LECITHINASE FROM BACILLUS ANTHRACIS

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When Bacillus anthracis is grown in egg yolk medium, a reaction occurs which is presumably due to lecinthinase (Colner, 1948; McGaughey and Chu, 1948; Chu, 1949). In liquid egg yolk medium the lecinthinase reaction is characterized by the formation of an opalescent suspension and flocculation of particles which rise to the top of the medium as a curd. A test such as this is usually referred to as the Nagler or lecitho-vitellin (LV) reaction (Macfarlane et al., 1941). The change in egg yolk broth is presumably due to the hydrolysis of lecithin. The enzymatic hydrolysis of lecithin results in the loss of its emulsifying properties and causes the separation of fatty substances in the egg yolk medium (Macfarlane and Knight, 1941).

Several strains of B. anthracis, both virulent and avirulent, were screened for lecinthinase activity on the basis of the egg yolk reaction. The most active of these were chosen for more detailed study of cell-free culture filtrates in attempts to isolate and determine some of the characteristics of the enzyme or enzymes involved in the egg yolk reaction. The results of some of these studies are presented in this paper.

MATERIALS AND METHODS

Cultures. Cell-free culture filtrates of virulent and avirulent strains of B. anthracis were used to obtain enzyme. Spore suspensions of each strain were prepared for use as inocula and were stored at -18 C. The avirulent strains M, R-1-NP, and Sterne (1937) were used for most of the experiments reported here. The virulent strains 99 and M36 were also tested. No correlation seemed to exist between the presence of lecinthinase and virulence of the organism since lecinthinase was present in both virulent and avirulent strains.

Cells were grown in 500-ml Erlenmeyer flasks containing 150 ml of nutrient broth (Difco) supplemented with 0.3 per cent yeast extract (Difco) at pH 6.9. Each flask was inoculated with approximately 1 × 10^8 spores and incubated at 37 C on a reciprocating shaker for 20 to 24 hr.

Enzyme preparation. The cultures from 12 to 16 flasks were centrifuged to remove most of the cells and the pooled supernatants were passed through a Seitz no. S-1 filter or a Selas porcelain candle. Filtration through the porcelain candle was much more rapid and convenient. The sterile filtrate was adjusted to pH 6.9 with HCl and brought to 75 per cent saturation with solid (NH₄)₂SO₄ in the cold. After standing for 2 hr, the solution was centrifuged and the supernatant was discarded. The precipitate was dissolved in 60 to 70 ml of cold, distilled water and one-half volume of saturated (NH₄)₂SO₄ solution was added. After standing 1 hr in the cold, the solution was centrifuged at 12,000 × G for 15 min to remove a dark precipitate. The supernatant was dialyzed against slowly running tap water in the cold for 24 hr, then against distilled water until the dialyze was free of (NH₄)₂SO₄.

Enzyme activity was preserved by lyophilization or dialysis against 50 per cent glycerol (Chu, 1949). Lyophilized preparations were used for the experiments reported here.

Substrates. Egg yolk broth was prepared according to the method of McGaughey and Chu (1948) and contained 5 per cent fresh egg yolk in nutrient broth. Partially purified, lecithin-containing substrates from vegetable (soybean) and egg sources were obtained from Nutritional Biochemicals Corporation. Neither of these lecithins was pure but both were mixtures of phosphatides containing mainly phosphatidylcholine (lecithin) and phosphatidylethanolamine (cephalin). For use in enzyme-substrate reaction mixtures, stable aqueous emulsions of the lecithins were prepared by treatment in a 10 kc Raytheon sonic oscillator for 15 min (Hayaishi, 1955). The lecithin emulsions contained Thiomerosal, N.F. (Lilly) 1:10,000 as a preservative which did not inhibit enzyme activity. The preparation of lecithin-emulsion substrate in this manner gave fairly consistent results from batch to batch.

Enzyme activity assay. Enzyme solution, sub-

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strate, CaCl₂, and 0.05 M barbital buffer at pH 7.6, varied as indicated in the results, were incubated in a water bath at 37°C for 3 hr. After incubation, cold 25 per cent trichloroacetic acid was added to a final concentration of 5 per cent in order to terminate the reaction and precipitate unhydrolyzed lecithin. The mixture was allowed to stand 30 min at room temperature and was filtered by gravity through Schleicher and Schuell no. 602 ED filter paper. Samples of the clear filtrate were assayed for total acid-soluble phosphorus by the method of King (1932) modified by employing sulfuric acid and hydrogen peroxide rather than perchorlic acid for digestion. All results were corrected for the small amount of acid-soluble phosphorus found in filtrates from substrates incubated without enzyme.

