THE PREPARATION AND PROPERTIES OF A LYSOPHOSPHOLIPASE FROM PENICILLIUM NOTATUM*

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In a previous communication (1) the name phospholipase was used to describe the enzyme in various venoms which converts lecithins and cephalins to lysolecithins and lysocephalins, with liberation of unsaturated fatty acids. The present work describes a powerful enzyme occurring in Penicillium notatum, which acts upon lysophospholipides to produce glycerylphosphorylcholine (or glycerylphosphorylethanolamine), with liberation of saturated fatty acids. This enzyme will be called lysophospholipase. Phospholipase and lysophospholipase are terms synonymous with lecithinase A and lecithinase B (2), respectively.

Investigation of the enzymatic degradation of the phospholipides has met with scant success when animal tissues have served as sources of the various enzymes concerned. This has not been due to absence of such enzymes from the tissues, for their existence has been amply proved through the study of autolytic processes (3, 4). In order to improve our understanding of the phospholipide-hydrolyzing enzymes, it has been considered advisable to look for sources of the individual enzymes which would make possible the study of each step in the degradation process. The classic example among such sources is, of course, the extremely active phospholipase of venoms, previously mentioned. In 1933 Contardi and Ercoli (5) described a lysophospholipase obtained from rice bran, rice embryos, and Aspergillus oryzae, whose activity was, however, quite low. Penicillium notatum has now been found to be an excellent source of this enzyme. Methods have been devised for the quantitative assessment of the reaction and for the determination of lysophospholipase activity. Certain properties of the enzyme have also been examined. The investigation has been discontinued.

EXPERIMENTAL

Enzyme—Penicillium notatum† was surface-cultured in 500 ml. Erlenmeyer flasks, on a corn steep, lactose-containing medium (6), for 5 to 8 days

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† The Penicillium strain used was kindly supplied by Dr. G. B. Reed.
at 25°. The mat of abundantly sporulating mold was then removed from
the culture medium, washed with water, and dried superficially by pressing
gently between filter papers. After crumbling the mat between the fingers,
drying was completed in vacuo over anhydrous calcium sulfate (Drierite).
Each culture yielded more than 1 gm. of dry mold, which was stored at 4°
until required.

Extracts of this material were prepared by pulverizing 5 gm. portions,
mixed with a little sand, adding 50 ml. of distilled water plus 5 drops of
toluene, and autolyzing for 24 hours at 20-25°. After filtration and wash-
ing with suction, the combined filtrates (60 ml.) were dialyzed for 24 hours
at 4°, with inside stirring, against 20 liters of running distilled water. After
dialysis, one of two procedures was followed: (1) The extract was diluted
to 75 ml. and stored at 4° under toluene; (2) the solution, in the dialysis sac,
was suspended in glycerol and concentrated to about 14 ml., after which it
was removed from the sac, diluted to 75 ml. with glycerol and stored at 4°.

Aqueous extracts lost about 80 per cent of their activity when stored at
4° for 2½ months. During the same period of time glycerol preparations
remained fully active.2 The nitrogen content of the dialyzed aqueous
extracts varied from 0.280 to 0.380 mg. per ml.

Substrate—Lysophospholipides were prepared from egg yolks by a sim-
plification of King’s procedure (7), in which troublesome concentration of
extracts in vacuo was eliminated. After formation of the lysophos-
pholipides by incubation of 60 yolks with moccasin venom (Agkistrodon pisci-
vorus), the reaction mixture (3 liters) was extracted with acetone (6 liters)
at 50°, and filtered rapidly while still hot. The residue was reextracted
twice with acetone (2 liters) and the combined filtrates cooled to 20°. To
this were then added 90 ml. of saturated aqueous cadmium chloride solution,
and the mixture was cooled to 0°. The copious precipitate was filtered off
rapidly and extracted 5 times in a Waring blender with 300 ml. portions of
cold acetone. It was then dried in vacuo, following which lysophospho-
lipides were isolated from the cadmium chloride complex by the usual
methods. Yield, 35 gm.; P 5.70 per cent, N 2.72 per cent, NH₂-N (8)
0.552 per cent, iodine value of constituent fat acids 4.

