METAMORPHIC ANTIGENS OF MOSQUITOES

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In this work, an attempt is made to apply a serological technique to the study of the phylogenetic and metamorphic relationships of five species of mosquitoes. The immediate aims were: (1) to determine the best source of mosquito antigens for inter- and intra-specific studies, and (2) to establish optimal conditions for a serological study utilizing mosquito antigens and rabbit antisera. An eventual aim is to prepare specific, stable and standard serological reagents for use in genetic and physiological investigations.

Lawlor (1949), Zaman and Chellappah (1963), as well as other previous workers, have shown that insect antigenic components do change during metamorphosis. Zaman (*loc. cit.*), working with *Culex pipiens fatigans,* found that fourth instar larval antiserum will react with antigens of second, third, and fourth instar larvae.

Telfer and Williams (1953) studied the chemical changes of insect metamorphosis and came to the conclusion that the reshaping of an organism must surely be accompanied by—or be an outward manifestation of—more fundamental changes in the protein constitution of the organism.

The endocrine glands of all holometabolous insects control the complex processes and changes which accompany development. The evidence indicates that the hormones express their effects by affecting gene activities which result in variations in protein synthesis. Support for this hypothesis is provided in the work of Laufer (1960, 1963) on the giant silk moths *Hyalophora cecropia* and *Samia cynthia* and with the blowfly *Phormia regina* in which immunodiffusion techniques were used to demonstrate changes in the constitution of proteins during ontogeny.

Because of these views, it was felt necessary in this preliminary investigation to study antigens prepared from as many stages as possible of each mosquito species and to determine the specificity and cross-reactivity of such antigens and their respective antisera. The full range of possible combinations is shown in Table 1.

MATERIALS AND METHODS

Five species of the family Culicidae were studied. These included four species of the subfamily Anophelinae, genus *Anopheles* namely, *A. albimanus, A. atroparvus, A. freeborni, A. quadrimaculatus* and one species of the subfamily Culicinae, *Aedes aegypti.* All of these stocks are kept in insectaries of the Department of Zoology, University of Illinois, Urbana, Illinois (Klassen *et. al.,* 1964).

All of the stocks have been identified and reidentified by morphological and/or chromosomal characteristics.

Young healthy male rabbits, about two months old at the beginning of study, were used for the preparation of antisera. They were housed under good conditions of temperature, humidity and light and were provided with all necessary food and water. These animals were kept in a room on a different floor from the insectaries.

PREPARATION OF ANTIGEN. Antigens were prepared from whole unfed adult mosquitoes, pupae, larvae and eggs. Insect material from the various stages was collected over a period of time, cleansed by rinsing in fresh tap and demineralized water at least three times, and stored at −20° C. until adequate quantities were available for extraction procedures.

Wet weight determinations were made and the insect material was then ground in a pre-cooled glass tissue grinder. Ice baths were used during grinding to keep the tissue cool and thus to prevent denaturation of the protein.

The extraction solvent was either physi-

TABLE 1.—Results of Ouchterlony agar-gel diffusion tests using antigens and antisera as indicated. Precipitation reactions are indicated by the following symbols: ‡=strong, +=moderate, —=weak, o=negative. The following symbols are used to indicate the various stages of mosquito used for preparation of antigen: ♂=adult male, ♀=adult female, P=pupa, 4=fourth instar, 3=third instar, 2=second instar, 1=first instar, E=egg.

