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# Haemolytic Activity in the Blood Clot of *Aedes aegypti*

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## *Abstract*

In the present study it was demonstrated that mosquito blood clots contain a haemolytic principle which achieves haemolysis of washed erythrocytes in an isotonic medium. Proteinases are thought to play a role in this process. No final proof could be given for the existence of phospholipase activity although some results suggest the presence of this group of enzymes.

## Introduction

The knowledge of the physiology of blood digestion in haematophagous insects is of special interest in studies on host/parasite relationships. All pathogens transmitted by blood-sucking insects spend some time in the gut of their vectors and might be influenced by digestive processes.

One of the questions in blood digestion is the mechanism of the break-down of erythrocyte membranes, a process necessary for rendering the haemoglobin accessible to digestive enzymes such as proteinases. Haemolysis may be induced by various factors such as environmental changes, phospholipases or direct lytic factors. Lytic substances have been described in venoms (CONDREA & DE VRIES 1965, HABERMANN 1972).

In order to obtain insight into the processes of blood digestion in mosquitoes, the haemolytic activity of the blood clot against washed erythrocytes was tested. In addition, attempts were made to identify a specific phospholipase which could indirectly induce haemolysis by forming lysolecithin. Phospholipase A and B have been previously described in mosquito homogenates, the highest activity being represented in larval stages (RAO & SUBRAHMANYAM 1969 a + b, 1970). No data, however, exist on the occurrence of these enzymes in blood fed females.

## Material and Methods

*Aedes aegypti* L., strain Segemaganga was used in this study.

### Haemolysis

The method described by LANKISCH and VOGT (1972) and by VOGT et al. (1970) was applied by using rabbit blood in an isotonic medium. The liberated haemoglobin was converted to cyano-methaemoglobin and measured photometrically at 540 nm. The effects were estimated as % haemolysis (Fig. 1). Forty blood clots were used in a final volume of 5 ml erythrocyte suspensions.

A second assay system contained 0.5 ml of a 1 : 30 dilution (v/v) of erythrocytes. The volume was made up to 3.5 ml with 0.01 M phosphate buffer pH 7.3 containing 0.15 M NaCl and 0.45 mM CaCl<sub>2</sub>. This mixture, after addition of

