Characterization of glycerophosphorylcholine, -ethanolamine, -serine, -inositol, and -glycerol hydrolytic activity in housefly larvae

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Abstract Homogenates of *Musca domestica* (housefly) larvae contain glycerophosphodiesterase activity, which is found in the supernatant fluid after centrifugation at 88,000 *g*. The phosphodiesterase is inhibited by EDTA and is stimulated by Mg^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+}. The pH optimum is 7.2. The enzyme is stable to heating at 50°C for 15 min and is insensitive to sulfhydryl inhibitors. Glycerophosphoryl diesters 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 × 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.

Supplementary key words glycerophosphodiesterase

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 of 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 enthanolamine, 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 *g* supernatant fraction from housefly larvae to yield glycerophosphate 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, *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 enzyme(s) is 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 vol of 0.025 M Tris, pH 8.0, for 1 min in a Waring Blendor. The homogenate was filtered through eight layers of

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

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cheesecloth and centrifuged at 40,000 *g* for 20 min using a Sorvall SS-34 rotor. The 40,000 *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°C. All of the above steps were done at 0–4°C. Assays were carried out using enzyme freshly dissolved in distilled water. 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 water.

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 $^{32}P_i$ 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 of GPI from a commercial preparation enriched in phosphatidylinositol.

Ion exchange separations

Glycerophosphoryl diesters were separated from the phosphorus-containing hydrolysis products on columns of Dowex 1-X8, 100–200 mesh, formate form. Unless otherwise indicated, columns measuring 6 × 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 P_i that was released during the incubation. Fraction A was collected during application of a 2.5-ml aqueous load, a 1.5-ml water rinse, and a 5-ml elution with 0.1 M ammonium formate. Fraction B was collected immediately after 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 water after loading the incubation mixture at pH 7. Inositol from GPI incubations was collected in the same manner as serine, and it was then chromatographed on Dowex 50-X8, H^+ form. The eluate was lyophilized and used for gas–liquid chromatographic analysis. When total phosphorus assays were performed on the substrate and product fractions, or when 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 water prior to loading onto the columns. When GPC and choline in [*Me*-^{14}C]choline labeling experiments were separated, the anion exchange columns were washed with water as for serine and inositol, and this fraction was loaded onto Dowex 50, H^+ form, columns. GPC was eluted using several volumes of water, and choline was recovered by elution with several column volumes of 4 N HCl.

Assay procedures

All incubations were carried out 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 4 min in a boiling water bath. The reaction volume (0.5 ml) was diluted with 2 ml of water and then thoroughly mixed (Vortex mixer) prior to loading onto anion exchange columns. When L-α-glycerophosphate was assayed directly (Table 4, experiments 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 was put on 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 percentage of total radioactivity recovered from 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 percentage distribution of radioactivity from anion exchange columns was ± 0.2%. In all results reported, a minimum of 2000 cpm of ^{32}P was incubated and recovered from the column fractionation. The assays routinely yielded 10–40% (above control values) of total radioactivity as product (fraction B). The effect of enzyme concentration on L-α-glycerophosphate production was determined using 0–0.7 mg of protein/ml. The assay was linear with protein to at least 0.7 mg/ml.

^{32}P-Cerenkov radiation was quantitated in a Packard Tri-Carb 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 were counted. The scintillator solution contained 4 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-[2-(phenyloxazolyl)]-benzene (POPOP) per liter of toluene. GPC samples containing [^{14}C]methyl-labeled choline were lyophilized and redissolved in a total volume of 0.5 ml. Aliquots from this solution were removed for counting.

Inositol enzymatically hydrolyzed from GPI was quantitated by gas–liquid chromatography (9) of the trimethylsilyl derivative. Aliquots of an internal standard, α-methylmannoside, were added to incubation mixtures prior to ion exchange purification, lyophliization, and derivatization. Ratios of inositol to phosphate in GPI preparations were determined to be 1 ± 0.1. Free inositol in 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. The ratio of phosphate to serine in a GPS acid hydrolysate 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 was more than 90% pure, and routine ion exchange purification of its deacylation product removed 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 after reduction of NAD^+ by L-α-glycerophosphate dehydrogenase (Sigma). The procedure for this assay was essentially that described for glycerol analysis (10), with the omission of glycerol kinase, Mg^{2+}, and ATP. Protein was determined according to Lowry et al. (11). Total phosphorus was determined as described by Bartlett (12).