Lysophospholipide sols were prepared as required, usually at concentra-
tions of 1 or 0.5 per cent, by shaking with water or buffer solution at 40-50°.
Such sols were stable at 30°, but precipitated slightly at 25° and heavily
at 4°. They were chemically stable, at pH 4.0, for a period of several weeks
and after storage at 4° were readily reformed by gentle heating.

2 As a result of the considerable stability of lysophospholipase in solution, exten-
sive efforts to purify the enzyme, or to produce dry preparations, were not made. It
could be precipitated partially from solution by saturated ammonium sulfate, by
acetone, and by acid (pH 3.0), but on the basis of nitrogen content, none of these
treatments increased the activity appreciably.
Reaction Mixture—Unless otherwise specified, the reaction mixture contained 1 ml. of 1 per cent lysophospholipides and 0.04 ml. of enzyme, in a total volume of 2.2 ml. All components of the final mixture were prepared in veronal-acetate buffer, pH 4.0. For the reaction, all components except enzyme were mixed in glass-stoppered test-tubes, and equilibrated for 20 minutes in a water bath maintained at 30°. The enzyme was added, and after 5 minutes a 1 ml. aliquot was removed into precipitation tubes as described below.

Under these conditions, the originally clear solution became quite turbid, owing to liberation of insoluble fatty acids, and if allowed to stand, formed a stiff gel. Degradation of the lysophospholipides varied from 15 to 50 per cent after 5 minutes, depending upon the age of the lysophospholipase used.

Quantitative Determination of Reaction—Preliminary experiments indicated that nephelometric or viscosimetric methods could not be applied as quantitative assessments of the reaction. Efforts were made, therefore, to separate the substrate from one or both of the reaction products. It was found that a satisfactorily complete separation of lysophospholipides and fatty acids from glycerylphosphorylcholine could be made by the colloidal iron-magnesium sulfate method of Folch and Van Slyke (9). Lysophospholipides in the precipitate were then determined by analysis for phosphorus.

Details of the procedure were as follows: 1 ml. aliquots of the reaction mixture were added to 12 ml. conical centrifuge tubes containing 2.8 ml. of veronal-acetate buffer, pH 9.2, plus 4.2 ml. of water, and stirred immediately. The alkaline pH of this mixture effectively stopped the reaction. To the tubes were then added with stirring 0.5 ml. of colloidal iron (Fe₂O₃, 5 per cent dialyzed) and 0.5 ml. of half saturated magnesium sulfate solution. Addition of these acid reagents lowered the pH of the mixture to about 6.5. The tubes were centrifuged for 3 minutes, the supernatant solution containing the GPC, decanted, and the precipitates washed with 10 ml. of 0.05 saturated magnesium sulfate solution. The centrifuge tubes were washed carefully with two 1 ml. portions of sulfuric acid (86 per cent by volume), and the washings transferred to the digestion flasks. Digestion was carried out by adding 0.200 gm. of potassium sulfate-copper sulfate mixture (9:1) and boiling for 1 hour. An acid-washed Hengar granule effectively overcame the tendency to bump. After digestion, any precipitate present was

The abbreviation GPC will be used to denote the mixed esters, glycerylphosphorylcholine and glycerylphosphorylethanolamine.
filtered off, and phosphorus was determined colorimetrically (10). The presence of iron, magnesium, copper, and potassium ions was without effect on the determination.

Identical amounts of lysophospholipides carried through the entire precipitation and digestion procedures showed a maximum variation in the analyses of ±1 per cent. The absolute amounts of phosphorus recovered in the precipitate, however, varied from 97 to 98 per cent of those found by direct analysis of lysophospholipides. There was, thus, a constant error of 3 per cent in the precipitation method. No correction was made for this loss.

