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## **In vitro damage of cultured ookinetes of *Plasmodium gallinaceum* by digestive proteinases from susceptible *Aedes aegypti***

R. F. GASS, R. A. YEATES

### **Summary**

After exposure to extracts from blood fed *A. aegypti* cultured ookinetes of *P. gallinaceum* were damaged to various, defined extents. Immature ookinetes were found to be more sensitive to damage than mature ones. The damage was dependent on the digestion time after which the *Aedes* extracts had been prepared and could be correlated with the proteolytic activity in the extracts. Control experiments demonstrated that the factors responsible for damage were neither present in unfed mosquitoes nor in blood alone and that the damage was not a result of osmotic stress. After the treatment of the *Aedes* extracts with lima bean trypsin inhibitor the ookinete damage was much less, indicating that the *Aedes* trypsin was the major agent of damage. These results were supported by experiments in which the tryptic activity of the extracts was eliminated by thermal denaturation.

It is concluded that in the mosquito midgut most of the ookinetes are damaged by digestive enzymes and that this is one factor leading to the discrepancy between the number of ingested macrogametocytes and the number of oocysts which is usually found in nature. It seems that the only ookinetes which have a chance of surviving are those which develop in the centre of the blood clot, away from the site of enzyme action.

*Key words:* host-parasite relationship; *Plasmodium gallinaceum*; ookinetes; culture; *Aedes aegypti*; blood digestion; proteinases; trypsin; proteinase inhibitor.

### **Introduction**

The role of blood digestion in the interrelationship between mosquitoes and malaria parasites is still poorly understood. Gooding (1972) has considered

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two possible ways of interaction. On the one hand blood digestion might provide nutrients to the developing parasites, a hypothesis which is supported by several investigations (reviewed by Gooding, 1972). On the other hand digestive enzymes might have a direct, damaging effect upon the parasites and might therefore be one factor determining the susceptibility of the mosquitoes. Strong support for the second hypothesis has been found in experiments with *Plasmodium gallinaceum* in double fed, susceptible *Aedes aegypti*, where the number of oocysts is strongly correlated with the level of mosquito trypsin (Gass, 1977). It was concluded that in susceptible mosquitoes digestive enzymes are largely responsible for the reduction of the parasite population in the gut and that this leads to the discrepancy between the number of ingested macrogametocytes and the number of oocysts (Darling, 1910; Eyles, 1951).

The intention of the present work was to support these findings and includes studies of the effects of extracts from blood fed mosquitoes on cultured ookinetes.

### Materials and methods

The parasite *P. gallinaceum* (strain obtained 1969 by Prof. Maegraith, Liverpool) and its vector *A. aegypti* (Rockefeller strain) were used in this study.

#### *Cultivation of ookinetes*

Ookinete cultures were carried out according to Ball and Chao (1960). Mosquitoes were dissected 2–3 h after their infective blood meal. The midguts, containing zygotes, were pooled in culture medium (5 midguts/10  $\mu$ l) and mashed up with a glass piston. 5  $\mu$ l of this suspension were transferred into 100  $\mu$ l of medium in microtitre plates. The cultures were carried out during 24–33 h in 25 mM HEPES (N-2-Hydroxyethylpiperazine N'-2-ethanesulphonic acid, Calbiochem) buffered medium 199 (Difco) containing 1000 units/ml penicillin, 1000  $\mu$ g/ml streptomycin and 2.5  $\mu$ g/ml Fungizone (Gibco) at pH 7.7 and 27° C. The ookinetes were examined in Giemsa stained smears.

#### *Crude mosquito extracts (CME)*

CME was prepared at various times during blood digestion and from unfed females. The whole insects were homogenized in culture medium (100  $\mu$ l/ml) and the homogenate was purified by 3 centrifugations (30 min, 26,000 g, 4° C). After adjustment of the pH to 7.7 the supernatant was sterile filtered and stored deep frozen till use.

Inactivation of tryptic activity in CME was achieved (i) by incubation with 10 mg/ml lima bean trypsin inhibitor (Sigma) for 3 h at pH 7.7 and room temperature and (ii) by thermal denaturation for 3 h at 50° C.

