Characterization of phospholipase B of *Culex pipiens fatigans*

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ABSTRACT Phospholipase B has been found in the mosquito *Culex pipiens fatigans*, and some of its properties have been studied. The enzyme had a high optimum temperature (45°C) and broad alkaline pH optimum (8–9). It was inactive toward diacylphospholipids, and hydrolyzed lysolecithin at a higher rate than lysophosphatidyl ethanolamine. The enzyme was heat labile, but lysolecithin protected it against heat inactivation. Phosphatidyl ethanolamine, phosphatidyl choline, deoxycholate, Fe^{+++}, and Hg^{++} inhibited the enzyme markedly.

The enzyme was present mainly in larvae; little enzyme activity was detected in pupae or adults. The total and specific activities were highest in IV instar (6 day) and I instar (1st day) larvae, respectively. It was localized in the microsomal fraction and was distributed mainly in the abdomen and thorax of the insect. The enzyme was present at much higher levels of activity in larvae of the mosquitoes *Anopheles stephensi* and *Aedes aegypti*.

SUPPLEMENTARY KEY WORDS mosquito vectors · developmental stages · lysophospholipids · alkaline pH optimum · high optimum temperature · deoxycholate · diacylphospholipid inhibition · heat lability · lysolecithin protection · *Anopheles stephensi* · *Aedes aegypti*

KNOWLEDGE OF THE metabolic pathways operating in insects, particularly those of importance in public health, is essential. Despite the recognized vital role lipids play in the physiology of insects (1), information on the metabolism of these constituents is fragmentary (2). Clements reported (3) that the mosquito *Culex pipiens fatigans*, the vector of bancroftian filariasis, has extensive stores of lipids which are utilized by the insect during its sustained flight. Further, lipids are believed to have a role in the development of resistance by certain insects to insecticides. An understanding of the steps involved in the catabolism of lipids is expected to throw light on the above aspects.

As part of an investigation on the metabolism of the principal lipid classes in *Culex pipiens fatigans* that are susceptible and resistant to insecticides, the nature of different phospholipases of the insects is being examined in this laboratory. In a previous work we have reported (4) the presence of phospholipases A and B (phosphatide acylhydrolase, EC 3.1.1.4 and 3.1.1.5, respectively) in the larvae of the mosquito, and the properties of the enzyme phospholipase A have been studied in considerable detail. The present work deals with the characterization of phospholipase B of the insects.

MATERIALS AND METHODS

Ovolecithin, lysolecithin, lysophosphatidyl ethanolamine, phosphatidic acid, and sphingomyelin were purchased from the Biochemicals Unit, Council of Scientific and Industrial Research, Delhi. The lysolipids were of the 1-acyl type, prepared by the use of snake venom enzyme on the diacyl phosphoglycerides purified from egg lipids. Cardiolipin was isolated from *Mycobacterium 607* as detailed elsewhere (5). PE was prepared from egg lipids by chromatography on columns of silicic acid (Mallinkrodt, 100–200 mesh; 15% methanol in chloroform fraction) and further purified by preparative TLC on Silica Gel G with chloroform–methanol–water 65:25:4. Analytical and TLC data revealed these compounds to be pure.

Phosphatidyl inositol from *M. tuberculosis* was a generous gift from Dr. M. C. Pangborn. Egg rafts of *Culex* and

Abbreviations: GPC, glyceryl phosphoryl choline; GPE, glyceryl phosphoryl ethanolamine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PCMPS, *p*-chloromercuriphenyl sulfonate; BSA, bovine serum albumin; TLC, thin-layer chromatography.

eggs of *Aedes aegypti* and *Anopheles stephensi*, obtained from the Medical Entomology Division of the Institute, were reared in yeast medium at 25–27°C, and the larvae were used in the enzyme assay. In the case of *Culex*, the enzyme activity was also measured in pupae and adults of both sexes collected within a few hours after their emergence.