In order to test the completeness with which lysophospholipide phosphorus and GPC phosphorus could be separated, varying amounts of the

<table>
<thead>
<tr>
<th>Table I</th>
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<td>Separation of Lysophospholipide Phosphorus from GPC Phosphorus</td>
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</table>

<table>
<thead>
<tr>
<th>$P$ found*</th>
<th>GPC $P$ added</th>
<th>GPC $P$ as per cent of total $P$</th>
<th>Recovery of lysophospholipide $P$ per cent</th>
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</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.270</td>
<td>0</td>
<td>3.7</td>
<td>97.5</td>
</tr>
<tr>
<td>0.272</td>
<td>0.0106</td>
<td>7.1</td>
<td>98.2</td>
</tr>
<tr>
<td>0.272</td>
<td>0.0212</td>
<td>16.0</td>
<td>98.2</td>
</tr>
<tr>
<td>0.270</td>
<td>0.0530</td>
<td>27.7</td>
<td>97.5</td>
</tr>
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<td>0.270</td>
<td>0.106</td>
<td>43.4</td>
<td>97.5</td>
</tr>
<tr>
<td>0.270</td>
<td>0.212</td>
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</tbody>
</table>

* Lysophospholipide $P$ by direct analysis (without precipitation by iron-magnesium mixture) = 0.277 mg. All values are averages of duplicate samples.

latter were added to a constant amount of lysophospholipides, following which the precipitation procedure was applied. As shown in Table I, the separation was complete.

The extent of the enzymatic reaction was calculated by comparison with the precipitated lysophospholipide phosphorus from a control tube. Since, in experimental tubes, the enzyme itself contained a small amount of precipitable phosphorus, an appropriate correction was made.

Under the conditions prescribed, addition of iron-magnesium to the precipitation tubes containing alkaline reaction mixture could be delayed 15 minutes, and after this addition the tubes could stand for at least 60 minutes before transfer of their contents to digestion flasks. In this way, it was possible to make twenty or more determinations in a single experiment.

Inorganic phosphate, if present, appeared in the precipitate with lyso-

A sample of pure racemic $\alpha$-glycerylphosphorylcholine was kindly supplied by Dr. Erich Baer.
phospholipides. Glycerophosphate was partially precipitated. Thus, the method was valid only in the virtual absence of enzymes liberating choline or inorganic phosphate, or both, from GPC. It was possible to show that the enzyme preparation used liberated appreciable amounts of inorganic phosphate in the reaction mixture, at pH 4.0, only when present in high concentration (10 to 100 times the concentration ordinarily used) and over a prolonged time (4 to 8 hours). Similarly, in mixtures containing 5 times

![Graph showing direct proportionality between first order constants (k) and lysophospholipase concentration, during the initial stages of reaction. The first point on the curve represents 7 per cent hydrolysis, the last point 53 per cent. Reaction time, 5 minutes.](http://www.jbc.org/)

the usual concentration of enzyme, incubated for 60 minutes, no free choline was determinable by the sensitive reineckate method. Under the conditions specified, therefore, the only reaction occurring to a significant extent was the conversion of lysophospholipides to GPC and fatty acids.

Measurement of Activity—During the initial stages of the reaction the decrease in lysophospholipide phosphorus, in a given time, was not directly proportional to enzyme concentration. Under certain conditions, however, a first order reaction was simulated, the appropriate constant (k) being proportional to enzyme concentration over a considerable range (Fig. 1).
These conditions were (1) a reaction temperature of 30° (2) a reaction time of 15 minutes or less, (3) a per cent reaction less than 40.