Estimations of proteolytic activity in CME were carried out in 25 mM HEPES buffer at pH 7.7 and room temperature. Total proteolytic activity was estimated with a modified Kunitz method (Laskowski, 1955): 500  $\mu$ l of diluted CME (1  $\mu$ l/ml) was mixed with 500  $\mu$ l of a 1% solution of casein and incubated for 2 h. The reaction was stopped with 1 ml of 5% trichloroacetic acid. The mixture was centrifuged (20 min, 1200 g, room temperature) and the digestion products in the supernatant were estimated spectrophotometrically at 280 nm. Trypsin esterase activity was determined spectrophotometrically according to Hummel (1959). 100  $\mu$ l of a 10 mM solution of TAME (Tosyl-L-arginine methylester HCl, Calbiochem) were added to 900  $\mu$ l of diluted CME (1–5  $\mu$ l/ml). The increase in extinction/time unit, due to hydrolysis of TAME, was recorded at 247 nm.





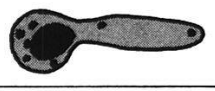

Valuation	Damage grade	Morphology	Giemsa staining	Appearance
↑ intact ookinetes ↓	1	Sickle shape. C: highly structured. P: spread all over the cell, often accumulated at the cell ends. V: regularly present	C: dark and light blue areas. N: red	
	2	a) Retort shape. Long cell protrusion. C: structured. N: in the spherical cell part or + in the protrusion. P: many granules in the spherical cell part, less in the protrusion. V: regularly present. b) As damage grade 1.	As damage grade 1. C: often faint.  C: dark blue N: red	 
↓ damaged ookinetes ↓	3	Sickle shape. C: + amorphous. P: as damage grade 1. V: rare.	C: dark or faint violet. N: red-violet	
	4	Retort shape. Long cell protrusion. C: + amorphous. N and P: as damage grade 2a. V: rare.	As damage grade 3.	
	5	Retort shape. Short cell protrusion. C: + amorphous. N and P: always in the spherical cell part. V: very rare.	As damage grade 3.	
	6	No ookinete development		

Table 1. Characterization of various damage grades of *P. gallinaceum* ookinetes after exposures to crude *A. aegypti* extracts. C = cytoplasm, N = nucleus, P = pigment, V = vacuoles

#### *Incubations of ookinetes with crude extracts (CME)*

2–3 h after the beginning of the culture, the ookinetes were incubated at 27° C over various times in microtitre plates in the presence of 100 µl of CME or CME with inhibited tryptic activity. To remove the CME after the incubation the ookinetes were washed once by centrifugation (10 min, 250 g, room temperature) and resuspended in 100 µl of culture medium. Control samples were treated in the same manner, but using exclusively culture medium in the whole procedure. The results of these experiments were checked after a total culture duration of 24 h on Giemsa stained smears. To examine differences in the susceptibility to CME between mature and immature ookinetes, parasites 25 h and 5 h after the beginning of the culture were incubated for 30 min or 8 h with CME (100 ♀♀/ml) obtained 25 h after blood meal (peak of proteinase activity). The results were checked after a total of 33 h of culture time in Giemsa stained smears.

## Results

Ookinetes which had been incubated for one day in CME prepared 25 h after blood meal exhibited clear indications of cell damage. At high concentrations of CME (70–100 ♀♀/ml) the parasites were totally lysed. At lower concentrations (10–60 ♀♀/ml) CME produced either mature, sickle shaped ookinetes or parasites whose development had been interrupted at an immature, retort shaped stage. In both cases the result of Giemsa staining was clearly altered. At