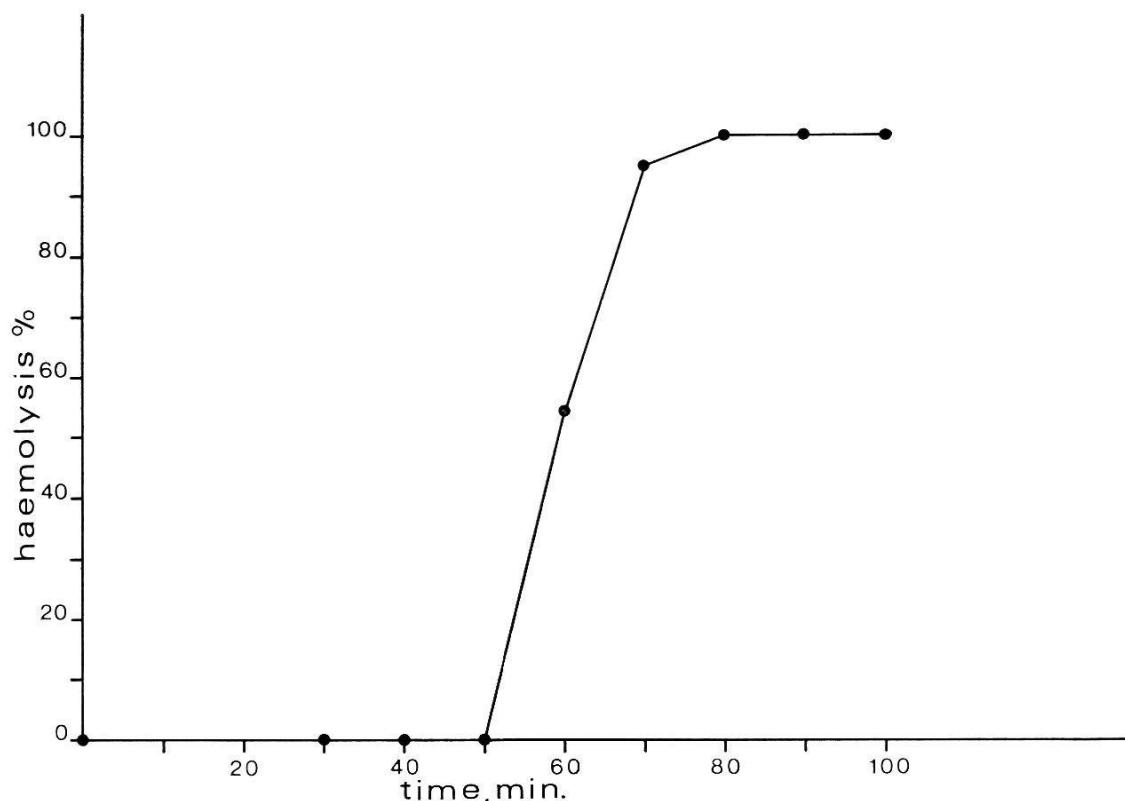


Fig. 1. The time course of haemolysis of washed erythrocytes by 24 hrs old mosquito blood clots.

0.25 ml of homogenate containing 40 blood clots was incubated in a heated cell at 37 °C in a Beckman M 25 spectrophotometer. The absorbancy change was registered directly with an attached recorder at 660 nm. The observed opacity of a blood cell suspension is proportional to the concentration of unlysed cells (COLLIER 1952).

In addition, haemolysis experiments were carried out on blood agar plates with blood clot homogenates sterilized through millipore filters (pore size 0.22  $\mu$ ). 20  $\mu$ l containing 6 blood clots were used and the plates incubated at 37 °C for 24 h.

Soybean trypsin inhibitor was used at a concentration of 11.6  $\mu$ g/blood clot in order to check the role of proteinases. Blood clots 24 h after a blood meal were tested.

### Clearing of egg yolk

The method described by MARINETTI (1965) for detection of phospholipases in snake venoms was used. The reaction was run at 37 °C in a heated cell in the spectrophotometer. Homogenate was added to the cuvette in different concentrations and the reaction mixture stirred. The recorder was put on after exactly 30 sec and the reaction course registered for 4 $\frac{1}{2}$  min. The clearing activity is expressed either as  $\Delta A$  (absorbancy change)  $\times 1000$  per mg blood clot per min, or as  $\Delta A \times 1000$  per blood clot per min. Blood clots were checked after various hours after a blood meal. TLC for detection of lysolecithin was performed on Silica gel aluminium sheets (Merck) with chloroform-methanol-acetic acid-water (25 : 15 : 4 : 2 v/v) as developing agent. The phospholipid spots were detected by iodine vapour (SKIPSI et al. 1964).

### Hydrolysis of lecithin

The assay system of RAO and SUBRAHMANYAM (1969a) was used with modifications: 3.2  $\mu$ moles of lecithin in 0.2 ml diethyl ether (or ethanol), 40 mg of deoxycholate in 1 ml of 0.05 M tris-maleate buffer pH 7.4 and 0.8 ml of the enzyme solution which contained 20 midguts. The reaction mixture was incubated at 38 °C for 90, 120, 150 min. The activity was followed by estimation of the decrease in acyl-ester content with a modified hydroxamate assay (AUGUSTYN & ELLIOTT 1969). Experiments were carried out with blood clots 24 h after a blood meal.

## Results and Discussion

### Haemolysis

The experiments proved that the blood clots of mosquitoes have specific haemolytic properties. Fig. 1 shows the breakdown of intact erythrocytes in an isotonic medium. After a lag period of 50 min complete haemolysis occurred within 30 min. Fig. 2 demonstrates the absorbancy change of a diluted reaction mixture incubated directly in a spectrophotometer. In this experiment, complete clearing of the erythrocyte solution was achieved in a short period of 15 min after a lag period of about 30 min. The reaction course of an incubation medium with soybean trypsin inhibitor is shown in Fig. 3. The lag period was greatly increased up to 2 h and the clearing reaction took 1 h 40 min. This shifting and slowing down of the haemolytic action suggests a participation of proteinases in this process.

The results of the haemolysis experiments carried out on blood agar plates are shown in Fig. 4. Haemolytic areas could be observed already after 4 h while with blood clots treated with trypsin inhibitor they appeared only after about 8 h. The results clearly show that bacteria do not contribute to haemolysis during blood digestion, a hypothesis which has been suggested by ARNAL (1950).

### Clearing of egg yolk

The clearing of an egg yolk suspension is a method originally described for the demonstration of phospholipase A (MARINETTI 1965). During blood digestion, midguts of mosquitoes also exhibit a clearing activity (Fig. 5). In the present study, however, it was not possible to demonstrate the appearance of lysolecithin by TLC after incubation; this proof would be indispensable for the confirmation of phospholipase action.