RESULTS

Distribution, purification, and stability of the glycerophosphodiesterase

Homogenization of housefly larvae in 0.025 M Tris, pH 8, and subsequent fractionation by differential centrifugation yielded fractions containing nuclei and mitochondria (250–9000 *g*), lysosomes and microsomes (9000–40,000 *g*), microsomes (40,000–88,000 *g*), and soluble components (88,000 *g* supernate). No significant glycerophosphorylcholine phosphodiesterase activity was found in any of the particulate fractions. All of the activity detectable in the 250 *g* supernatant fluid (Table 1) was recovered in the 40,000 *g* supernatant fraction. The greater activity in the 250 *g* supernatant fraction than in the homogenate was not always observed. More than 70% of the activity could be recovered in an 88,000 *g* supernatant fluid, but the limited purification obtained did not warrant this high-speed centrifugation.

TABLE 1. Isolation of glycerophosphodiesterase from housefly larvae

Fraction	Total Protein	Specific Activity	Activity Recovered	Purification
	mg	*nmoles/min/mg protein*	%	
Homogenate	536	4.4	100	1.0
250 *g* supernate	345	7.8	115	1.8
40,000 *g* supernate	226	12.0	115	2.7
35–50% $(NH_4)_2SO_4$ precipitate	72	35.0	106	8.0

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

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

In order to determine whether the phosphodiesterase activity occurred only in housefly larvae entering the

TABLE 2. Effects of heating and EDTA on glycerophosphodiesterase activity

Enzyme Experiment	Additions	α-Glycerophosphate Formed
		nmoles
A.		
Unheated	15 mM $MgCl_2$	330
Unheated	2.5 mM EDTA	10
Heated	15 mM $MgCl_2$	260
B.		
Heated	None	150
Heated	15 mM $MgCl_2$	210
Heated	2.5 mM EDTA	40
Heated	2.5 mM EDTA[a] 15 mM $MgCl_2$	210

All incubation mixtures contained approximately 2 mg of 40,000 *g* supernatant protein unheated or heated for 15 min at 50°C, 0.15 M sucrose, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4, in a final volume of 2 ml. Incubation was at 30°C 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.

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

pupal stage of development or was present in other developmental stages, eggs and 1–2-day-old pupae were homogenized and assayed for phosphodiesterase 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 contaminants were present that could interfere with glycerophosphodiester assays. All of the phosphomonoester released was L-α-glycerophosphate, as determined by L-α-glycerophosphate dehydrogenase assays. Phosphatases active against L-α-glycerophosphate were not detected by paper chromatographic analysis of products of GP-X hydrolysis. The enzyme preparation did not liberate $^{32}P_i$ 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. At pH 7.2, 0.93 nmole of P_i/min/mg of protein was released from *p*-nitrophenylphosphate, and 21.3 nmoles of L-α-glycerophosphate was released from [^{32}P]GPE in the same experiment. 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% of the rate observed with [^{32}P]GPE. This activity was apparently different from the [^{32}P]GPE hydrolytic activity, because cleavage of the synthetic substrate was not inhibited by [^{32}P]GPE, and [^{32}P]GPE 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 within ± 0.5 pH units when [^{32}P]GPC is the substrate, as shown in Fig. 1. With [^{32}P]GPE as substrate, the pH optimum was 7.4, but the curve was nearly flat from pH 7.0 to pH 7.8. The rates of [^{32}P]GPC cleavage above 40 nmoles/min/mg of protein may be slightly low due to the substrate becoming limiting during the incubation period. The maximal rates for [^{32}P]GPE cleavage shown in Fig. 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 [^{32}P]GPS was similar to [^{32}P]GPE.