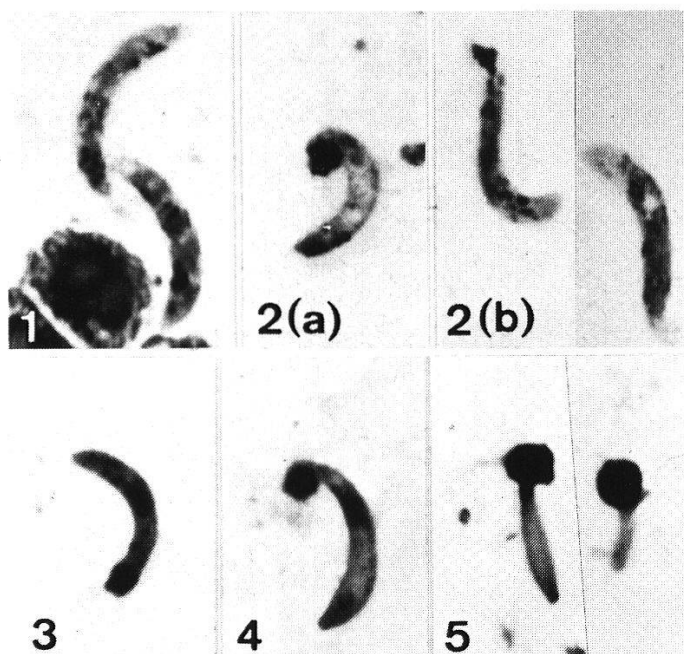


Fig. 1. Microphotographs of damage grades 1–5 of *P. gallinaceum* ookinetes (compare to Table 1).  $\times 1800$ .

Table 2. Mean damage grades  $\pm$  standard errors (S.E.) of *P. gallinaceum* ookinetes after one day of incubation in various concentrations of crude *A. aegypti* extracts (CME) prepared 25 h after blood meal

CME conc. (♀♀/ml)	No. of exp.	Mean damage grade
0 .....	15	$1.25 \pm 0$
5 .....	5	$1.25 \pm 0$
10 .....	13	$2.7 \pm 0.3$
20 .....	13	$3.8 \pm 0.1$
30 .....	13	$4.2 \pm 0.1$
40 .....	13	$4.5 \pm 0.1$
50 .....	12	$5.1 \pm 0.2$
60 .....	12	$5.6 \pm 0.2$
70 .....	10	$6 \pm 0$
80 .....	10	$6 \pm 0$
90 .....	11	$6 \pm 0$
100 .....	12	$6 \pm 0$

concentrations of 5 ♀♀/ml no damage was observed and the cells were comparable to control ookinetes which had developed in medium 199.

The dependence of the damage on the CME concentration allowed us to classify various grades of damage (Table 1, Fig. 1). The experiments did not usually produce ookinetes of only one damage grade, but a mixture of various forms. The results were therefore evaluated as an approximate mean of the frequencies of the single forms found; e.g. controls usually led to many oo-