By expressing  $\Delta A$  per blood clot, maximal activity can be observed

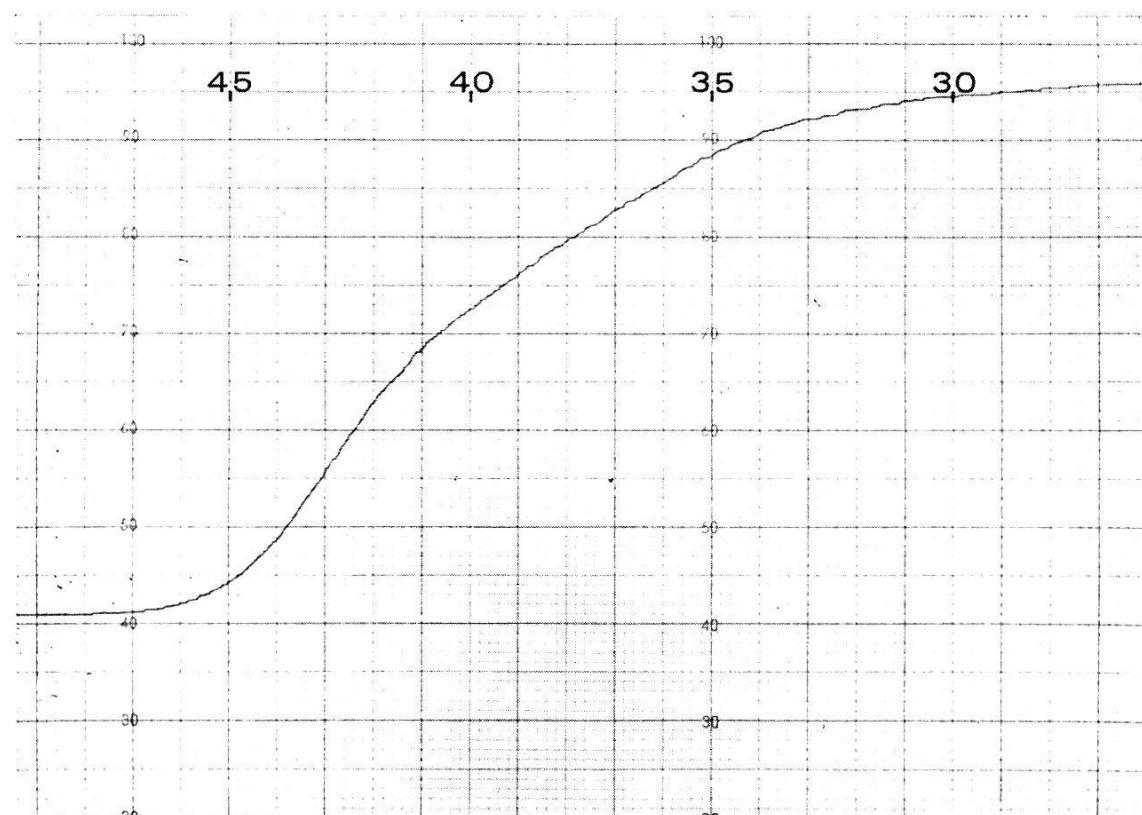


Fig. 2. Spectrophotometrical recording of the opacity change of an erythrocyte solution after incubation with mosquito blood clots. Numbers = incubation time in minutes.