Preparation of 2-Acyl GPC and 2-Acyl GPE

In view of the specificity of phospholipase A of certain species of mosquitoes in splitting the 1-fatty acids of PC (6) and PE (R. H. Rao and D. Subrahmanyam, unpublished observations), the insect enzyme has been used in the preparation of 2-acyl GPC and 2-acyl GPE. A 0.4% homogenate of *Aedes aegypti* larvae in 0.05 M Tris–maleate buffer of pH 7.4 was prepared in a Potter–Elvejhem glass homogenizer. The extract was centrifuged at 13,000 *g* in the cold for 20 min in an International refrigerated centrifuge, and the supernatant served as the enzyme source. The incubation mixture contained 16.1 μmoles of phospholipid in 0.5 ml of ethanol, 50 mg of sodium deoxycholate, and 4 ml of the enzyme solution in a final volume of 5 ml. The mixture was incubated for 90 min at 37°C. The reaction was then stopped by the addition of 20 ml of chloroform, 10 ml of methanol, and 1 ml of 4.2% KCl, thus making up a Folch mixture (4). The mixture was then vigorously shaken and centrifuged. The lower layer was concentrated, and the lysolipids were isolated by preparative TLC in chloroform–methanol–ammonia 65:25:4. The lysolipids, located by marker spots run simultaneously, were rapidly eluted from the silica gel with suitable aliquots of chloroform–methanol 2:1, chloroform–methanol 1:1, and methanol. The TLC on Silica Gel G plates and the elution of the lipid were conducted at 5°C in order to minimize acyl migration (7) from the 2 to the 1 position of the lysolipid. The purity of the lipids thus isolated was checked by TLC and other analytical methods (5).

Enzyme Assay

The assay system, unless otherwise indicated, consisted of 0.8 μmoles of 1-acyl GPC in 0.1 ml of water and 0.4 ml (6.6 μg protein) of the 13,000 *g* supernatant of 0.1% *Culex* homogenate, made up to a final volume of 1.0 ml with 0.05 M Tris–maleate buffer of pH 6.5. The mixture was incubated in Teflon-stoppered tubes for 90 min at 37°C, and the enzyme activity was stopped with 4 ml of chloroform, 2 ml of methanol, and 0.2 ml of 4.2% KCl to make up a Folch mixture (4) ("extraction mixture"). The tubes were thoroughly mixed and centrifuged. The upper aqueous layer containing the released GPC was washed with 3 volumes of chloroform–isobutanol 2:1 to remove any contaminating lysolecithin (8). The amount of phosphorus in the supernatant fraction thus obtained was taken as a measure of GPC released.

Lysophosphatidyl ethanolamine, phosphatidic acid, cardiolipin, and PE were emulsified in buffer by sonication in the cold by the method of Gammack, Perrin, and Saunders (9) before addition to the assay system. PC and sphingomyelin were dissolved in ethanol and added at a final concentration of ethanol of 5% in the incubation mixture. GPC and GPE, as products of hydrolysis of lysolecithin and lysophosphatidyl ethanolamine, respectively, were identified by cochromatography (on Whatman No. 1 paper) of the aqueous fraction with authentic compounds, with phenol saturated with 0.1% ammonia or isopropanol–ammonia (25%, w/w)–water 70:6:30 as developing solvents.

Phosphorus was determined by the method of Bartlett (10). Protein in the enzyme extracts was estimated according to the method of Lowry, Rosebrough, Farr, and Randall (11). Subcellular fractions were prepared from a 10% homogenate in 0.25 M sucrose in a Spinco model L preparative ultracentrifuge at 2°C at 1000 *g*, 13,000 *g*, and 105,000 *g* for the separation of nuclei, mitochondria, and microsomes, respectively. The purity of the fractions has been checked by specific enzyme assays (12, 13).

RESULTS

Rate of Hydrolysis of Lysolecithin

The rate of hydrolysis of lysolecithin was studied at different time intervals of incubation and in another experiment with increasing concentrations of the enzyme preparation. The enzyme was inactivated at the end of incubation by the addition of the extraction mixture. The rate of hydrolysis was constant up to 120 min (0.08 μmoles of GPC formed per hour from 0.8 μmoles of lysolecithin) and linear with respect to the enzyme content of the incubation mixture up to 0.8 ml of homogenate, i.e., 13.2 μg of protein (0.02 μmoles of GPC formed from 0.8 μmoles of lysolecithin per 0.1 ml of homogenate per hr).

Effect of Substrate Concentration

The rate of hydrolysis as a function of 1-acyl GPC concentration was studied and the optimal substrate concentration was found to be about 0.8 μmoles/ml, which was the concentration used throughout the experiments.