Divalent cation requirements of the glycerophosphodiesterase

Mg^{2+} stimulates the phosphodiesterase activity, even though the enzyme preparations were not dialyzed or

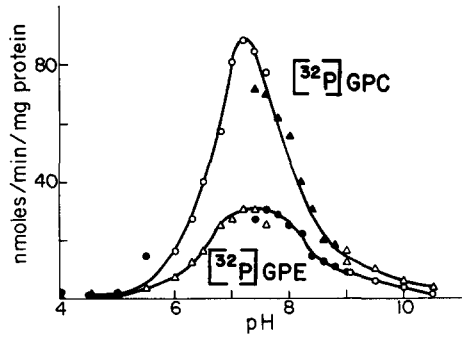

FIG. 1. pH dependence of GPC and GPE hydrolysis by the glycerophosphodiesterase. Incubation mixtures contained 2 mM $MgCl_2$, 40 mM buffer, 1 mM [^{32}P]GPC or [^{32}P]GPE, and 0.14 mg of protein. Incubation was for 20 min at 30°C. Buffers for [^{32}P]GPC were acetate (●), imidazole (○), Tris (▲), and glycine (△). Buffers for [^{32}P]GPE were acetate (▲), imidazole (△), Tris (●), and glycine (○). Assays were carried out as described in Methods. [^{32}P]GPC is glycero[^{32}P]phosphorylcholine; [^{32}P]GPE is glycero[^{32}P]phosphorylethanolamine.

treated with EDTA. Small amounts of glycerophosphodiesterase activity were obtained in the absence of added Mg^{2+}, but this low activity varied from enzyme preparation to preparation and was probably due to differences in residual divalent cations. As shown in Table 2A, 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 2B).

Table 3 shows that the divalent cation requirement is quite specific. The highest activity was obtained with Mg^{2+} and Co^{2+}. Mn^{2+} and Ni^{2+} gave approximately 50% as much activity as Mg^{2+}. The variation in salt concentration in Table 3 suggests that the major portion of

TABLE 3. Effects of divalent cations on glycerophosphodiesterase activity

Substrate:	[^{32}P]GPE			[^{32}P]GPC
mM of the Salt:	0.5	2.5	10	10
	nmoles α-glycerophosphate/min/mg protein			
Salt				
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 [^{32}P]GPC or [^{32}P]GPE, 40 mM imidazole buffer, pH 7.2, and 0.17 mg of protein. Samples were incubated at 30°C for 30 min and the assays were performed as described in Methods. [^{32}P]GPE = glycero[^{32}P]phosphorylethanolamine; [^{32}P]GPC = glycero[^{32}P]-phosphorylcholine.

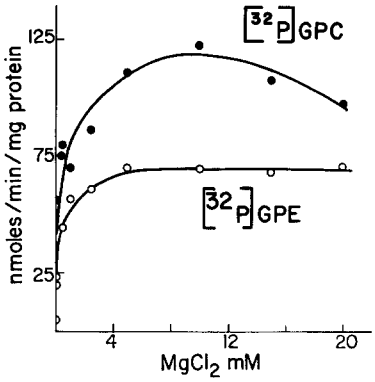

FIG. 2. Effect of Mg^{2+} concentration on the enzymatic hydrolysis of GPE and GPC. The incubation mixtures contained $MgCl_2$, as indicated, as well as 40 mM imidazole, pH 7.2, 0.17 mg of protein, and [^{32}P]GPE or [^{32}P]GPC in a final volume of 0.5 ml. [^{32}P]GPE (2.8 mM) was incubated at 30°C for 30 min, and [^{32}P]-GPC (3.0 mM) was incubated at 30°C for 15 min. The assays were performed as described in Methods. [^{32}P]GPE is glycero[^{32}P]-phosphorylethanolamine; [^{32}P]GPC is glycero[^{32}P]phosphoryl-choline.

cation stimulation occurs at concentrations below 0.5 mM. As shown in Fig. 2, submillimolar concentrations of Mg^{2+} give a definite stimulation. The activity leveled off above 4 mM with [^{32}P]GPE. The optimum Mg^{2+} concentration for [^{32}P]GPC cleavage was approximately 10 mM.

