STUDIES ON A HOUSEFLY PHOSPHODIESTERASE ACTING ON VARIOUS BIOLOGICAL GLYCEROPHOSPHODIESTERS

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This is to certify that the

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ABSTRACT

STUDIES ON A HOUSEFLY PHOSPHODIESTERASE ACTING ON VARIOUS BIOLOGICAL GLYCEROPHOSPHODIESTERS

By

George R. Hildenbrandt

Housefly larvae metabolize phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol by a microsomal phospholipase A to the respective monoacylglycerophosphatides. The monoacylglycerophosphatides are deacylated by a lysophospholipase to the respective glycerophosphoryl derivatives. An 88,000 x g soluble enzyme hydrolyzed glycerophosphoryl (GP)-ethanolamine (E), -choline (C), -β-methylcholine (βMC), -inositol (I), -serine (S), and -glycerol (G) to ℓ-α-glycerolphosphate and the respective free base. The phosphodiesterase is stable to heating at 50° for 15 min and is not inhibited by sulfhydryl reagents. It is stable to treatment with some proteases and the reaction products are not inhibitory. The pH optimum is 7.2-7.4 for GPC and GPE. Added Mg^{++} greatly stimulates activity. EDTA completely inhibits activity, but the inhibition is reversed by Mg^{++}. Co^{++} is as stimulatory and Mn^{++} and Ni^{++} are about 50% as stimulatory as Mg^{++}. Other divalent cations tested show no stimulation. Mutual inhibition of hydrolysis by pairs of substrates incubated together suggests a single enzyme acting on all of the substrates. GPC is the preferential substrate on the basis of most favorable competition for hydrolysis, a 70% higher rate of hydrolysis than any other substrate (90 vs 50 ± 5 nmoles/min/mg protein), and the lowest K_m. The K_m's for GPC, GPE, and GPI are 2×10^{-4}, 4×10^{-4}, and 2×10^{-3} M respectively. A tightly bound enzyme-glycerolphosphate intermediate is indicated by the following results. Labeled choline is exchanged into GPC and GPI but ℓ-α-glycerolphosphate is not exchanged into GPC. Net reversal of the reaction is not observed.

STUDIES ON A HOUSEFLY PHOSPHODIESTERASE ACTING ON VARIOUS BIOLOGICAL GLYCEROPHOSPHODIESTERS

By

George R. Hildenbrandt

A THESIS

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DEDICATION

This thesis is dedicated to the beginning of the reassumption by people of the power unreasonably or ineffectually assumed by institutions. All power is in the people--may we grow to exercise it well.

ACKNOWLEDGMENTS

Without substantial tolerance and good faith on the part of Loran Bieber, this thesis would not have been possible. My deepest gratitude is extended to the beautiful people with whom I have associated this past year through the Lansing Area Peace Council. Without the joy, love, and strength gained from these relationships, the absurdity of the past year would certainly have driven me from this doctoral program.

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LIST OF ABBREVIATIONS

EDTA	ethylene diaminetetra-acetic acid
GP	glycerophosphate
GPβMC	glycerophosphoryl-β-methylcholine
GPC	glycerophosphorylcholine
GPE	glycerophosphorylethanolamine
GPG	glycerophosphorylglycerol
GPI	glycerophosphorylinositol
GPS	glycerophosphorylserine
GP-X	ℓ-α-glycerophosphoryl derivative of X
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
X	ethanolamine, choline, β-methylcholine, inositol, glycerol, or serine

INTRODUCTION

INTRODUCTION

Statement of problem

The work reported in this thesis was undertaken as part of an ongoing study of housefly (*Musca domestica* L.) phospholipid metabolism. It has been established (1 and 2) in our laboratory that housefly larvae catabolize phosphatidyl-β-methylcholine, phosphatidylcholine, and phosphatidylethanolamine by microsome bound phospholipase A_1 and lysophospholipase to the respective glycerolphosphoryl compounds (GP-X). Glycero-^{32}P-phosphoryl-β-methylcholine ($G^{32}P\beta MC$) was found to be hydrolyzed by a 40,000 x g soluble larval enzyme predominately to glycerol-^{32}P-phosphate ($G^{32}P$). My task became the characterization of the glycerophosphodiesterase responsible for this hydrolysis.

The minor glycerophosphatides of the housefly, phosphatidyl-inositol, -serine, and -glycerol, were studied to determine whether they were deacylated by the microsomal phospholipase A_1-B system to yield glycerophosphoryl derivatives. In view of the alternative pathways of catabolism for phosphatidylinositol (PI) in some animals (3), it was of interest to establish the pathway(s) existing in housefly larvae. Having determined what GP-X compounds are produced by the larvae, I undertook to demonstrate the presence of glycerophosphodiesterase activity capable of hydrolyzing all of these compounds to reutilizable components. Because of the substrate selectivity and particulate nature of the rat kidney enzyme (4), I attempted to characterize the larval activity and determine whether one or several enzymes are involved.

Introduction to glycerophosphatide catabolism

It is beyond the scope and intent of this thesis to review phospholipid biosynthesis and function, but a general introduction to diacylglycerophosphatide catabolism and a comprehensive review of glycerophosphodiesterases will be given to orient the reader. Glycerophosphatides are well established as being integral parts of virtually all biological membranes and are implicated in a rapidly growing list of biochemical reactions, especially those associated with membrane systems. Some peculiarities of phospholipid metabolism in houseflies making them particularly interesting to study will be discussed later. For more extensive background on phospholipids and their metabolism the reader is referred to one of the following reviews from which the subsequent discussion is taken: Ansell and Hawthorne, 1964 (5); van Deenen and de Haas, 1966 (6); and Rossiter, 1968 (3).

The four major hydrolytic activities that can catabolize a 1,2-diacyl-sn-glycerol-3-phosphatide are designated phospholipase A, B, C, and D. Phospholipase D is documented only in plants and catalyzes the exchange or cleavage of the moiety (e.g., choline, inositol, etc.) esterified to the phosphate of diacylglycerophosphate. Phospholipase C has been studied mainly in bacteria but has been reported to occur in some mammalian tissues. It hydrolyzes the phosphodiester linkage between the glycerol and phosphate moieties yielding, 1,2-diacylglycerol and phosphorylcholine or other phosphoryl derivative. The major catabolic pathway for diacylglycerophosphatides in animals is the hydrolysis of the two acyl groups, one at a time, to yield monoacylglycerophosphatide and finally GP-X. Phospholipases A_1 and A_2 are the enzymes catalyzing the hydrolysis of the first fatty acid at the 1 or 2 position respectively. The resulting 2- or 1-acyl-sn-glycero-3-phosphatides are deacylated by phospholipase B, also designated lysophospholipase. One still encounters in the literature the term phospholipase B in reference to activity which removes both fatty acids without the demonstrable accumulation of any lyso derivative. Such activity is generally considered to represent sequential removal of the two fatty acids by one or several enzymes since to date no substantial evidence for the simultaneous cleavage of both fatty acids has been obtained. The phospholipase A and B pathway seems to hydrolyze phosphatidyl-choline (PC), -ethanolamine (PE), -serine (PS), and -glycerol (PG) as well as cardiolipin in most systems that have been thoroughly studied. Phosphatidylinositol (Pl) is a major exception to this scheme in many systems and will be discussed later.

As indicated above, there are different phospholipase A's which can cleave either the 1 or 2 fatty acyl group. These enzymes are generally associated with a membraneous fraction in animal cells and are difficult to purify. Phospholipase A also occurs in snake and insect venoms as a soluble enzyme and is secreted as a zymogen by the pancreas. Early work with phospholipase A was done using the readily obtained and purified Ca^{++} dependent snake venom enzyme which was shown in 1963 to hydrolyze exclusively the 2 (sometimes designated β) fatty acid. In 1964, using substrates labeled with ^3H or ^{14}C specifically in the 1 or 2 fatty acid, phospholipase A activity cleaving the 1 (or α) fatty acid of PC was found in rat liver and other tissues. These tissues also contain the enzyme hydrolyzing the 2 fatty acid since both lyso compounds were demonstrated to accumulate in rat liver. Studies (7, 8, and 9) on the subcellular distribution of phospholipases A_1 and A_2 in rat liver have shown that mitochondria contain only A_2 and microsomes contain only A_1 while lysosomes contain both A_1 and A_2. The lysosomal activities (9) occur at acid pH and are distinct from the pH 8 A_2 of mitochondria and the neutral to alkaline A_1 of microsomes. The lysosomal pH 4.5 activity is substantially inhibited by Ca^{++} and activated by EDTA as is the microsomal A_1 activity at pH 7.4. The A_2 activity at pH 4.5 may or may not be inhibited by Ca^{++}, but the lysosomal A_2 activity at pH 7 is stimulated by Ca^{++}. Mitochondria contain small amounts of pH 7 A_2 but contain mainly pH 8.8 A_2 which is substantially stimulated by Ca^{++}. These results and similar findings in other systems support a more common occurrence of Ca^{++} independent phospholipase A_1 in microsomes and Ca^{++} dependent A_2 in mitochondria. Lysosomes appear to have the capacity to completely hydrolyze diacylglycerophosphatides by the combined action of phospholipases A_1 and A_2 since A_2 will also hydrolyze the 2-lyso compound resulting from A_1 activity. Lysophospholipase activity may also occur in lysosomes as well as in the other particulate fractions. It has also been reported as a soluble enzyme in some tissues.

The questions of distinguishing lysophospholipase activity from phospholipase A_2 activity which is known to act on the 2-lyso compound and of distinguishing between one and several enzymes where complete deacylation occurs without production of a detectable lyso intermediate are still inadequately answered. Except for soluble venom and pancreatic phospholipase A_2's, purification has been nearly impossible due to the membraneous nature of the enzymes. Differences in heat stability and detergent effects have been the main tools used to assay and separate the different proposed activities. Another problem in the study of phospholipases has been getting the hydrophobic lipid substrates into a form that is accessible to the enzyme and into a form that approaches the situation found in vivo. Hanahan found in early work that ether enhanced snake venom phospholipase A_2 activity on PC presumably by loosening the micelle structure to make the substrate more accessible. Detergents are frequently used to disperse the substrates. This often stimulates activity on a substrate not hydrolyzed in the absence of detergents. Mixtures of phospholipids also have been found to have a similar stimulatory effect on activity by altering the charge on the lipid micelle. When studying phospholipases in membranes, particularly of intact organelles, endogenous phospholipids may be cleaved at different rates than exogenous lipids due to poor penetration of substrate unless detergents or other disruptive measures are employed.

As mentioned previously, PI is metabolized in many systems by a pathway other than phospholipase A and B deacylation. This is a phospholipase C generally requiring Ca^{++} and acid pH which is specific for PI. The products are diglyceride and inositolphosphate. See Appendix A for references and discussion of the distribution of the two PI catabolic pathways. In a recent report (10), White et al. have shown that guinea pig pancreas contains a microsomal phospholipase A_1 having high activity with PI. This phospholipase A_1 is inhibited by Ca^{++}. A lysophospholipase is also present yielding GPI as the final product. In contrast to this system, Dawson and co-workers (11 and 12) reported that thyroid gland of the pig metabolizes PI by the diglyceride pathway yielding cyclic inositolmonophosphate. This system is active at acid pH and is stimulated by Ca^{++}. The pancreatic phospholipase A_1 of White (10) is also active on PC but is more active on PI suggesting involvement in the enhanced PI turnover occurring during secretion.

Insect glycerophosphatide metabolism

The following discussion is taken largely from a review by Fast, 1970 (13) on insect lipids. Phospholipase D activity has been proposed in certain dipterous insects but has not been well established. The exchange of X in diacyl GP-X probably occurs suggesting an activity that is similar to phospholipase D but is unable to cause accumulation of phosphatidic acid. A phospholipase A_1 not requiring Ca^{++} has been reported in three species of mosquitoes. Rao and Subrahmanyam have studied this mosquito phospholipase A_1 (14) and have partially purified a phospholipase B activity away from the A_1 activity (15). The mosquito enzymes are microsomal, have optimum activity at basic pH, and the A_1 activity is inhibited by Ca^{++}. The housefly has been found by Kumar et al. (2) to have a very similar phospholipase A_1-B system. The mosquito system is reported (14) to be inactive on PI and phosphatidic acid but active on PE, PC, and cardiolipin. The housefly system was shown (2) to be active on PE, PC, and phosphatidyl-β-methylcholine. Phospholipase A activity has been reported by Khan and Hodgson (16) in housefly and blowfly mitochondria as well as microsomes.

To the extent phospholipid catabolism is known in insects it is similar to that in other animals. However, there are some interesting peculiarities in the total phospholipid story in dipterous insects, particularly houseflies. The predominate phospholipid is PE rather than PC which predominates in most insects and animals. It is known that houseflies cannot synthesize choline but require it as a dietary component for growth. Evidence is accumulating in this laboratory that houseflies have a normal one carbon metabolism utilizing Me-^{14}C-methionine but have a lesion in the system that methylates ethanolamine to form choline. If dietary choline is substituted for by choline analogs such as β-methylocholine, these bases will be incorporated into phospholipids in place of choline. Many of these analogs (17) do not provide for completely normal development and reproduction in houseflies. Findings of this nature suggest that choline plays an important role in normal insect development and functioning. Essentially all the choline present in the insect is in the form of PC making it very likely that the critical role of choline is some structural or functional role of phosphatidylcholine. There may be some step in the metabolism of PC that fails to function adequately when an analog is substituted for choline.

The matter of metabolic handling of choline is of particular interest in the insect. When houseflies metamorphose from larvae to adults, they pass through a pupal stage during which they can ingest and excrete nothing with the exception of some gas exchange. In this closed system the tissues of the larva are broken down extensively and the components are reformed into the cells and tissues of the adult. Since choline cannot be synthesized, it seems crucial that the organism be able to efficiently reutilize the choline from the PC of the larva to synthesize the new membrane systems in the cells of the adult tissues. Since it is known that there is extensive phospholipid turnover, presumably by phospholipase A and B deacylation, in the late larval stage and in the pupal stage, it seems likely that there would be an enzyme such as glycerophosphodiesterase to release choline from glycerophosphorylcholine (GPC) for reutilization in PC synthesis. Reacylation of GPC has not been detected and its accumulation has not been observed during PC breakdown. LysoPC does accumulate during pupation but not during the turnover in the late larval stage. It may not be a satisfactory candidate for storage of choline and reacylation to PC in newly formed adult tissues and other lyso derivatives do not accumulate.

Review of glycerophosphodiesterase literature

Hayaishi and Kornberg (18) isolated a strain *Serratia plymuthicum* capable of growing on glycerophosphorycholine (GPC), yeast extract and mineral salts. They partially purified a glycerophosphodiesterase from this bacterium that hydrolyzes GPC to ℓ-α-GP and choline. The enzyme was stable to heat for 5 min. at 50°, had a pH optimum of from 8 to 9, and was inhibited 90% by 1 mM Mg^{++} or Mn^{++} or 0.1 mM EDTA. EDTA inhibition was not reversed by metal ions. GPC and glycerophosphorylethanolamine (GPE) gave K_m's of $1.2 \times 10^{-3}M$ and $2.5 \times 10^{-3}M$ respectively, and they each showed competitive inhibition of the hydrolysis of the other. In a brief communication, Prasad and Benson (19) reported that the *S. plymuthicum* enzyme hydrolyzes glycerophosphorylserine (GPS), glycerophosphorylinositol (GPI), and glycerophosphorylglucose as well as GPC and GPE.