The affinity of the enzyme for different lysolipids was studied by determining the activity after 60 min incubation at pH 7.1 with various amounts of the substrates. The Lineweaver–Burk plots of the results are shown in Figs. 1 and 2. The calculated Michaelis–Menten constants (K_s), are 1.03×10^{-4} M, 1.05×10^{-4} M, $5.82 \times$

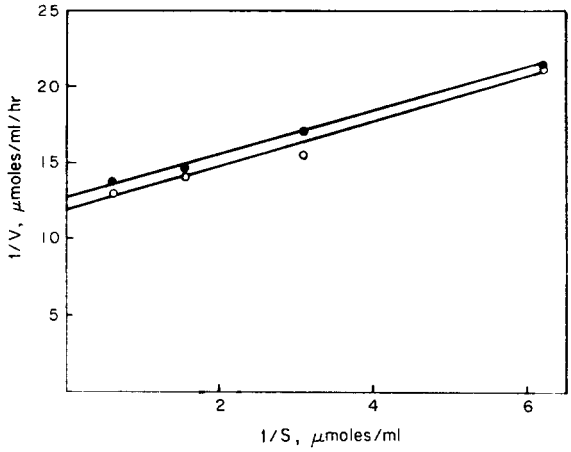

FIG. 1. Lineweaver–Burk plots with 1-acyl GPC (●—●) and 2-acyl GPC (○—○) as substrates. Standard assay conditions with various amounts of lipids, with 0.3 ml (5 μg of protein) of enzyme preparation and incubation for 60 min.

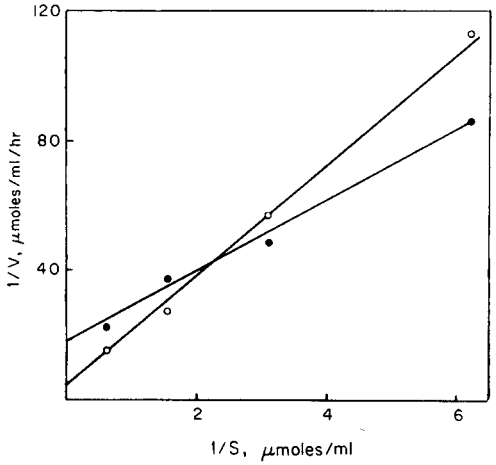

FIG. 2. Lineweaver–Burk plots with 1-acyl GPE (●—●) and 2-acyl GPE (○—○) as substrates. Conditions for assay were as in Fig. 1.

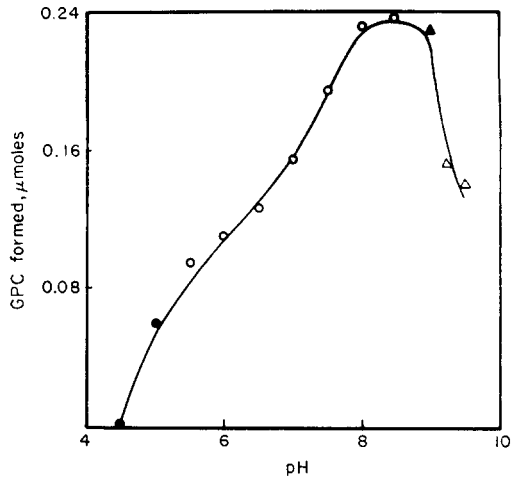

FIG. 3. Effect of pH on enzyme activity. The incubation mixture was as in Methods except that 0.2 M buffers of appropriate pH were used (acetate, ●—●; Tris–maleate, ○—○; Tris, ▲; and carbonate–bicarbonate, △).

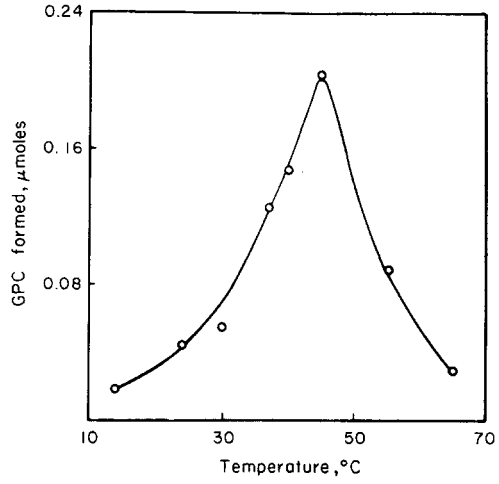

FIG. 4. Effect of temperature of incubation on phospholipase B activity. Conditions for assay were as in Methods.

10^{-4} M, and 3.33×10^{-3} M for 1-acyl GPC, 2-acyl GPC, 1-acyl GPE, and 2-acyl GPE, respectively. In view of the limitations in solubility of the lipid substrates and physical nature of the solutions, these values were considered approximate.

Optimum pH

The effect of pH on the enzyme activity was studied in 0.2 M buffers (Fig. 3). The enzyme had a broad alkaline pH optimum (8.0–9.0).