Quantitation and identification of the glycerophosphodiesterase reaction products

When GPS, GPE, GPI, GPC, or GPG was 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 column assay procedure (compare the nmoles of L-α-glycerophosphate with the nmoles of ^{32}P-labeled product in experiments 3, 4, 5, 8, and 9 of Table 4). The molar ratio of L-α-glycerophosphate determined enzymatically to the glycerophosphate determined by the column assay was between 0.89 and 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 of serine with 260 nmoles of L-α-glycerophosphate in experiment 1 and 388 nmoles of serine with 340 nmoles of L-α-glycerophosphate in experiment 2 of Table 4).

Quantitative analysis of inositol hydrolyzed from [^{32}P]GPI compared very closely to the amount of ^{32}P-labeled product formed; i.e., in experiment 6 of Table 4, 350 nmoles of ^{32}P-labeled L-α-glycerophosphate and 356 nmoles of inositol were released. In addition, experiments 6 and 7 show that the values are quite reproducible for identical incubations (compare 350 with 333 nmoles of ^{32}P-labeled product and 356 with 320 nmoles of inositol).

TABLE 4. Identification and quantitation of products from hydrolysis of glycerophosphodiesters by larval phosphodiesterase

Expt. No.	Substrate	L-α-GP[a]	^{32}P-Labeled Product	X-OH[b]	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	[^{32}P]GPX (8.0 mM)	440	464		7.3[c]
4	[^{32}P]GPE (5.0 mM)	910	1020		17[c]
5	[^{32}P]GPC (4.2 mM)	800	900		80
6	[^{32}P]GPI (1.7 mM)		350	356	41[d]
7	[^{32}P]GPI (1.7 mM)		333	320	40[d]
8	[^{32}P]GPI (8.0 mM)	955	1020		15[c]
9	[^{32}P]GPG (8.0 mM)	800	890		8.9[c]

All incubations were done at 30°C with a final volume of 0.5 ml. Imidazole buffer, pH 7.2, was used. Compositions of mixtures and incubation times were as follows: experiments 1 and 2, 40 mM buffer, 2 mM $MgCl_2$, 0.23 mg of protein, 30 min; 3, 50 mM buffer, 4 mM $MgCl_2$, 1 mg of protein, 63 min; 4, 40 mM buffer, 4 mM $MgCl_2$, 1 mg of protein, 60 min; 5, 40 mM buffer, 2 mM $MgCl_2$, 0.5 mg of protein, 20 min; 6 and 7, 40 mM buffer, 2 mM $MgCl_2$, 0.14 mg of protein, 60 min; 8, 50 mM buffer, 4 mM $MgCl_2$, 1 mg of protein, 63 min; 9, 50 mM buffer, 4 mM $MgCl_2$, 1 mg of protein, 90 min. L-α-Glycerophosphate was assayed enzymatically, and the ^{32}P-labeled product was determined by the column fractionation assay. X-OH was assayed for serine (amino acid analysis) and for inositol (gas–liquid chromatography). See Methods for details of the assays. The values in parentheses in the substrate column indicate the concentration of substrate used.

[a] L-α-GP = L-α-glycerophosphate.

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

[c] Low specific activity enzyme preparation was used.

[d] V = nmoles ^{32}P-labeled product/min/mg protein.

Experiment 9, Table 4, shows that [^{32}P]GPG 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 [^{32}P]GPG.

Glycerophosphoryldiesterase apparent K_m and V_{max} values

Apparent K_m values for two of the substrates are indicated by the data in Fig. 3. The 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 *g* super-

