AN EVALUATION OF THE EGG-YOLK REACTION AS A TEST FOR LECITHINASE ACTIVITY

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Received for publication July 2, 1956

Bacterial lecithinases are of special interest because of the possible role these enzymes play in pathogenicity (reviewed by Macfarlane, 1955, and Van Heyningen, 1950, 1955). In studying lecithinase distribution and function, the egg-yolk reaction, so called because lecithinase acting on an egg-yolk emulsion causes turbidity to appear and a curd of fat to rise to the surface (Macfarlane et al., 1941; Macfarlane and Knight, 1941), has been widely used, both taxonomically (Colmer, 1947; McClung and Toabe, 1947; McGaughey and Chu, 1948; Knight and Proom, 1950; Toumanoff, 1953; Smith, 1955; and others) and for quantitative measurements of lecithinase activity (Van Heyningen, 1941; Chu, 1949). Heimpel (1955) used the egg-yolk reaction to study the relationship between bacterial production and pathogenicity for insects of different strains of Bacillus cereus. In connection with further studies of this problem, the validity of the egg-yolk reaction as a quantitative measure of lecithinase activity was examined. The results obtained are reported here.

Preparations possessing lecithinase activity have been found, thus far, to give the egg-yolk reaction, and there is little doubt that lecithinase will produce turbidity in an egg-yolk emulsion. The lecithinase preparations used, however, have not in general been highly purified, so that this association of activity is not proof that both lecithin breakdown and turbidity production in egg-yolk saline are entirely caused by the same enzyme and that one process is a quantitative measure of the other. The relationship between turbidity production and lecithinase activity was investigated by studying the comparative heat stability of each process. Bacterial lecithinases are relatively heat-stable (Macfarlane and Knight, 1941; Chu, 1949), and if both turbidity and lecithin breakdown are caused by the same enzyme, the heat stabilities of the two processes should be the same. If the processes are caused by different enzymes, however, some quantitative differences between their heat stabilities might be expected.

MATERIALS AND METHODS

Enzymes. Two lots of lecithinase from Clostridium perfringens were investigated, one a crude filtrate of C. perfringens Type A (Lederie Laboratories, Pearl River, N. Y.), and the other a purified Type A α-toxin (Wellcome Research Laboratories, Beckenham, Kent). The strains of B. cereus used were maintained on nutrient agar, and broth cultures were prepared by growing them in nutrient broth for 20 hr at 37 C. The bacteria were removed by centrifuging at 25,000 × G. For concentrating lecithinase from B. cereus, the method described by Chu (1949) was employed, using 30 L of a culture of strain A. The enzyme, stored in the cold after dialysis against 50 per cent glycerol, retained its activity for over 1 year.

Egg-yolk saline. The yolk of one egg was washed with saline and beaten up with 200 ml of either 0.2 or 0.9 per cent NaCl. After the heavier par

1 Contribution No. 314, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.
2 The term is used here to refer to the enzyme produced by C. perfringens and B. cereus, that splits lecithin into phosphoryl choline and a diglyceride. Other lecithinases have been reviewed by Zeller (1951).
3 In view of the work reported here, Dr. Heimpel and I have reexamined this relationship using specific chemical methods for lecithinase assay, and have reached essentially the same conclusions as before. These results are now being prepared for publication.
4 The strains of B. cereus, supplied by Dr. A. M. Heimpel, were isolated as insect pathogens of different degrees of pathogenicity (Heimpel, 1955). After maintenance in the laboratory some of their culture characteristics changed, and they are now arbitrarily called Strains A–F.
articles had been sedimented, the supernatant was passed through a Seitz filter and the filtrate stored in the cold. One ml of egg-yolk saline (0.2 per cent NaCl) contains 260 µg total phosphorus, consisting of 14 µg acid-soluble phosphorus, 242 µg lipid phosphorus, and 4 µg residual (nucleic acid and protein) phosphorus (analyzed by the method of Schneider (1945)).

Lecithin. Purified egg lecithin was prepared by the method of Hanahan et al. (1951); it contained 4.1 per cent phosphorus and had a phosphorus-to-choline molar ratio of 1.05:1.00.

Analytical method. Total phosphorus was determined, after ashing with H2SO4 and H2O2, by the method of Fiske and SubbaRow (1925).

