a fairly wide occurrence among E. coli strains.

Relevant to this discussion is the relatively recent and interesting finding that certain strains of E. coli (about 20% of those examined) are hemolytic, and that one of the hemolysins present, α hemolysin is secreted into the medium, is proteinaceous and requires Ca for its action (99). It would be interesting to see if whether or not this hemolytic factor possesses phospholipase activity, since some of the properties of α hemolysin of E. coli seem to parallel those of the hemolytic α toxin of Cl. welchii which is known to possess phospholipase C activity (100).

Substrate specificity of phospholipase A

Although in our present investigation the substrate specificity was not studied in detail, in our early work performed at pH 7.4, we obtained evidence that both PE and PC are degraded to their respective monoacyl analogues. Further studies at pH 8.4 by Mrs. E. Caertner of our group have shown that phospholipase A can degrade ^{14}C-labelled PG, synthesized from liver PC by the transphosphatidylation reaction with phospholipase D and glycerol, or (7) ^{32}P-labelled PG isolated from E. coli. Thus phospholipase A activity at alkaline pH seems to have fairly broad substrate specificity. The specificity at acid pH has not yet been studied in detail.

Cation Requirements

Interesting is our finding that Ca and Mg are required for phospholipase activity at either pH. That Ca be needed for phospholipase A1 activity appears to be an unusual feature particular to the E. coli enzyme. Phospholipase A1 activity of animal tissues is not activated by calcium and at higher concentrations, this ion is inhibitory (50).

Mode of action, positional specificity and other properties of phospholipase A

Whether the A1 activity detected at pH 5 is due to a different enzyme than the one responsible for A1 activity at alkaline pH is a difficult question to answer at present. Besides differences in their pH optima, the activities seem to display different susceptibilities towards detergent or heat treatment. Susceptibility to heat at alkaline pH depends however on the presence of detergent and we attribute this to the likely possibility that the detergent solubilizes the enzyme and renders it more labile. No comparison can be made with respect to the susceptibility of both activities to heat treatment since, at pH 5, detergent inhibits phospholipase A. Furthermore we have at present no arguements disproving the possibility that the action of SDS is merely to shift the pH optimum from 5 to 8.4, although physico-chemical explanations of such a phenomenon would be difficult. On the other hand, lysophospholipase activity increases from pH 5 upwards and could account for the fall of phospholipase A activity as measured by lysoPE accumulation between pH 5 and 6 in the absence of detergent.

It is uncertain whether alkaline phospholipase A displaying both A1 and A2 activities represents one or two enzymes. In most other organisms studied, A1 and A2

activities have been resolved as separate enzymes (57) but it is possible that a single enzyme having preference for certain types of fatty acyl groups might be operating at alkaline pH in E. coli. In any event it is certain that a phospholipase B as described for P. Notatum, (83) is not involved here since the accumulation of lysophosphoglyceride can be clearly demonstrated in the presence of detergents.

The suggestion has been made that A1 activity may be due to lipase with broad specificity rather than to a distinctive phospholipase (6). Recent work by Van Deenen's group (personnal communication to Dr. Proulx) has shown that partially purified phospholipase A1 from pancreas is much more active on phosphoglycerides than on triglycerides and is likely to be an enzyme distinct from lipase. Furthermore lipase activity has never been clearly characterized in E. coli and is definitely absent in one strain studied (101). Nevertheless this point would need further clarification using various types of tri, di-, and monoglycerides as substrates to see if first of all lipase activity can be detected in our strains.

Any discussion on the specificity and mode of action is obviously complicated at this point by the presence of lysophospholipase, the need for an inhibitor of this enzyme and the presence of two likely distinct enzymes, phospholipase A1 and A2.

On the purification procedures for phospholipase A

Purification of the enzyme (s) concerned with the activities at acid and alkaline pH will be most fruitfully made using substrate labelled with ^{14}C at either acyl positions. Using ^{14}C substrate we have detected alkaline phospholipase A1 and A2 in the particulate fraction mainly. Preliminary results in this laboratory have indicated that the acid phospholipase is also particulate bound. This subcellular fractionation would seem a useful initial step in the purification of these enzymes. Likewise, heat treatment studied with ^{14}C-labelled substrates instead of ^{32}P-labelled substrates might allow definite conclusions to be made as to which activity is susceptible under these conditions and might favor purification of one of the enzymes. The finding that SDS and DOC enhance activity at alkaline pH will no doubt prove to be useful in the purification of the particulate enzyme since it may permit its solubilization without loss of activity.