Chromatography. Paper chromatography of the phosphorus-containing hydrolytic products of enzyme action on lecithin was performed according to the method of Huennekens et al. (1954). The method of Lea and Rhodes (1954) and Lea et al. (1955) was used for purification of soybean lecithin by silicic acid column and paper chromatography of column fractions. Authentic standards of phosphorylecholine and phosphorylethanolamine were synthesized by the procedures of Baer (1947) and Outhouse (1936).

A synthetic, saturated lecithin (1α-dimyristoyl lecithin) was obtained from the LaMotte Chemical Products Co. for use as a reference standard for chromatography.

Samples of acid-soluble hydrolytic products were prepared for chromatography by vigorous shaking of the trichloroacetic acid filtrates twice with 1 volume of chloroform followed by extraction of the aqueous phase with 1 volume of ether. These extractions served to remove any remaining lecithin and diglyceride end products as well as most of the trichloroacetic acid. The aqueous phase was dried in vacuo over calcium chloride. The residue was dissolved in a minimum quantity of distilled water for spotting the chromatogram. The best results were obtained with an acidic sample (pH 5 to 6).

Phosphorus-containing hydrolytic products were located on chromatograms with the acid molybdate spray of Hanes and Isherwood (1949) followed by irradiation with ultraviolet light (Bandurski and Axelrod, 1951). Amino compounds were located with 0.5 per cent ninhydrin in butanol. Nitrogen-containing phospholipids were stained with the phosphomolybdic acid reagent of Levine and Chargaff (1951). The nitrogen content of lecithin samples and hydrolytic products was determined with a micro-Kjeldahl method.

Manometric method. The manometric method of measuring carbon dioxide released from sodium bicarbonate buffer as a result of lecithinase activity was essentially the same as that reported by Zamecnik et al. (1947). For phosphorus determination, cold trichloroacetic acid was added to the flask contents and samples were taken from the filtrate.

Estimation of ester bonds in lecithin. The number of ester bonds in the purified lecithin substrate was determined by the method of Rapport and Alonzo (1955). Ferric hydroxamic acid derivatives of lecithin were prepared and their molar extinction (530 μM) calculated based on the total amount of phosphorus present. Assuming a molar extinction value (E₅₃₀) of 1000 for each fatty acid ester bond based on the total phosphorus content, the theoretical value for lecithin would be 2000.

Gelatinase determination. The presence of gelatinase was determined by incubating 0.1 ml of an enzyme sample on X-ray film in a moist atmosphere at 37°C (Block et al., 1955). After incubation overnight, the X-ray film was carefully washed in cold tap water. Where gelatin hydrolysis occurred, the emulsion was easily washed off the film leaving a pit in the area where the drop of enzyme solution was located.

RESULTS

Egg yolk activity. No activity was observed in 5 ml of egg yolk broth when 2 to 5 ml of culture filtrate was used. However, after concentration by ammonium sulfate precipitation and lyophilization, the equivalent of 10 ml of original filtrate caused turbidity in egg yolk broth in 3 to 4 hr and formation of a fatty layer in 18 to 24 hr. This activity was completely lost when solutions of the concentrate were boiled.

Lecithinase activity. Lecithinase activity was determined with substrates from various sources including one sample of egg lecithin which had been purified further on an alumina column (Hanahan et al., 1951). The data in table 1 show that under the conditions used, only lecithin of soybean origin was hydrolyzed to yield significant quantities of acid-soluble phosphorus. No inorganic phosphorus or free choline was detectable. The enzyme preparation alone
contained no detectable amount of phosphorus and heated enzyme (100 C for 15 min) was totally inactive. Since only soybean lecithin was hydrolyzed, the subsequent experiments were performed with this substrate unless stated otherwise. A more detailed study of the soybean lecithin hydrolysis was undertaken in an attempt to explain the apparent substrate specificity.

The effect of substrate concentration on the release of acid-soluble phosphorus from soybean lecithin is shown in table 2. The enzyme was saturated by approximately 40 mg (50 μmoles) of substrate since further increase in concentration indicated no increase in the release of acid-soluble phosphorus. No inhibition of activity was observed with higher concentrations of substrate.