ANTIGENS		ANTISERA				
		Anopheles albimanus ♂♀P 4 3 2 1 E	Anopheles atroparvus ♂♀P 4 2 1 E	Anopheles freeborni P E	Anopheles quadrimaculatus ♂♀P 2 1 E	Aedes aegypti ♂♀P 4 E
Anopheles albimanus	♂	+ - - + + + o o	+ + + o + o +	+ o	+ - + + + o	o - o o o
	♀	+ + + + + + + +	+ + + o + o +	+ o	+ + + + + o	+ + + - o
	P	+ + + ‡ ‡ + + +	+ ‡ + ‡ + - +	‡ o	‡ + ‡ + + -	+ + + + o
	4	+ + + ‡ ‡ + + +	+ + + ‡ ‡ + +	‡ o	‡ + + + + o	+ + + + o
	3	+ + + + ‡ + + +	+ + + ‡ ‡ + +	+ o	+ + + + + o	+ + o + o
	2	- o o - + + + +	+ + o o ‡ + +	o o	+ - + + + o	+ - o o o
	1	- o o o + + + +	+ + o o ‡ o +	o o	+ o + + + o	o - o o o
	E	- o o o o o + ‡	o o o o - o ‡	o o	o o o o + -	o o o o o
Anopheles atroparvus	♂	+ o o + + o + o	+ + o o + o +	o o	+ o + + + -	+ o o o o
	♀	o o o + + + + o	+ + o o ‡ o +	o -	+ + + + + -	+ + o o o
	P	+ + + + ‡ + + +	+ ‡ ‡ ‡ ‡ + +	‡ -	‡ + ‡ + + +	o + + + o
	4	+ + + + ‡ + + +	+ ‡ + ‡ ‡ + +	+ o	‡ + ‡ + + +	+ + + + o
	2	o o o + + o o o	+ + o o + o +	o o	+ o + + + o	+ o o o o
	1	o o o + + o o o	+ + o o + o +	o o	+ o + + + o	+ o o o o
	E	o o o o o o o ‡	o + o o - + ‡	o +	o o o - + + -	o o o o o
Anopheles freeborni	P	+ + + + ‡ + o +	‡ ‡ ‡ ‡ + + +	‡ o	‡ + ‡ + + +	+ ‡ + ‡ o
	E	o o o o o o o ‡	o o o o o o ‡	o +	o o o o +	o + o o o
Anopheles quadrimaculatus	♂	+ + + + + + o -	+ + + + o o +	+ o	+ + + + + +	+ + o + o
	♀	+ + + + + + + +	+ + + + o o +	+ o	+ + + + + +	+ + + + o
	P	+ o + + ‡ + + +	+ ‡ ‡ ‡ + + +	‡ o	‡ + ‡ + ‡ +	o ‡ + + o
	2	o o o + + - + -	+ + o o ‡ + +	o o	+ o + + + o	o + o o o
	1	o o o + + o + -	+ + o o + o +	o o	+ o + + + +	o o o o o
	E	o o o o o o - ‡	+ + o o o + ‡	o +	o o + + ‡ +	o o o o -
Aedes aegypti	♂	o o o + + + o +	+ + o o + + o	o o	+ o + - + o	‡ + o + +
	♀	+ o o + + o o +	+ + o + + + o	o o	+ o + - + o	+ ‡ + + +
	P	+ - - + ‡ - o +	+ + - ‡ o o o	+ o	+ + ‡ - + o	+ ‡ ‡ ‡ +
	4	+ o o + + o o -	+ + - + o + o	o o	o o + o o o	+ ‡ + ‡ +
	E	o o o o o o o o	o - o o o o o	o o	o o o o o o	o o o o ‡

ological saline or 1/50 molar phosphate buffer used in a dilution ratio of 1:5 to 1:100, weight to volume. Extraction was allowed to take place overnight at 4° C. After centrifugation the clear supernatant was collected and frozen in small quantities for future use. Protein content was determined by use of the biuret method. (See Table 2 for protein determinations).