Glycerophosphodiesterase activity has also been studied in the rat. Dawson (20) reported an enzyme in an acetone powder of rat liver that hydrolyzes GPC to GP and choline. GPE is also hydrolyzed and is a competitor of GPC cleavage. The pH optimum is 7.5 and 1 mM EDTA or Zn^{++} causes complete inhibition. 1 mM Mg^{++} stimulates activity, but higher concentrations abolish activity. Fowler and de Duve (21) have confirmed Dawson's findings and established that the GPC diesterase is not lysosomal or mitochondrial but primarily soluble. Webster et al.

(22) studied the glycerophosphodiesterase activity of rat brain and other nervous tissues. The dialyzed rat brain homogenate cleaves GPC to choline and GP optimally at pH 9.5 without added divalent cations. EDTA inhibits completely and the inhibition can be overcome with Mg^{++} or Mn^{++}. Zn^{++} causes no inhibition or reversal of EDTA inhibition. A rat kidney microsome bound enzyme has been reported by Baldwin and Cornatzer (4). This enzyme hydrolyzes GPC and GPE to ℓ-α-GP and free base. GPI and GPS apparently are not hydrolyzed by this kidney microsomal preparation. The pH optimum is 9.5 and the K_m's for GPE and GPC are 11.5×10^{-3}M and 2.2×10^{-3}M respectively. The enzyme was stable to heating for 5 min. at 50° and was not inhibited by either N-ethylmaleimide or iodoacetamide. At concentrations from 1 to 10 mM ethanolamine and its mono-, di-, and tri-N-methylated analogs show inhibition of GPC hydrolysis. Inhibition increased with concentration over this range and increases with degree of methylation. Choline, 10 mM, gives over 60% inhibition. The kidney enzyme shows no divalent cation stimulation even after dialysis. However, 2 mM EDTA completely inhibits activity. It has been reported (23) that EDTA inhibition can be reversed by several cations including Zn^{++}; however, these cations are inhibitory to the enzyme prior to dialysis against EDTA. The enzyme is thought to contain a metal ion,

possibly Zn^{++}, which is lost only when a chelator such as EDTA is present.

EXPERIMENTAL PROCEDURES AND RESULTS

The results of my work on this thesis problem have been drawn up as two manuscripts for journal publication. A reprint of the published manuscript and the final draft of the other manuscript are included in this thesis as Appendices. The reader is referred to these Appendices for the presentation of methodology and experimental results discussed in the following section.

Appendix A is a reprint of a journal article, Lipids 6, 508 (1971), consisting of work I did independently on the metabolism of phosphatidyl-inositol, -serine, and -glycerol. T. Abraham and L. L. Bieber investigated the metabolism of ceramide phosphorylethanolamine. Only my work will be discussed in the following section. The manuscript presented in Appendix B, describing the glycerophosphodiesterase of housefly larvae, is entirely my work. This work will be submitted for publication.

A brief outline of the methods used in Appendices A and B follows. Phospholipase A and B activity were assayed using ^{32}P labeled phospholipids prepared from housefly larvae reared on ^{32}P-inorganic phosphate. The water soluble products were extracted for paper chromatographic identification. The lysophosphatides and unreacted diacylglycerophosphatides were separated by thin layer chromatography. The fractions were scraped from the plate and quantitated by counting ^{32}P Cerenkov radiation. For assaying glycerophosphodiesterase activity $G^{32}P$-X substrates were prepared by deacylation of ^{32}P-phospholipids with methanolic KOH. The products of the incubations were separated by anion exchange chromatography. The ^{32}P radioactivity was quantitated for the unreacted substrate fraction and the glycerolphosphate product fraction.

DISCUSSION OF RESULTS

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Diacylglycerophosphatide catabolism in housefly larvae

Appendix A presents data indicating that phosphatidylglycerol (PG) (Appendix A, Figure 1), phosphatidylserine (PS) (Appendix A, Figure 2), and phosphatidylinositol (PI) (Appendix A, Figure 3) are metabolized to monoacylglycerophosphatides and to GP-X derivatives by larval microsomes. It had been established (2) in our laboratory that phosphatidyl-ethanolamine, -choline, and -β-methylcholine are metabolized by larval microsomes to yield the lysoglycerophosphatides and GP-X compounds. It was also shown that the lysoglycerophosphatides were 2-acyl-glycerophosphatides and that the lyso compounds are precursors of the GP-X derivatives. It was not verified that the lysoPI, lysoPG, and lysoPS are 2-monoacylglycerophosphatides. It was shown that lysoPI is a precursor of GPI by following the course of PI breakdown with time (Appendix A, Figure 4).

The catabolism of PI in housefly larvae was found to be exclusively by phospholipases A and B to yield GPI. No significant amounts of breakdown by other pathways were detected in subcellular fractions other than microsomes at acidic or basic pH.

Properties of housefly larval glycerophosphodiesterase

Appendix B presents results indicating the presence of a glycerophosphodiesterase in housefly larvae that is not bound to any subcelluar particle (Appendix B, Table I). This hydrolytic activity is stable to heating at 50° for 15 min (Appendix B, Table II). The optimum pH for hydrolysis of GPC and GPE is 7.2-7.4 (Appendix B, Figure 1). As indicated in Appendix B, Tables II and III and Figure 2, an ammonium sulfate fractionated enzyme preparation has little activity without added divalent cations and attains optimal activity with 4-10 mM Mg^{++}. EDTA completely inhibits the enzyme. This inhibition is reversed by adding Mg^{++} during incubation. There is no significant inhibition of GPC or GPE hydrolysis by Mg^{++} up to 20 mM. Co^{++} is nearly as stimulatory as Mg^{++}. Mn^{++} and Ni^{++} showed only half as much stimulation and other divalent cations showed none. Appendix B, Table VI shows that the enzyme is not inhibited by agents reactive with sulfhydryl groups and is not highly sensitive to proteolytic activity. The major difference between the above findings and results of similar studies on the bacterial and rat enzymes is that the larval enzyme requires added Mg^{++} for activity and is not inhibited by high Mg^{++} concentrations.

Appendix B, Table IV shows that the larval enzyme cleaves GPC, GPE, GPS, GPI, and GPG to yield 90% of the phosphate released as ℓ-α-GP (sn-glycero-3-phosphate).

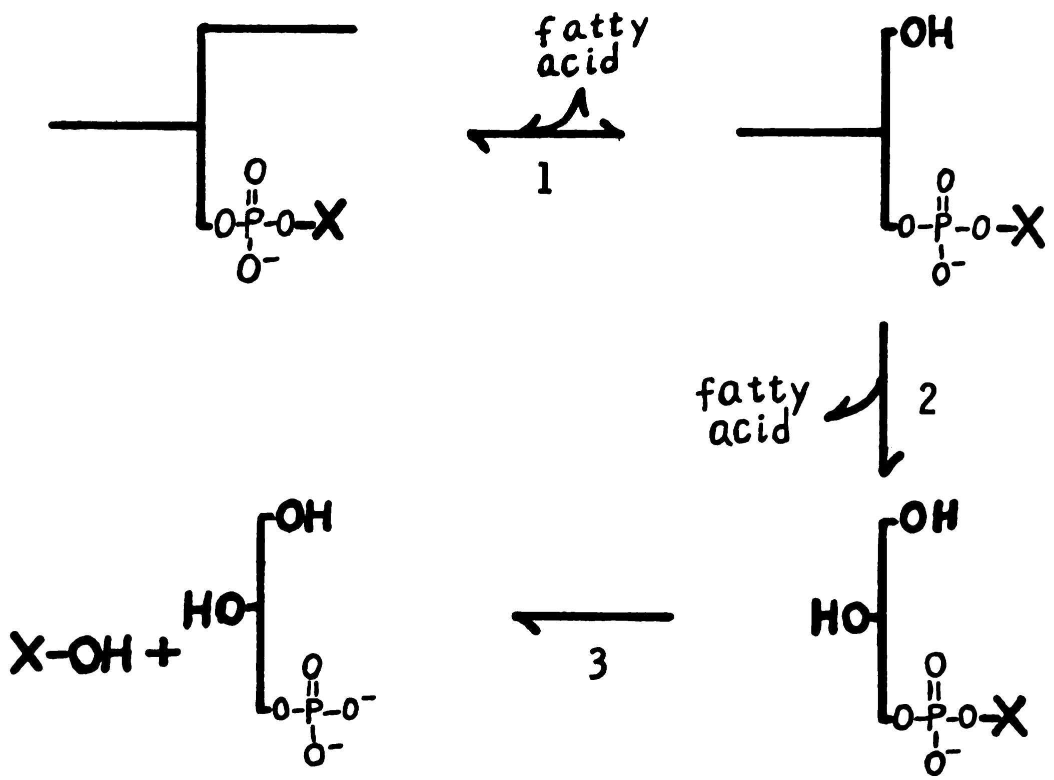
Stoichiometric amounts of serine and inositol are also released from GPS and GPI respectively. One enzyme apparently hydrolyzes the above substrates and GPβMC. Appendix B, Figure 4 shows that when pairs of substrates are incubated together they are both hydrolyzed but at rates lower than when incubated separately. The total hydrolysis of both substrates does not exceed the hydrolysis of the most active substrate incubated alone. The two cases (Appendix B, Figure 4, A and B) where combined hydrolysis slightly exceeds maximum hydrolysis of either single substrate may be accounted for by low rates for cleavage of $G^{32}PE$ (A) and GPC (B) when incubated alone. This may be due to some apparent inhibitor in these particular substrate preparations that does not affect the other substrate in coincubations. GPC, which is hydrolyzed 70% faster than any of the other substrates (90 <u>vs</u> 50 ± 5 n moles/min/mg protein), competes much more favorably than GPE or GPI when coincubated with either of these substrates. Appendix B, Table V indicates that there is no extensive inhibition of GPC hydrolysis by any of the products of GP-X cleavage. In particular, choline shows no inhibition in sharp contrast to its 60% inhibition (at similar concentrations) of GPC cleavage by the rat kidney enzyme (4). Appendix B, Figure 3 gives an apparent K_m determination of $2 \times 10^{-4}M$ for GPC and $2 \times 10^{-3}M$ for GPI. GPE and GPβMC have K_m's near that of GPC but K_m's for GPS and GPG appear to be at least as high as the K_m for GPI.

The findings outlined in the above paragraph suggest that housefly larvae have a single glycerophosphodiesterase enzyme with broad substrate specificity. The synthetic phosphodiesterase substrate bis-p-nitrophenylphosphate does not inhibit GPC cleavage but is cleaved by other phosphodiesterases in the preparation. Appendix B, Table VIII shows that GPC cleavage is virtually irreversible starting with choline and ℓ-α-GP and that ℓ-α-G^{32}P cannot be exchanged into GPC. Since Me-^{14}C-choline does exchange into GPC or GPI, it is suggested that the ℓ-α-GP moiety of GP-X is firmly bound to the enzyme. After hydrolysis, the base moiety (e.g., choline or inositol) is free to exchange. The rate of release of the GP moiety is less than the rate at which the base can exchange and reformed GP-X can be released. The failure of ℓ-α-GP to exchange suggests that the formation of an enzyme-GP intermediate from free enzyme and GP is very unfavorable. The exchange of Me-^{14}C-choline into GPI and GPC is additional support for the proposed single enzyme active on all GP-X compounds.

CONCLUSIONS

CONCLUSIONS

From the results presented in this thesis and the available literature, the following conclusions are drawn: The microsomal phospholipase A_1-B system of housefly larvae deacylates PI, PS, and PG, as well as PC, PE, and phosphatidyl-β-methylcholine (2) to the respective GP-X derivatives (see Figure 1). This system differs from the mosquito microsomal system in being active with PI. The catabolism of PI by the microsomal phospholipase A_1-B system is very similar to the PI metabolizing pancreatic microsomal system of White et al. (10). It remains to be established what the fate of GPI is in the pancreas. It may be metabolized by a glycerophosphodiesterase activity located in some fraction other than the microsomes. This would be in contrast to the microsomal enzyme found in rat tissues (4). It is possible that houseflies contain minor amounts of a specialized PI metabolic pathway such as the diglyceride-cyclic inositolphosphate reaction of thyroid gland (12). Any such activity could have been masked by the activity observed which is probably responsible for the extensive turnover of phospholipids in the late larval stage.



X-OH = CHOLINE, ETHANOLAMINE, β-METHYLCHOLINE, INOSITOL, SERINE or GLYCEROL

Figure 1. Diacylglycerophosphatide catabolic pathway in housefly larvae.

In considering the extensive turnover of phospholipids occurring in late larval stage and particularly during pupation, it would be interesting to know what phospholipases are present in housefly lysosomes. If there is a system of phospholipases in housefly lysosomes similar to that in rat lysosomes (9 and 21), this system is probably very active during pupation when other lysosomal activity is causing extensive histolysis prior to formation of the adult fly.

The soluble glycerophosphodiesterase reported on in Appendix B may be the only such activity functioning during pupation. There is no similar enzyme found in lysosomes from rat liver (21) and I found none in larval lysosome fractions. If this enzyme is functional during the histolysis of early pupation, as preliminary results suggest, a single enzyme active with many substrates and stable toward proteolysis would seem to be of physiological importance. GPC was found to be the best substrate of those tested with the glycerophosphodiesterase. It was cleaved at a higher rate, had a lower K_m, and competed more favorably for cleavage when incubated with another substrate. These observations may be of significance when one considers the necessity of efficient reutilization of choline in the formation of PC for adult tissues in late pupation.

Figure 1 summarizes the findings of my work and previous work (2) in this laboratory on diacylglycerophosphatide metabolism in housefly larvae. Reaction 1 is reversible in the sense that 2-lyso compounds can be reacylated, as can 1-lyso compounds, to yield diacylglycerophosphatides.

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APPENDICES

APPENDIX A

Reprinted from LIPIDS, Vol. 6, No. 7, Pages: 508-516 (1971)

Metabolism of Ceramide Phosphorylethanolamine, Phosphatidylinositol, Phosphatidylserine and Phosphatidylglycerol by Housefly Larvae[1]

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ABSTRACT

Microsome preparations (40,000-90,000 g sediment) from *Musca domestica*, housefly, larvae convert exogenous ^{32}P-labeled phosphatidylinositol, phosphatidylserine and phosphatidylglycerol to the respective lysoglycerophosphatides and, ultimately, to the glycerophosphoryl derivatives. These data, combined with previous results, demonstrate that housefly larvae can convert their normal diacylglycerophosphatides to the respective glycerophosphoryl derivatives. Experiments utilizing exogenous ^{3}H-labeled, ^{32}P-labeled and ^{14}C-labeled ceramide phosphorylethanolamine demonstrate that particulate preparations from housefly larvae convert ceramide phosphorylethanolamine to ceramide, phosphorylethanolamine, sphingosine and fatty acid. The presence of ceramide phosphorylethanolamine phosphohydrolase and ceramidase activity in housefly larvae is consistent with the conclusion that ceramide phosphorylethanolamine is metabolized to ceramide and phosphorylethanolamine and the ceramide is then hydrolyzed to sphingosine and fatty acid. Thus, metabolism of ceramide phosphorylethanolamine by these insects is analogous to the metabolism of sphingomyelin by mammalian systems.