Figure 1 shows the effect of calcium concentration on lecithinase activity. There was stimulation of activity to a concentration of 5 X 10^{-3} M CaCl₂, then a slight inhibition as the concentra-

\[ \text{Total Acid-Soluble Phosphorus Released} \]

<table>
<thead>
<tr>
<th>Incubation Time</th>
<th>Soybean lecithin</th>
<th>Ovolecithin</th>
<th>Beef brain lecithin</th>
<th>Purified ovolecithin</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
<td>µg</td>
</tr>
<tr>
<td>244</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Experimental conditions: Lyophilized enzyme, 5 mg; emulsified lecithin, 10 mg; CaCl₂, 5 X 10^{-3} M; barbital buffer, pH 7.6, to a total volume of 3.0 ml.

\[ \text{Effect of substrate concentration on the liberation of acid-soluble phosphorus from soybean lecithin} \]

<table>
<thead>
<tr>
<th>Lecithin (mg)</th>
<th>Acid-Soluble Phosphorus Released (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>86</td>
</tr>
<tr>
<td>40</td>
<td>117</td>
</tr>
<tr>
<td>60</td>
<td>110</td>
</tr>
<tr>
<td>80</td>
<td>114</td>
</tr>
<tr>
<td>100</td>
<td>124</td>
</tr>
</tbody>
</table>

Experimental conditions: Lyophilized enzyme, 2 mg; emulsified lecithin as indicated; CaCl₂, 3 X 10^{-3} M; barbital buffer, pH 7.0, to a total volume of 3.0 ml; incubated for 3 hr at 37 C.

Figure 1. Effect of calcium concentration on the activity of lecithinase from Bacillus anthracis. Duplicate tubes for each calcium level contained: Lyophilized enzyme, 2.0 mg; emulsified soybean lecithin, 40 mg; barbital buffer, pH 7.6; total volume, 3.0 ml; incubated for 3 hr at 37 C.

The pH range for lecithinase activity was broad with an optimum at pH 7.4 to 7.6. Figure 2 shows that there is measurable activity from pH 5.5 to 8.5 and that the activity decreases more sharply above the optimum than below it. Michaelis buffers of the same ionic strength were used at each pH value. Buffers which would chelate or form insoluble salts with calcium had to be avoided because of the calcium requirement of the system. Tris(hydroxymethyl)-aminomethane (Tris) or Tris-maleate buffers at the same pH were inhibitory.

The lecithinase from B. anthracis was relatively stable to heat. Figure 3 shows the heat stability of the enzyme in 0.05 m barbital buffer, pH 7.6, at three temperatures. Twenty-five per cent of the activity remained after heating at 60 C for 2 hr,
Table 3
Effects of various cations on the activity of lecithinase from Bacillus anthracis

<table>
<thead>
<tr>
<th>Cation</th>
<th>Acid-Soluble Phosphorus Released (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>40</td>
</tr>
<tr>
<td>Ca++</td>
<td>240</td>
</tr>
<tr>
<td>Ca++ + 0.005 M citric acid</td>
<td>32</td>
</tr>
<tr>
<td>Ca++ + 0.005 M NaF</td>
<td>264</td>
</tr>
<tr>
<td>Mg++</td>
<td>288</td>
</tr>
<tr>
<td>Mn++</td>
<td>208</td>
</tr>
<tr>
<td>Ba++</td>
<td>176</td>
</tr>
<tr>
<td>Co++</td>
<td>20</td>
</tr>
<tr>
<td>Cu++</td>
<td>0</td>
</tr>
</tbody>
</table>

Experimental conditions: Lyophilized enzyme, 1 mg; emulsified soybean lecithin, 40 mg; barbital buffer, pH 7.6; total volume 3.0 ml; incubated for 3 hr at 37°C.

Figure 2. Effect of pH on the activity of lecithinase from Bacillus anthracis. Duplicate tubes at each pH level contained: Lyophilized enzyme, 2.0 mg; emulsified soybean lecithin, 40 mg; CaCl₂, 5 × 10⁻³ M; Michaelis buffer at all pH values; total volume, 3.0 ml; incubated for 3 hr at 37°C. Controls containing substrate alone at each pH level showed no nonenzymatic hydrolysis.

whereas at 100°C, activity was completely lost in 5 min. The enzyme was stable in 0.05 M barbital buffer at pH 7.6 for 2 months at 5°C.

