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Non-identity of the Phospholipase of *Bacillus anthracis* with the Anthrax Toxin

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SUMMARY: Filtrates from suitable cultures of *Bacillus anthracis* contain a phospholipase which slowly hydrolyses the phospholipid in egg-yolk broth but has no action on free egg-lecithin. This enzyme does not appear to be the anthrax toxin.

McGaughey & Chu (1948) showed that *Bacillus anthracis* produced an opalescence when growing in a medium containing egg yolk, but the effect was less than that produced by *B. cereus* and *B. mycoides*. *B. anthracis* growing in the blood of infected guinea-pigs produces an extracellular toxin which kills the host in secondary shock (Smith, Keppie, Stanley & Harris-Smith, 1955; Smith, Keppie & Stanley, 1955); a lethal amount of this toxin is only produced by the growth of a large number of organisms in the blood during the terminal phase of anthrax. Lecithinases, responsible for the egg-yolk reaction of certain bacteria, are involved in their toxigenicity (MacFarlane, 1955) and when injected can produce the symptoms of shock (Berg, Levinson & Wang, 1951). A possible relationship between the egg-yolk reaction and the toxin of *B. anthracis* has therefore been investigated. First, the egg-yolk reaction was examined to see whether the opalescence produced by *B. anthracis* was due to an extracellular product of the organism and whether this product was a lecithinase. Then, the toxic plasma of infected guinea-pigs was investigated for the presence of such an enzyme and for a connexion between it and the toxic action.

METHODS

Organism. A spore suspension of *Bacillus anthracis*, strain N.P., as used in all previous work (Smith *et al.* 1955) was the source of organisms.

Egg-yolk broth (E.Y.B.). Two egg yolks/l. 1% peptone solution containing 0.5% NaCl and at pH 7.4.

Plasma containing the anthrax toxin. This was collected from guinea-pigs dying of anthrax as described by Smith *et al.* (1955).

Anthrax antiserum. This was prepared in the horse by injecting the 'Sterne' strain of *Bacillus anthracis* (Belton & Strange, 1954).

Test for phospholipase activity. A sterile mixture of the solution to be tested (2 ml.) and E.Y.B. (2 ml., containing c. 50 μ g. ether-soluble P) was incubated at 37° and pH 7.4. After cooling the mixture, it was extracted with ether (3 ml.) and the P content of the ethereal extract determined by the method of Fiske & Subbarow (1925). Activities are expressed as the percentage decrease in the ether-soluble P produced by the test materials in a stated time. Controls incubated for 72 hr. at 37° did not show a decrease in ether-soluble P.

RESULTS

The egg-yolk reaction with the growing organism

E.Y.B. was inoculated with *Bacillus anthracis* and incubated at 37° for 4 days. The culture was then opalescent; centrifugation deposited the bacteria and produced a layer of fat on top of the clear medium. In three experiments the ether-soluble P of the medium was decreased 90 % by the growth of the organism.

Culture filtrates with phospholipase activity

Preparation. Media (E.Y.B., 1 % peptone or tryptic meat broth; 100 ml.) were inoculated with 6×10^7 spores and incubated at 37° and pH 7.4 in a flask (250 ml.) for 2 days. After filtration through 'Millipore' filters (Lovell Chemical Co., Mass., U.S.A.) the sterile filtrate was stored at 0°.

Selection of medium. Table 1 shows the results of tests for phospholipase activity on filtrates from cultures of *Bacillus anthracis* in E.Y.B., 1 % peptone and tryptic meat broth. Filtrates from cultures in E.Y.B. and 1 % peptone were consistently active but cultures in tryptic meat broth T.M.B. had a much smaller and variable activity. Filtrates from cultures in 1 % peptone were used in the following work.

Table 1. *Phospholipase activity of culture filtrates from different media*

Culture medium		Phospholipase activity*	
		Decrease (%) in ether soluble P during	
		24 hr.	48 hr.
E.Y.B.	(1)	61	81
	(2)	83	90
Peptone	(1)	90	95
	(2)	79	90
T.M.B.	(1)	6	9
	(2)	14	34

* For details of test for phospholipase activity see text.

Activity. Table 2 shows the slow progressive decrease with time of the ether-soluble P of E.Y.B. when the culture filtrate was examined in the test for phospholipase activity.

Effect of pH. In 8 hr. a culture filtrate decreased the ether-soluble P of E.Y.B. by 10, 14, 35, 35 and 18 % when examined at values of pH 5.6, 6.7, 7.4, 8.0 and 8.8, respectively, in the test for phospholipase activity.