INTRODUCTION

Phosphatidylcholine and phosphatidylethanolamine are the principal phospholipids of many insects (1). Housefly (2) and blowfly (3) larvae also contain minor phospholipids such as phosphatidylserine and phosphatidylinositol as well as ceramide phosphorylethanolamine-type sphingolipids (2,4-6). Particulate preparations, containing microsomes, from larvae of the housefly (*Musca domestica*) (7,8), the blowfly (*Phormia regina*) (7) and the mosquito (*Papiens fatigans*) (9,10), contain phospholipase A^{1} and A^{2} activity towards phosphatidylcholine and phosphatidylethanolamine. Little is known concerning the metabolism of the minor lipids of insects. We will demonstrate that particulate preparations containing microsomes from housefly larvae deacylate phosphatidylserine, phosphatidylinositol and phosphatidylglycerol to the respective lysophosphatides and, ultimately, to the water soluble glycerophosphoryl derivatives. Ceramide phosphorylethanolamine is cleaved to ceramide and phosphorylethanolamine, and the ceramide is cleaved to fatty acids and sphingosines.

METHODS

Isolation of Phospholipids and Ceramide Derivatives

^{32}P-Labeled phosphatidylinositol, phosphatidylglycerol, phosphatidylserine and ceramide phosphorylethanolamine were isolated from housefly larvae that had been reared on a ^{32}Pi-containing diet as described previously (6,8). The lipid extract containing phosphatidylinositol, phosphatidylserine, phosphatidylglycerol and other phospholipids was chromatographed on silicic acid to remove neutral lipids and lecithin as described elsewhere (8,11). The remaining phospholipid classes were resolved by column chromatography using DEAE cellulose essentially as described by Rouser et al. (12). Column eluates containing acetic acid or formic acid were dripped directly into NH_4HCO_3 solutions to neutralize the acid. These preparations were then partitioned between water and chloroform and the chloroform phase evaporated to dryness. The dried lipid samples were dissolved in warm tertiary butyl alcohol, lyophilized and stored dry at -20 C. After chromatography on DEAE cellulose, phosphatidylglycerol and phosphatidylinositol were separated by preparative thin layer chromatography (TLC) using Brinkman preparative plates (Silica Gel F-254) and the solvent system, chloroform-methanol-H_2O (65:35:4). Occasionally, the other phospholipids were further purified by preparative TLC using the solvent system, chloroform-methanol-H_2O (65:35:4) or chloroform-methanol-conc NH_4OH (65:35:4). The phospholipids were at least 95% pure before use, as determined by monitoring the ^{32}P distribution

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[1] Paper No. 5250 from the Michigan Experiment Station.

with a Packard Model 7201 radiochromatogram scanner after TLC in one or more solvent systems. In experiments (unpublished data), G.R. Hildenbrandt has shown that the phosphorus-inositol ratio and the phosphorus-serine ratio of the glycerophosphoryl derivatives prepared from these preparations of phosphatidylinositol and phosphatidylserine are 1.0 ± 0.1. ^{32}P in column eluates was monitored with a recording Geiger Muller apparatus.

Tritiated ceramide phosphorylethanolamine was prepared according to the procedure described above for preparation of ^{32}P-labeled lipids except that larvae were reared on a diet containing 1 curie of tritiated water. ^{14}C-labeled ceramide phosphorylethanolamine was prepared similarly except that larvae were reared on diets containing uniformly labeled L-serine from New England Nuclear. Ceramide was prepared from bovine heart sphingomyelin by treatment with 2-5 mg of phospholipase C from Worthington, *Clostridium Welchii*, as follows. The sphingomyelin (400 mg) in chloroform was adsorbed onto 1 g of Celite using a rotary evaporator and the reaction was run overnight with stirring at room temperature in 20 ml of 0.025 *M* Hepes (N-2-hydroxypiperazine-N'-2-ethane sulfuric acid), pH 7.3, containing 3 mM $CaCl_2$. The chloroform soluble-products were resolved on silicic acid (8). Ceramide was detected on thin layer chromatograms utilizing a Chloroxbenzidine spray reagent (13). Sphingosine standards were prepared by hydrolyzing bovine brain sphingomyelin in 1 N methanolic HCl for 18 hr at 105 C. Phospholipids were deacylated by adding 1.0 ml of 0.5 N KOH in 95% methanol to 2 ml of phospholipid in chloroform. After stirring for 10 min at room temperature, excess Dowex-50 resin, H^+ form, was added and the samples were partitioned between equal volumes of chloroform and water. The resin was removed and the aqueous phase neutralized immediately with NaOH. Glycerophosphate and Pi were removed by column chromatography on Dowex-1, formate form and the water soluble glycerophosphoryl derivatives were then chromatographed on Dowex-50 H^+ as described below.

Enzyme Preparations and Assays

Microsomes were prepared from housefly larvae and phospholipase assayed as described by Kumar et al. (8). ^{32}P-labeled diacylglycerophosphatides were separated from the respective monoacylglycerophosphatides by TLC as described in the figure legends. The water soluble portion of the reaction products, which contained the glycerophosphoryl derivatives, was passed through columns of Dowex-50 resin, H^+ form, 100-200 mesh, 0.8 x 10 cm to remove cations. The column eluates (containing the glycerophosphorylinositol, -serine, or -glycerol) were lyophilized, dissolved in a small quantity of water (<0.5 ml), neutralized, and then chromatographed on paper as described in the legends of Figures 1-3. ^{32}P in water, in organic solvents and on silicic acid was quantitated utilizing Cerenkov radiation as described by Haviland and Bieber (14). Ceramide phosphorylethanolamine phosphohydrolase was prepared and measured as described in the legend of Figure 5.

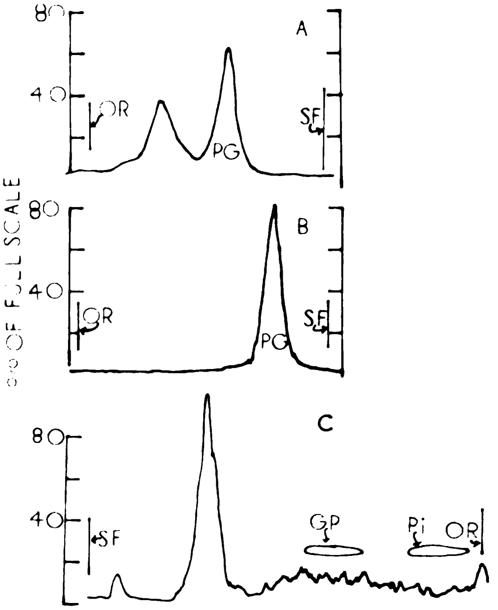

FIG. 1. Tracings of the radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylglycerol. Larval microsomes, 0.6 mg protein, were incubated with 1 μmole of ^{32}P-labeled phosphatidylglycerol (44,000 CPM) for 1 hr at 31 C in 1 ml of 50 mM Tris HCl, pH 7.2. Assays were performed as described in Methods. A contained 2.5 mM lauryl sulfate. Lauryl sulfate and microsomes were not added in B. A and B are radiochromatogram scans of the chloroform phases that were chromatographed on Silica Gel F-254 Brinkman thin layer plates in the solvent system, chloroform-methanol-H_2O (65:35:4). 73% of total counts were in the chloroform phase of A and 100% of total counts were in the chloroform phase of B. C is a radiochromatogram scan of the water soluble fraction from A (27% of total counts) that were chromatographed on paper in the solvent system, methanol-conc. NH_4OH-H_2O (12:2:3). OR, origin; SF, solvent front; PG, phosphatidylglycerol; GP, α-glycerophosphate; Pi, inorganic phosphate.

^{32}Pi was determined by the method of Lindberg and Ernster (15). Pi was precipitated from

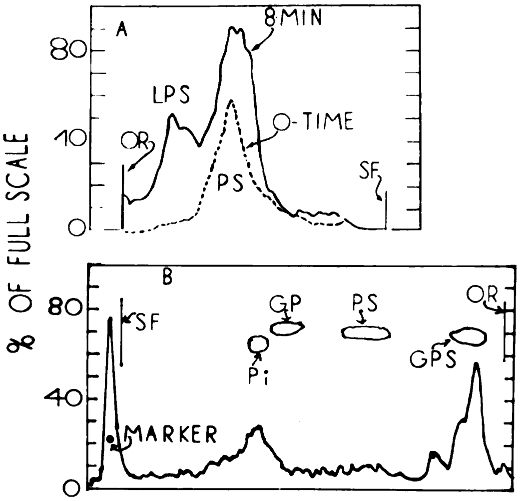

FIG. 2. Tracings of the radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylserine. Microsomes, 2.9 mg protein, were incubated for 8 min in 2.5 ml of a solution containing 0.05 M imidazole buffer, pH 7.4, 2.5 mM lauryl sulfate, 1 mM $HgCl_2$, and 2.75 μmoles of phosphatidylserine (64,400 CPM). The reaction was terminated and assayed as described in the Methods. The aqueous phase contained 7,762 CPM. SF, solvent front; OR, origin. For A, the chloroform soluble extract was chromatographed as described for Figures 1A and B. LPS, lysophosphatidylserine; PS, phosphatidylserine. For B, the water soluble fraction was chromatographed on paper in the solvent system, picric acid-tertiary butyl alcohol-water (4 g:80:20). GP, α-glycerophosphate; GPS, glycerophosphorylserine; PS, phosphorylserine; Pi, inorganic phosphate.

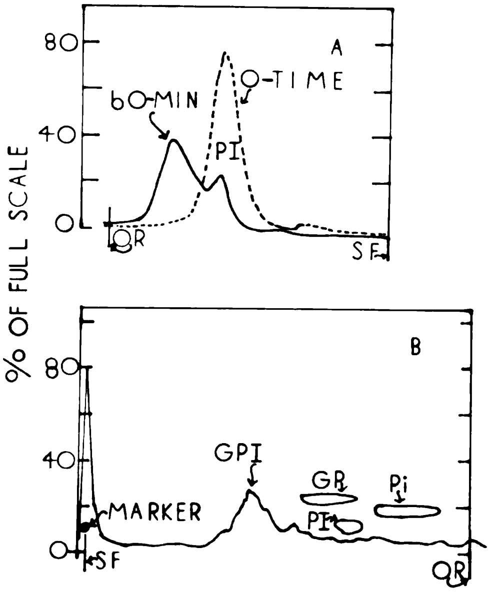

FIG. 3. Tracings of radiochromatogram scans of chromatograms of the chloroform soluble and water soluble extracts obtained from larval microsomes incubated with ^{32}P-labeled phosphatidylinositol. Incubations and assays were identical to those described for Figure 1 including quantity and specific radioactivity of substrate. For A, the lipid extract was chromatographed as in Figures 1A and B. The dashed curve (- - - -) is a tracing of the radiochromatogram scan obtained with the lipid extract (100% of total counts) from an incubation without microsomes, and the solid curve (——) is a tracing of the radiochromatogram scan of the lipid extract (66% of total counts) from microsomes that were incubated 60 min with 2.5 mM lauryl sulfate. PI, phosphatidylinositol; 0.7 mg protein per sample was used. For B, an aliquot of the water soluble portion (53% of total counts) of a 90 min incubation, as described for Part A, was chromatographed on paper in the solvent system described for Figure 1C. B is a tracing of the radiochromatogram scan. GPI, glycerophosphorylinositol; GP, α-glycerophosphate; PI, phosphorylinositol; and Pi, inorganic phosphate. In A and B, SF, solvent front; OR, origin.

aqueous solutions as the triethylamine phosphomolybdate complex by the method of Sugino and Miyoshi (16). Serine was quantitated using an automated amino acid analyzer as described by D.C. Robertson, H.B. Brockman, W.I. Wood and W.A. Wood (personal communication). Protein was determined by the method of Lowry et al. (17).

Inositol was quantitated by gas chromatography of the TMSi derivatives as described by Wells et al. (18).

RESULTS

Microsomal Deacylation of Phosphatidylinositol, Phosphatidylserine, and Phosphatidylglycerol

When microsomes from housefly larvae were incubated with ^{32}P-labeled phosphatidylserine and phosphatidylglycerol, TLC of the chloroform soluble fraction demonstrated the presence of the respective ^{32}P-labeled lysophospholipids and paper chromatography of the water soluble fractions demonstrated production of glycerophosphoryl derivatives (Fig 1 and 2). The thin layer chromatograms for Figures 1A and B were run in the solvent at different times. Thus, the difference in the Rf of phosphatidylglycerol in Fig. 1A and B is probably due to slightly different solvent composition or different activation of the plates. Some inorganic phosphate was present in the water soluble fraction when phosphatidylserine was the substrate (Fig. 2B). The inorganic phosphate was probably produced by the phosphatase(s) which are present in the enzyme preparations. Glycerophosphate, a phosphatase substrate, could be produced enzymatically

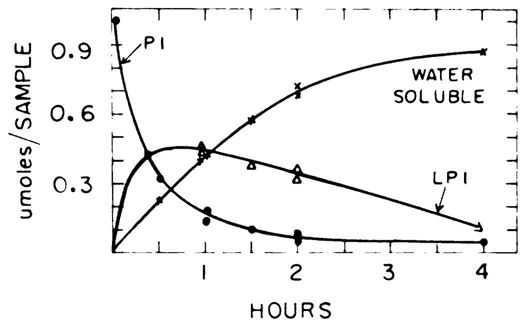

FIG. 4. Time course for deacylation of ^{32}P-labeled phosphatidylinositol by housefly larvae microsomes. Each sample of 1 ml, taken from an 8 ml incubation mixture at the indicated times, contained: 1 μmole ^{32}P-labeled phosphatidylinositol at zero time; 50 μmoles Tris Cl$^-$, pH 8.0 and 2.5 μmoles lauryl sulfate. Incubation was at 31 C, and assays were performed as described in Methods. PI, phosphatidylinositol, ●—●; LPI, lysophosphatidylinositol, △—△; water soluble, ^{32}P-labeled glycerophosphorylinositol formed, x—x. The protein concentration was 0.7 mg/ml.