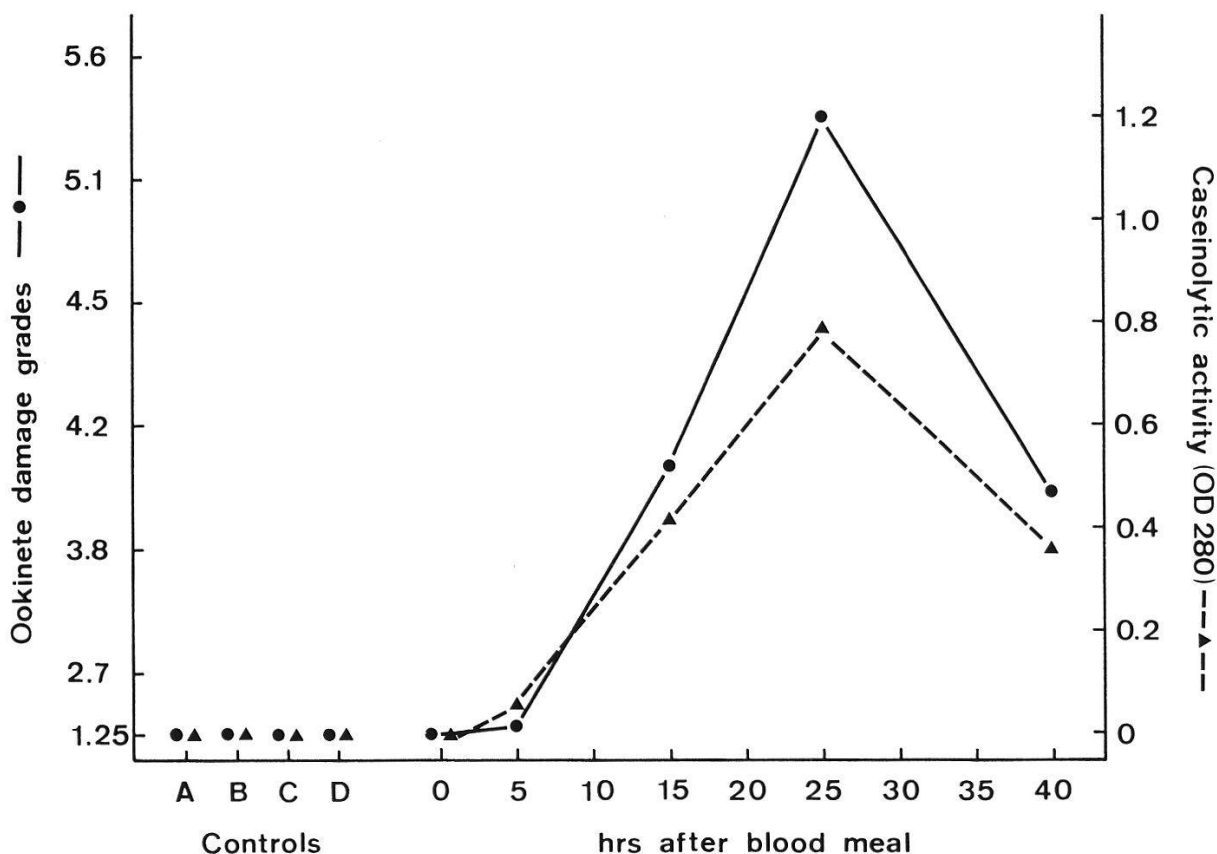


Fig. 2. Caseinolytic and ookinete damaging activity of crude *A. aegypti* extracts (50 ♀♀/ml) prepared at various times after blood meal (means of 5 independent experiments). The damage grade scale is set up according to Table 2. Controls: A = TC 199, B = TC 199 with increased osmolarity, C = unfed female *A. aegypti*, D = chicken blood.

kinetes of grade 1 and significantly fewer forms of grade 2 and this was then evaluated as damage grade 1.25.

The results of the incubation experiments expressed in mean damage grades are summarized in Table 2. The use of a defined series of CME concentrations with each corresponding to a certain mean damage grade allowed a quantitative estimation of the steps between the single damage grades; e.g. CME of 40 ♀♀/ml causes damage of grade 4.5; CME of 20 ♀♀/ml causes damage of grade 3.8. Therefore damage grade 4.5 represents twice the damage of 3.8. As such quantitative estimations are only possible when the ookinetes are not totally destroyed (damage grade less than 6), the CME concentration of later incubation experiments were chosen accordingly. This relationship between the damage grades was used to set up a scale for later experiments (Figs. 2, 3) in which different degrees of damage were to be quantitatively compared.

Comparative incubations of immature and mature ookinetes with CME (100 ♀♀/ml) revealed clear differences between the two development stages in their sensitivity to destruction. After 8 h of incubation (5 experiments) immature ookinetes were always lysed. Mature ookinetes on the other hand resisted lysis, although damage could be demonstrated by Giemsa staining. Decreasing