PREPARATION OF ANTIBODY. Silverman (1963) points out that the best immunization schedule for the stimulation of good antisera must be determined for each antigen by experimentation. After several variations were tested to determine optimum antiserum stimulation, the rabbits were injected according to the following schedule. B = bleeding, I = injection:

14 days 7 days 7 days
$B_0\ I_1$ —— $B_1\ I_2$ —— $B_2\ I_3$ —— B_3
I_4 (if necessary) ——

One milliliter of antigen was given at each injection. B_0 in the above sequence was taken from the marginal ear vein of the rabbit (as were all the bleedings) before the injection of any foreign protein in order to obtain normal rabbit serum for use as a control in agar-gel diffusion tests. About one hour after blood was taken, the clot was separated from the collecting vial walls and allowed to retract overnight at 4° C. The serum was pipetted off the following day and nonclotted red cells removed by centrifugation. When the desired antibody titre was obtained, about 30 ml. of blood were collected. This yielded about 15 ml. of serum for subsequent tests. In some cases, persistently low antibody titre prompted the use of adjuvants. Both aluminum hydroxide and Freund's complete adjuvant were used to enhance antigenicity.

TABLE 2.—Protein concentration of stock antigens. Protein content is given in milligrams/milliliter of equivalent bovine serum albumin, fraction V. (b.s.a.)

Species	Stage	Mg. b.s.a. equivalent
Anopheles albimanus	adult male	1.2
	adult female	3.0
	pupa	4.5
	fourth instar larva	14.0
	third instar larva	4.7
	second instar larva	6.1
	first instar larva	3.0
	egg	3.0
Anopheles atroparvus	adult male	1.0
	adult female	5.5
	pupa	13.6
	fourth instar larva	8.0
	second instar larva	3.3
	first instar larva	3.0
	egg	3.8
Anopheles freeborni	pupa	12.0
	egg	6.5
Anopheles quadrimaculatus	adult male	2.8
	adult female	4.0
	pupa	10.0
	second instar larva	5.0
	first instar larva	4.0
	egg	6.5
Aedes aegypti	adult male	4.0
	adult female	7.5
	pupa	10.7
	fourth instar larva	16.0
	egg	1.6

TESTING OF SYSTEMS. The Ouchterlony (1948) technique of agar-gel diffusion was used for testing antigen-antibody complexes in this investigation. After being set up, the plates were kept refrigerated and observed each day for changes in the reactions. This was continued for at least seven days. Reactions were sketched into a record book and when they reached optimum visibility (usually from 1 to 3 days) the plates were photographed. Staining was used in some cases to enhance faint reaction lines and to preserve the plates for future reference

RESULTS AND DISCUSSION

The full range of tests involved the preparation of several hundred plates. The photographic presentation of these results is a formidable task and an attempt was made to summarize the data in Table 1.

In tests with the pupal systems, specific and homologous reactions were found to occur at the generic level in some cases (Fig. 1 and 2). Later tests, however, revealed some inter-generic cross reactions (Fig. 3 and 4). Another observation from pupal studies was that there appears to be a difference in reactivity depending upon whether the antigen or antiserum for a particular species is used (Fig. 1 and 2). The amount and complexity of cross-reactivity between pupal systems can be related in part to the relatively high protein content of the pupal antigens used in this study (see Table 2).

At least one case of a specific and homologous reaction on the species level was observed in the egg system of *A. atroparvus* (Fig. 5). Due to the proximity