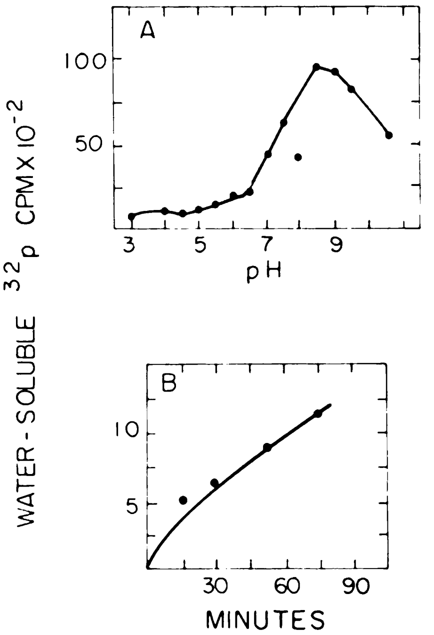

FIG. 5. pH Optimum and Time course for hydrolysis of ^{32}P-labeled ceramide phosphoryl-ethanolamine. In A, 0.2 ml of the 10,000-40,000 g fraction (0.6 mg protein) in 0.1 M KCl was added to 0.8 ml of 0.05 M buffer, and 0.1 ml of ^{32}P-labeled ceramide phosphorylethanolamine in 5% Triton X-100. The final concentration of Triton X-100 was 1% and ceramide phosphorylethanolamine was 0.25 mM = 32,140 CPM of ^{32}P. The samples were incubated at 31 C for 30 min, and the reaction was terminated by adding 6 ml of chloroform-methanol (1:2). After mixing for 15 min, 5 ml of chloroform and 5 ml of 0.02 M $MgCl_2$ were added. The samples were mixed and the layers separated by centrifugation. The water soluble fraction was counted and used as a measure of the extent of reaction. The water soluble CPM are plotted in A and B. The buffers were formate at pH 3.1 and 4.1 · acetate at pH 4.5 and 5.5; citrate at pH 5.0; imidazole at pH 6.0, 6.5 and 7.0; Tris Cl$^-$ at pH 7.5, 8.5 and 9.5; phosphate at pH 8.0, and glycine at pH 9.0 and 10.5. In B, 1 ml of the 10,000-40,000 g pellet in 0.05 M KCl was added to 4 ml of 0.05 M Tris HCl, pH 9.5, buffer containing sufficient Triton X-100 and ceramide phosphorylethanolamine to make a final concentration of 1% Triton X-100 and 0.3 mM ^{32}P-labeled ceramide phosphorylethanolamine. The sample was incubated at 31 C. 1.0 ml Aliquots were taken at the times indicated and assayed as described for part A. Each assay contained 0.6 mg protein. The 10,000-40,000 g fraction was prepared by homogenizing larvae in 0.15 M Tris, pH 7.5 (5ml/g larvae) in a micro Waring blendor for 30 sec at maximum speed. The homogenate was squeezed through several layers of cheesecloth and a 10,000-40,000 g fraction was collected and suspended in 0.1 M KCl or the appropriate buffer.

from glycerophosphorylserine by traces of phosphodiesterase. A large amount of this phosphodiesterase activity is present in the 90,000 g supernatant fluid from larvae and occasionally small amounts remain with the microsome preparations. Attempts to detect production of lysophosphatidylserine were unsuccessful until lauryl sulfate and $HgCl_2$ were added to the reaction mixture. It was shown previously (8) that lauryl sulfate greatly stimulates monoacylglycerophosphatide production from the diacylglycerophosphatides. Apparently, in the absence of lauryl sulfate and $HgCl_2$, housefly microsomes deacylate lysophosphatidylserine at a much greater rate than phosphatidylserine, preventing accumulation of the lyso-intermediate. When ^{32}P-labeled phosphatidylinositol was the substrate, after 1 hr, microsome preparations had converted much of the ^{32}P to a phospholipid which had the Rf on thin layer chromatograms of lysophosphatidylinositol (Fig. 3A). A water soluble compound was also formed. It had an Rf corresponding to that of glycerophosphorylinositol rather than glycerophosphate, inorganic phosphate or phosphorylinositol (Fig. 3B).

Since phosphatidylinositol is cleaved to phosphorylinositol and diglyceride in some systems, its cleavage by the insect system was further investigated. The microsomal system from housefly larvae is capable of deacylating most of the added phosphatidylinositol, as shown by the time course in Figure 4. At the end of the experiment, 4 hr, the concentration of exogenous phosphatidylinositol had decreased from 1 mM to 0.04 mM. The amount of lysophosphatidylinositol increased rapidly and then decreased with time, while the glycerophosphorylinositol only increased with time, indicating a product-precursor relationship.

Alternate pathways of phosphatidylinositol

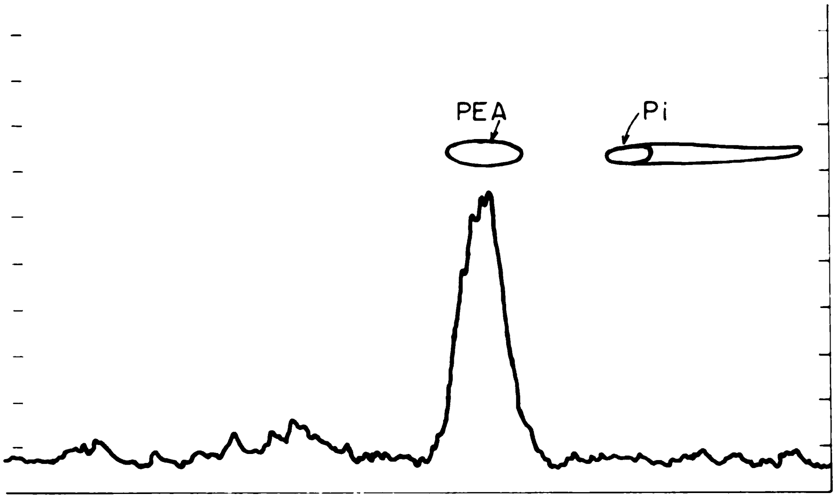

FIG. 6. Identification of ^{32}P-labeled phosphorylethanolamine as a product of the cleavage of ceramide phosphorylethanolamine. To a 10,000-40,000 g fraction, 0.6 mg protein, in 0.5 ml of 0.05 M KCl was added 0.5 ml of 0.5 M Tris Cl, pH 9.0 and 0.2 ml of ^{32}P-labeled ceramide phosphorylethanolamine (0.43 μmole) in 1% Triton X-100. The sample was incubated at 31 C for 30 min. The reaction was terminated by adding 3 ml of liquid phenol; see reference 39 for description of this method. The phenol was washed two times with 2 ml water, and the combined aqueous phases were applied to a Dowex-1, HCO_3^- column. The column was eluted with water, then 0.25 M triethylammonium bicarbonate, pH 7.5, followed by 1 M triethylammonium bicarbonate, pH 7.5. Two peaks were obtained as described in the text. The first radioactive peak was chromatographed on paper using the solvent system, methanol-conc NH_4OH-H_2O (12:2:3). Figure 6 is a tracing of a portion of a radiochromatogram scan of the paper chromatogram. One radioactive peak was detected; it had an Rf identical to phosphorylethanolamine. PEA, phosphorylethanolamine; Pi, inorganic phosphate.

metabolism were not detected in housefly larvae. Attempts to detect production of phosphorylinositol using whole homogenates as well as particulate fractions containing microsomes plus lysosomes and mitochondria were unsuccessful. Assays were performed at pH 5.0 and 8.0. Such results indicate that cleavage of phosphatidylinositol to diglyceride and phosphorylinositol is not a major pathway of metabolism in housefly larvae. It should be noted that deacylation of phosphatidylinositol was detected at pH 5.0 with particulate preparations that should contain lysosomes. The water soluble fraction from the pH 5.0 incubation contained inorganic ^{32}P and glycerophosphorylinositol-^{32}P. The precursor of ^{32}Pi was not investigated and could have been phosphorylinositol.

In these experiments, the ^{32}P-labeled water soluble products were not rigorously characterized; however, paper and column chromatography demonstrated that the principal water soluble ^{32}P was not inorganic phosphate, glycerophosphate, phosphorylinositol or phosphorylserine. The water soluble ^{32}P had paper chromatographic properties expected for the glycerophosphoryl derivatives of inositol, serine and glycerol. Authentic glycerophosphoryl derivatives of inositol, serine and glycerol were prepared from the respective diacylglycerophosphatides by mild alkaline hydrolysis. Portions of these derivatives were used as standards for paper chromatography and as substrates for the phosphodiesterase that cleaves these compounds to glycerophosphate and free hydroxyl compound.

Cleavage of Ceramide Phosphorylethanolamine by Particulate Preparations Obtained From Housefly Larvae

When whole homogenates as well as the 0-800 g, 800-9,000 g, 9,000-40,000 g, 40,000-90,000 g and the 90,000 g soluble fractions from housefly larvae were incubated with ^{32}P-labeled ceramide phosphorylethanolamine at pH 7.5 and 9.5, water soluble ^{32}P was released. These experiments demonstrated that these fractions cleaved ceramide phosphorylethanolamine. The soluble fraction and the 0-800 g fraction contained the lowest specific activities. The ratio of the specific activities of the 800-9,000 g, 9,000-40,000 g and 40,000-90,000 g fractions varied from preparation to preparation. The specific activity differences appeared to be related to the stage of

TABLE I

Fraction	Total CPM	Distribution of $CHCl_3$-soluble material, %
Free fatty acid	13,320	10.2
Ceramide	2,500	1.9
Sphingosines	18,600	14.3
Ceramide phosphorylethanolamine	95,520	73.5

[a] Amounts of 0.5 mg of ^3H-ceramide phosphorylethanolamine plus 4 mg of ceramide were dissolved in 1 ml of a solution of 1% Triton X-100 and 0.05 M glycine, pH 9.0. The 8,000-40,000 g particulate fraction in 2 ml of 0.05 M glycine buffer, pH 9.5, from 21 g of larvae was added to the substrate solution and incubated at 31 C for 15 min. The reaction was terminated by adding 8 ml of chloroform-methanol, 1:2, and stirring for 15 min. Then 5 ml of 0.02 M $MgCl_2$ and 5 ml of chloroform were added. The chloroform layer was washed three times with 5 ml of 0.02 M $MgCl_2$. The aqueous washes were combined and counted. The aqueous layer contained 8,430 CPM. The chloroform fraction was evaporated to dryness, and the fatty acids, sphingosine, ceramide and ceramide phosphorylethanolamine separated by TLC as follows. The chloroform soluble material was streaked onto a Silica Gel F-254 (Brinkman) plate and developed in chloroform-glacial acetic acid (90:10). The plate was dried at approximately 50 C and placed in iodine vapors. The zones with Rf's of ceramide and oleic acid were marked. The plate was then placed into the solvent system, chloroform-methanol conc NH_4OH (65:35:4) and developed until the solvent front reached the ceramide zone. This solvent system separated ceramide phosphorylethanolamine from the sphingosines. The zones having Rf's of sphingosines, fatty acids and ceramide were scraped from the plates. The scrapings were put into small columns and eluted with methanol. Aliquots of the methanol solution were transferred to scintillation vials and the samples counted for ^3H. The remainder of the methanol was evaporated to dryness and the residue was dissolved in chloroform and applied to thin layer plates. The plates were developed in the solvent systems A, chloroformglacial acetic acid (96:4), and B, chloroform-methanol-conc, NH_4OH (65:35:4). For each, the zones with Rf's of sphingosine, fatty acid and ceramide were scraped from plates, eluted and counted as described above. The rechromatography was essential because the ceramide and sphingosine overlapped slightly on the initial chromatography. In solvent A, the Rf's of ceramide phosphorylethanolamine, sphingosine, ceramide and free fatty acids were 0.0, $<$ 0.02, 0.26, and 0.59, respectively. In solvent B, the Rf's of ceramide phosphorylethanolamine and sphingosine were 0.42 and 0.88, respectively. The tritiated samples were dissolved in 1 ml of methanol or 1 ml of water. Then 0.4 ml of Triton X-100 and 10 ml of toluene scintillation solution (4 g PPO and 100 mg POPOP per liter toluene) were added and the samples were counted. 13.9 x 10^4 DPM were used in the experiment.

larval development and were not investigated further. Since all three particulate fractions contained ceramide phosphorylethanolamine phosphohydrolase activity, a 10,000-40,000 g particulate fraction was used for most of the following investigations. In all of the preparations, ^{32}P-labeled phosphorylethanolamine as well as ^{32}Pi were detected when ^{32}P-labeled ceramide phosphorylethanolamine was the substrate.

pH Optimum and Time Course

Four pH optimum curves were run on 10,000-40,000 g preparations. In all four determinations, the greatest activity was obtained near pH 9.0; however, in two of the runs, considerably more activity at pH 4-5 and at 7-8 was obtained than is shown in Figure 5A.

At pH 9.5, hydrolysis of ceramide phosphorylethanolamine was nearly linear for over an hour, as shown in Figure 5B.

Identification of Phosphorylethanolamine as a Reaction Product

When ^{32}P-labeled ceramide phosphorylethanolamine was incubated with the 0-800 g, 800-9,000 g, 9,000-40,000 g, 40,000-90,000 g, or the 90,000 g supernatant fraction, water soluble ^{32}P was released. From 10% to 45% of the water soluble radioactivity from each fraction was inorganic phosphate as determined by partitioning the phosphomolybdate complex between sulfuric acid and isotubanol-benzene (15). When the water soluble fraction from a 10,000-40,000 g fraction was exchanged onto Dowex-1 in the bicarbonate form and the column eluted successively with 0.25 M and 1 M triethylammonium bicarbonate, pH 7.5, two radioactive peaks wer obtained. The first peak had chromatographic properties on Dowex-1 of authentic phosphorylethanolamine. It also had paper chromatographic properties of phosphorylethanolamine as shown in Figure 6. The second peak obtained from the Dowex-1 column was Pi as determined by paper chromatography and by partitioning radioactivity as the molybdate complex into isobutanol-benzene.

Inorganic phosphate would be expected in these preparations from the action of phosphatase on phosphorylethanolamine. Each of the fractions contained phosphatase activity as determined by release of Pi from glucose 6-phosphate and release of p-nitrophenol from p-nitrophenyl phosphate.

When the water-soluble ^{32}P was partitioned between water and chloroform as described by Hirschberg et al. (19), negligible radioactivity was detected in the chloroform layer. Since the partition coefficient in this system for phosphoryl sphingosines is approximately 0.73 (19), some radioactivity should have partitioned into the organic phase if phosphoryl sphingosines were present.

Identification of Ceramides as a Product of Ceramide Phosphorylethanolamine Cleavage by Larvae Particulate Preparations

When ^{14}C-labeled ceramide phosphorylethanolamine was incubated with a 10,000-40,000 g particulate fraction, ^{14}C-labeled sphingosine and ^{14}C-labeled phosphorylethanolamine were detected; however, ceramide was not detected in the organic soluble fraction, nor was ^{14}C detected on thin layer chromatograms at the Rf corresponding to ceramide standards. ^{14}C was anticipated in ethanolamine and sphingosine because the ^{14}C-labeled ceramide phosphorylethanolamine was isolated from larvae that were reared in the presence of uniformly labeled L-serine. The data mentioned above indicated that either ceramide was not a reaction product or the preparations contained considerable ceramidase activity. To distinguish between these possibilities, 0.5 mg of tritiated ceramide phosphorylethanolamine—both the sphingosine and fatty acid portion of the substrate contained tritium— was combined with 4 mg of ceramide and incubated with an 8,000-40,000 g fraction. Carrier ceramide was added to dilute any radioactive ceramide that would be produced. The organic soluble reaction products were separated by TLC in three solvent systems, as described in the legend of Table I. Very weak iodine-absorbing spots were detected in the ceramide regions of the thin layer chromatograms although samples equivalent to 1 mg of initial ceramide were applied to the thin layer chromatograms indicating that the particulate preparations contained ceramidase activity. Compounds with Rf's of sphingosine and fatty acids were detected. Although very little, if any, ceramide was detected by iodine vapors on the thin layer chromatograms, some tritium was detected in the ceramide area with the three solvent systems. used. When the tritiated material in the ceramide region of the thin layer chromatograms was eluted and rechromatographed, as described in the legend of Table I, the tritium again migrated with ceramide. Thus, small amounts, approximately 2% (Table I) of the chloroform soluble tritiated material obtained from the reaction media had chromatographic properties of ceramide. Large amounts of sphingosine and fatty acids were produced, presumably from ceramide via the ceramidase activity.

The appearance of 2% of the radioactivity of tritiated ceramide phosphorylethanolamine in the ceramide fraction when carrier ceramide was added to the incubation media is significant, as indicated by the following results. When ^{14}C-labeled ceramide phosphorylethanolamine was incubated with the 10,000-40,000 g particulate fraction in the absence of carrier ceramide, only 0.02% of the initial ^{14}C was detected in ceramide. This amount is 1% of the radioactivity that accumulated in the presence of a large pool of cold ceramide.

Ceramide phosphorylethanolamine might be initially deacylated to sphingosine phosphorylethanolamine and fatty acids, but the data do not indicate such cleavage. ^{32}P-labeled material with thin layer chromatographic properties expected for sphingosine phosphorylethanolamine was not detected in the experiments described for Table I.