Lecithinase activity was followed manometrically as well as by determination of the

Figure 3. Effect of heat at pH 7.6 on the activity of lecithinase from Bacillus anthracis. Lyophilized enzyme in barbital buffer at pH 7.6 was heated in a water bath for the indicated time then cooled immediately to 37°C before adding it to the substrate. All tubes were incubated 4 hr from the time of adding enzyme to substrate. Duplicate tubes contained: Lyophilized enzyme 2.0 mg; emulsified soybean lecithin, 40 mg; CaCl₂, 5 × 10⁻³ M; barbital buffer, pH 7.6; total volume 3.0 ml.

Figure 4. Comparison of two methods used to determine the activity of lecithinase from Bacillus anthracis. The solid line represents manometric measurement of CO₂ evolved from bicarbonate buffer as a result of lecithinase activity. The broken line represents the total acid-soluble phosphorus released, calculated as equivalents of CO₂ in µL. The side arms contained 2.0 mg of lyophilized enzyme in 0.5 ml of distilled water. The flask contained purified soybean lecithin, 10 mg; NaHCO₃, 2.3 × 10⁻³ M; total liquid volume, 2.5 ml; pH 7.6 in an atmosphere of 5 per cent CO₂ : 95 per cent N₂; incubated at 37°C.
release of acid-soluble phosphorus as shown in figure 4. Based on the assumption that 1 mole of CO₂ was evolved from the NaHCO₃ buffer per mole of phosphorus released, a good correlation was obtained between the two methods. A conversion of 72.5 per cent of the total phosphorus to acid-soluble phosphorus was obtained. The soybean lecithin substrate used for this manometric study was purified by column chromatography.

Substrate purification. Paper chromatograms of enzyme reaction mixtures with crude soybean lecithin substrate revealed two major end products containing phosphorus. In order to identify these end products, purification of the crude substrate was necessary. This purification was accomplished satisfactorily with a silicic acid-celite column and CHCl₃:CH₃OH (4:1, v/v) solvent. The eluate contained two major nitrogen-containing phospholipid fractions. The compound in each fraction migrated as a single, well-defined spot on chromatograms run on silicic acid-impregnated paper (Lea et al., 1955).

The first fraction (I) from the column was presumed to be cephalin although no authentic compound was available with which to compare it. Fraction I reacted with ninhydrin and stained weakly with phosphomolybdic acid which is typical of cephalin. When incubated with enzyme, fraction I was hydrolyzed to give one major phosphorus-containing compound which reacted strongly with ninhydrin and migrated identically on paper chromatograms with an authentic phosphorylethanolamine sample. One other amino compound was observed but it did not contain phosphorus and was not identified.

The second major fraction (II) from the column migrated identically with an authentic lecithin sample on paper chromatograms. Fraction II did not react with ninhydrin and had an N:P molar ratio of 1.01. Carboxyl ester determinations of fraction II based on the molar extinction of ferric hydroxamic acid derivatives indicated that two ester bonds were present. The molar extinction value based on phosphorus (E₂₅) for fraction II was 2409 which approaches the theoretical value of 2000 for lecithin. When the lecithin fraction (II) was incubated with enzyme, a single phosphorus-containing end product was obtained which was subsequently identified as phosphorylethanolamine.

The diglycerides resulting from lecithin and cephalin hydrolysis were not isolated or identified since the identification of the phosphorus-containing end product from purified substrate was sufficient to establish the point of hydrolysis.

Identification of hydrolytic end products. The phosphorus-containing enzymatic hydrolysis products from purified lecithin and cephalin were identified by paper chromatography as phosphorylcholine and phosphorylethanolamine, respectively. Evidence for the identification of phosphorylcholine is shown in figure 5. Synthetic phosphorylcholine and the unknown compound from lecithin hydrolysis behaved identically on paper chromatograms developed descending for 17 hr in 85 per cent ethanol. When the authentic sample and the unknown were hydrolyzed in 6 N HCl for 2 hr in an autoclave at 122 C, both compounds were split to yield inorganic phosphorus and choline. The choline resulting from acid hydrolysis was chromatographically identical with authentic choline as can be seen in figure 5. Inorganic phosphorus does not show up on the chromatogram.