Substrate Specificity—Repeated attempts were made to secure a reaction between lysophospholipase and lecithins or cephalins, all of them unsuccessful. The following phospholipide preparations were used: egg yolk and brain lecithins purified according to Pangborn (11), petroleum ether-soluble egg yolk phospholipides (lecithins and cephalins), and the acetone-insoluble fraction of a commercial soy bean lecithin. With quantities of enzyme which extensively decomposed lysophospholipides (60 to 70 per cent) within 10 minutes, no action on lecithins was observed even after 1 hour.

Since the lysophospholipides in routine use as substrate for the enzyme contained 20.3 per cent of their total nitrogen as amino nitrogen, it was of interest to discover whether lysocephalins were reactive. In the absence of a method for obtaining pure lysocephalins, an indirect approach to the problem was made. 192 mg. of lysophospholipides (containing 1.11 mg. of amino nitrogen) were incubated for 1 hour with excess enzyme, at which time the reaction was 84 per cent complete. After separation of the residual lysophospholipides by the usual method, the GPC-containing solution was dried in vacuo, and analyzed for ethanolamine nitrogen (12). 0.573 mg. of ethanolamine nitrogen was found, corresponding to 52 per cent of the total amino nitrogen available for reaction. Lysophospholipase, therefore, acted upon lysocephalins as well as upon lysolecithins. Serine nitrogen (12) was not present in the substrate, and the enzyme itself contained neither ethanolamine nor serine nitrogen.

pH Optimum—The activity of lysophospholipase was determined at hydrogen ion concentrations varying from pH 2.88 to 6.43. The veronal-acetate buffer used exhibits constant ionic strength over this range (13). Sufficient enzyme was added to produce 31 per cent reaction at the most favorable hydrogen ion concentration. From the results (Fig. 2) it is seen that the optimum acidity for the reaction was rather sharply defined in the pH range 3.8 to 4.4. Contardi and Ercoli (5) found an optimum at pH 3.5 for the corresponding enzyme from Aspergillus oryzae.

Heat Stability—Fig. 3 contains a group of curves illustrating the resistance of lysophospholipase to various temperatures and hydrogen ion concentrations. In establishing these curves, solutions containing 0.6 ml. of enzyme extract per ml., at varying hydrogen ion concentrations, were heated at a given temperature (41°, 50°, or 61°) for a given time (15 or 30 minutes). The solutions were then cooled quickly, and their activity measured and compared with untreated enzyme. It is evident from the figure that the lysophospholipase exhibited a maximum heat stability near pH 4.5 and
was rapidly inactivated at a pH greater than 7.0, at temperatures of 41° or higher.

Activation and Inhibition—In general, lysophospholipase was resistant to most of the common enzyme inhibitors, and no activator was discovered.

FIG. 2. pH-activity curve for lysophospholipase

![Graph showing pH-activity curve for lysophospholipase]

FIG. 3. Stability of lysophospholipase to heat and hydrogen ion concentration. Per cent inactivation = 100 (1 - (k (treated)/k (control))

Cyanide was the most effective inhibitor among those tested (Table II), a 0.01 M concentration suppressing the reaction almost completely. Silver and copper ions were less effective. Calcium, magnesium, and cobalt ions did not inhibit the reaction, nor did sodium azide, cysteine, hydrogen sulfide, or hydrogen peroxide. The enzyme solution was slowly inactivated, pre-
sumably by surface denaturation, when air or nitrogen was bubbled through it.

As products of the reaction, GPC and fatty acids might be considered as inhibitors, since the reaction in the presence of the usual concentration of enzyme proceeded only to 60 to 70 per cent completion. Palmitic and linoleic acids (the latter a liquid acid not actually occurring in the substrate), added to the reaction mixture in amounts approximating their maximum concentration in the substrate, slightly inhibited the reaction. GPC, on the other hand, exerted no effect. Quite possibly the reaction was effectively suppressed before a chemical equilibrium was reached, as a result of