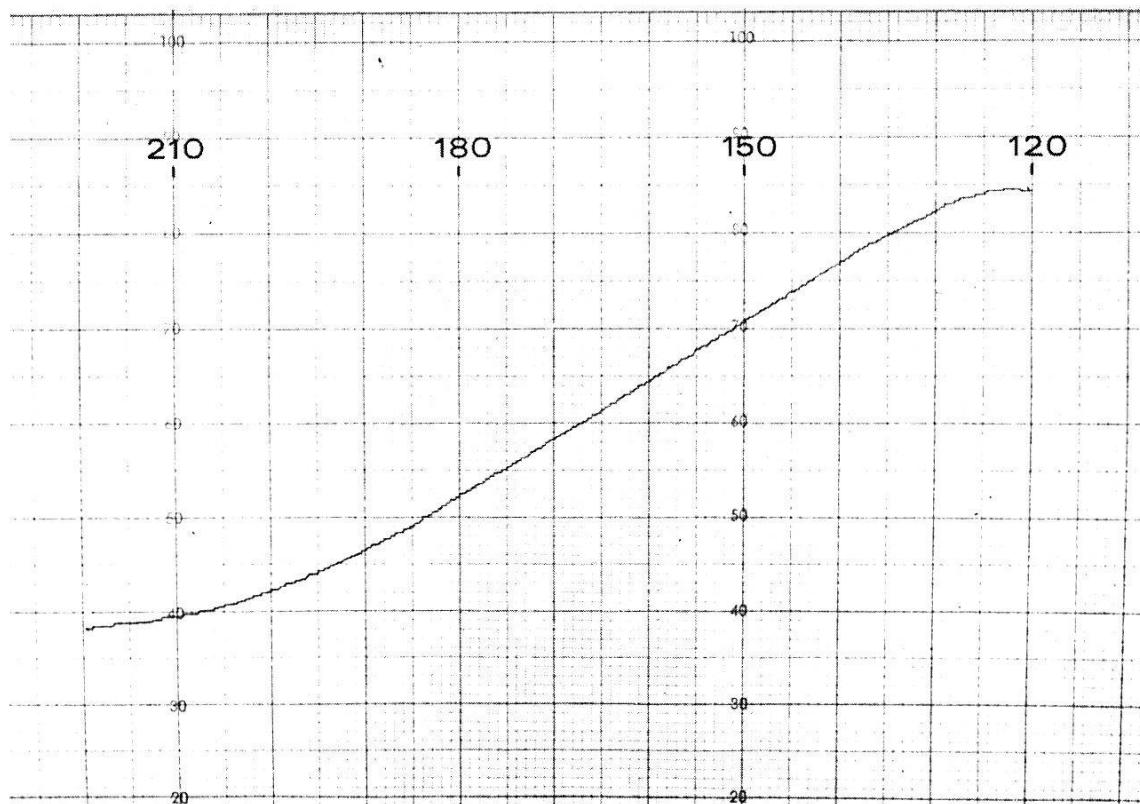


Fig. 3. Spectrophotometrical recording of the opacity change of an erythrocyte solution after incubation with mosquito blood clots treated with soybean trypsin inhibitor. Numbers = incubation time in minutes.

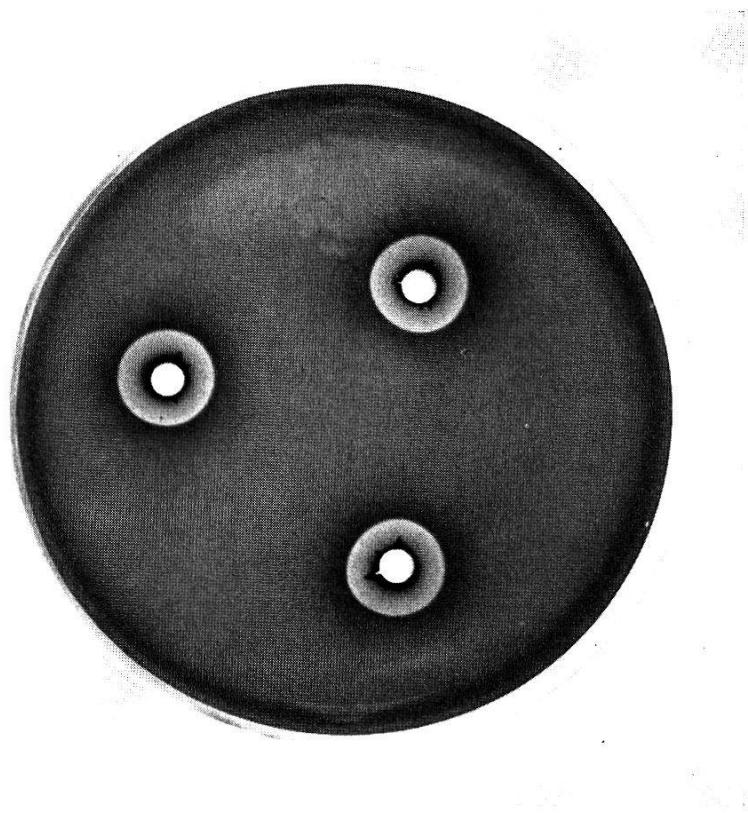


Fig. 4. Haemolysis on blood agar plates by mosquito blood clots after 24 hrs incubation.

24 h after a blood meal, by expressing  $\Delta A$  per mg blood clot the maximal activity, however, is shifted to 32 h. Linear decrease of blood clot size takes place between 10 and 48 h.