DISCUSSION

The data show that a microsome-enriched fraction from housefly larvae converts phosphatidylinositol, phosphatidylserine and phosphatidylglycerol to lysophospholipids and to the respective glycerophosphoryl derivatives. A typical product-precursor relationship was observed for lysophosphatidylinositol (Fig. 4). Previous investigations (8) demonstrated that phosphatidylcholine, phosphatidylethanolamine and phosphatidyl-β-methylcholine are deacylated similarly by microsome-containing preparations. Thus, housefly larvae have the capacity for completely deacylating their principal and minor diacylglycerophosphatides; cardiolipin and phosphatidic acid, both occurring in larvae, have not been investigated. Presumably, this deacylation system can also remove the fatty acids from the numerous abnormal glycerophosphatides that are formed by housefly larvae (20-24).

Formation of glycerophosphorylinositol from phosphatidylinositol occurs in mammalian systems such as ram seminal fluid (25), ox pancreas (26), rat liver (27) and rat prostate (29), and in the microorganism, *penicillium notatum* (26). Phosphatidylinositol is also cleaved by phosphoinositide inositolphosphohydrolase to diglyceride and phosphorylinositol by ram spermatozoa (25), ox pancreas (26), rat liver (27), guinea pig intestine (28) and guinea-pig brain (40). Our studies indicate that housefly larvae contain little, if any, phosphatidylinositol phosphohydrolase activity. When ^{32}P-labeled phosphatidylinositol was incubated with larval microsomes, no phosphorylinositol was detected in the reaction mixture (Fig. 3B). Some glycerophosphate was found (Fig. 3B), but this undoubtedly was caused by a larval phosphodiesterase that cleaves glycerophosphoryl derivatives (unpublished results) to glycerophosphate and the hydroxyl compound. Phosphorylinositol was not detected when the soluble or particulate fractions were used as the enzyme source. It should be noted that small amounts of ^{32}Pi were detected in some of the reactions. Such results indicate that third instar *M. domestica* larvae contain little, if any, phospholipase C type activity towards phosphatidylinositol.

Ceramide phosphorylethanolamine is a phospholipid in several species of flies (4-6, 30,31) as well as in honey bees (32), scorpions (32), fresh water mollusks (33) and pond snails (34). Our data are consistent with the conclusion that housefly larvae can metabolize ceramide phosphorylethanolamine, as follows:

1. Ceramide phosphorylethanolamine + HOH → ceramide + phosphorylethanolamine
2. Ceramide + HOH → fatty acids + sphingosines

The above-mentioned pathway is supported by the following results:

1. Phosphorylethanolamine was the principal water soluble product when ^{32}P-labeled ceramide phosphorylethanolamine was the substrate. ^{32}Pi was also detected, but it most likely was cleaved from phosphorylethanolamine by a phosphomonoesterase.

2. Tritiated ceramide was detected in the reaction mixture when tritiated ceramide phosphorylethanolamine was the substrate. Tritiated sphingosine and fatty acid were also detected, but these products could be caused by the action of ceramidase on the ceramide.

3. The particulate enzyme preparations contained ceramidase activity. The ceramidase activity was greater than the ceramide phosphorylethanolamine phosphohydrolase activity.

Thus, ceramide phosphorylethanolamine is metabolized by housefly larvae similar to the metabolism of ceramide phosphorylcholine, sphingomyelin, in mammals. In mammals, sphingomyelin is hydrolyzed to ceramide and phosphorylcholine (35,36) and ceramide is cleaved to fatty acids and sphingosines (37,38).

Although phosphatidase C and D activity was not detected with any of the ^{32}P-labeled diacylglycerophospholipids, the metabolism of diacylglycerophosphatides via pathways other than those described above has not been rigorously excluded. Similarly, pathways involving conversion of ceramide phosphorylethanolamine to sphingosine-phosphorylethanolamine or to phosphorylceramide, especially by nonmicrosome fractions, were not investigated and could occur in housefly larvae.

ACKNOWLEDGMENT

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APPENDIX B

CHARACTERIZATION OF GLYCEROPHOSPHORYL-CHOLINE, -ETHANOLAMINE, -SERINE, -INOSITOL, AND -GLYCEROL HYDROLYTIC ACTIVITY IN HOUSEFLY LARVAE

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SUMMARY

Homogenates of *Musca domestica* (housefly) larvae contain glycerophosphodiesterase activity which fractionates into the 88,000 x g supernatant fluid. The phosphodiesterase is inhibited by EDTA and is stimulated by Mg^{++}, Ni^{++}, Co^{++}, and Mn^{++}. The pH optimum is 7.2. The enzyme is stable to heating at 50° for 15 minutes and is insensitive to sulfhydryl inhibitors. Glycerophosphoryldiesters of choline, ethanolamine, inositol, serine, glycerol, and β-methylcholine are hydrolyzed to the common product, ℓ-α-glycerophosphate, and the appropriate free alcohol. The rate of glycerophosphorylcholine hydrolysis is 70% greater than the rate of hydrolysis of the other glycerophosphodiesters. Apparent K_m values for glycerophosphorylcholine, glycerophosphorylethanolamine, and glycerophosphoryl-β-methylcholine are $2 - 4 \times 10^{-4}$ M and for glycerophosphorylinositol, 2×10^{-3} M. Competitive studies using various pairs of substrates as well as the exchange of free choline into both glycerophosphorylcholine and glycerophosphorylinositol suggest that a single enzyme cleaves all substrates. Product inhibition and reversal of the reaction were not detected. Choline, but not ℓ-α-glycerophosphate exchanges into glycerophosphorylcholine and glycerophosphorylinositol.

Introduction

Catabolism of glycerophosphatides in animals is generally thought to follow the pattern of phospholipase A and B hydrolysis of the two fatty acyl groups yielding free fatty acids and glycerophosphoryl-alcohols (GP-X[1] compounds); see review by Rossiter (1). Phosphatidylinositol is an exception since it is also catabolized by an additional or alternative pathway to diglyceride and inositol phosphate in several mammalian tissues (1). Investigations previously reported from this laboratory (2-4) showed that in housefly larvae, the glycerophosphatides of ethanolamine, choline, β-methylcholine, serine, glycerol, and inositol can be catabolized by microsomal phospholipase A_1 and lysophospholipase to yield GP-X compounds. It was also reported (3) that glycerophosphoryl-β-methylcholine is hydrolyzed by a 40,000 x g supernatant fraction from housefly larvae to yield glycerol-P and β-methylcholine.

The limited literature on glycerophosphodiesterase activity in animals does not clearly document the metabolic fate of GPS, GPG, and GPI. Baldwin and Cornatzer (5) reported that rat kidney microsomal glycerophosphodiesterase does not cleave GPI or GPS. In contrast,

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[1] GP-X = sn-glycero-3-phosphoryl-X
X = choline (C) ethanolamine (E), inositol (I), serine (S), glycerol (G), or β-methylcholine.

Serratia plymuthicum apparently can hydrolyze all of its GP-X compounds (6). The work reported herein was undertaken to determine whether housefly larvae can hydrolyze all GP-X compounds and whether a single or multiple enzymes are involved.

Materials and Methods

Enzyme Preparation: *Musca domestica* larvae were grown aseptically to the third instar (mature, wandering) stage on the diet described by Monroe (7). Larvae free of medium were weighed and homogenized at full speed in 2 - 3 volumes of 0.025 M Tris, pH 8.0, for 1 min in a Waring blender. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 40,000 x g for 20 min using a Sorvall SS-34 rotor. The 40,000 x g supernatant fluid was fractionated with solid ammonium sulfate to yield a 35-50% saturation insoluble fraction. This fraction, after resuspension in homogenizing buffer, was lyophilized and stored at -20°. All of the above steps were done at 0-4°. Assays were done using enzyme freshly dissolved in distilled H_2O. Most of the investigations reported herein were done with a single enzyme preparation. Where indicated, a preparation with about one-third the specific activity of the normal preparation was used. This preparation was redissolved in 0.05 M imidazole, pH 7.2, rather than H_2O.

Substrate Preparation: ^{32}P-labeled substrates were used for the routine assay of GP-X phosphodiesterase activity. Substrates were prepared by rearing housefly larvae as described above except that ^{32}Pi was added to the diets. The phospholipids were extracted and purified as previously described (2-4). Glycerophosphoryl-derivatives were prepared by alkaline deacylation (4) of the various pure phospholipids. Glycerophosphoryl-β-methylcholine was prepared from phosphatidyl-β-methylcholine, which was isolated from larvae reared on choline-free, carnitine-supplemented diets (3). The same procedures were followed for preparation of unlabeled GPS from bovine brain lipids and GPI from a commercial preparation enriched in phosphatidylinositol.

Ion Exchange Separations: Glycerophosphoryldiesters were separated from the phosphorus-containing hydrolysis products on columns of Dowex 1 - 8x, 100-200 mesh, formate form. Unless otherwise indicated, columns 6 x 0.5 cm were used. Paper chromatography (4) of enzyme reaction products and other criteria (see Results) demonstrated that GP-X hydrolysis yielded no phosphoryl-X compounds. A batch elution procedure was used to elute unhydrolyzed substrate into fraction A, and glycerophosphate into fraction B. Fraction B also contained any Pi that was released during the incubation. Fraction A was collected during application of a 2.5 ml aqueous load, a

1.5 ml H_2O rinse, and a 5 ml elution with 0.1 M ammonium formate. Fraction B was collected immediately following A by application of 10 ml of 0.4 M ammonium formate-0.2 M formic acid. Serine from GPS incubations was separated from phosphate-containing compounds on the above described columns by elution with 5 ml of H_2O after loading the incubation mixture at pH 7. Inositol from GPI incubations was collected the same as serine and then run over Dowex 50 x 8, H^+ form. The eluate was lyophilized and used for gas chromatographic analysis. When total phosphorus assays were done on the substrate and product fractions or ℓ-α-glycerophosphate analysis was done on fraction B, the fractions were passed through Dowex 50, H^+ form, columns to convert formate salts to formic acid and to remove cations. The acidic column eluates were immediately frozen and lyophilized to remove formic acid. In the exchange and reversal experiments larger columns were used and the incubations were diluted to 10 ml with H_2O prior to loading onto the columns. When GPC and choline in methyl-^{14}C-choline labeling experiments were separated, the anion exchange columns were washed with H_2O as for serine and inositol, and this fraction was loaded onto Dowex 50, H^+ form, columns. GPC was eluted using several volumes of H_2O and choline was recovered by elution with several column volumes of 4 N HCl.

Assay Procedures: All incubations were done in 12 ml conical glass centrifuge tubes. Incubations were initiated by adding enzyme to the temperature equilibrated reaction mixtures. The reactions were terminated by heating for four minutes in a boiling water bath. The 0.5 ml reaction volume was diluted with 2 ml of H_2O and vortexed prior to loading onto anion exchange columns. When ℓ-α-glycerophosphate was assayed directly (Table IV, exp. no. 3, 8, and 9), the boiled incubation mixtures were lyophilized and resuspended in a known volume of distilled water and aliquots were taken for the ℓ-α-glycerophosphate assay. The remainder of the mixtures was put onto anion exchange columns. Controls were run for each experiment and each substrate preparation. Identical incubation mixtures and conditions as for experimental tubes were used for the controls except that the enzyme was inactivated by boiling prior to the addition of substrate. The per cent of total radioactivity recovered off anion exchange columns in the B fraction (phosphate-containing hydrolysis products of GP-X) for control incubations was always less than 2%. This blank value largely represents breakdown products from the substrate, as similar values were obtained when samples of substrate were applied to columns without incubation. The precision of per cent distribution of radioactivity from anion exchange columns is ± 0.2%. In all results reported a minimum of 2,000 cpm of ^{32}P were incubated and recovered from the column fractionation. The assays routinely yielded 10-40% (above control values) of total radioactivity as product (fraction B).

^{32}P Cerenkov radiation was quantitated in a Packard Tricarb scintillation counter for 10 ml aqueous samples in polyethylene vials according to Haviland and Bieber (8). For monitoring anion exchange columns, the 10-ml substrate and product fractions were collected directly into polyethylene vials and counted. For quantitating ^{14}C, 0.1 ml aqueous aliquots in 10 ml of a 1:1 mixture of Triton X-100:toluene scintillator[2] were counted. GPC samples containing methyl-^{14}C-labeled choline were lyophilized and redissolved to 0.5 ml from which aliquots were counted.

Inositol enzymatically hydrolyzed from GPI was quantitated by gas chromatography (9) of the trimethylsilyl derivative. Aliquots of an internal standard, α-methylmannoside, were added to incubation mixtures prior to ion exchange purification, lyophilization, and derivatization. Ratios of inositol to phosphate in GPI preparations were determined to be 1±0.1. Free inositol in

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[2] Toluene scintillation mixture:

4 g 2,5-diphenyloxazole (PPO)
100 mg 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP)
1 ℓ toluene

boiled enzyme control incubations was less than 0.5% of GPI-bound inositol.

Serine hydrolyzed from GPS was quantitated with an automated amino acid analyzer.[3] The ratio of phosphate to serine in a GPS acid hydrolyzate was found to be 1.1/0.85. This low value for serine is due to the manipulations involved in the serine preparation. The phosphatidylserine source was greater than 90% pure, and routine ion exchange purification of its deacylation product removes essentially all non-GPS phosphate. Boiled enzyme control incubations showed negligible amounts of free serine in the GPS preparation.

ℓ-α-glycerophosphate was determined fluorometrically or spectrophotometrically following NAD^+ reduction by ℓ-α-glycerophosphate dehydrogenase (Sigma). The protocol for this assay was essentially that described for glycerol analysis (10) with the omission of glycerol kinase, Mg^{++}, and ATP. Protein was determined according to Lowry et al. (11). Total phosphate was determined as described by Bartlett (12).

Results

Distribution, Purification, and Stability of the Glycerophosphodiesterase:

Homogenization of housefly

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[3] D. C. Robertson, H. B. Brockman, W. I. Wood, and W. A. Wood, personal communication.

larvae in 0.025 M Tris, pH 8, and subsequent fractionation by differential centrifugation yielded fractions containing nuclei and mitochondria (250-9,000 x g), lysosomes and microsomes (9,000-40,000 x g), microsomes (40,000-88,000 x g), and soluble components (88,000 x g supernatant). No significant glycerophosphorylcholine phosphodiesterase activity was found in any of the particulate fractions. All of the activity detectable in the 250 x g supernatant fluid (Table I) is recovered in the 40,000 x g supernatant fraction. The greater activity in the 250 x g supernatant fraction than in the homogenate was not always observed. Greater than 70% of the activity can be recovered in an 88,000 x g supernatant fluid, but the limited purification obtained did not warrant this high-speed centrifugation.

The phosphodiesterase activity in the 40,000 x g supernatant fluid is 75-80% stable to heating for 15 min at 50° (Table II), and is stable to freezing and thawing. Fractionation of the 40,000 x g supernatant fraction with ammonium sulfate, 35-50% saturation, routinely yielded 90% of the total activity in the precipitate with a 2- to 5-fold purification. This 35-50% fraction was dissolved in 0.05 M imidazole, pH 7.2, buffer, lyophilized, and stored at -20° for up to 2 years with less than 15% loss of activity.

TABLE I

Isolation of glycerophosphodiesterase from housefly larvae

Fraction	Total Protein	Specific Activity	Activity Recovered	-fold Purified
	mg	nmoles/min/mg protein	%	
Homogenate	536	4.4	100	1.0
250 x g Supernatant	345	7.8	115	1.8
40,000 x g Supernatant	226	12.0	115	2.7
35-50% $(NH_4)_2SO_4$ Precipitate	72	35.0	106	8.0

12.5 g of larvae were homogenized for 1 min in 30 ml of 0.025 M Tris buffer, pH 8.0. All manipulations were carried out at 0-4° as described in Methods. ^{32}P-labeled glycerophosphorylcholine was the substrate. Assays for ^{32}P-labeled glycerophosphate were performed as described in the Methods section.