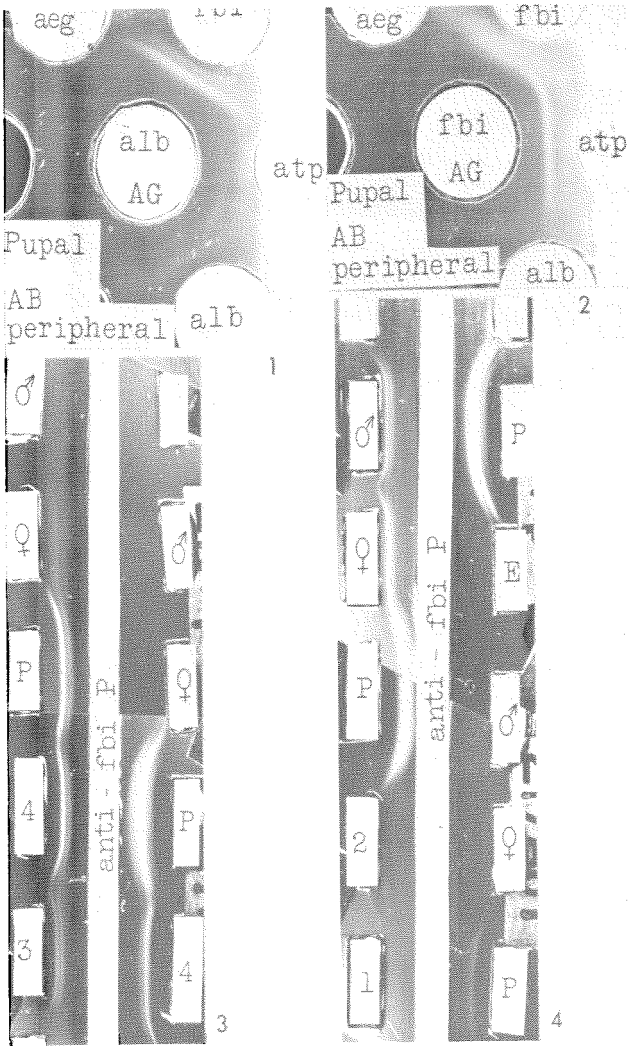

FIG. 1.—Showing agar-gel diffusion precipitation reactions between pupal antigen (AG) and antisera (AB), prepared against *Aedes aegypti* (aeg), *Anopheles freeborni* (fbi), *A. atroparvus* (atp), *A. albimanus* (alb) and *A. quadrimaculatus* (quad). *A. albimanus* antigen in center well and designated antisera in peripheral wells.

FIG. 2.—Showing agar-gel diffusion precipitation reactions of *A. freeborni* antigen with its homologous antiserum and with *A. atroparvus* antiserum.

FIG. 3.—Showing agar-gel precipitation reactions bettween *A. freeborni* pupal antiserum in center trough of the agar-gel plate and antigens prepared from various stages of *A. albimanus* (on left) and *A. atroparvus* (on right).

FIG. 4.—Showing agar-gel diffusion precipitation reactions between *A. freeborni* pupal antiserum in the center trough of the agar-gel plate and antigens prepared from various stages of *A. quadrimaculatus* (on left), *A. freeborni* eggs and pupae (upper right) and various stages of *A. aegypti* (lower right).

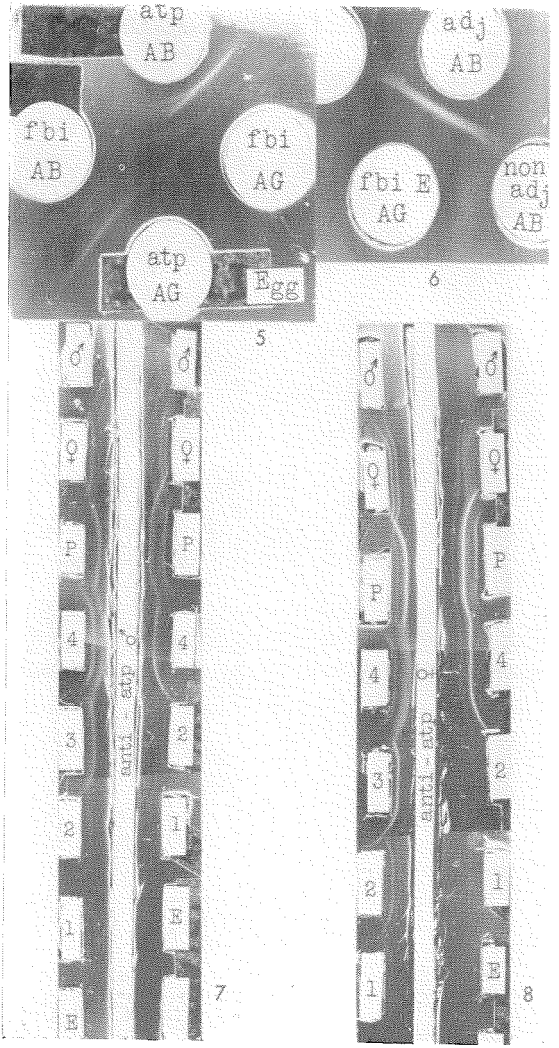

FIG. 5.—Showing agar-gel diffusion precipitation reactions between *A. atroparvus* and *A. freeborni* egg antigens and their respective homologous antisera.