One significant aspect of the work discussed so far is that it has defined the complexity of phospholipase A activity in E. coli and has given at least preliminary information on some of the general properties of the enzyme (s) which will no doubt be useful in designing purification procedures.

Purifying phospholipase A1 from any source whatever would be an important achievement in itself. It would also be very useful as a Biochemical tool for the preparation of 2-monoacyl-phosphoglycerides. Coupled with purified lysophospholipase it could help determine the positional specificity of the latter enzyme and thus avoid some of the separation-procedures which enhance acyl migration. E. coli appears to be a rich source of phospholipase A1 and poorer source of phospholipase A2. Purification of phospholipase A1 from E. coli rather than animal tissues which contain a variety of equally or more active lypolytic enzymes would seem a logical choice.

pH optimum of lysophospholipase

We have confirmed the presence of lysopholipase activity in E. coli. The enzyme appears to be active over a wide range of pH and displays optimal activity at pH 10, under the conditions used. It resembles the lysophopholipase of snake and bee venoms with respect to its pH requirements (79). Although we have presented evidence that the 1-acyl lysophosphoglyceride is hydrolysed, it is not known whether the 2-acyl analogues are also attacked by this enzyme. Van Deenen and De Haas (70) have suggested that lysophospholipase activity might infact be the result of A1 or A2

activity on lysophosphoglycerides. We have no evidence completely against such on hypothesis although in E. coli at least, the pH optima of the phospholipase A activities are all below that of lysophospholipase.

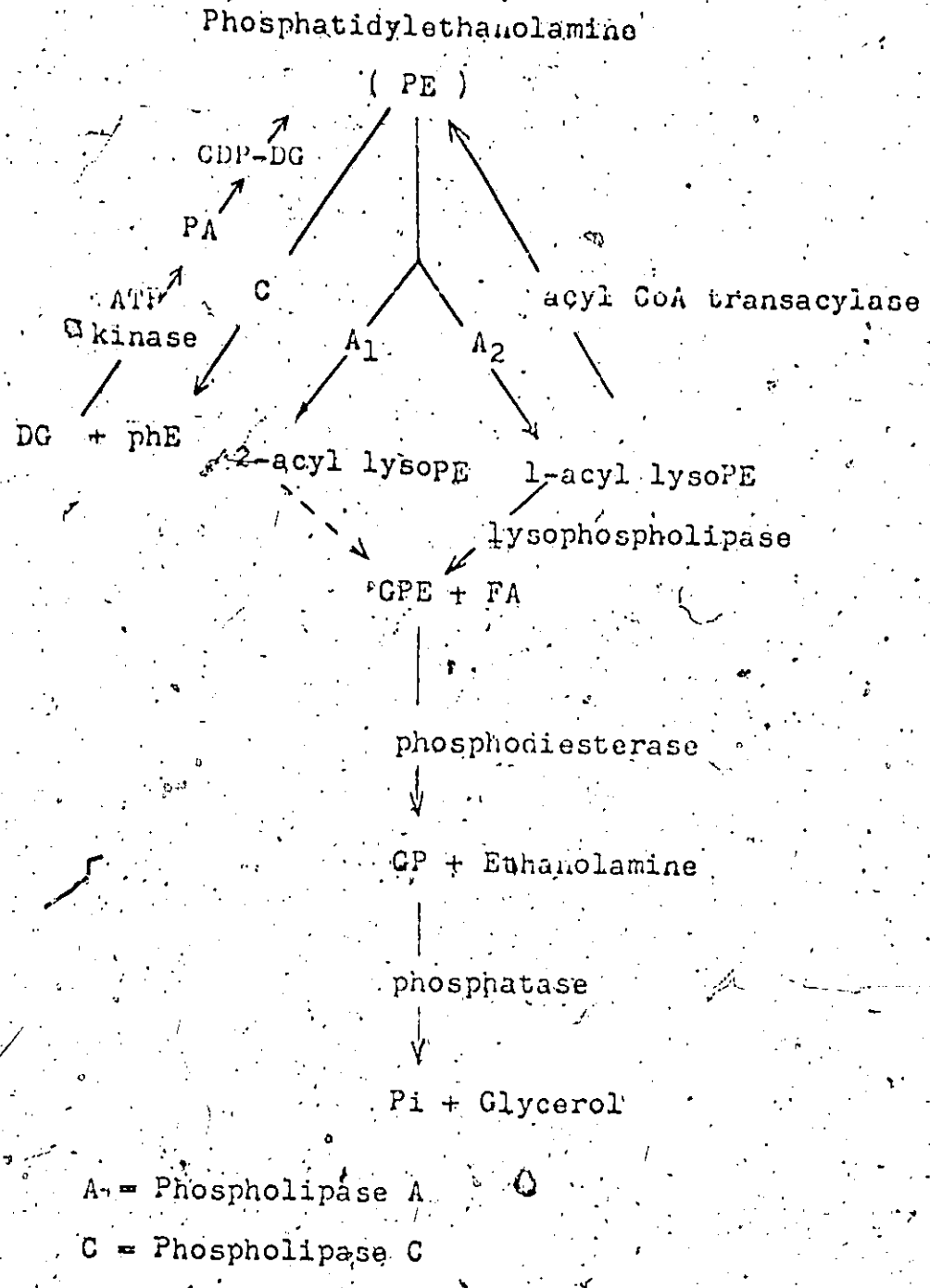
The possible role of phospholipase A in E. coli

