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BIOCHEMICAL INVESTIGATIONS OF THE HOST-PARASITE RELATIONSHIP OF MOSQUITOS AND THE PARASITE FUNGUS, 'LAGENIDIUM GIGANTEUM'

North Carolina University, Chapel Hill

1 July 1976

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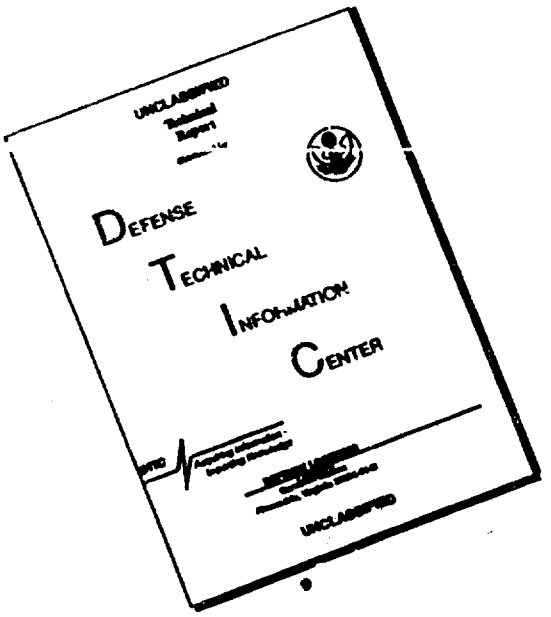

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BIOCHEMICAL INVESTIGATIONS OF THE HOST-PARASITE RELATIONSHIP OF MOSQUITOS AND THE PARASITE FUNGUS, LAGENIDIUM GIGANTEUM

ANNUAL SUMMARY REPORT

A. J. DOMNAS

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Biochemistry Laboratory, Botany Department
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ABSTRACT

Biochemical Investigations of the Host-Parasite Relationship of Mosquitoes and the Parasite Fungus, Lagenidium giganteum

The study of the utilization of sterols by the fungus Lagenidium giganteum has been continued and expanded. The organism uses a wide range of natural sterols and synthetic sterols, which induce zoospore formation. The antibiotics nystatin and amphotericin have been shown to repress zoospore production. The study of sterol utilization and metabolism has been initiated, and work on general fungal physiology has been continued. It has been noted that lipoidal substances seem to be required for utilization of ammonium ion. The fungus protease has also been further isolated and purified and it seems to belong to the serine, alkaline trypsin class.

Mosquito proteases have been further analyzed from Culex pipiens and Aedes epactuis. On the basis of further examination and study, it appears that a new trypsin like alkalophilic enzyme is present. A new, more reliable purification procedure has been developed.

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Title: Biochemical investigations of the host-parasite relationship of mosquitos and the parasite fungus, Lagenidium giganteum.

Dear Sirs:

The discovery that phytosterols are necessary for zoospore production of the fungal mosquito parasite has stimulated and expanded our research area. The implications in this research are many, extending from enzyme study to morphogenesis and cancer. At this time we have been concentrating on acquiring more information on the application and utilization of these sterols in zoospore production as is shown in our annual report.

1. Phytosterols and zoospore production

a. Natural sterols

The identification of the phytosterols as the principal (and only) requirement for the production of zoospores in the fungus Lagenidium giganteum facilitated our work. We found, that to a large extent, nearly all sterols and sterols derivatives were utilizable by the fungus. Table 1, which was excerpted from a paper submitted for publication summarizes the data in a concise manner. However, in order to avoid disputation concerning the complexity of the PYG medium, we ran two repeat sets using a defined medium (Gleasons) and the results of these experiments are listed in Table 2.

Furthermore, GLC analysis of PYG media showed no sterols present, but only when sterols were added. The data is totally convincing, and there is no doubt that this fungus must have sterols of some kind in order to produce zoospores.

b. Synthetic sterols

A number of synthetic sterols have been tested as shown in Table 3, plus a sitosteryl glucoside. From this data it can be seen that the organism utilize cholesteryl and sitosteryl phosphates, and can use steryl glucosides as well. The lag period would indicate the hydrolysis of the latter substance and its transformation from sitosterol to x, an unknown compound. The nature of the transformations will be reported on in a later section.

c. Effect of antibiotics

During our study we showed that nystatin and amphotericin inhibited zoospore production as shown by Table 4. Hendrix (personal communication) feels that the quantities are rather high, but Fowlkes et al have used these concentrations, and obtained satisfactory results.

d. Sterol metabolism in the fungus

We are now able to observe zoospore production of the fungus grown on solid medium. During our zoospore study we noted that sugars such as glucose, fructose, sucrose, maltose or trehalose at concentrations of less than 0.1M induced sporangia and zoospores and these were readily discernible by low power microscopic scanning. Zoospores were forthcoming only on sterol supplemented media since PYG grown Lagenidium only produced the sporangia extensions, and this indicates, inferrentially that the sterol is primarily involved with the zoospore.

