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Lysozyme Retention by Cockroach *Periplaneta americana* L.

Denis R. A. Wharton

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Abstract. Blood from the cockroach *Periplaneta americana* usually does not react with a suspension of *Micrococcus lysodeikticus*. When lysozyme is added to the mixture it causes an immediate reduction in the optical density at 450 nanometers which is soon followed by a strong rise. Blood from roaches injected with lysozyme causes a similar series of changes in the optical density of a suspension of *M. lysodeikticus*, an indication that lysozyme has been retained in the blood. It may persist for many days or weeks.

The bacterial symbiont of the cockroach (*Periplaneta americana*) is intimately connected with the egg (1) and with mycetocytes of the fat body (2). This association suggests that it may have an influence on reproduction and growth, and indeed its removal proves to be injurious to these functions (3). It is by no means sure, however, whether such injury is due to the loss of the symbionts or to the sterilizing treatment by heat (4), antibiotics (2, 3), or lysozyme (3).

Use of the first of these two has entailed considerable mortality. However, Malke (3) has sterilized the cockroach *Periplaneta americana* with egg-white lysozyme, 0.5 mg of which destroyed most of the bacteroids within a week and eventually completely disintegrated them. Such a dose is not lethal to the insect, and even the injection of 22 successive doses at 3-day intervals is not toxic (3). If this is so, it would aid in understanding the physiology of growth and reproduction in this and similar associations.

It might be feasible to detect the enzyme in 20 μ l of blood, the volume expected from an individual male. We used egg-white lysozyme which was crystallized three times, dialyzed, and lyophilized; it had an activity of 30,000 sigma units (5) per milligram. To reduce coagulation of the blood, we used as

diluting fluid 0.2 percent potassium oxalate solution (6) in 0.066M potassium phosphate buffer (pH 6.2) in the ratio of 4:1. Twenty microliters of blood were added to a reaction volume of 2.6 ml, except for the experiment shown in Fig. 1b, in which 50 μ l of normal blood pooled from several insects was used to attain an appropriate optical density (O.D.). This increase in concentration of blood increased its tendency to clot. Reactions were conducted at room temperatures. The addition of 0.1 ml of a solution of lysozyme (0.1 mg/ml) to 2.5 ml of a suspension of *Micrococcus lysodeikticus*, having an O.D.₄₅₀ of about 0.6, caused a pronounced and uninterrupted decrease in the absorbance until lysis was completed and the suspension became clear (Fig. 1a). The addition of the same amount of lysozyme to 50 μ l of normal blood in 2.5 ml of potassium oxalate buffer showed no significant effect (Fig. 1b) (the slight decline in absorbance is attributed to developing coagulation and can be considerably reversed by inverting the container).

The addition of 20 μ l of normal blood to 2.6 ml of a suspension of *M. lysodeikticus* generally shows no effect (Fig. 1c). However, some blood samples do react. This is shown after a delay of from 1 to 2 minutes by a strong rise in absorbance over a period of 6 to 8 minutes, to a value that may be two to three times the original absorbance to complete a sigmoid curve (Fig. 2a). On the other hand, the addition of 0.1 ml of lysozyme solution to a mixture of 2.5 ml of bacterial suspension and 20 μ l of normal blood caused an immediate and sharp reduction in absorption, followed in about 1 minute by an even greater rise (Fig. 1d). This evidently was the type of curve to expect from blood with high lysozyme activity. If the injected lysozyme were uniformly distributed throughout the insect, a 0.5-mg dose in an 800-mg male cockroach would yield approximately 0.06 mg of the enzyme per 100 ml of blood. Twenty microliters

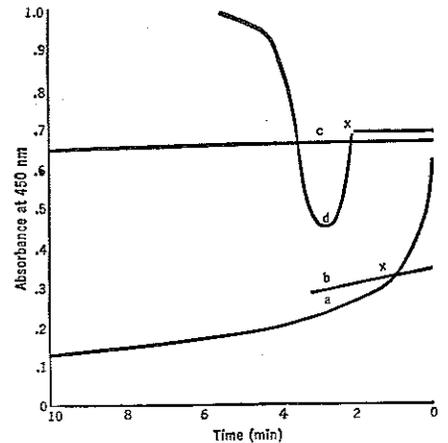


Fig. 1. (a) Action of lysozyme on suspension of *Micrococcus lysodeikticus*. (b) Action of lysozyme on normal blood. The lysozyme was added to the blood sample at time X, and produced no significant change in absorbance. The slight decrease in absorbance was due largely to coagulation of the blood which, in this sample of 50 μ l, was greater than in the samples of 15 to 20 μ l used in the other experiments. (c) Apparent lack of action of normal blood on *M. lysodeikticus*. (d) Action of lysozyme on a mixture of normal blood and *M. lysodeikticus*. Lysozyme added at time X.

of such blood added to 2.6 ml of a suspension of *M. lysodeikticus* would thus

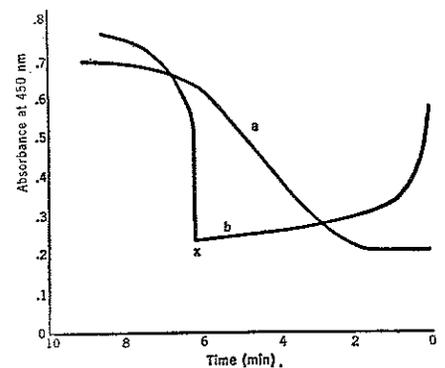


Fig. 2. (a) Reaction of suspension of *M. lysodeikticus* and blood from certain normal males. Note delayed rise in absorbance. (b) Immediate increase in absorbance and continuing rise after addition, at time X, of normal blood to a reacted mixture of lysozyme and *M. lysodeikticus*.