Effect of calcium. The mixture of E.Y.B. and peptone culture filtrate used in the phospholipase test contained *c.* 0.001 M-Ca⁺⁺. Increase of the Ca⁺⁺ concentration to 0.003, 0.006, 0.01 and 0.05 M did not increase the phospholipase activity, although addition of a high concentration of sodium citrate (0.1 M) decreased it.

Stability. There was no loss in phospholipase activity on storage at 0–4° for several weeks. No appreciable loss in activity occurred after heating the culture filtrate to 60° for 1 hr., but heating at 100° for the same period destroyed the activity. After shaking with ballotini beads and air for 16 hr. at 0–4° the activity was decreased about 50 %.

Table 2. *The progressive decrease of ether soluble P when a peptone culture filtrate was incubated with egg-yolk broth at pH 7.4 and 37°*

Time of incubation (hr.)	Decrease (%) of ether soluble P
4	9
8	26
12	47
16	53
18	66
21	76
24	79
32	88
41	98

Specificity. Active culture filtrates did not liberate inorganic phosphate from solutions (0.1 %) of sodium α - and β -glycerophosphates or adenylic acid. Sodium monophenyl phosphate was slowly hydrolysed; under the conditions of the test for phospholipase activity in which the E.Y.B. was replaced by a solution (0.1 %) of sodium monophenyl phosphate, the P set free as inorganic phosphate in 72 hr. (about 15 % of the total P) was far less than the decrease of ether-soluble P in a normal phospholipase test.

The culture filtrates did not hydrolyse either lecithin (egg) or cephalin (sheep brain). The tests were carried out as in the test for phospholipase activity, i.e. at pH 7.4 and 37° for 48 hr. with E.Y.B. replaced by aqueous emulsions (0.2 %) of lecithin or cephalin. The addition of Ca^{++} (0.002 M and 0.005 M) did not affect hydrolysis of the lecithin. No haemolysis or increase in fragility of guinea-pig erythrocytes was produced when they were incubated with culture filtrates for 24 hr. at 37°.

Table 3. *The effect of anthrax antiserum on phospholipase activity*

Material	Phospholipase activity	
	Decrease (%) in ether soluble P during 24 hr.	
	No addition	With antiserum (0.3 ml.)*
Control of normal plasma (0.3 ml.)	8	19
Toxic plasma (0.3 ml.)	98	100
E.Y.B. culture filtrate (2 ml.)	91	98
Peptone culture filtrate (2 ml.)	82	95

* This quantity of antiserum neutralized 9 ml. of toxic plasma (Smith, Keppie & Stanley, 1955).

Absence of a connexion between the phospholipase and the anthrax toxin

The active culture filtrates did not produce oedema when injected into the skin of a guinea-pig as did the toxic plasma of guinea-pigs dying of anthrax. The toxic plasma had a higher phospholipase activity than the non-toxic culture filtrates; 0.3 ml. of toxic plasma had approximately the same activity as 2 ml. of culture filtrate. However, Table 3 shows that neither the phospholipase activity of the toxic plasma nor that of the culture filtrates was inhibited by an amount of anthrax antiserum which was in excess of that required to completely neutralize the toxic action of the plasma.

DISCUSSION

McGaughey & Chu (1948) could not detect an enzyme responsible for the egg-yolk reaction in culture filtrates of *Bacillus anthracis*. Contrary to this, we have found that filtrates from cultures in suitable media do contain such an enzyme, although the activity is small. The enzyme did not attack purified egg-lecithin. The hydrolysis of phospholipid by the enzyme may be dependent on the 'combined' state of the substrate in E.Y.B. It is relevant to point out here that lecithinase preparations from *Bacillus cereus* and *Clostridium welchii*, which hydrolysed equal amounts of free phospholipid, differed greatly in their ability to hydrolyse lipovitellenin (Chu, 1949). The phospholipase of *B. anthracis* resembles bacterial lecithinases in being fairly stable to heat and sensitive to surface denaturation.

Plasma containing the anthrax toxin also contains a phospholipase, but the two are probably not connected since anthrax antitoxin did not inhibit the phospholipase activity of the toxic plasma or of culture filtrates. In the case of lecithinase preparations from *Clostridium welchii* and *Bacillus cereus*, both the egg-yolk reaction and the toxicity are inhibited by the same homologous antiserum (MacFarlane, 1955; Chu, 1949).

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