| Table II |
| Inhibition of Lysophospholipase* |

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration mole per l.</th>
<th>Inhibition per cent</th>
<th>Inhibitor</th>
<th>Concentration mole per l.</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>0.0094</td>
<td>18</td>
<td>AgNO₃</td>
<td>0.01</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>0.00094</td>
<td>4</td>
<td></td>
<td>0.002</td>
<td>13</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.0089</td>
<td>11</td>
<td></td>
<td>0.001</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>0.00089</td>
<td>6</td>
<td></td>
<td>0.0001</td>
<td>4</td>
</tr>
<tr>
<td>GPC</td>
<td>0.0007</td>
<td>2</td>
<td>KCN</td>
<td>0.01</td>
<td>97</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>0.05</td>
<td>51</td>
<td></td>
<td>0.0002</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>36</td>
<td></td>
<td>0.0001</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>0.0001</td>
<td>21</td>
<td></td>
<td>0.00002</td>
<td>0</td>
</tr>
</tbody>
</table>

* Copper, silver, and cyanide ions were incubated with lysophospholipase for 30 minutes at 30° before addition to the substrate. During this time their concentration was 11 times that indicated in the table.

the gel formation which occurred after some 60 per cent of the lysophospholipides were decomposed.

Francioli (14) reported the complete inhibition by physostigmine chloride of a comparable enzyme found in wasp venom. In our experiments physostigmine sulfate did not inhibit in a concentration as high as 1.0 mg. per ml., which was some 50 times greater than the concentration employed by Francioli. Since neither physostigmine chloride nor wasp venom was available to us for direct test, no explanation of this discrepancy in results can be advanced.

DISCUSSION

The name lysophospholipase implies a certain specificity of the enzyme for lysophospholipides. Contardi and Ercoli (5) and Francioli (14), after examining the corresponding enzyme derived from rice bran and embryos,
Aspergillus, and wasp venom, concluded that phospholipides also served as substrate. The former workers, however, did not demonstrate in their preparations the absence of a true phospholipase. The results of Francioli, on the other hand, were based upon evidence acquired through the use of physostigmine as a lysophospholipase inhibitor. Our preparations, which were many times more active than those hitherto described, were completely inactive with respect to lecithins and cephalins, nor was it possible to demonstrate any inhibition of the reaction by physostigmine. Lysophospholipase from Penicillum may be regarded, therefore, as being specific for lysophospholipides.

Failure of Penicillium extracts to liberate inorganic phosphate from lysophospholipides or GPC was due not to lack of the acid phosphatase abundantly present in molds, but to a deficiency of GPCase, without which glycerophosphate could not be formed. Extracts of Aspergillus oryzae, prepared in the laboratory or obtained commercially, contained considerable GPCase.

The lysophospholipase preparation used was relatively pure, i.e., free of dialyzable substances, and low in nitrogen content. Each reaction tube, as ordinarily prepared, contained only 0.010 to 0.016 mg. of nitrogen added as enzyme. Efforts directed towards further purification of lysophospholipase should be rewarding.

**SUMMARY**

Lysophospholipase, a highly active enzyme specific in its action for lysophospholipides, was prepared from Penicillium notatum. The products of the reaction were saturated fatty acids and glycerylphosphorylcholine or glycerylphosphorylethanolamine (GPC).

Quantitative methods for determining the extent of the reaction were based upon the separation of residual lysophospholipides and liberated fatty acids from GPC by precipitation of the former with colloidal iron-magnesium sulfate mixture.

Lysophospholipase activity was determined, under prescribed conditions, by evaluation of the first order reaction constant.

The enzyme was readily inactivated by heat at a slightly alkaline reaction, by cyanide, and, less readily, by heavy metal ions. No activator was discovered. Optimum activity was at pH 4.0.

Grateful acknowledgment is made to Miss Katherine Justus for many of the analyses, and to Miss Eve Minovitch for the determinations of ethanolamine and serine nitrogen.

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6 By courtesy of Mr. F. F. Taylor, Takamine Laboratory, Inc., Clifton, New Jersey.
LYSOPHOSPHOLIPASE

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