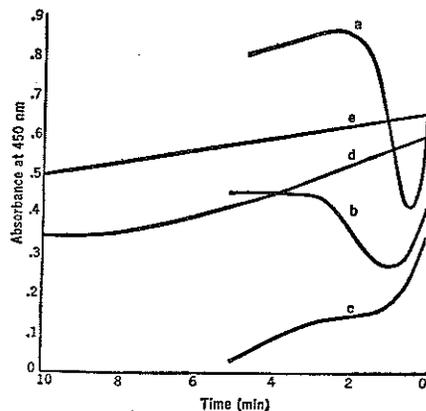


Fig. 3. (a to d) Action on *M. lysodeikticus* of blood taken from insect injected (a) 5 days (compare with Fig. 1d), (b and c) 13 days, and (d) 18 days previously with 0.05 ml of 2 percent lysozyme solution. (e) Action on *M. lysodeikticus* of blood taken from insect injected 18 days previously with 0.05 ml of 1 percent lysozyme solution.

result in a reaction mixture containing about 0.012 mg of lysozyme, approximating the 0.01 mg of enzyme used in the reference assay (Fig. 1a).

Adult male cockroaches were injected with 0.05 ml of 1 or 2 percent lysozyme in 0.5 percent NaCl solution and examined at intervals of several days. Blood (15 to 20 μ l) was rapidly withdrawn from the chilled insect and mixed with 2.6 ml of bacterial suspension; the mixture was analyzed in a Beckman linear recording spectrophotometer at a wavelength of 450 nm. The reaction obtained with blood withdrawn 5 days after injection of 2 percent lysozyme (Fig. 3a) duplicates in all essentials that of lysozyme in a mixture of normal blood and micrococci (Fig. 1d). Blood withdrawn 13 days after injection of 2 percent lysozyme also reproduces this curve (Fig. 3b), although indications of lower lysozyme concentration are seen in this and still more in another specimen (Fig. 3c). Activity is still evident 18 days after injection (Fig. 3d), even after injection of 0.05 ml of only 1 percent lysozyme (Fig. 3e). It may then be concluded that the indicated activity is due to the presence of residual lysozyme.

The addition of 20 μ l of normal blood to a reacted mixture of lysozyme and *M. lysodeikticus* results in an immediate and sharp increase in absorbance, which continues as a gradually rising curve (Fig. 2b). In view of this and of the sharp increase that follows the initial reduction in absorbance in a suspension of the bacteria to which has been added either normal blood and

then lysozyme (Fig. 1d), or blood from a cockroach injected with lysozyme (Fig. 3a), it appears that lysis of the bacterial cell wall enables the lysed bacteria to react with the blood to produce highly absorbing products. Thus, lysis must first begin; but since the change in absorbance does not equal that produced by lysozyme acting on the bacteria alone, it seems that the bacterial products are already reacting with the blood before lysis is completed. It is therefore not feasible to assay the lysozyme concentration in the presence of blood. The foregoing phenomenon may explain the reaction of certain normal bloods with the bacteria (Fig. 2a). The blood of *P. americana* is weak in native lysozyme and pH 3.5 is the optimum for activity of this enzyme (7). With a low concentration and less than optimum pH, lysozyme could be conceived as having a weakly lytic action on the bacteria, which is made all the more cryptic by the competing absorbance of the developing reaction between bacterial cell products and blood; hence the lack of a decline in absorbance and the delayed increase. We have no proof, however, that the observed reaction is the consequence of lysozyme action.

It is interesting that the injected lysozyme should persist in the insect for a considerable time, for notwithstanding Malke's view (3) that the egg-white lysozyme used was identical with the insect's lysozyme and therefore incapable of causing a foreign body reaction, Powning and Irzykiewicz (7) have shown that they differ. The observed evidence of its toxicity in the cockroach (8) is probably due to both its difference and its persistence.

DENIS R. A. WHARTON

Pioneering Research Laboratory,
U.S. Army Natick Laboratories,
Natick, Massachusetts 01760

References and Notes

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3. H. Malke, *Nature* **204**, 1223 (1964); — and W. Schwartz, *Arch. Mikrobiol.* **53**, 17 (1966).
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5. One sigma unit is that amount of enzyme which will cause a change in absorbance at 450 nm (O.D.₄₅₀) of 0.001 in a *Micrococcus lysodeikticus* suspension in 1 minute at pH 6.24, in 2.6 ml of reaction mixtures, and with a light path of 1 cm.
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