The identification of phosphorylethanolamine as the phosphorus-containing compound from enzymatic cephalin hydrolysis was done in much the same way as the phosphorylcholine identification. The unknown compound migrated identically with an authentic phosphorylethanolamine sample on descending paper chromatograms developed for 17 hr in 85 per cent ethanol. The unknown and the authentic samples were hydrolyzed by adding an equal volume of concentrated HCl to each and drying over NaOH in vacuo at room temperature. Acid hydrolysis split the compounds to inorganic phosphorus and ethanolamine. The ethanolamine from acid hydrolysis of the known and unknown compounds was chromatographically identical with an authentic ethanolamine sample. The Rf values for the compounds and their acid hydrolysis products are shown in table 4. A known serine sample was included in the chromatogram to rule out the presence of phosphatidylserine as the nitrogen-containing phospholipid and in turn, phosphorylserine as an enzymatic hydrolysis product. No compound matching chromatographically with serine was observed.

Activity on egg lecithin. Although it had been established that the enzyme being studied had lecithinase activity on soybean lecithin, the question still remained as to why there was no activity on lecithin, pure or crude, derived from
Figure 5. Identification of phosphorylcholine. From right to left the compounds are: Synthetic phosphorylcholine standard (SPC); phosphorylcholine from enzymatic hydrolysis of lecithin (EPC); synthetic choline standard (SC). The last two compounds are the choline resulting from the acid hydrolysis of SPC and EPC. The inorganic phosphorus resulting from acid hydrolysis does not show on the chromatogram.

egg yolk. It was standard procedure to add CaCl₂ to any enzyme-substrate test mixture since the requirement for a divalent cation such as calcium or magnesium was well known. If CaCl₂ was not added to the test system, egg lecithin was slowly hydrolyzed by the enzyme to give phosphorylcholine as a hydrolytic product. Chromatographic comparison of crude egg lecithin with crude soybean lecithin revealed no major qualitative differences. Neither purified egg lecithin nor the crude sample was hydrolyzed in the presence of the divalent cation. In the absence of the divalent cation the reaction proceeded slowly, much the same as the egg yolk broth reaction. The data shown in table 5 are typical for egg lecithin hydrolysis. The reason for the difference in activity between egg and soybean lecithin substrate is still not known but it might help to explain why B. anthracis does not give a rapid egg yolk broth reaction.

Gelatinase activity. It was found that the lecithinase preparation also had gelatinase activity. Attempts to separate the two activities by (NH₄)₂SO₄ or ethanol fractionation were unsuccessful. However, the presence of gelatinase did not interfere with soybean lecithin hydrolysis and did not decrease the enzyme stability in solution. Concentrations of ethylenediaminetetraacetic acid, disodium salt (EDTA) which inhibited lecithinase had no effect on the gelati-
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TABLE 4
Identification of phosphorylethanolamine by paper chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Rf</th>
<th>Amino compounds</th>
<th>Phosphorus-containing compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolamine standard</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine standard</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorylethanolamine from enzymatic hydrolysis of cephalin</td>
<td>0.21*</td>
<td>0.21*</td>
<td></td>
</tr>
<tr>
<td>Acid hydrolyzate of standard phosphorylethanolamine</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid hydrolyzate of P-containing end product of cephalin hydrolysis</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chromatograms of acid hydrolyzates were developed ascending for 24 hr in 76 per cent aqueous phenol. Amino compounds were located with 0.5 per cent ninhydrin in butanol. Acid molybdate spray was used to locate phosphorus-containing compounds.

* The Rf values for phosphorylethanolamine and the unknown compound were obtained from papers developed descending in 85 per cent ethanol.

TABLE 5
Effect of various additives on the activity of lecithinase from Bacillus anthracis with ovolecithin

<table>
<thead>
<tr>
<th>Additive</th>
<th>Total Acid-Soluble Phosphorus Released (µg/tube)</th>
<th>Inhibition of Maximum Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Ca++</td>
<td>85</td>
<td>57</td>
</tr>
<tr>
<td>Mg++</td>
<td>115</td>
<td>42</td>
</tr>
<tr>
<td>K+</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>Ca++, Mg++, K+</td>
<td>85</td>
<td>57</td>
</tr>
<tr>
<td>Fe**</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine (0.001 M)</td>
<td>210</td>
<td>0</td>
</tr>
</tbody>
</table>

Experimental conditions: Lyophilized enzyme, 2 mg/tube; emulsified ovolecithin, 20 mg/tube; barbital buffer pH 7.6; total volume 4.0 ml; incubated 21 hr at 37 C. Salts were supplied as chlorides in a final concentration of 5 X 10^{-7} M.