TABLE II

Effects of heating and EDTA on glycerophosphodiesterase activity

	Enzyme Experiment	Additions	α-Glycerophosphate formed
			nmoles
	Unheated	15 mM $MgCl_2$	330
A	Unheated	2.5 mM EDTA	10
	Heated (15 min at 50°)	15 mM $MgCl_2$	260
	Heated	None	150
	Heated	15 mM $MgCl_2$	210
B	Heated	2.5 mM EDTA	40
	Heated	2.5 mM EDTA[a] 15 mM $MgCl_2$	210

[a] The sample was incubated with EDTA for 10 min prior to addition of $MgCl_2$ and then the incubation was continued for 20 minutes.

All incubation mixtures contained approximately 2 mg of 40,000 x g supernatant protein unheated or heated for 15 min at 50°, 0.15 M sucrose, and 15 mM Hepes, pH 7.4, in a final volume of 2 ml. Incubation was at 30° for 20 min. The glycero-^{32}P-phosphoryl-β-methylcholine substrate was 0.56 mM in experiment A and 0.28 mM in experiment B. Assays were performed as described in the Methods section.

In order to determine whether the phosphodiesterase activity occurred only in housefly larvae entering the pupal stage of development or was present in other developmental stages, eggs and 1- to 2-day old pupae were homogenized and assayed for activity. Results (not shown) demonstrated that both of these developmental stages had the same activity on a live weight basis as third instar larvae.

Because the preparation was not pure, it was essential to know if contaminating activities were present that could interfere with glycerophosphodiester assays. All of the phosphomonoester released was ℓ-α-glycerphosphate as determined by ℓ-α-glycerophosphate dehydrogenase assays. Phosphatases active on ℓ-α-glycerophosphate were not detected by paper chromatographic analysis of products of GP-X hydrolysis. The enzyme preparation did not liberate ^{32}Pi from ^{32}P-labeled glycerophosphate. This was determined by partitioning the reaction products into isobutanol-benzene (13). Traces of phosphatase activity were detectable using p-nitrophenylphosphate (14) as substrate. This activity at pH 7.2 was less than 2% that of the phosphodiesterase. Slightly higher p-nitrophenylphosphate hydrolysis was detected at pH 4.5 and pH 9.0. Biologically occurring phosphodiesters that do not contain glycerol were not tested as competitive inhibitors of GP-X hydrolysis, but the synthetic phosphodiesterase substrate, bis-p-nitrophenylphosphate, was assayed (15). The synthetic substrate was cleaved at about 25% the rate observed with $G^{32}PE$. This activity was apparently different than the $G^{32}PE$ hydrolytic activity because cleavage of the synthetic substrate was not inhibited by $G^{32}PE$, and $G^{32}PE$ cleavage was not inhibited by the synthetic substrate.

Effect of pH on Glycerophosphodiesterase Activity: The glycerophosphodiesterase has a pH optimum of 7.2 that falls markedly within ± 0.5 pH units when $G^{32}PC$ is the substrate, as shown in Figure 1. With $G^{32}PE$ as substrate, the pH optimum is 7.4, but the curve is nearly flat from pH 7.0 to pH 7.8. The rates of $G^{32}PC$ cleavage above 40 nmoles/min/mg protein may be slightly low due to the substrate becoming limiting during the incubation period. The maximal rates for $G^{32}PE$ cleavage shown in Figure 1 are lower than sometimes observed with the same enzyme preparation. This was undoubtedly due to the presence of an impurity in the substrate for this determination. In data not shown, the pH optimum for $G^{32}PS$ was similar to $G^{32}PE$.

Divalent Cation Requirement of the Glycerophosphodiesterase: Mg^{++} stimulates the phosphodiesterase activity. Small amounts of glycerophosphodiesterase activity were obtained in the absence of added Mg^{++}, but

Figure 1

pH Dependence of GPC and GPE Hydrolysis by the Glycerophosphodiesterase. Incubation mixtures contained 2 mM $MgCl_2$ 40 mM buffer, 1 mM $G^{32}PC$ or $G^{32}PE$, and 0.14 mg protein. Incubation was for 20 min at 30°. Buffers for $G^{32}PC$ were acetate ●, imidazole ○, tris ▲, and glycine △. Buffers for $G^{32}PE$ were acetate ▲, imidazole △, tris ●, and glycine ○. Assays were done as described in the Methods.

$G^{32}PC$ is glycero-^{32}P-phosphorylcholine; $G^{32}PE$ is glycero-^{32}P-phosphorylethanolamine.

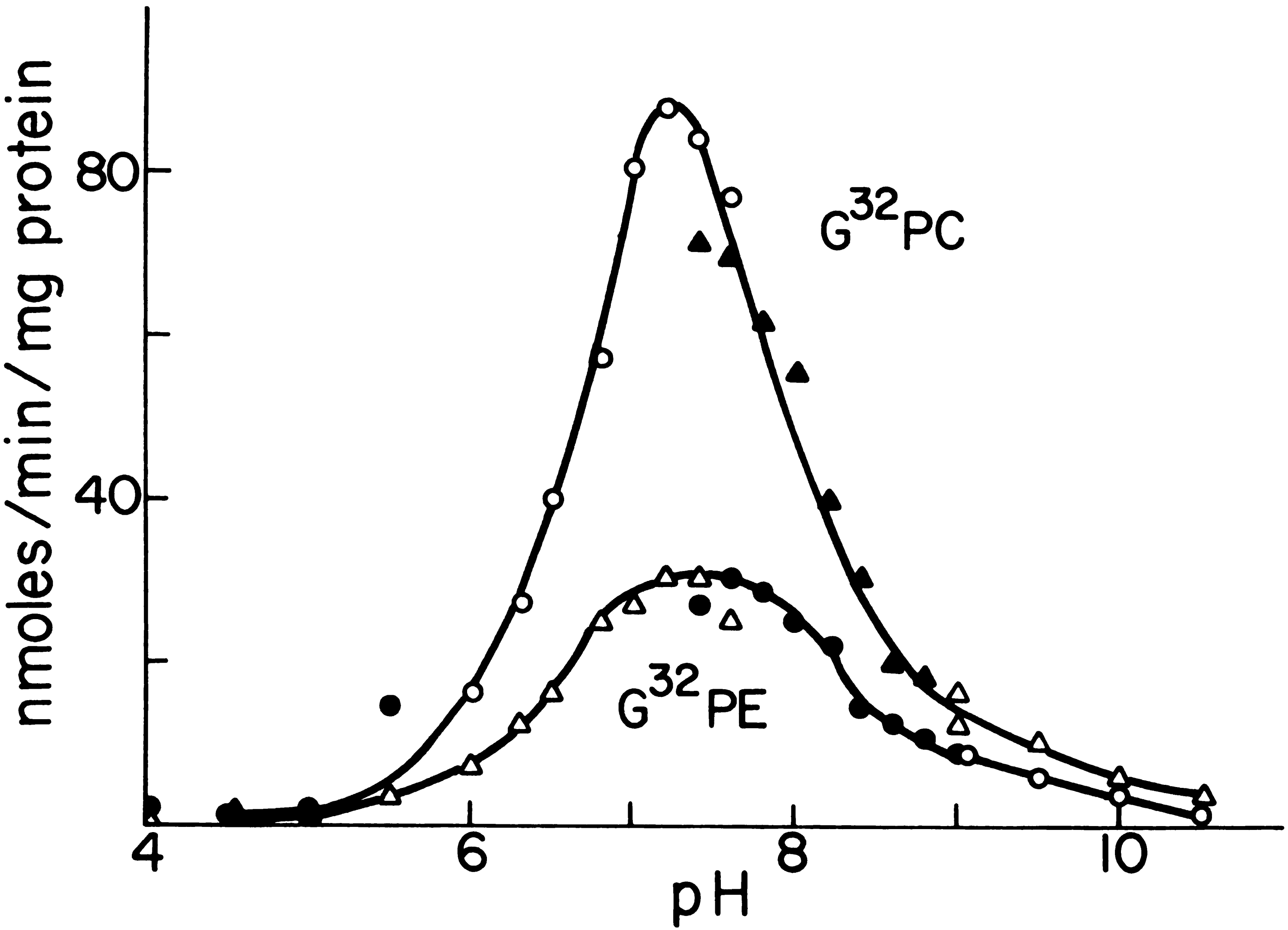

Figure 1

this low activity varied from enzyme preparation to preparation and was probably due to differences in residual divalent cations. As shown in Table IIA, EDTA inhibits the phosphodiesterase activity. The inhibition by EDTA was usually greater than 95%. Addition of excess $MgCl_2$ overcomes the EDTA inhibition and restores the activity to that of the control (see Table IIB).

Table III shows that the divalent cation requirement is quite specific. The highest activity was obtained with Mg^{++} and Co^{++}. Mn^{++} and Ni^{++} gave approximately 50% as much activity as Mg^{++}. The variation in salt concentration in Table III suggests that the major portion of cation stimulation occurs at concentrations below 0.5 mM. As shown in Figure 2, submillimolar Mg^{++} concentrations give a definite stimulation. The activity leveled off above 4 mM with $G^{32}PE$. The optimum Mg^{++} concentration for $G^{32}PC$ cleavage was approximately 10 mM.

Quantitation and Identification of Glycerophosphodiesterase Reaction Products: When either GPS, GPE, GPI, GPC, or GPG were used as substrate, the only phosphomonoester detected was ℓ-α-glycerophosphate. Phosphoryl monoesters of serine, ethanolamine, choline, and inositol were not detected using column and paper chromatographic techniques. For all of the above-mentioned substrates, the amounts of ℓ-α-glycerophosphate determined enzymatically were very similar to the values obtained using the

TABLE III

Effects of divalent cations on glycerophosphodiesterase activity

	Substrate	$G^{32}PE$			$G^{32}PC$
Salt	mM of the salt	0.5	2.5	10	10
	nmoles α-glycerophosphate/min/mg protein				
None		3.6			4.8
$MgCl_2$		50	52	58	80
$MgSO_4$		54	51	61	97
$Mg(NO_3)_2$		41	51	52	84
$CoCl_2$		55	57	48	94
$MnSO_4$		29	31	30	30
$NiCl_2$		18	22	29	55
$Zn(C_2H_3O_2)_2$		3.5	0.7	0	0
$Ca(NO_3)_2$		3.9	0	0	1.4
$CdCl_2$		3.7	1.9	0.3	1.9
$CuCl_2$		0	0.8	0.3	0

The incubation mixture contained the indicated salt, and 2.8 mM $G^{32}PC$ or $G^{32}PE$, 40 mM imidazole buffer, pH 7.2, and 0.17 mg of protein. Samples were incubated at 30° for 30 min and the assays were performed as described in Methods.

$G^{32}PE$ = glycero-^{32}P-phosphorylethanolamine; $G^{32}PC$ = glycero^{32}P-phosphorylcholine.

Figure 2

Effect of Mg^{++} Concentration on the Enzymatic Hydrolysis of GPE and GPC. The incubation mixtures contained $MgCl_2$ as indicated as well as 40 mM imidazole, pH 7.2, 0.17 mg protein, and $G^{32}PE$ or $G^{32}PC$ in a final volume of 0.5 ml. $G^{32}PE$ (2.8 mM) was incubated at 30° for 30 min and $G^{32}PC$ (3.0 mM) was incubated at 30° for 15 min. The assays were performed as described in the Methods.

$G^{32}PE$ is glycero-^{32}P-phosphorylethanolamine; $G^{32}PC$ is glycero-^{32}P-phosphorylcholine.

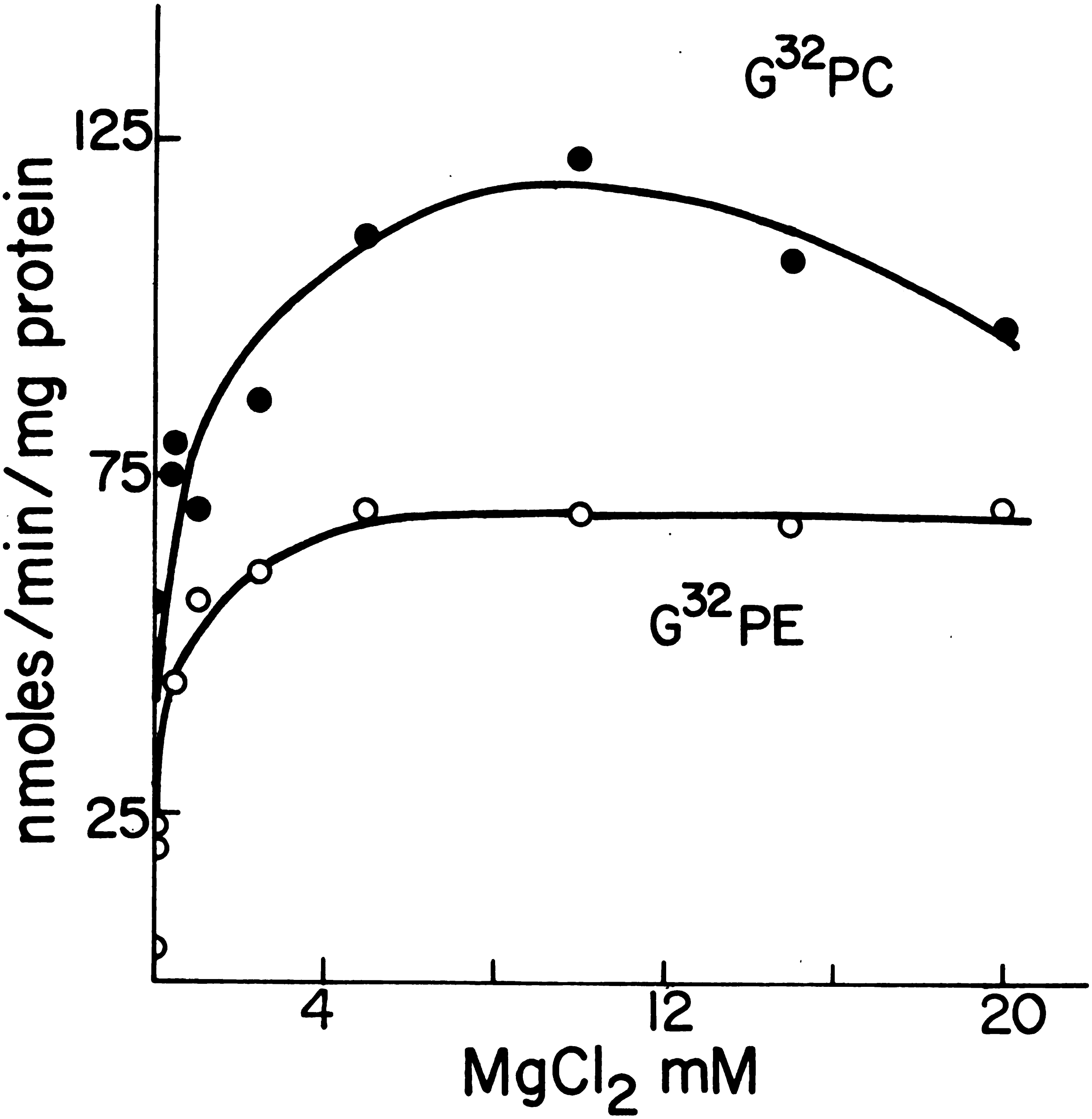

Figure 2

column assay procedure; compare the nmoles of a ℓ-α-glycerophosphate to the nmoles of ^{32}P-labeled product in experiments 3, 4, 5, 8, and 9 of Table IV. The molar ratio of ℓ-α-glycerophosphate determined enzymatically to the glycerophosphate determined by the column assay was between 0.89 to 0.95.