FIG. 6.—Showing agar-gel diffusion precipitation reactions between *A. freeborni* egg antigen and antisera from injections of adjuvanted and non-adjuvanted antigens.

FIG. 7.—Showing agar-gel diffusion precipitation reactions between *A. atroparvus* adult male antiserum in the center trough of the agar-gel plate and antigens prepared from various stages of *A. albimanus* (on left) and *A. atroparvus* (on right).

FIG. 8.—Showing agar-gel diffusion precipitation reactions between *A. atroparvus* adult female antiserum in the center trough of the agar-gel plate and antigens prepared from various stages of *A. albimanus* (on left) and *A. atroparvus* (on right).

of the wells containing heterologous antigens and antisera, the cross-reacting lines were the first to form. The lines (or spurs in this case) showing homologous reactions might have progressed further through the gel if there had been no interference due to the migration fronts of the heterologous reactions.

Adjuvants were found to be required with certain antigens in the production of an antibody titre adequate for use in these studies (Fig. 6). Comparative tests with aluminum hydroxide and Freund's complete adjuvant yielded similar results. Freund's adjuvant was found to be more convenient to use and was adopted as the standard adjuvant when one was required.

Having established that cross reactions occur within a species between egg and pupal systems, other stages in the life cycles of these mosquitoes were examined to attempt to find systems of greater specificity. The tests with male and female adult antisera showed wide ranges of reactivity (Fig. 7 & 8). Antisera against fourth instar larval antigens reacted with most stages of the life cycles against which they were tested, but did not react with extracts of eggs or early larval instars (Fig. 9). Similar results were obtained with the only third instar larval antiserum tested, i.e. *A. albimanus*. First and second instar larval extracts were widely reactive in both homologous and heterologous test systems. On the other hand, in some tests with antisera to first instar larval extracts, there was no visible detection of the homologous antigen (Fig. 10). Protein content of some of the antigens was low and might be the reason for the lack of certain reactions (see Table 2). Freezing and thawing of some of the antigens might have had a deleterious effect and this aspect needs to be examined.

During the course of experimentation when extensive cross reactions were being observed, it was thought that there might be a protein denaturing enzyme in the mosquito extracts, e.g., a protease, which might be responsible for some of the apparent nonspecific reactions. In the agar-gel plate, denaturation of antisera would be indistinguishable from antigen-antibody complexing if this occurred at optimal concentration levels of enzyme and substrate and if it were dependent on a gradient concentration. The "equivalence zone" of the denaturation precipitin line might result in a distinct migration front similar to that obtained with antigen-antibody complexing. If such a denaturing enzyme were present in these antigens, repeated freezing and thawing of the antigens should at least partially destroy its reactivity.

One reactive antigen of high protein content, *A. aegypti* fourth instar larval extract, was frozen and thawed ten to twelve times and then tested against its homologous antiserum. A replicate test, similar except that the antigen in the latter test had been frozen and thawed only once, was set up adjacent to the first test. No distinct differences between the two tests were observed (Fig. 11). However, before this possibility can be completely ruled out, further tests need to be conducted.

CONCLUSIONS

According to Frizzi and De Carli (Kitzmiller, 1963), *Anopheles freeborni* and *A. atroparvus* are closely related to one another phylogenetically, and both are related, but less closely, to *A. quadrimaculatus*. This has been shown by salivary chromosome studies and has been verified by the work of Kitzmiller and Baker and others (personal communication). *A. albimanus* is somewhat removed in relationship, and *Aedes aegypti* is much further removed, being a species within another genus. Our serological results fit in with these phylogenetic relationships.