The lecithinase produced by B. anthracis released phosphorylcholine and phosphorylethanolamine from purified lecithin and cephalin of soybean origin. For this activity a divalent cation such as calcium or magnesium was required. The same enzyme preparations gave an egg yolk broth reaction but did not hydrolyze free egg lecithin when CaCl2 was present. Conversely, the hydrolysis of free egg lecithin proceeded in the absence of CaCl2 but more slowly than that of soybean lecithin with CaCl2. The same phosphorus-containing end product was observed from the enzymatic hydrolysis of lecithin from both sources. The slow hydrolysis of isolated egg lecithin by B. anthracis lecithinase suggests an explanation for the slow egg yolk broth reaction which is typical of this organism. The complexity of egg yolk, however, makes it very difficult for one to draw sound conclusions based on reactions observed with isolated components. Closely related species such as Bacillus cereus give rapid egg yolk broth reactions and have a more active lecithinase. From the data obtained on the general characteristics of B. anthracis lecithinase, it seemed to be similar to the lecinthinases of B. cereus and Clostridium perfringens (α toxin) (Chu, 1949; Hanahan and Vercamer, 1954) with regard to heat stability and pH range for activity. The activity of B. cereus lecithinase on egg lecithin was also inhibited by the addition of CaCl2 but some hydrolysis occurred even in its presence.

The possibility that lecithinase has a role in the disease of anthrax has not been extensively investigated. Zwartouw and Smith (1956) stated that B. anthracis culture filtrates hydrolyzed phospholipid in egg yolk broth but did not hydrolyze free egg lecithin and that the phospholipase was not anthrax toxin. Preliminary toxicity tests performed with lecithinase from an avirulent strain have indicated that B. anthracis lecithinase was nontoxic to mice and that it may not be a contributing factor in the damage of
host tissue in anthrax. Sheep and rabbit red blood cells were hemolyzed in vitro by B. anthracis lecithinase and hemolytic activity was completely inhibited by ethylenediaminetetraacetic acid. It was tentatively concluded that lecithinase and not gelatinase was responsible for hemolysis since ethylenediaminetetraacetic acid inhibited hemolysis but did not affect gelatin hydrolysis.

It is known that lecithin can be split enzymatically at four different positions in the molecule presupposing the existence of four different lecithinases. One of these lecithinases hydrolyzes the ester bond between fatty acid and the α carbon of the glycerol moiety. Another enzyme hydrolyzes the ester bond between fatty acid and the β carbon of glycerol. A third hydrolyzes the phosphate ester bond at the α' carbon of glycerol. The fourth enzyme hydrolyzes the nitrogenous base (choline) from the molecule leaving a phosphatidic acid.

Only one of the enzymatic reactions on lecithin was observed in lecithinase preparations from B. anthracis, i.e., the removal of an intact phosphorylated base from the lecithin molecule. This is supported by the good correlation between data obtained manometrically and by acid-soluble phosphorus determination. The identification of phosphorylecholine and phosphorylethanolamine from the enzymatic hydrolysis of lecithin and cephalin is evidence for this conclusion. The presence of other lecithinases was unlikely since the phosphorylecholine moiety of lecithin was removed intact and a mole for mole correlation was obtained between acid-soluble phosphorus released and CO₂ evolved from bicarbonate buffer. No inorganic phosphorus or free choline was found indicating that the phosphorylated base was not hydrolyzed by the enzyme. This enzyme was not specific for lecithin but would remove phosphorylethanolamine from cephalin.

ACKNOWLEDGMENTS

The capable assistance of Mr. S. Marvin Friedman and Mr. Dale Van Donsel during the course of this study is gratefully acknowledged. The author is also indebted to Dr. Curtis B. Thorne for helpful suggestions and encouragement.

SUMMARY

A lecithinase preparation obtained by (NH₄)₂SO₄ precipitation of cell-free culture filtrates of Bacillus anthracis is described. Lyophilized enzyme preparations gave a typical lecithinovitellin reaction in egg yolk broth and hydrolyzed purified soybean lecithin. A divalent cation was required for activity on soybean lecithin. Egg lecithin was more slowly hydrolyzed than soybean lecithin and was inhibited by the addition of divalent cation. The enzyme apparently was not specific for lecithin but would slowly hydrolyze soybean cephalin as well.

The point of cleavage of the lecithin molecule was established by identification of the phosphorus-containing end product of enzymatic hydrolysis. The end products of soybean lecithin and cephalin hydrolysis were identified by paper chromatography as phosphorylecholine and phosphorylethanolamine, respectively.

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