Three processes were measured: the production of turbidity in egg-yolk saline, the breakdown of phospholipids of egg-yolk saline (phospholipase activity), and the hydrolysis of purified lecithin. With enzymes from C. perfringens and the enzyme treated with glycerol from B. cereus, turbidity production was measured by a modification of the method of Chu (1949): to the enzyme was added 0.05 M borate buffer (pH 7.1), 1.0 ml; 2 per cent NaCl, 0.55 ml; and water to a volume of 5.5 ml. One-half ml of the egg-yolk saline (0.2 per cent NaCl) was added; after 20 min at room temperature (23 to 25° C) the optical density was read at wave-length 650 µm against a blank made up in the same way but containing no enzyme. The enzyme preparations were not turbid. Addition of the 2 per cent NaCl was necessary, since mixing egg-yolk saline with water caused immediate precipitation, possibly of lipoproteins more soluble in saline than in water (Aklerton and Fevold, 1945; Fevold and Lausten, 1946).

With supernatant fluids from B. cereus, turbidity production was measured both by this method and by a method essentially the same as that described by Heimpel (1955): borate buffer (pH 7.1), 1.0 ml; culture supernatant, 6.0 ml; and egg-yolk saline (0.9 per cent NaCl), 0.5 ml, were mixed, incubated at 34 C for 30 min, and the optical density was measured at wave-length 575 mµ. Substances present in nutrient broth prevented extensive precipitation despite the absence of added NaCl.

The modified system of Chu was also used in measuring phospholipase action against egg-yolk saline. After incubation for 2 hr at 27 C, 1.0 ml of 20 per cent trichloroacetic acid was added, the precipitate centrifuged down, and the increase in total phosphorus in the supernatant was measured and compared to a control to which trichloroacetic acid had been added immediately after adding the egg-yolk saline. All acid-soluble phosphorus released from egg-yolk saline was found to come from the lipid phosphorus fraction. For the three bacterial enzymes, in the concentration range used, there was an approximately linear relationship between enzyme concentration and both turbidity production (measured in the modified Chu system) and acid-soluble phosphorus release.

Lecithinase from C. perfringens was measured nanometrically (Zameenik et al., 1947). Each Warburg vessel contained x NaHCO3, 0.2 ml; 0.1 M CaCl2, 0.1 ml; 8 per cent lecithin, 0.7 ml; and H2O, 0.5 ml; in the side arm was the enzyme, diluted with water to a volume of 0.5 ml. The vessels were gassed with 100 per cent CO2 and incubated at 27 C. After tipping in the contents of the side arm, CO2 production was followed for 1 hr. There was a direct proportionality between the amount of enzyme and CO2 production up to 0.2 ml of filtrate from C. perfringens and up to 0.4 mg of the purified α-toxin.

With enzymes from C. perfringens, activity remaining after heat treatment was calculated in percentages from appropriate enzyme concentration-activity curves, or as per cent of CO2 evolution remaining in the case of enzyme action against purified lecithin.

The enzymes from both C. perfringens and B. cereus are much more active against lecithin in egg yolk than against purified lecithin. This may be due to limited contact between the enzymes and the emulsion of purified lecithin. Kates (1953) found that plant plastid enzymes attack purified lecithin only if ether or certain other organic solvents are added. Hanahan and Vercamer (1954) observed that perfringens lecithinase can act on lecithin dissolved in 98 per cent ether-2 per cent alcohol. The activity of the small amounts of lecithinase produced by B. cereus could be measured only in the presence of added organic solvents. The reaction mixture consisted of 0.25 M borate buffer, pH 7.1, 0.1 ml; 0.1 M CaCl2, 0.05 ml; enzyme; water, to a volume of 1.5 ml; 2.5 per cent lecithin, 0.5 ml; toluene, 0.2 ml; and ethyl ether, 0.2 ml; in a glass-stopped tube. Incubation was carried out for 2 hr at 27 C. Two ml of 5 per cent trichloroacetic acid were added, and the contents of the tube cooled and filtered.
The filtrate was extracted three times with about 5 ml of ether, heated for 5 min at 55 to 60 C to drive off residual ether, and analyzed to determine the increase in acid-soluble phosphorus.

RESULTS

*Perfringens* enzymes. The filtrate and purified toxin from *C. perfringens* appear identical in heat stabilities of turbidity production, of lecithinase activity, and of phospholipase activity (table 1). Within the limits of experimental error, each heat treatment led to the same relative decrease in all three activities. In the culture filtrate, all processes showed remarkable heat stability. A curious finding was that heating the filtrate for 5 min at 60 C destroyed more of each activity than did heating for the same time at 100 C. This phenomenon was not observed in the purified toxin, the lecithinase activity of which was much more sensitive to 100 C than was that of the culture filtrate. Possibly a lecithinase-destroying enzyme, present in the filtrate but not in the purified toxin, was activated at 60 C and destroyed at 100 C.