Effect of Temperature on Enzyme Activity

The effect of temperature on the enzyme activity is shown in Figs. 4 and 5. The optimum temperature (Fig. 4) is 45°C. The enzyme was found to be stable for several months at 0°C but heat labile at higher temperatures (Fig. 5). The enzyme lost 80% of its activity when kept at 55°C for 10 min at pH 4.5 or 7.0. 1-acyl GPC at a concentration of 1.6 μmoles/ml of the incubation mixture protected the enzyme from heat inactivation. In this experiment the enzyme was brought to pH 4.5, at which it was essentially inactive, mixed with the substrate, and treated at 55°C for 10 min. The mixture was cooled, the pH was adjusted to 6.5, and the incubation was continued for 90 min.

Effect of Metal Ions

A number of metal ions ranging in concentration from 1 to 10 mM were examined for their effect on the enzyme activity. For this study the enzyme was dialyzed overnight in the cold against 0.05 M Tris–maleate buffer at

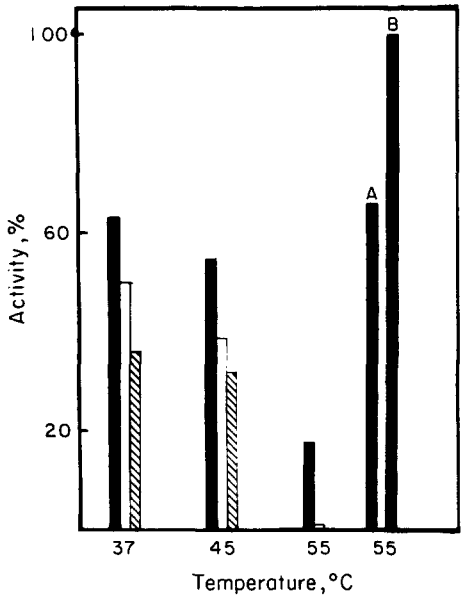

FIG. 5. Heat lability of the enzyme preparation. The enzyme was heated for 10 min, ■; 30 min, □ and 60 min, ▨ at the temperatures indicated (pH 6.5) and chilled before assay at 37°C. At A and B, the enzyme was subjected to heat treatment at pH 4.5 in the presence of 0.8 and 1.6 μmoles, respectively, of 1-acyl GPC.

pH 6.5 containing EDTA (0.001 M) and was subsequently freed from EDTA by further dialysis against the buffer. The enzyme activity was unaffected on dialysis under the conditions and the results of the experiment are given in Table 1. It is evident from the Table that no metal was required for enzyme activity. At 10 mM concentration, the enzyme was strongly inhibited by Zn^{++} and Cu^{++} and completely inhibited by Fe^{+++} and Hg^{++}. Similar total inhibition of the enzyme by Hg^{++} was observed with 2-acyl GPC as the substrate. Ca^{++} and Co^{++} had no effect on the enzyme activity although they were found to inhibit phospholipase A of the insects by about 30% even at 1 mM concentration (4).

Effect of Reagents

A number of reagents listed in Table 2 have been used to study their effect on the enzyme. F^-, PCMPS, and iodoacetate had no effect on the activity at the concentrations used. F^- at 10 mM was, however, found to inhibit 60% of phospholipase A activity of the mosquito (4). Triton (0.1%) and ethanol (20%) inhibited the enzyme activity by 33 and 84%, respectively. Sodium deoxycholate (0.1%) completely inhibited the enzyme under the assay conditions; it has been shown to be an activator of phospholipase A of the insect species (4). Similar results were obtained when the enzyme activity was determined with 2-acyl GPC in presence of ethanol and deoxycholate.

TABLE 1 EFFECT OF METAL IONS ON PHOSPHOLIPASE B ACTIVITY OF *Culex pipiens fatigans*

Metal (10 mM)	Activity
	%
None	100
Ca^{++}	100
Mg^{++}	100
Mn^{++}	100
Ba^{++}	82
Zn^{++}	36
Cu^{++}	18
Fe^{++}	49
Fe^{+++}	0
Hg^{++}	0
Co^{++}	100
Cd^{++}	77
Ni^{++}	100

TABLE 2 EFFECT OF SOME REAGENTS ON PHOSPHOLIPASE B ACTIVITY OF *Culex pipiens fatigans*