In several investigations we have endeavored to trace the metabolism of the applied sterol, but we have had no success in detecting any metabolites stemming from the substrate. The big problem is that the fungus needs very little sterol to affect its transformation to zoospore induction. There is also a problem in timing, that is to say that the fungus must be actively producing zoospores before any transformation of sterol substrate occurs. Using a halophilic Lagenidium sp. which is parasitic on Paeneid shrimp, we have detected several unknown substances that appear to be of a steroidal nature. By parallel analogy we hope to find these substances in Lagenidium giganteum. The use of tracers is also indicated in these studies which are being vigorously pushed.

TABLE 1

Effect of various treatments and lipid supplements on zoospore production of L. giganteum as measured by bioassay.

Nature of medium	% Infection		
	Day 1	Day 2	Day 3
WHS	30	90	nc
WSB	6	82	nc
WHS - lipid depleted	0	0	5
WHS + WHS oil reconstituted	2[illegible]	80	nc
methyloleate	0	0	0
tristearin	0	0	0
squalene	0	0	2
phytol	0	0	10
soy oil	50	80	nc
peanut oil	0	37	[illegible]
WHS oil	0	62	[illegible]7
PYG control	[illegible]	0	0

0.1 ml of methyloleate, tristearin, and oils were added to the hot PYG agar medium. Phytol and squalene were added at 0.05% levels. WHS, WSB extracts were prepared as described and contained 1 mg protein/ml. Test organism was Aedes epactius. nc - not counted.

TABLE 2

Effect of various sterols on zoospore production by *L. giganteum* as measured by bioassay.

Nature of Medium	# of Trials	% Infection Range (3 day period)
Sterols from WHS	3	50-88
Sterol depleted WHS oil	3	0
Sterols from WSB oil	4	28-88
Sterol depleted soy oil	2	0
Lanosterol	1	30
Cholesterol	10	40-78
Desmosterol	1	45
Cholestadiene	1	25
Ergosterol	4	52-87
Campesterol	6	72-90
Sitosterol	6	70-90
Stigmasterol	3	5-10
Campesterol:stigmasterol 1:1	2	23-53
Stigmasterol:sitosterol	2	0
Sitosterol:campesterol: stigmasterol 3:1:1	2	32-38

0.1% sterol depleted oils were added to PYG agar. Digitorin precipitated sterols were dissolved in either $CHCl_3$ or MeOH to give approximately 30 µg sterol/ml media. All authentic sterols were added at the level of 30 µg/ml PYG media. Test larvae were *Aedes* sp.

TABLE 3

The effect of sterols on zoospore production on L. ziganteum grown on defined medium.

Nature of sterol supplement	% Infection 3rd day
none	0
sitosterol	22
campesterol	32
sitosterol:campesterol: stigmasterol 3:1:1	45
soy oil sterols	28
soy oil	36

Authentic sterols and oils added as indicated in the text. Test species Culex.

TABLE 4

The effect of amphotericin and nystatin on zoospore production as measured by infection against Culex sp.

Antibiotic and Amount	% Infection 3rd day
0	85
100 mg/ml Amphotericin	68
200 " "	44
300 " "	22
100 mg/ml Nystatin	38
200 " "	24
300 " "	11

L. giganteum was grown on PYG agar supplemented with soy oil and the various drugs were added directly in the agar.

TABLE 5

The effect of sterols on zoospore production by *L. giganteum* measured by bioassay on *Culex* sp.

Nature of sterol supplement	# of trials	% Infection 3rd day
cholestan 3β-ol	2	14.2, 38
cholesteryl disodium phosphate	2	29, 83
cholesteryl dihydrogen phosphate	2	16.2, 28
cholestan 3β, 6α diol	1	4.5
sitosteryl glucoside	3	35, 65, 68
sitosteryl triol	1	73
sitosteryl dihydrogen phosphate	1	55
sitosteryl triol diformate	1	70

Substances were added as described previously in text.
See also Table 2.