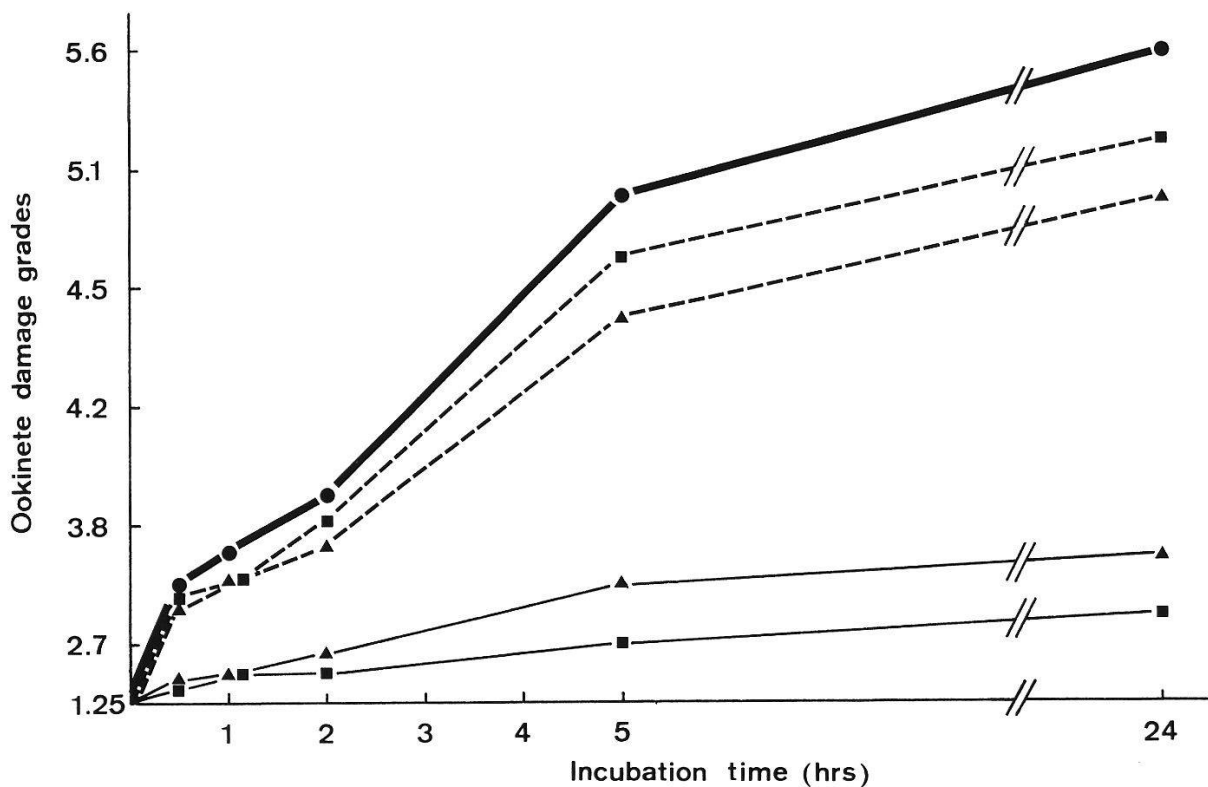


Fig. 3. Damage of *P. gallinaceum* ookinetes with crude *A. aegypti* extracts (50 ♀♀/ml) which are either treated with lima bean trypsin inhibitor or by thermal denaturation (means of 5 independent experiments). The damage grade scale is set up according to Table 2. Legend: —● = damage due to untreated extracts; --■ = damage due to factors sensitive to thermal denaturation; —■ = damage due to factors resistant to thermal denaturation; --▲ = damage due to factors sensitive to lima bean trypsin inhibitor; —▲ = damage due to factors resistant to lima bean trypsin inhibitor.

the incubation time demonstrated that mature ookinetes were still abnormally stained after an exposure to CME of only 30 min. Under these conditions, however, we usually obtained a mixture of damaged and intact cells and in one experiment no damage at all could be observed. Immature ookinetes on the other hand were always heavily damaged after 30 min of incubation; 2 experiments led to lysis of the parasites while 8 experiments produced an average damage grade of 4.6.

A first step towards characterization and identification of the factors in CME which produced the described ookinete damage was carried out by incubating ookinetes with crude extracts prepared at various times during blood digestion. The effect upon the parasites after 24 h of exposure was compared with 4 control incubation media: (i) medium 199, (ii) medium 199 which had been concentrated to increase its osmolarity. The osmolarity was adjusted to 409 mOsmol which corresponded to CME (50 ♀♀/ml) prepared 25 h after blood meal, (iii) CME from unfed females (50 ♀♀/ml) and (iv) suspension of homogenized chicken blood in medium 199 (150 µl/ml). None of the controls produced any effect upon the ookinetes, while crude extracts (50 ♀♀/ml) prepared during blood digestion clearly led to damage. The damage was correlated with the

Table 3. Inhibition of proteolytic activity in crude *A. aegypti* extracts prepared 25 h after blood meal (means of 5 independent experiments,  $\pm$  S.E.)