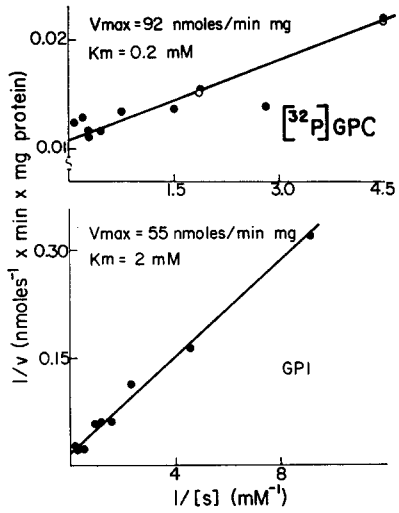

FIG. 3. Determination of K_m values for GPC and GPI. Samples containing [^{32}P]GPC contained 2 mM $MgCl_2$, 40 mM imidazole, pH 7.2, and 0.084 mg of protein in 0.5 ml. The samples were incubated at 30°C for 10 min. Mixtures containing GPI contained 4 mM $MgCl_2$, 40 mM imidazole, pH 7.2, and 0.23 mg of protein/ml in a final volume of 1 or 3 ml. The incubations were carried out at 30°C for 15 or 30 min. Hydrolysis of GPI was quantitated by measuring the inositol released. Quantitation of inositol using gas–liquid chromatography and assay of [^{32}P]GPC are described in Methods. [^{32}P]GPC is glycero[^{32}P]phosphorylcholine; GPI is glycerophosphorylinositol.

natant 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-methylcholine behaves as a competitive inhibitor of [^{32}P]GPC hydrolysis with a K_i of 7×10^{-4} M. Complete data are not available for K_m values of GPG or GPS, but they appear to be on the order of GPI rather than GPC. Experiments 1 and 2 of Table 4 indicate that the K_m for GPS is at least as large as that 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 Fig. 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 had apparent V_{max} values of 50 ± 5.

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 those of the substrates.

Other data not shown 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 Biochemicals) and pronase (Calbiochem) to inhibit the activity after 50 min of incubation 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 a single or a multiple enzyme is involved in hydrolysis of the various substrates, experiments involving incubation of pairs of substrates separately and in combination were performed. Fig. 4 shows that the hydrolysis of substrate

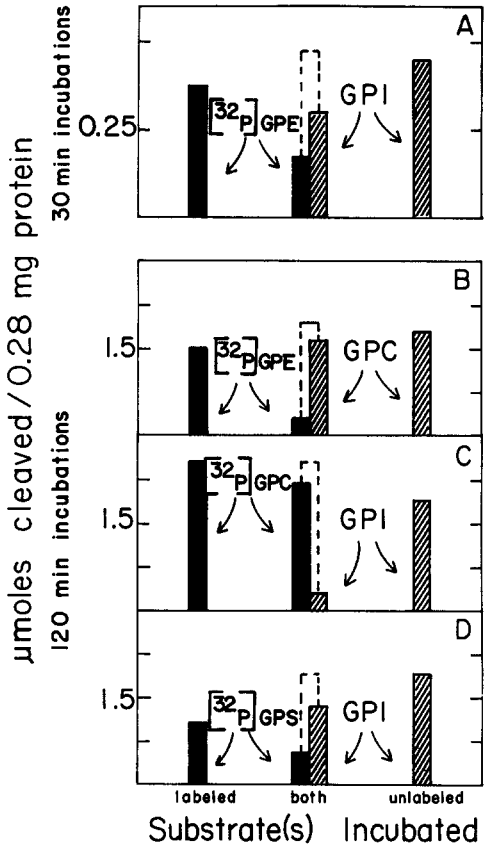

FIG. 4. Mutual inhibition of enzymatic hydrolysis by pairs of glycerophosphodiesters. All incubation mixtures contained 4 mM $MgCl_2$, and 65 mM imidazole, pH 7.2, in addition to enzyme and substrate in a final volume of 0.5 ml. Incubation temperature was 30°C. The substrate concentrations were: for experiment *A*, [^{32}P]GPE, 4.5 mM, GPI, 10 mM; for experiment *B*, [^{32}P]GPE, 5 mM, GPC, 5 mM; for experiment *C*, [^{32}P]GPC, 5 mM, GPI, 10 mM; and for experiment *D*, [^{32}P]GPS, 9.6 mM, GPI, 10 mM. Hydrolysis of the labeled substrates was measured by counting fractions from the anion exchange columns, and hydrolysis of unlabeled substrate and combined substrates was measured by quantitating the total phosphate in the substrate and product fractions. The open bar in the column for both substrates indicates total L-α-glycerophosphate produced. [^{32}P]GPE is glycero[^{32}P]-phosphorylethanolamine; GPI is glycerophosphorylinositol; GPC is glycerophosphorylcholine; [^{32}P]GPC is glycero[^{32}P]phosphorylcholine; [^{32}P]GPS is glycero[^{32}P]phosphorylserine.