2. Fungal metabolism

a. Ammonium sulfate utilization

Mr. H. Moosa, an honors biology student, has been studying the problem of ammonium ion utilization. This has turned out to be a rather tricky one, since often enough we get no growth on ammonium sulfate even after 28 days, although it has grown in a time period as short as ten days. This problem is of some importance because the organism can survive in open waters provided it has enough catalytic material. We are uncertain at this time of the nature of "factor X" the "catalytic material" but we are proceeding with a number of substances. Of interest is the fact that yeast extract helps this growth and it may be related to a vitamin. More recent studies demonstrate that oil is extremely helpful for nitrogen assimilation.

b. Fungal exocellular protease

Mr. D. Dean has isolated the enzyme by disc gel electrophoresis and shown it to be a complex of seven different proteins. Only one band shows enzymic activity which is very helpful for its further isolation and identification. Further work has shown that the artificial substance TAME, (Tosyl L-arginine methyl ester) is hydroluzed by the enzyme and in addition the enzyme is inhibited by soy bean trypsin inhibitor. In fact all the details, properties and characteristics of this enzyme show that this enzyme can also be classed as a "trypsin-like" substance. Work is proceeding on this subject with the intention of characterization and determination of its structure.

3. Mosquito proteases

a. Purification

A new purification procedure has been evolved which gives a far better yield of proteases, and higher specific activity. The procedure is applicable to Aedes epactius or Culex pipiens with equal ease, and the assay procedure involves use of Azocoll, and an insoluble dye which is solubilized by the enzyme to a red color measured at 520 nm. A unit is defined as that quantity of enzyme which gives a change of 0.1 at 520 nm in 30', at 30°; specific activity is units/mg protein. Typical fractionation is shown in Table 5 for Aedes sp.

b. Nature of the protease

The purified preparation from above is extraordinarily potent at pH 11.05, which is its optimum activity, and at this pH there is absolutely no classical chymotrypsin or trypsin activity (see Yang and Davies). Both of these enzymes had been demonstrated previously by us and their activity was found to be restricted between pH range of 6.8 to 8.0 It turns out now that this is a new enzyme(s), which has previously been unreported. The enzyme is very stable, since it has been kept in water solutions for several months at -7°. Its temperature of denaturation is 60° without substrate, and ca 90° in the presence of substrate. Both Aedes and Culex spp. have been shown to possess this enzyme.

c. Effect of inhibitors

The information supplied here in is applicable to enzyme from either species and a pH of 11.05 was used throughout (see Table 6).

At this time, the data indicates that this is a trypsin like enzyme which does not hydrolyze TAME, an arginine derivative. Furthermore the properties of this enzyme are very similar to the hornet midgut protease reported by Hagenmaier in that the enzyme is strongly alkaline and is inhibited by trypsin inhibitors. Table 7 shows the substrates which are hydroloyzed by this enzyme.

d. Development in growing mosquito populations

Both chymotrypsin and trypsin activities were followed in the developing Aedes larvae from first instar to pupation. Both enzymes attain a high level/larvae at late third instar to middle fourth instar and then drop to a non-measurable level. The alkaline trypsin like enzyme shows identical patterns in both Culex pipiens and Aedes epactius. There is no evidence as

yet that the fungal enzyme is involved in infection to any degree. In fact, in the infected larvae of Aedes and Culex, the "trypsin-like" protease stays very high and is not significantly different from controls. The fungal enzyme has an 8.5 pH optimum and shows no activity at pH 11.05; we are thus forced to conclude that it has no role in the post-infection pathology. That it may be involved in initiation of infection by zoospore penetration through the cuticle is yet to be demonstrated.

Table 6. Inhibitors of the alkaline protease of Culex sp. and Aedes sp. larvae.

Substance	% Inhibition
0	0%
EDTA - (0.001M)	58
EDTA - (0.005M)	81
cysteine HCL - (0.001M)	0
" " - (0.01M)	20
Phenylmethane sulfonyl fluoride (0.001M)	44
Soy bean trypsin inhibitor (1mg/ml)	100
tosyl- L-ϕ-alanyl chloromethane (TPCM - 0.001M)	0

Table 7. Substrates of the alkaline protease of *Culex* sp. and *Aedes* sp. larvae.

Substance
Azocoll
Casein
Hemoglobin
Benzoyl L-arginyl p-nitroamlide

4. Mosquito sterols

We have initiated investigations into the nature of the steroids present in third and fourth instar mosquito larvae. This work has never been done to the best of our knowledge and is of critical importance from the standpoint of infection. At this time we have detected two unknown steroids and a sterol which is similar to cholesterol but not the same substance. Also, we have detected no difference in steroidal character in infected vs. uninfected larvae.

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