Reagent	Concentration	Activity
	mM	%
None	—	100
NaF	10	100
PCMPS	1	100
Iodoacetate	1	100
KCN	10	100
EDTA	1	100
Oleic acid	1	100
Cysteine	10	100
Glutathione	10	100
Sodium deoxycholate	2.42	0
BSA	0.5%	100
Triton	0.1%	67
Ethanol	5%	100
	20%	16
Protamine sulfate	1%	75

TABLE 3 EFFECT OF SOME PHOSPHOLIPIDS ON PHOSPHOLIPASE B ACTIVITY OF *Culex pipiens fatigans*

Substrate (0.8 μmoles)	Phospholipid Added	Amount Added	Activity
		μmoles	%
1-Acyl GPC	None	—	100
	Phosphatidyl choline	1.6	42
		6.4	0
	Phosphatidyl ethanolamine	1.6	0
	Phosphatidic acid	0.8	100
	Phosphatidyl inositol	0.8	100
	Cardiolipin	0.8	82
	Sphingomyelin	0.8	80
1-Acyl GPE	None	—	100
	Phosphatidyl choline	1.6	0
	Phosphatidyl ethanolamine	1.6	0

Effect of Different Phospholipids

The results on the effect of different phospholipids on the activity of the enzyme are presented in Table 3. PE and, to a lesser extent, PC were potent inhibitors of phospholipase B activity while cardiolipin and sphingomyelin

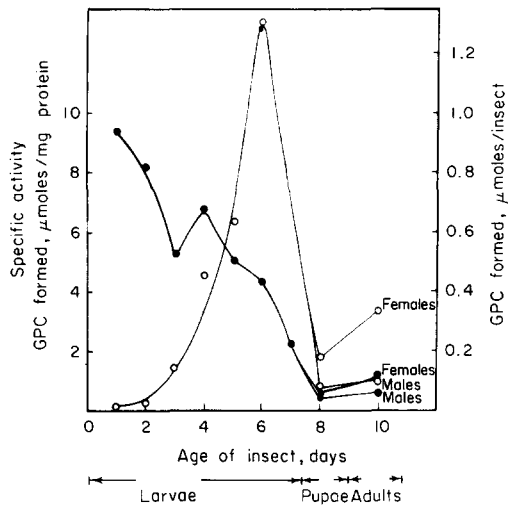

FIG. 6. Enzyme activity in different developmental stages of the insect. Total activity per insect O—O; specific activity, ●—●. Assay conditions as in Methods.

were partially inhibitory at the concentrations used. Phosphatidic acid and phosphatidyl inositol had no effect on the enzyme at a concentration of 0.8 mM. None of the diacyl phospholipids was hydrolyzed by the enzyme under the experimental conditions.

Enzyme Activity in Certain Other Species

The activity of the enzyme under similar conditions in different species of mosquito was determined (Table 4). Larvae of *Aedes aegypti* and *Anopheles stephensi* contained the enzyme at higher levels than *Culex pipiens fatigans*. Each value was an average of three determinations.

Enzyme Activity in Different Developmental Stages of Culex

The activity of the enzyme in different developmental stages was studied, and the results are shown in Fig. 6. Egg rafts possessed no enzyme activity while low activity was detected in pupae and adults, the females having slightly more than males. The total enzyme activity was highest in 6-day larvae (IV instar), but the specific activity continuously declined with age of the insects, except for slightly higher activity in 4-day (III instar) than the 3-day larvae (early III instar).

Regional and Subcellular Distribution of the Enzyme

The anatomical distribution of the enzyme activity is given in Table 5. The activity was found to be distributed in the abdomen and thorax.

The subcellular distribution of the enzyme activity was studied (Table 6), and the maximum activity was found almost exclusively in the microsomes, whereas microsomal supernatant contained very little activity. Identical results were obtained with 2-acyl GPC as the substrate.

TABLE 4 PHOSPHOLIPASE B ACTIVITY IN DIFFERENT SPECIES OF MOSQUITOES

Species	Specific Activity
	μmoles GPC formed/mg protein
Culex pipiens fatigans	16.3
Aedes aegypti	60.0
Anopheles stephensi	45.6

TABLE 5 REGIONAL DISTRIBUTION OF PHOSPHOLIPASE B ACTIVITY IN LARVAE OF *Culex pipiens fatigans*

Fraction	Specific Activity
	μmoles GPC formed/mg protein
Whole homogenate	3.57
Head	Traces
Thorax	2.11
Abdomen	2.46

TABLE 6 SUBCELLULAR DISTRIBUTION OF PHOSPHOLIPASE B ACTIVITY IN *Culex pipiens fatigans*