Method	% inhibition of activity against casein	%inhibition of activity against TAME
Lima bean trypsin inhibitor .....	57.7 $\pm$ 2.2	100
Thermal denaturation	67.9 $\pm$ 0.8	100

proteolytic activity measured in the extracts and both showed a maximum with CME made up 25 h after blood meal (Fig. 2).

Further information on the factors which damaged ookinetes in CME was obtained by eliminating the tryptic activity of the extracts (prepared 25 h after blood meal) before ookinete incubation. This was achieved either with the specific inhibitor from lima bean or by thermal denaturation. With both methods hydrolysis of TAME was completely suppressed, which indicated total elimination of tryptic activity in CME (Table 3). Assays for total proteolytic activity (hydrolysis of casein) demonstrated the presence of non-tryptic proteinases which resisted the two inactivation methods. The inhibition of caseinolytic activity after thermal denaturation was somewhat greater than that obtained with lima bean inhibitor.

Incubations of ookinetes in extracts (50 ♀♀/ml, prepared 25 h after blood meal) with inhibited tryptic activity produced markedly less damage than that with untreated CME. This effect was particularly obvious after short incubation times (30 min–2 h), when damage was almost prevented (damage grade 1.5–2.3). After 24 h of exposure the remaining damage was more evident – damage grade 3.3 with lima bean inhibitor and 3.0 after thermal denaturation. However, this damage was still much less than that obtained with untreated CME (damage grade 5.6). Control experiments demonstrated that no damage was produced from lima bean inhibitor itself.

These experiments allowed us to distinguish between two groups of factors which caused ookinete damage. The first group consisted of factors which were resistant to lima bean inhibitor and to thermal denaturation. Their effect upon ookinetes was directly measured by the incubation experiments. The second group consisted of factors which were sensitive to the two treatments and included mosquito trypsin. Their effect was calculated as the difference in damage between ookinetes exposed to treated and to untreated CME. The ookinete damage produced by these two factors and by untreated CME after various periods of incubation are presented in Fig. 3. The graph shows clearly that most of the damage is caused by factors of the second group.

## Discussion

Our study has demonstrated in vitro damage of ookinetes of *P. gallinaceum* after exposure to CME prepared from their blood fed vector *A. aegypti*. The damage is dependent on the digestion time after which CME is made up and can be correlated with the proteolytic activity in the CME. Control experiments have shown that the factors responsible for damage are neither found in chicken blood nor in unfed mosquitoes and that the damage is not a result of osmotic stress. This demonstrates that in our in vitro system ookinetes are damaged by factors which are related to blood digestion and that proteolytic enzymes are likely to play a role. This also supports earlier in vivo findings with double fed mosquitoes which revealed a close correlation between oocyst production and proteolytic activity during blood digestion and which therefore suggested that ookinetes are sensitive to destruction by proteolytic enzymes (Gass, 1977).

Ookinete damage is characterized at high concentrations of CME by lysis of the cell. At lower concentrations CME interrupts ookinete development at the stage of an immature, retort shaped cell and leads to altered Giemsa staining. These effects are a result of cytoplasmic disorganization, as demonstrated by ultrastructural investigations (Gass [7]). These studies also provided a possible explanation for the different susceptibility to damage of immature and mature ookinetes. It was suggested that the pellicle – a reinforcement of the cell envelope which is typical of extracellular plasmodial stages – might have a protective function for the cell. The increased susceptibility of immature ookinetes might then be caused by inadequate protection by the pellicle, which has been shown to cover their cell body only partially.