When GPS was the substrate, the amount of serine liberated was similar to the amount of ℓ-α-glycerophosphate released. Compare 238 nmoles serine to 260 nmoles ℓ-α-glycerophosphate in experiment 1 and 340 nmoles serine to 388 nmoles ℓ-α-glycerophosphate in experiment 2 of Table IV.

Quantitative analysis of inositol hydrolyzed from G^{32}PI compared very closely to the amount of ^{32}P product formed; i.e., in experiment 6 of Table IV, 350 nmoles ^{32}P-labeled α-glycerophosphate and 356 nmoles inositol were released. In addition, experiments 6 and 7 show that the values are quite reproducible for identical incubations. Compare 350 to 333 nmoles ^{32}P product and 356 to 320 nmoles inositol.

Experiment 9, Table IV, shows that G^{32}PG is, within experimental error, completely hydrolyzed to the ℓ-α-glycerophosphate, indicating that the d isomer is not formed. The control contained less than 2% as much ℓ-α-glycerophosphate as the experimental product fraction. No attempt was made to assay free glycerol released from G^{32}PG.

TABLE IV

Identification and quantitation of products from hydrolysis of glycerophosphodiesters by larval phosphodiesterase

Exp. No.	Substrate	ℓ-α-GP*	^{32}P-labeled product	X-OH	V
		nmoles	nmoles	nmoles	nmoles ℓ-α-GP*/ min/mg protein
1	GPS (5.5 mM)	260		238	38
2	GPS (11 mM)	340		388	49
3	G^{32}PS (8.0 mM)	440	464		7.3[a]
4	G^{32}PE (5.0 mM)	910	1020		17[a]
5	G^{32}PC (4.2 mM)	800	900		80
6	G^{32}PI (1.7 mM)		350	356	41[b]
7	G^{32}PI (1.7 mM)		333	320	40[b]
8	G^{32}PI (8.0 mM)	955	1020		15[a]
9	G^{32}PG (8.0 mM)	800	890		8.9[a]

[a] Low specific activity enzyme preparation was used.

[b] v = nmoles ^{32}P product/min/mg protein.

X-OH = serine (experiments 1 and 2) or inositol (experiments 6 and 7).

* ℓ-α-GP = ℓ-α-glycerophosphate.

All incubations were done at 30° with a final volume of 0.5 ml. The buffer was imidazole, pH 7.2. Composition of mixtures and incubation times were as follows: 1 and 2, 40 mM buffer, 2 mM MgCl$_2$, 0.23 mg protein, and 30 min; 3, 60 mM buffer, 4 mM MgCl$_2$, 1 mg protein, and 63 min; 4, 40 mM buffer, 4 mM MgCl$_2$, 1 mg protein, and 60 min; 5, 40 mM buffer, 2 mM MgCl$_2$, 0.5 mg protein, and 20 min; 6 and 7, 40 mM buffer, 2 mM MgCl$_2$, 0.14 mg protein, and 60 min; 8, 50 mM buffer, 4 mM MgCl$_2$, 1 mg protein, and 63 min; 9, 50 mM buffer, 4 mM MgCl$_2$, 1 mg protein, and 90 min. ℓ-α-glycerophosphate was assayed enzymatically, and the ^{32}P product was determined by the column fractionation assay. X-OH was assayed by amino acid analyzer for serine (experiments 1 and 2) and by gas chromatography for inositol (experiments 6 and 7). See Methods for details of the assays. The values in parentheses in the substrate column indicate the concentration of substrate used.

Glycerophosphoryldiesterase Substrate Affinities and V_{max}'s: The affinity of the enzyme(s) for two of the substrates is indicated by the data in Figure 3. The K_m for $G^{32}PC$ is an order of magnitude lower than that for GPI. The apparent K_m for GPC was 2×10^{-4} M and for GPI was 2×10^{-3} M. From data not shown, values for GPE and glycerophosphoryl-β-methylcholine were similar to the K_m for GPC; the variation was between 2 and 4×10^{-4} M. Hydrolysis of glycerophosphoryl-β-methylcholine by a 40,000 x g supernatant fraction from housefly larvae has been previously reported by this laboratory (3). Complete characterization of the products has not been done, but kinetically, glycerophosphoryl-β-methlycholine behaves as a competitive inhibitor of $G^{32}PC$ hydrolysis with a K_i of 7×10^{-4} M. Complete data is not available for GPG or GPS K_m's, but they appear to be on the order of GPI rather than GPC. Experiments 1 and 2 of Table IV indicate that the K_m for GPS is at least as large as the K_m for GPI.

Values for V_{max} with various enzyme and substrate preparations varied, but the V_{max} for GPC was consistently 70% higher than that for GPI (see Figure 3). With the enzyme preparation used for most of these studies, the V_{max} (nmoles/min/mg protein) for GPC was near 90 and for GPI was 50-55. GPE, GPS, GPG, and glycerophosphoryl-β-methylcholine all had apparent V_{max} values of 50 ± 5.

Figure 3

Determination of K_m Values for GPC and GPI. Samples containing $G^{32}PC$ contained 2 mM $MgCl_2$, 40 mM imidazole, pH 7.2, and 0.084 mg protein in 0.5 ml. The samples were incubated at 30° for 10 min. Mixtures containing GPI contained 4 mM $MgCl_2$, 40 mM imidazole, pH 7.2, and 0.23 mg protein/ml in a final volume of 1 or 3 ml. The incubations were done at 30° for 15 or 30 min. Hydrolysis of GPI was quantitated by measuring the inositol released. Inositol was quantitated using gas chromatography as described in the Methods. $G^{32}PC$ was assayed as described in the Methods.

$G^{32}PC$ is glycero-^{32}P-phosphorylcholine; GPI is glycerophosphorylinositol.

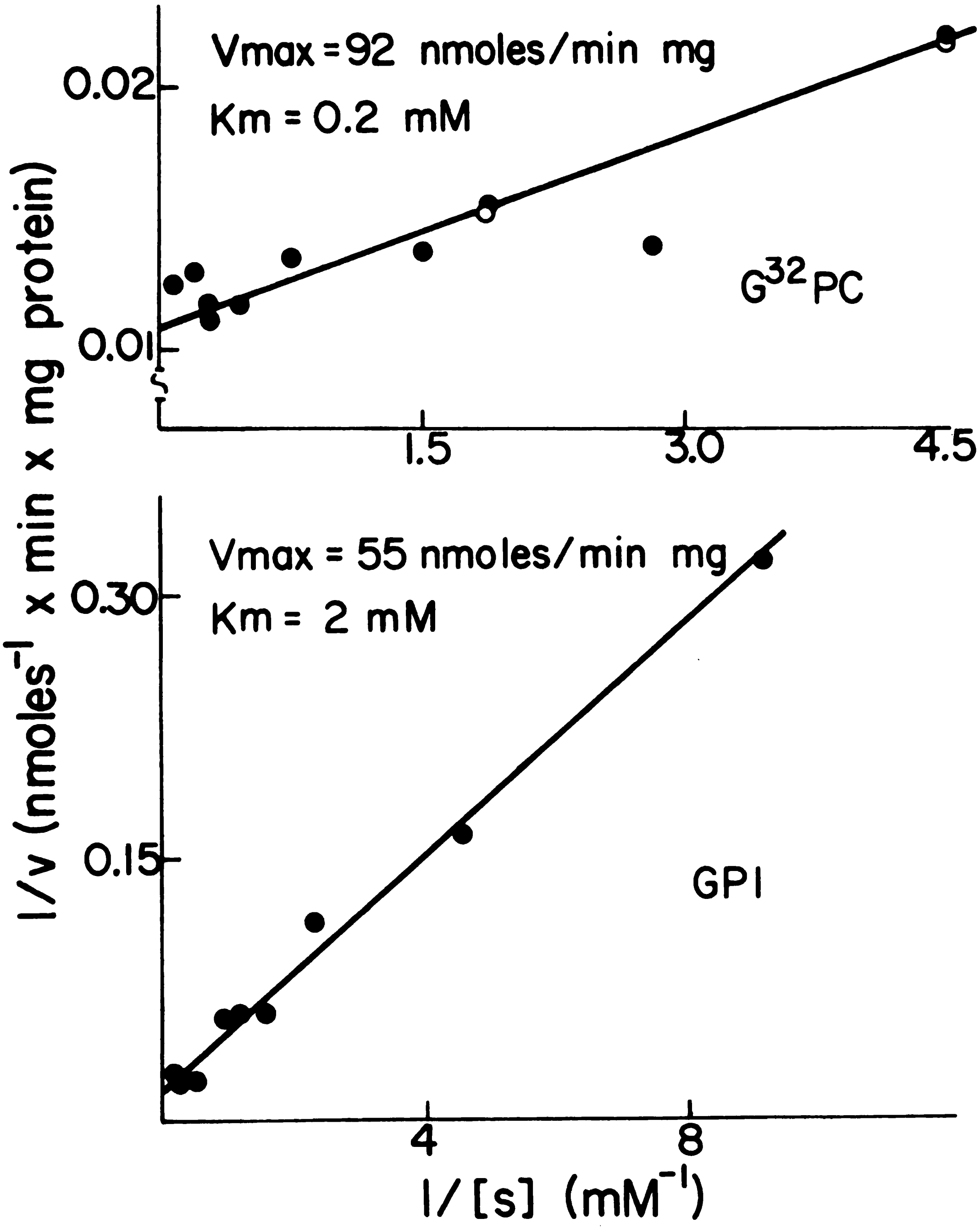
Vmax=92 nmoles/min mg
Km = 0.2 mM
G^{32}PC
0.02
0.01
1.5
3.0
4.5
Vmax = 55 nmoles/min mg
Km = 2 mM
GPI
0.30
0.15
4
8
1/v (nmoles^{-1} x min x mg protein)
1/[s] (mM^{-1})

Figure 3

Effects of Potential Inhibitors and Reaction Products on Glycerophosphodiesterase Activity: The phosphodiesterase activity was not significantly affected by the reaction products, choline, ethanolamine, inositol, and glycerophosphate at concentrations near that of the substrates (see Table V). It should be noted that the hydrolysis in the control is about 10% below values obtained in other experiments. This may partially account for the apparent stimulation at 1 mM by most of the compounds tested. In data not shown, 25 mM concentrations were also used. These data are in close agreement with the 5 mM values except for choline and ethanolamine which give 75 and 20% of the control rate, respectively.

The data in Table VI indicate that the phosphodiesterase activity is not dependent on catalytic sulfhydryl groups since N-ethylmaleimide, iodoacetamide, and p-chloromercuribenzoate were not inhibitory. The failure of trypsin (Nutritional Biochemical Co.) and pronase (Cal Biochem) to inhibit the activity after 50 min of incubation (Table VI) or even after 80 min (not shown) indicates that the phosphodiesterase enzyme(s) is not highly sensitive to these types of protease activity.

Mutual Inhibition Between Pairs of Glycerophosphodiester Substrates: To obtain some indication as to whether or not a single or multiple enzymes are involved

TABLE V

Effects of potential inhibitors and reaction products on the glycerophosphodiesterase activity

Compound added	1 mM compound nmoles ℓ-α-GP/ min/mg protein	1 mM compound %	5 mM compound nmoles ℓ-α-GP/ min/mg protein	5 mM compound %
None	72	100	72	100
dℓ-α-GP	80	111	60	83
β-GP	76	106	67	93
Choline	81	113	72	100
Ethanolamine	83	115	67	93
Inositol	82	114	88	122
Phosphorylcholine	83	115	76	106

Incubation mixtures containing 3.1 mM glycero-^{32}P-phosphorylcholine, 4 mM $MgCl_2$, 40 mM imidazole, pH 7.2, and 0.17 mg of protein in 0.5 ml final volume were incubated at 30° for 30 min. See Methods for assay procedures.

GP = glycerophosphate.

TABLE VI

Effects of potential enzyme inhibitors on glycerophosphodiesterase activity

Inhibitor	nmoles ℓ-α-glycerophosphate/min/mg protein
None	21.3
NEM[a], 2.0 mM	20.3
IAA[b], 2.0 mM	22.7
PCMB[c], 0.04 mM	27.6
Trypsin, 0.025 mg	20.9
Pronase, 0.025 mg	22.9

[a] NEM, N-ethylmaleimide.

[b] IAA, iodoacetamide.

[c] PCMB, p-chloromercuribenzoate.

Incubation mixtures containing inhibitor, 4 mM $MgCl_2$, 50 mM imidazole, pH 7.2, and 1 mg of protein of low specific activity in 0.5 ml final volume were preincubated at 30° for 10 min (20 min with pronase and trypsin). 5 mM glycero-^{32}P-phosphorylcholine was added and incubation was continued at 30° for 30 min. See Methods for assay procedure.

in hydrolysis of the various substrates, experiments involving incubation of pairs of substrates separately and in combination were performed. Figure 4 shows that the hydrolysis of substrate pairs was not significantly greater than the most active substrate incubated alone. For each substrate pair, both substrates were hydrolyzed and the rates were less than the rates of hydrolysis for the individual substrates. For example, as shown in Figure 4A, $G^{32}PE$ hydrolysis in the presence of GPI was about half the control rate. This and the fact that the GPI hydrolysis was inhibited by one-third suggests that $G^{32}PE$ competes slightly less favorably than GPI for hydrolysis when coincubated. Figure 4D indicates that GPI may be a slightly better competitor than the $G^{32}PS$ and is hydrolyzed at a higher rate than $G^{32}PS$. In evaluating Figure 4B and C, it is noted that the GPC preparation used in B gave rates about 35% below normal (e.g., $G^{32}PC$ V_{max}, Figure 3). Even considering this limitation for GPC hydrolysis, it is apparent from Figure 4B and C that GPC competes much more favorably for hydrolysis than does GPI or GPE. All data obtained are consistent with a single enzyme hydrolyzing all of the substrates.

Reversal of the Glycerophosphodiesterase Reaction: Attempts to demonstrate formation $G^{32}PC$ from ^{32}P-labeled ℓ-α-glycerophosphate and choline were unsuccessful (experiments I and II of Table VII), even at very high (0.1 M)

Figure 4

Mutual Inhibition of Enzymatic Hydrolysis by Pairs of Glycerophosphodiesters. All incubation mixtures contained 4 mM $MgCl_2$ and 65 mM imidazole, pH 7.2, in addition to enzyme and substrate in a final volume of 0.5 ml. Incubation temperature was 30°. The substrate concentrations were: for Experiment A, $G^{32}PE$, 4.5 mM and GPI, 10 mM; for Experiment B, $G^{32}PE$, 5 mM and GPC, 5 mM; for Experiment C, $G^{32}PC$, 5 mM, and GPI, 10 mM; for Experiment D, $G^{32}PS$, 9.6 mM, and GPI, 10 mM. Hydrolysis of the labeled substrates was measured by counting the anion exchange column fractions and hydrolysis of unlabeled substrate and combined substrates was measured using total phosphate assay of the substrate and product fractions. The open bar in the column for both substrates indicates total ℓ-α-glycerophosphate produced.