These observations are consistent with, and lend additional support to, the belief that in *C. perfringens* preparations turbidity production in egg yolk, phospholipase action on egg yolk, and lecithinase activity are all caused by the same enzyme.

*Cereus* enzymes. In the case of the enzyme from *B. cereus*, treated with glycerol, turbidity production and lecithinase activity had markedly different heat stabilities. This is seen in the results given in table 2. Autoclaving completely destroyed both lecithinase and phospholipase activity. One ml of the unheated enzyme liberated 24 µg acid-soluble phosphorus from lecithin and 17.5 µg from egg-yolk saline in 2 hr, but after autoclaving liberated none. The autoclaved enzyme, however, still produced turbidity in egg-yolk saline. The appearance of turbidity was a continuous process in both heated and unheated enzymes.

A similar situation existed in supernatant fluids from cultures of *B. cereus*. In a number of strains, comparison was made of turbidity production by unheated and by autoclaved culture supernatants (table 2). Nutrient broth itself caused some turbidity to appear. After the growth of each strain, turbidity production was increased. Autoclaving of most culture supernatants reduced the turbidity below that produced by nutrient broth alone. In strain D, however, significant turbidity production remained after autoclaving, and the greater part of the high turbidity production of strain C was heat-stable. With the latter strain, turbidity appeared rapidly: nearly the maximum was reached in 2 min. In other strains, turbidity appeared in a slower and steadier fashion. Separate experiments

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**Table 1**

The effect of heat on the turbidity production, phospholipase activity, and lecithinase activity of a Clostridium *perfringens* filtrate and a purified α-toxin

<table>
<thead>
<tr>
<th>Material</th>
<th>Treatment*</th>
<th>Per Cent Activity Remaining†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Turbidity in egg yolk</td>
</tr>
<tr>
<td>Filtrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min at 60 C</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>5 min at 100 C</td>
<td>54</td>
<td>59</td>
</tr>
<tr>
<td>10 min at 100 C</td>
<td>48</td>
<td>46</td>
</tr>
<tr>
<td>20 min at 100 C</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>α-Toxin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min at 60 C</td>
<td>88</td>
<td>89</td>
</tr>
<tr>
<td>5 min at 100 C</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

* The heat treatment was applied to the undiluted filtrate and to the α-toxin in 1 mg/ml concentration. The enzymes were then diluted for assay.

† Figures represent the mean percentage residual activities of the following amounts of enzyme: Turbidity production, 0.02 and 0.04 ml filtrate, 10 and 20 µg α-toxin; acid-soluble phosphorus release from egg yolk, 0.01 and 0.02 ml filtrate, 5 and 10 µg α-toxin; lecithinase, 0.2 ml filtrate, 200 µg α-toxin (single determination in each experiment.)

Typical 100% activities:
- Turbidity produced by 0.02 ml filtrate
- Turbidity produced by 10 µg α-toxin
- Phospholipase activity of 0.02 ml filtrate
- Phospholipase activity of 10 µg α-toxin
- Lecithinase activity of 0.2 ml filtrate
- Lecithinase activity of 200 µg α-toxin

Conditions as described in the text.
TABLE 2

The effect of heat on turbidity production by Bacillus cereus enzymes

<table>
<thead>
<tr>
<th>Material</th>
<th>Method</th>
<th>Turbidity Produced in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20 min</td>
</tr>
<tr>
<td>Glycerinated enzyme</td>
<td>Chu (modified)*</td>
<td>0.244</td>
</tr>
<tr>
<td>Unheated</td>
<td></td>
<td>0.112</td>
</tr>
<tr>
<td>Autoclaved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture supernatants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (sterile nutrient broth)</td>
<td>Chu (modified)*</td>
<td>0.043</td>
</tr>
<tr>
<td>Strain A</td>
<td></td>
<td>0.132</td>
</tr>
<tr>
<td>Strain B</td>
<td></td>
<td>0.052</td>
</tr>
<tr>
<td>Strain C</td>
<td></td>
<td>0.078</td>
</tr>
<tr>
<td>Control (sterile nutrient broth)</td>
<td>Heimpel</td>
<td>0.159</td>
</tr>
<tr>
<td>Strain A</td>
<td></td>
<td>0.380</td>
</tr>
<tr>
<td>Strain B</td>
<td></td>
<td>0.192</td>
</tr>
<tr>
<td>Strain C</td>
<td></td>
<td>0.064</td>
</tr>
<tr>
<td>Strain D</td>
<td></td>
<td>0.258</td>
</tr>
<tr>
<td>Strain E</td>
<td></td>
<td>0.430</td>
</tr>
<tr>
<td>Strain F</td>
<td></td>
<td>0.199</td>
</tr>
</tbody>
</table>