Our results together with previously reported evidence indicate that all the enzymes necessary for complete breakdown of phosphoglycerides are present in E. coli (see scheme No.1). The initial steps in the breakdown are catalized by phospholipase A (8;39,40,41).

The enzymes for the de novo synthesis of phosphoglycerides have also been well characterized in vitro (36). Thus it is highly probable that turnover of the phosphatides de novo is a normal process in E. coli. Nevertheless one can suppose that the extent of turnover de nove would be influenced by the environmental conditions of growth and probably the age of the cells.

A difficulty is now apparent from our results for it was shown by several investigators that PE in contrast to the other phosphatide does not turnover under normal conditions of growth and yet in vitro our results show that PE can be degraded by phospholipase A. The work of Okuyama clarifies this point somewhat. He showed

Scheme 1. The Possible Role of Phospholipase A in E. coli.



A = Phospholipase A

C = Phospholipase C

that PE as well as the other phosphatides do turnover during a five hour lag period associated with the adaptive processes of E. coli exposed to cold. Furthermore that PE is a stable pool under normal growth conditions has been shown with chase experiments on cells labelled only with ^{32}P. There is therefore, no evidence to date precluding partial turnover of PE or other phosphoglycerides even under normal conditions or turnover de novo of PE under altered conditions.

A close examination of the results of Okuyama (23) reveals that during the 5 hour lag period associated with cold exposure, there was a net synthesis of 5 mu moles of phosphoglyceride which proceeded via de novo process. There was yet another pool of 8 mu moles of phosphatide which turned over during this period. One cannot ascertain whether a de novo process or a partial turnover process was involved in this case since his strain of E. coli also possessed phospholipase C and DG kinase. Chase experiments performed on the ^{14}C-labelled cells during the lag did not further clarify this point although they did indicate that ^{14}C-labelled phospholipids turned over at a different rate than the ^{32}P-labelled phosphoglycerides. Thus as Okuyama himself concludes, although phospholipase A is likely to be a key enzyme in the de novo turnover of phosphoglycerides

in E. coli it may also function in the partial turnover of acyl groups as part of the Land's cycle. Furthermore partial turnover might be affected via phospholipase C present in certain E. coli strains. It is not yet possible to assign quantitative significance to anyone turnover pathway at present although it is reasonable to assume that phospholipase A is important in such processes.

With respect to the possibility of phospholipase A involvement in the partial turnover of acyl groups, there is only very indirect evidence that such a process might occur in E. coli. Although lysophosphoglyceride acylating activity has been detected in vitro (41) there is to date no evidence that both this enzyme and phospholipase A can operate in a cycle in vivo. An analogous case exists in animal tissues where both these enzymes may be present in a tissue, but are both enzymes present in the same types of cells or is the subcellular distribution of these enzymes favorable for the operation of a cycle? These questions still remain largely unanswered.

In the case of E. coli the answer should be easier to obtain. By labelling the acyl groups of the phosphoglycerides, with ^{14}C and after a chase, by degrading the phosphatides with snake venom phospholipase A, one can obtain information as to the rate of turnover in the

1 and 2 ester positions. If these rates were different as was the case for H. Parainfluenzae (43) then the Lands cycle would be functional.

Conclusion

In summary, one can conclude that the fore-discussed experiments have clearly demonstrated the activities of phospholipase A1 and A2 as well as lysophospholipase in E. coli and preliminary studies have also revealed the gross properties of these lypolytic enzymes. These properties will no doubt be very useful in designing procedures for their purification, a condition which must be fulfilled before further understanding their specificity and mode of action. Our results and discussion also point to the importance of establishing more clearly the pathways involved with the turnover of phosphoglycerides present in this organizm and to reveal more clearly the role(s) of phospholipase A.

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