$G^{32}PE$ is glycero-^{32}P-phosphorylethanolamine; GPI is glycerophosphorylinositol; GPC is glycerophosphorylcholine; $G^{32}PC$ is glycero-^{32}P-phosphorylcholine; $G^{32}PS$ is glycero-^{32}P-phosphorylserine.

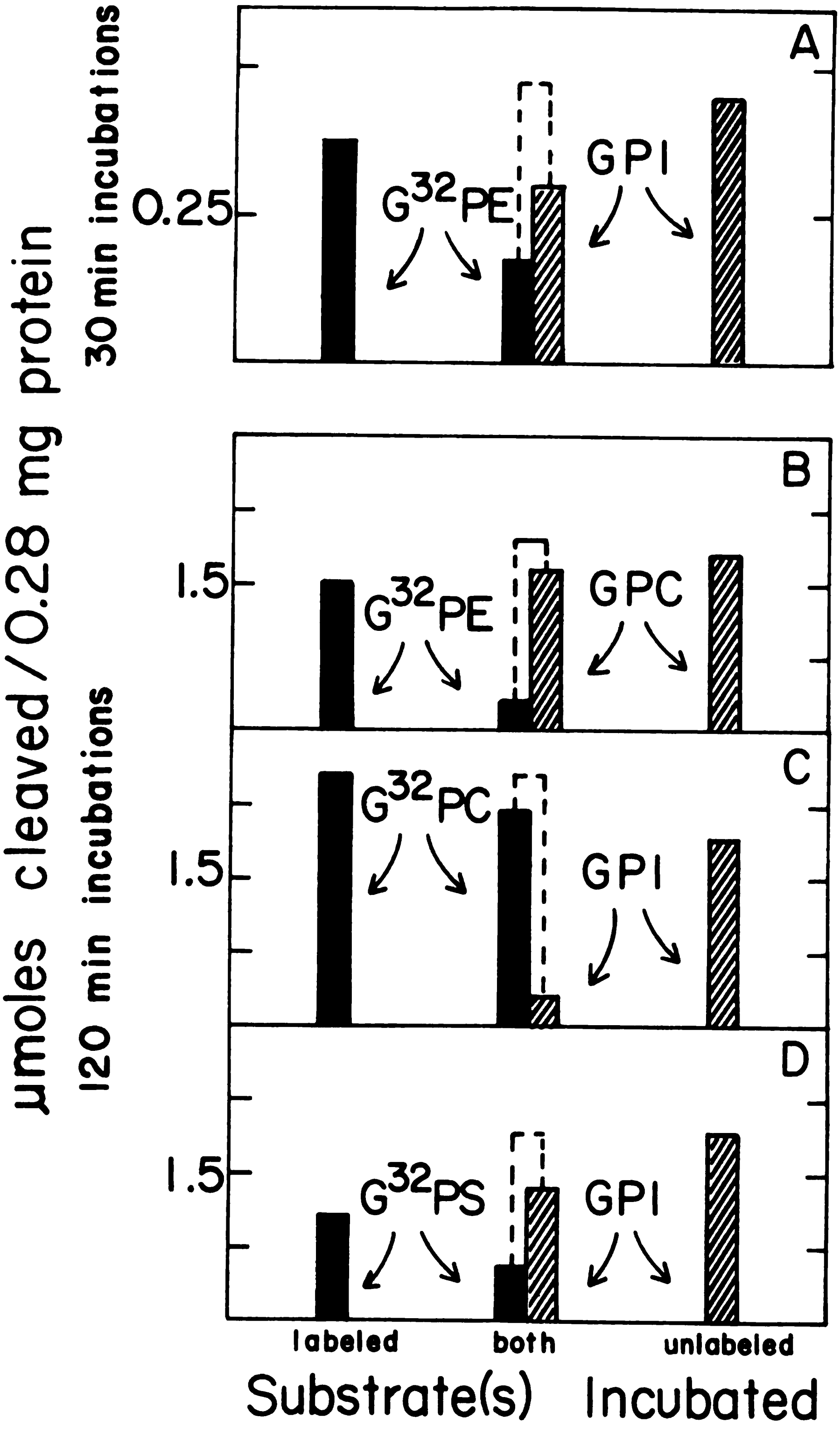
µmoles cleaved/0.28 mg protein
30 min incubations
120 min incubations
A
0.25
G^{32}PE
GPI
B
1.5
G^{32}PE
GPC
C
1.5
G^{32}PC
GPI
D
1.5
G^{32}PS
GPI
labeled
both
unlabeled
Substrate(s) Incubated

Figure 4

TABLE VII

Reversal of enzymatic hydrolysis of GPC and exchange of Me-^{14}C-choline or ℓ-α-glycerol-^{32}P into GPC and GPI

Exp.	Radioactive compound used	Other Reactants	Incubation time	Radioactivity recovered in GPC	
			min.	cpm	% x 10^2
I.	ℓ-α-glycerol-^{32}P (43 x 10^6 cpm, 100 mM)	GPC 100 mM	90	2.7 x 10^4[a]	6.3
				1.9 x 10^4	4.4
		Choline[b] 100 mM	90	3.0 x 10^4[a]	7.0
				1.4 x 10^4	3.3
II.	ℓ-α-glycerol-^{32}P(7.5 x 10^6 cpm, 15 mM)	Choline[c] 10 mM	90	75[a]	0.10
				100	0.13
III.	Me^{14}C-choline (1.2 x 10^6 cpm, 10 mM)	GPC 10 mM	60	100[a]	0.83
				2060	17.0
		GPI 10 mM	90	225[a]	1.9
			30	630	5.3
			60	1020	8.5
			90	1170	9.7

[a] Boiled enzyme control.

[b] 50 μmoles of carrier GPC were added after stopping the reaction.

[c] 5 μmoles of carrier GPC were added after stopping the reaction.

All reaction mixtures contained, in addition to components indicated, 4 mM $MgCl_2$, 0.5 mg of low specific activity protein, and 65 mM imidazole, pH 7.2 (reactants were adjusted to pH 7.0) in a final volume of 0.5 ml. Assay for ^{32}P-labeling of GPC was by anion exchange columns of a sufficient capacity to separate the larger than usual quantities of anionic material. The incorporation of Me^{14}C-choline into GPC was determined by cation exchange separation of GPC and choline on Dowex 50 H^+ columns. GPC = glycerophosphorylcholine; GPI = glycerophosphorylinisitol.

concentrations of ℓ-α-glycerophosphate and choline. Exchange of ^{32}P-labeled ℓ-α-glycerophosphate into GPC was also not detected as shown by the data given in experiment I of Table VII. The incubations employing 0.1 M reactants were adjusted to pH 7.0 with NaOH and the choline was made up in imidazole buffer, pH 7.2. Thus, the effective buffering capacity was higher than indicated in the legend of Table VII.

When methyl-^{14}C-choline was incubated with either GPC or GPI, a significant percentage (on the order of 0.1%) of the total activity was recovered as GP^{14}C. In the exchange of methyl-^{14}C-choline into GPI, the choline incorporation appeared to be approaching a steady level after 90 min of incubation. This suggests that the rate of exchange of choline for inositol is being approached by the rate of hydrolysis of the GP^{14}C produced. Total GP^{14}C concentration approached 0.01 mM after 90 min of incubation. For the experiment involving exchange of methyl-^{14}C-choline into GPC, after 60 min, 1/600 (0.17%) of the label appeared in GPC which is 20 times the amount of ^{14}C in the blank. Approximately 20% of the initial GPC was hydrolyzed during the incubation. Thus, 40% of the total choline in the mixture was present in the GPC fraction at the end of the experiment. The data strongly indicate that choline was exchanged into GPC and GPI.

Discussion

Subcellular Distribution: Our results show that housefly larvae contain glycerophosphodiesterase activity which is soluble in a 88,000 x g supernatant fraction. The particulate fractions, including microsomes, contain little, if any, of the phosphodiesterase activity. This is in contrast to the report by Baldwin and Cornatzer (5) that rat kidney glycerophosphodiesterase is microsome-bound.

Divalent Cation Effects: The glycerophosphodiesterase is greatly stimulated by the divalent cation Mg^{++} and is also stimulated by Ni^{++}, Co^{++}, and Mn^{++}. The optimum Mg^{++} concentration is 4 mM for GPE and 4-10 mM for GPC (Figure 2). EDTA can completely inhibit the activity and the inhibition by EDTA can be overcome by excess Mg^{++}. This is in contrast to the enzyme from Serratia plymuthicum (16) which is inhibited by mM concentrations of divalent cations and shows an EDTA inhibition that is irreversible with metal ions. EDTA also inhibits the rat kidney glycerophosphodiesterase (5), but this EDTA effect is reversed with equimolar Zn^{++} (17) and partially inhibited by higher Zn^{++} concentrations. Like rat kidney microsomes rat brain homogenates (18) show no loss of activity on dialysis unless EDTA is added. This inhibition is reversed by several metal ions in both tissues. Rat liver

GPC diesterase (19) is stimulated slightly by Mg^{++} after dialysis but is inhibited at concentrations above 3 mM. The rat liver diesterase is also inhibited by Zn^{++} and Ca^{++}. The larval enzyme appears to be quite different from all the rat tissue glycerophosphodiesterases and the _S. plymuthicum_ enzyme. It is nearly inactive, even in undialyzed preparations, unless divalent cations are added. The specificity for Mg^{++} (Table III) and the failure of Mg^{++} to inhibit at 10 mM concentration are in sharp contrast to the bacterial and rat enzymes.

pH Optima and Heat Stability: The larval enzyme is 80% stable to heating 50° for 15 min. This is very similar to the results reported for rat kidney (5) and _S. plymuthicum_ (16). pH optima for the bacterial, rat kidney, and rat brain (18) enzyme are about pH 9. Larval activity is optimal at pH 7.2 which is similar to the rat liver preparation (pH 7.5) of Dawson (19).

Substrate Specificity: Housefly larval glycerophosphodiesterase is active on _sn_-glycero-3-phosphorylethanolamine, -choline, -serine, -inositol, -1'_sn_-glycerol, and -β-methylcholine. A similar spectrum of substrates has been reported (6) for the _S. plymuthicum_ enzyme. The products of enzyme hydrolysis are without exception ℓ-α-glycerophosphate (_sn_-glycerol-3-phosphate) and free alcohol moiety. Our results for the hydrolysis of _sn_-glycerol-3-phosphoryl-1'_sn_-glycerol (Prepared by deacylating housefly larval phosphatidylglycerol) show the product to be all _sn_-glycerol-3-phosphate and not _sn_-glycerol-1-phosphate. This agrees with the results for _S. plymuthicum_ (6).

_K_m and V_{max}:_ Housefly larval glycerophosphodiesterase has an apparent K_m of 2.0×10^{-4} M for GPC and 2×10^{-3} M for GPI. GPE and glycerophosphoryl-β-methylcholine have K_m's in the range of $2 - 4 \times 10^{-4}$ M. The limited data available suggests that GPG and GPS have K_m's on the order of 10^{-3} M rather than 10^{-4} M. The reported values for GPC and GPE K_m's with _S. plymuthicum_ enzyme (16) are 2.5×10^{-3} and 1.2×10^{-3} M, respectively. Baldwin and Cornatzer (5) determined the rat kidney K_m's to be 2.2×10^{-3} M for GPC and 11.5×10^{-3} M for GPE.

V_{max} values for the larval enzyme preparation used for most of this work show that GPC is hydrolyzed (92 nmoles/min/mg protein) about 80% faster than any of the other substrates studied (GPG, GPE, GPI, GPS, and glycerophosphoryl-β-methylcholine in the range 50± 5 nmoles/min/mg protein). GPC is cleaved about 2.5 times as fast as GPE by the rat kidney enzyme (5) and about 30% faster by the _S. plymuthicum_ enzyme (16).

Other: Sulfhydryl groups apparently are not required for activity since N-ethylmaleimide, p-chloromercuribenzoate, and iodoacetamide are not inhibitory. Similar findings have been reported for the mammalian and bacterial enzymes.

The larval enzyme shows no significant inhibition of GPC hydrolysis by the reaction products at 1 or 5 mM concentrations and only slight (25%) inhibition by 25 mM choline. This is in contrast to the rat kidney enzyme (5) which was inhibited up to 60% by choline at 1-10 mM concentrations. This product inhibition and the apparent substrate specificity for GPC and GPE suggest that the rat kidney enzyme differs from the larval enzyme and probably the bacterial enzyme in that the larval enzyme is not as specific. It does not readily distinguish ethanolamine and choline from serine, inositol, and glycerol.

Our results indicate, but do not prove, that all GP-X compounds are cleaved by one larval enzyme. When pairs of substrates were co-incubated, both were hydrolyzed but at rates below noncompetitive values (Figure 4). GPI competes slightly more favorably for hydrolysis (i.e., it is less inhibited relative to noncompetitive rates) when co-incubated with GPE or GPS. GPC is a better competitor for hydrolysis than GPE or GPI. GPC cleavage is inhibited only 10-15% in the presence of GPI or GPS, but GPC inhibits hydrolysis of GPI and GPS 80-85%. Total cleavage of any pair of substrates does not exceed significantly the cleavage of the single most active substrate in the pair. The bacterial enzyme (16) and the rat liver enzyme (19) exhibit competitive kinetics for hydrolysis of GPC in the presence of GPE. In results not published, the larval enzyme deviates from competitive kinetics when GPE is used as the inhibitor of GPC hydrolysis but glycerophosphoryl-β-methylcholine is a competitive inhibitor of GPC.

Hydrolysis of GPC by the larval glycerophosphodiesterase is virtually irreversible; however, ^{14}C-methyl-labeled choline did exchange slowly into GPC and GPI. These observations suggest that the ℓ-α-glycerophosphate is released from the enzyme slower than the alcohol moiety. Release of ℓ-α-glycerophosphate is nearly irreversible since ^{32}P-labeled ℓ-α-glycerophosphate was not exchanged into GPC. The exchange of choline into GPI and GPC indicates, but does not prove, that some type of enzyme-phosphoryl-glycerol intermediate is formed during the enzymatic conversion of glycerophosphodiesters to ℓ-α-glycerophosphate and XOH. A plausible reaction sequence that is consistent with our limited data is:

1. GPX + enzyme ⇄ glycerophosphorylenzyme + XOH
2. Glycerophosphoryl-enzyme → ℓ-α-glycerophosphate

$$\begin{array}{ccc} \text{Glycerophosphoryl-enzyme} & \rightarrow & \ell\text{-}\alpha\text{-glycerophosphate} \\ + & & + \\ \text{HOH} & & \text{enzyme} \end{array}$$

The exchange of free choline into GPC and GPI, the only phosphodiesters tested, is strong evidence that a single enzyme hydrolyzes both GPC and GPI.

From the above discussion, it is apparent that the housefly larval enzyme has several significant differences from both the bacterial and the rat kidney microsomal enzymes. The properties observed for the larval enzyme indicate that it can hydrolyze all the GP-X compounds that are known (2 and 4) to be major catabolic products of glycerophosphatides in housefly larvae. The amount of activity, about 200 nmoles/min/g of larvae, would be sufficient to turn over the 7 μmoles of lipid phosphate/g of larvae[4] in less than an hour. Insects in the mature larval stage that are entering the pupal stage undergo extensive breakdown of phospholipids and changes in phospholipid composition (20). It is likely that glycerophosphodiesterase activity is essential during this phase of metamorphosis when the organism, operating as a nearly closed system, extensively hydrolyzes its cellular constituents and reassembles the components into the tissues of the adult organism. The apparent insensitivity of the enzyme to protease may be important during the autolysis of pupation.

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[4] L. L. Bieber, unpublished observation.

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