	Specific Activity
Fraction	*μmoles GPC formed/mg protein*
Crude homogenate	3.57
Cell debris and nuclei (1000 *g*, 10 min)	0
Mitochondria (13,000 *g*, 20 min)	0
Microsomes (105,000 *g*, 60 min)	31.89
Supernatant	4.49

DISCUSSION

Phospholipase B has been detected and characterized from a variety of sources. However, information on the presence of this enzyme in insects is scanty. Khan and Hodgson (14) recently found phospholipases A and B in houseflies and studied the subcellular distribution of the enzymes. The present investigation and those of earlier studies (4) show that *Culex pipiens fatigans* contains phospholipases A and B. The different substrate requirements and the differential effect of deoxycholate, F^-, Ca^{++}, and Co^{++} on the enzyme activities reveal that these two are distinct enzymes present in high concentrations in larvae of the species. In addition, it has been possible to isolate phospholipase A free of B activity by heating the insect homogenate at 45°C for 1 hr in the presence of deoxycholate, which protected phospholipase A. The enzyme after exhaustive dialysis to remove deoxycholate did not detectably hydrolyze 1-acyl GPC. Phospholipase A could, however, be inactivated by heating the enzyme mixture at 60°C for 15 min in presence of 1-acyl GPC at a concentration of 1.6 mM, under which conditions B was completely protected.

The phospholipase B from *Culex pipiens fatigans* is different from that identified in molds (15, 16), bacteria

(17), or animal tissues (18, 19) in having a high optimum temperature (45°C) and alkaline pH optimum (8–9). F^-, CN^-, and Mg^{++}, which inhibit rat liver enzyme (19), had no effect on the insect enzyme at 10 mM concentration. The enzyme did not hydrolyze diacyl phospholipids with (20) or without emulsification by sonication (21) or even in the presence of auxiliary lipids (16), unlike the one isolated from *Penicillium notatum*. On the other hand, the enzyme activity appeared to be regulated by PE and PC, which are the major phospholipids in the insect (D. Subrahmanyam and R. H. Rao, unpublished observations), as these lipids inhibited the hydrolysis of lysolecithin or 1-acyl GPE. The activity of the insect enzyme was not influenced by the nature of the fatty acids in the lysolipid molecule, as it hydrolyzed 1-acyl GPC containing predominantly saturated fatty acids at the same rate as the unsaturated 2-acyl GPC. However, the insect enzyme hydrolyzed 1- or 2-acyl GPE more slowly than lysolecithin. Of the two acyl GPE, 1-acyl seemed to be a better substrate than 2-acyl GPE.

The insect phospholipase B was similar to that identified in vibrio *El Tor* (8) in its specificity for lysolipids and alkaline pH optimum. However, it differs from the vibrio enzyme in having a broad optimum pH and in its heat lability.

The enzyme differs from that reported in housefly by Khan and Hodgson (14) in its distribution. The housefly enzyme was active in adults and was detected in all subcellular fractions. *Culex* enzyme was present in larvae in maximum amounts and little activity could be found in adults. The enzyme was localized predominantly in the microsomal fraction (105,000 *g* pellet).

The enzyme activity was not studied at the optimum conditions of temperature and pH; we stayed as close to the physiological range as possible in order to compare the properties of the phospholipase with those from other sources and also to ensure stability of the reagents and cofactors whose effects were being studied. Deoxycholate was found to be an effective inhibitor under the assay conditions, and in this respect the enzyme resembles the mammalian enzyme (22). Iodoacetate and PCMPS (1 mM) did not affect the activity, and this suggests that the enzyme is not -SH dependent.

The data of this investigation do not conclusively prove that the phospholipase B activity on 1- and 2-acyl lipids is due to a single enzyme. However, the similarity in the distribution of the enzyme activity in different subcellular fractions as seen with 1- and 2-acyl GPC as substrates and the same degree of inhibition of hydrolysis found with the two substrates in the presence of inhibitors such as Hg^{++}, deoxycholate, and ethanol suggest that the activity may be due to a single enzyme.

The enzyme was present at high levels in abdomen and thorax but was only in detectable amounts in the heads. This distribution is similar to that of phospholipase A (unpublished observations) in the insect species. The enzyme, together with phospholipase A, may be involved in digestion of dietary lipids and in the turnover of the insect phospholipids and the released fatty acids may be utilized as energy source by the mosquito. The enzyme may also have a role in preventing the accumulation of lysolipids to cytotoxic levels in the insects.

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