The best method to investigate the role of mosquito digestive proteinases in destruction of ookinetes would be to incubate the parasites with purified enzymes. However, purified enzymes are not yet available and we therefore have been forced to choose an indirect method – the elimination of certain enzymes from CME before incubating ookinetes with it. Together with a metalloproteinase, trypsin is the major proteolytic enzyme in *A. aegypti* and constitutes 30–40% of the proteolytic activity during blood digestion (Yeates, 1978). After the elimination of the trypsin either by lima bean inhibitor or by thermal denaturation, CME is much less effective against ookinetes. Besides the trypsin, lima bean inhibitor inactivates a minor proteinase which constitutes only around 10% of the proteolytic activity (Yeates, 1978) and forms a “front band” after polyacryamide gel electrophoresis (Kunz, 1978). Similar results to those with lima bean inhibitor have been obtained with trypsin inhibitor from ovomucoid and with chicken serum (Gass, unpublished experiments), both of which are known to inhibit mosquito trypsin (Huang, 1971; Kunz, 1978). From these results we consider that most of the ookinete damage is due to proteinases which are sensitive to lima bean inhibitor and which include trypsin as a major component.

Factors resistant to lima bean inhibitor produce relatively weak ookinete damage which is evident only after more than 2 h of incubation. Besides non-proteolytic factors these include as a major component a metalloproteinase which is sensitive to EDTA (Yeates, 1978). Unfortunately we did not succeed in investigating the role of this enzyme as with trypsin. Prior to ookinete incubation the toxic EDTA had to be removed from CME and subsequent addition of culture medium led to reactivation of the metalloproteinase. However, we have some evidence that proteinases resistant to lima bean inhibitor might cause some ookinete damage. Thermal denaturation inactivates more of the caseinolytic activity of CME (= total proteolytic activity) than lima bean inhibitor and must therefore be effective against lima bean resistant proteinases. Corresponding to that difference, after thermal denaturation CME produces less ookinete damage than CME treated with lima bean inhibitor.

The malaria parasite has to overcome several barriers during its development in the mosquito host and we have provided very strong evidence that one of these barriers is destruction of ookinetes by the digestive enzymes of the host: Together with decreased gametocyte infectivity (Vanderberg et al., 1977) we consider this to be a plausible explanation for the considerable loss of parasites which occurs prior to oocyst formation (Darling, 1910; Eyles 1951). For a realistic assessment of the *in vivo* situation one should take into account that in the mosquito midgut the ookinetes are exposed to an enzyme concentration at least ten times that in our *in vitro* system (CME, 100 ♀♀/ml). It therefore seems to us that the only chance for ookinetes to survive in the gut is provided by the unequal distribution of digestive activities in the blood clot at the beginning of digestion. Blood digestion proceeds only slowly from the periphery towards the centre of the blood clot and will undoubtedly destroy all parasites exposed. However, one day after blood meal digestion has not yet reached the clot centre (Stohler, 1957) and this time is sufficient for centrally located ookinetes to achieve maturity and motility without exposure to digestive enzymes. These stages are to some small extent resistant to digestive activities, perhaps due to a complete pellicle, and have to make their way out towards the gut epithelium by crossing digestive zones. This migration must be achieved within a short time as mature ookinetes have been shown to survive only about 30 min in CME. As no chemotactic effect seems to guide the ookinetes, their migration very probably involves extensive deviations (Freyvogel, 1966) and we therefore consider that only a small percentage of ookinetes reaches the gut epithelium unaffected by blood digestion.

The question remains open of whether destruction of ookinetes by blood digestion has any significance for the immunity of mosquitoes to certain species of plasmodium. It is known that sporogonic development in resistant mosquitoes is interrupted at different phases and sites, depending on the mosquito-plasmodium combination. An important site of interruption is undoubtedly the haemolymph, as demonstrated for *P. gonderi* in *Anopheles maculipennis* (Garn-

ham, 1966) and *P. gallinaceum* in *Culex pipiens* (Weathersby and McCall, 1968). However, in certain cases – such as *P. berghei* in *Aedes aegypti* – interruption of development occurs in the midgut (Yoeli, 1973) and it seems reasonable that here blood digestion is involved in the mechanism of resistance.

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