ABSORPTION OF PROTONS DURING THE CLOTTING OF *LIMULUS* LYSATE INDUCED BY ENDOTOXIN

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1. Introduction

A solution of the lysed white blood cells of the horseshoe crab *Limulus polyphemus* slowly polymerizes in the presence of trace quantities (10^{-12}g/ml) of endotoxin and may form a loose gel or 'clot' [1–4]. Since the rate and extent of this polymerization were found related to the quantity of endotoxin present [1,3] the polymerization reaction is used as the basis of the important, recently described, clinical test for endotoxin [4,5]. However, the mechanism of this polymerization is poorly understood. The lysate consists of a crude mixture of only partially characterized proteins whose relative proportions vary from preparation to preparation. It has been suggested that endotoxin binds to a specific enzyme present in the lysate thus activating it, and the complex formed subsequently hydrolyzes a different soluble protein known variously as 'clottable protein', 'coagulogen' or 'alpha clot protein' [6–9]. This 21 000 dalton alpha clot protein is believed to be split into at least a small soluble fragment and a 14 000 dalton highly insoluble 'beta clot protein' which polymerizes and forms the gel matrix [7–11]. Until now the principal methods for measuring the polymerization of *Limulus* lysate have been by means of light scattering or viscosity methods which depend mainly upon the polymerization of beta clot protein but not at all on earlier intermediate reactions. We report here a new method for observing the clotting of *Limulus* lysate based on pH measurements which in principle, may be used in the elucidation of earlier reactions between endotoxin, specific enzyme and alpha clot protein.

2. Materials and methods

Limuli were obtained from Woods Hole, Massachusetts waters or from a colony in Eilat originally obtained from Panacea, FL (Gulf Specimen Co.). All manipulations were done under pyrogen-free conditions. Lysate was prepared by the methods [4,12]. The lysate was dialyzed against several changes of distilled water and then centrifuged in sterile tubes before use.

The reaction mixture consisting of *Limulus* lysate (0.6 mg/ml) in 0.001 M $CaCl_2$, was contained in standard 1 cm disposable plastic optical cuvettes. Following a 10 min period of temperature equilibration at 37°C the reaction was started by the addition of endotoxin (10^{-6} g/ml, *E. coli* 055:B5, DIFCO) to the cuvette. Optical density and pH were simultaneously and continuously recorded until the end of the experiment by means of a Gilford 240 spectrophotometer equipped with thermoregulator and magnetic stirrer, and Beckmann SS-1 pH meter set to expanded scale using a microelectrode extending into the reaction mixture.

3. Results

After the addition of endotoxin, the pH increased slowly for 8–10 min (fig.1) then rapidly for about 5 min followed by an additional slow period until completion. The half-time for the pH increase was 11.5 min at 37°C and the total change was completed with in approx. 25 min. The net pH change was

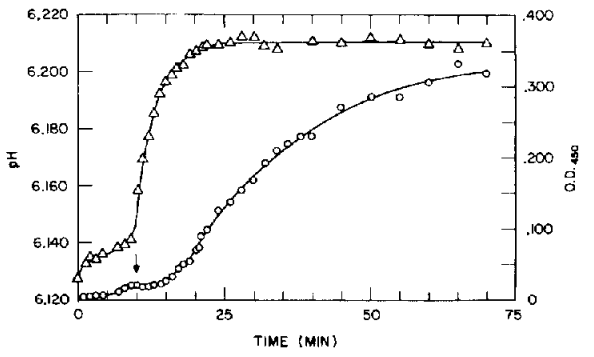

Fig.1. pH and turbidity changes during the clotting of *Limulus* lysate. Reaction mixture: *Limulus* lysate (0.6 mg/ml) in 0.001 M $CaCl_2$ at 37°C, with magnetic stirring. Endotoxin (10^{-6} g/ml) added at time zero. A large rapid pH change (triangles) occurred several minutes before the main *A* increase (circles) but concommitant with a small *A* (arrow).

+0.084 ± 0.004. This change occurred several minutes before the main increase in turbidity which occurred with a half-time of approx. 28 min. The magnitude of the pH increase was similar when the reaction was run at 27°C but the rate of change of pH and turbidity was reduced to approx. half.

Although the pH change reported here is small, it occurred in every experiment and it preceded the main rise in turbidity. In order to observe this pH change one must record the pH continuously. A brief removal of the electrode from the reaction mixture or a change of the scale of the pH meter results in a large artefact which obscures the small pH change otherwise found. An earlier report concerning the lack of pH change during the lysate polymerization is probably due to a lack of continuous and simultaneous measurement of pH and optical density, as well as to the smallness of the change [7].

4. Discussion

The main advantage of a pH measurement compared to viscosity or turbidity measurement is that it has theoretical quantitative importance and is not merely a crude indicator of polymerization.

A 'latent period' appears in fig.1 in the optical density curve during which endotoxin acts on the lysate and causes a pH change, but before significant polymerization occurs. During this period, at minimum the following types of reactions are suggested to take place:

(i) Endotoxin binds to the specific enzyme.
(ii) This complex diffuses to and binds with alpha clot protein.
(iii) Alpha clot protein is split into beta clot protein and fragment.
(iv) The endotoxin–enzyme complex detaches from beta clot protein or fragment and then binds to another alpha clot protein.
(v) The concentration of beta clot protein increases and it begins to polymerize.

The pH increase reported here cannot yet be attributed specifically to any one of the above reactions. It is to be hoped that the finding of conditions or poisons which inhibit the polymerization reaction but not the pH change, will help elucidate the problem. It may be significant that just prior to or concommitant with the maximum rate of change of pH there is a small but consistent shoulder or peak in the turbidity time curve (arrow). The cause of this turbidity may be quite different from that of the later turbidity rise which is due to the polymerization of beta clot protein.

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