* One ml of glycerinated enzyme, 2.0 ml of nutrient broth or 2.0 ml of culture supernatant were used in the modified method of Chu.
† The optical density of nutrient broth and of all the culture supernatants before the addition of egg-yolk saline was 0.050.

showed that autoclaving completely destroyed lecithinase and phospholipase activity in these supernatants.

The heat-stable component(s) that produces turbidity appears to be a large molecule, since prolonged dialysis (up to 6 days against running water) failed to remove it. It was destroyed by ashing with H₂SO₄ and H₂O₂. The turbidity produced by it was due partly to fat formation and partly to lipoprotein precipitation. After a time, fat globules rose to the top, although the final amount of fat produced was less than the definite curd appearing after true lecithinase action. A precipitate was also formed that, after being collected by centrifuging, was found to contain phosphorus; the phosphorus was soluble in ethanol but not in ether.

DISCUSSION

Chu (1949) found that the rate of liberation of acid-soluble P and the development of turbidity were almost parallel with lecithinase from *B. cereus*, and McGaughey and Chu (1948) observed that in several strains of *B. cereus* and *B. mycoides* yolk reactivity ran in parallel with the phospholipase activity. Macfarlane and Knight also showed that, in a number of Type A antisera from different sources, ability to inhibit the egg-yolk reaction paralleled ability to inhibit lecithinase activity. The results presented here add further indirect evidence to the existing indirect evidence that turbidity production in an egg-yolk emulsion may, in certain cases, be a quantitative index of lecithinase activity.

It is hardly surprising, however, that in such a complex mixture as egg-yolk saline a factor or factors other than lecithinase can also produce turbidity (see Van Heyningen, 1950, p. 31). The presence of such a factor in bacterial cultures may lead to serious errors if turbidity is assumed to be due to lecithinase activity alone. The degree of error would, of course, vary with the amount of lecithinase present. A given amount of the factor would produce less relative error in the presence of high than in the presence of low lecithinase activity.
Although the heat-stable factor may produce considerable turbidity, it does not lead to the formation of a curd of fat. Only preparations with lecithinase activity led to this curd formation. Curd formation, then, appears thus far to be a valid qualitative or semi-quantitative index of lecithinase activity.

The lack of complete specificity of the egg-yolk reaction in no way renders it valueless. Though a false positive result is possible, it is highly unlikely that a false negative one would be. Cultures possessing lecithinase activity may almost certainly be depended upon to give the egg-yolk reaction. With its simplicity and its great sensitivity, the reaction should still serve in screening a large number of organisms for lecithinase activity. Its value will be enhanced if organisms apparently possessing lecithinase activity are then reexamined, as was done here, by more specific chemical methods.

ACKNOWLEDGMENTS

This work was carried out with the careful technical assistance of Miss F. Elliott. I am indebted to Drs. J. F. Monroe and W. E. van Heyningen and Mrs. I. Batty for supplying the enzymes from _C. perfringens_ used in this study; to Dr. A. M. Heimpel for assistance in preparing the _B. cereus_ enzyme and for much helpful discussion; and to Drs. S. G. Smith and R. M. Belyea for help in preparing the manuscript for this paper.

SUMMARY

A test of the validity of the egg-yolk reaction as a measure of lecithinase activity was made by comparing the heat stabilities of (1) turbidity production in egg-yolk saline, (2) phospholipase activity against egg-yolk saline, and (3) lecithinase activity.

In a culture filtrate and a purified α-toxin of _Clostridium perfringens_ the three processes were found to possess quantitatively the same heat stability.

In a concentrated enzyme from _Bacillus cereus_ and in culture supernatants of certain _B. cereus_ strains, the phospholipase and lecithinase activities were much more sensitive to heat than was turbidity production. A highly heat-stable substance(s) was present that produced turbidity without having any lecithinase activity.

The egg-yolk reaction appears to be a quantitative measure of lecithinase activity in _C. perfringens_, but not in _B. cereus_. Care must be used in interpreting the egg-yolk reaction to avoid a false positive test for lecithinase activity.

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