Heating the homogenate for 10 min at 80 °C destroyed the clearing activity which points to a heat labile substance responsible for the reaction.

In contrast to the haemolysis experiments, trypsin inhibitor did not interfere with the reaction so that a participation of trypsin like proteinases may be excluded. Maximal clearing activity, however, coincides with maximal proteinase activity in *Aedes aegypti* (GOODING 1972).

#### Hydrolysis of lecithin

In all experiments, a decrease in acylester bonds could be followed which was independent of self hydrolysis in the blood clot. The activity was increased after including ethyl ether into the reaction mixture. By judging these results, phospholipase is likely to occur in blood clots. The lecithin reaction, however, could not be standardized; a fact which suggests an interference by other substances of the crude homogenate.

In order to be able to make a clear statement on phospholipase

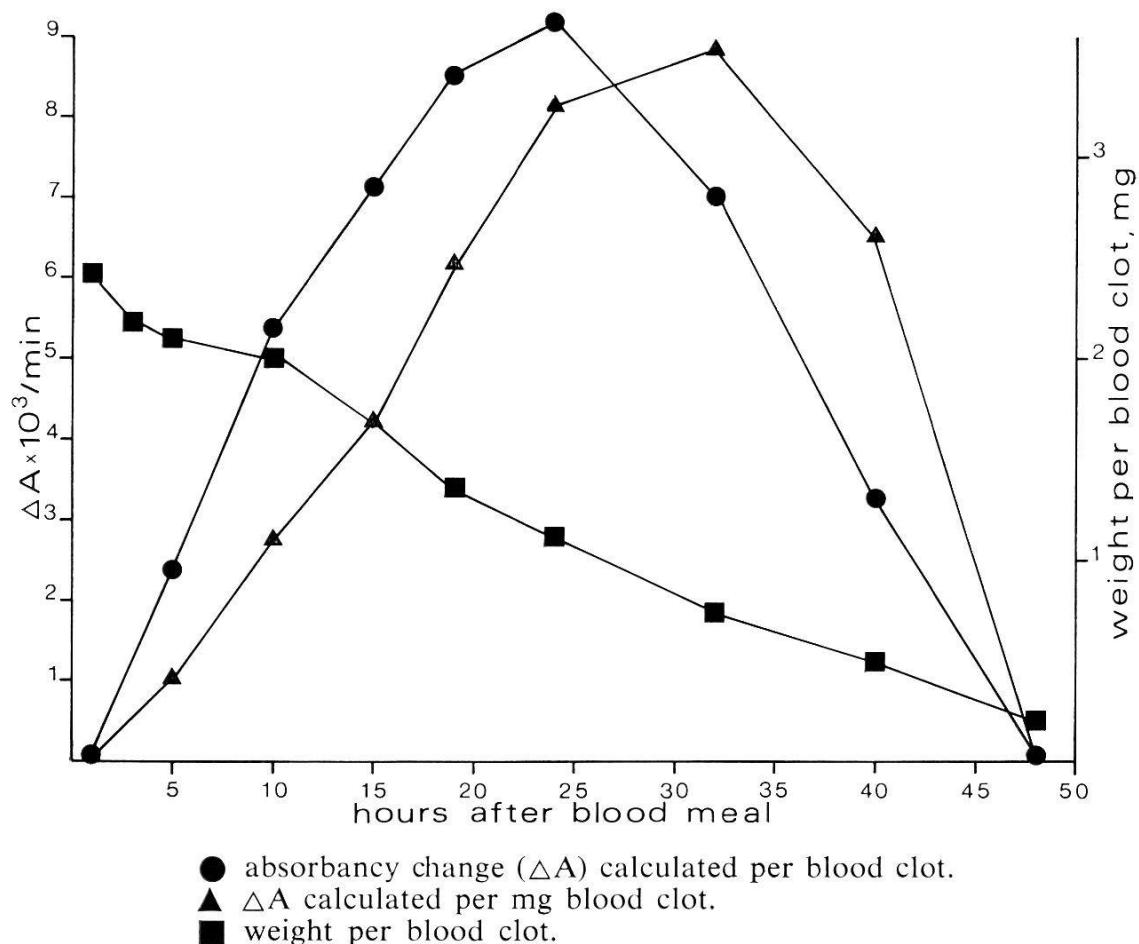


Fig. 5. Clearing activity against an egg yolk suspension of the mosquito midgut during digestion.

activity or on any other haemolytic principle in mosquitoes it is essential to test the digestive enzymes in a purified state. Further studies will be concerned with this problem.

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