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 Fig. 4*A*, hydrolysis of [^{32}P]GPE in the presence of GPI was about one-half the control rate, and GPI hydrolysis was inhibited by one-third. Fig. 4*D* indicates that GPI may be a slightly better competitor than [^{32}P]GPS and is hydrolyzed at a higher rate than [^{32}P]GPS. In evaluating Fig. 4*B* and *C*, it is noted that the GPC preparation used in *B* gave rates about 35% below normal (e.g., [^{32}P]GPC V_{max}, Fig. 3). Even considering this limitation for GPC hydrolysis, it is apparent from Fig. 4*B* 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 of [^{32}P]GPC from ^{32}P-labeled L-α-glycerophosphate and choline were unsuccessful (experiments 1 and 2 of Table 5), even at very high (0.1 M) 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 1 of Table 5. 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 5.

TABLE 5. Reversal of enzymatic hydrolysis of GPC and exchange of [*Me*-^{14}C]choline or L-α-glycerol[^{32}P]phosphate into GPC and GPI

Expt. No.	Radioactive Compound	Other Reactants	Incubation Time	Radioactivity Recovered in GPC	
			min	*cpm*	*% × 100*
1	L-α-Glycerol[^{32}P]-phosphate (43 × 10^6 cpm, 100 mM)	GPC 100 mM	90	2.7 × 10^{4a}	6.3
				1.9 × 10^4	4.4
		Choline[b] 100 mM	90	3.0 × 10^{4a}	7.0
				1.4 × 10^4	3.3
2	L-α-Glycerol[^{32}P]-phosphate (7.5 × 10^6 cpm, 15 mM)	Choline[c] 10 mM	90	75[a]	0.10
				100	0.13
3	[*Me*-^{14}C]Choline (1.2 × 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

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.

[a] Boiled enzyme control.

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

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

When [*Me*-^{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 [^{14}C]GPC. In the exchange of [*Me*-^{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 approaches the rate of hydrolysis of the [^{32}P]GPC produced. Total [^{14}C]GPC concentration approached 0.01 mM after 90 min of incubation. For the experiment involving exchange of [*Me*-^{14}C]choline into GPC, after 60 min, 1/600 (0.17%) of the label appeared in GPC; this 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

It is apparent that the soluble phosphodiesterase from housefly larvae has some properties that are different from those of enzymes isolated from other sources. The apparent K_m values for GPC and GPE are about an order of magnitude lower for the larval enzyme than for the enzyme from *Serratia plymuthicum* (16) and kidney microsomes (5): 2–4 × 10^{-4} M for the larval enzyme and > 1 × 10^{-3} M for the bacterial and kidney enzyme. The response of the larval enzyme to various concentrations of Mg^{2+} and to EDTA is different from that of the enzymes from *Serratia plymuthicum* (16), rat kidney (17), brain (18), and liver (19). The larval enzyme is quite specific for Mg^{2+} (Table 3) and is not inhibited by 10 mM Mg^{2+}, which is unlike the bacterial or kidney enzyme.

The properties observed for the larval enzyme indicate that it can hydrolyze all the GP-X compounds that are known to be major catabolic products of glycerophosphatides in housefly larvae (2, 4). 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[2] 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 constit-

[2] Bieber, L. L. Unpublished observation.

uents 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.

Our results indicate, but do not prove, that all GP-X compounds are cleaved by one larval enzyme. When two substrates were incubated together, both were hydrolyzed, but at rates below noncompetitive values (Fig. 4). Total cleavage of any pair of substrates does not exceed significantly the cleavage of the single most active substrate in the pair. 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.

Hydrolysis of GPC by the larval glycerophosphodiesterase is virtually irreversible; however, [*Me*-^{14}C]-choline did exchange slowly into GPC and GPI. These observations suggest that the L-α-glycerophosphate is released from the enzyme more slowly than is 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–phosphorylglycerol intermediate is formed during the enzymatic conversion of glycerophosphodiesters to L-α-glycerophosphate and X-OH.

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