In the systems studied here there are differences in reactivity between two species depending upon whether antigen or antibody is used as the reagent in heterologous tests (see Fig. 2, 3 and Table 1).

The appearance of spur formations in studies with some of the egg antigens

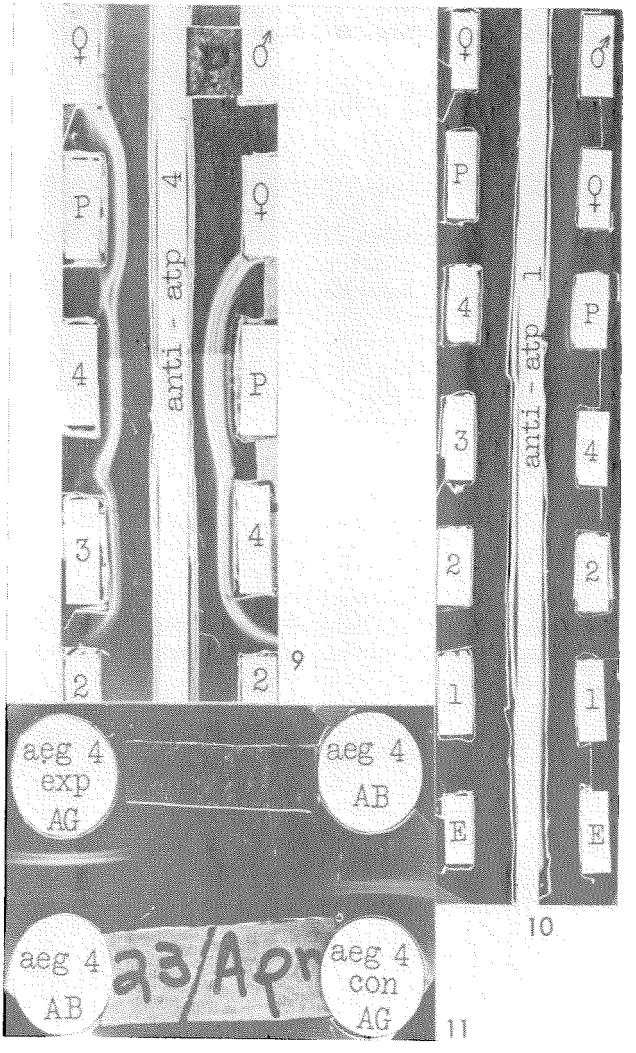

FIG. 9.—Showing agar-gel diffusion precipitation reactions between *A. atroparvus* fourth instar larval antiserum in the center trough of the agar-gel plate and antigens prepared from various stages of *A. albimanus* (on left) and *A. atroparvus* (on right). There were no reactions with extracts from second or first instar larvae or with eggs and thus these portions of the plate were not photographed.

FIG. 10.—Showing agar-gel diffusion precipitation reactions between *A. atroparvus* first instar larval antiserum in the center trough of the agar-gel plate and antigens prepared from various stages of *A. albimanus* (on left) and *A. atroparvus* (on right).

FIG. 11.—Showing agar-gel diffusion precipitation reactions between *A. aegypti* fourth instar larval antigen and its homologous antiserum after ten (10) freezings and thawings (on left) and after two (2) freezings and thawings (on right).

(Fig. 5) indicated that it is possible to detect specific reactions which have not been observed with the precipitin method used by Lawlor (1949) in which reactions were measured in a liquid rather than a semisolid medium.

Studies with adjuvanted antigens show that adjuvant does enhance the production of antibody, and thus helps in the production of antisera which are sufficiently reactive to exhibit precipitin reactions which had not been previously seen (Fig. 6).

Mosquito egg antisera exhibited a wide range of antigen detection of all stages as well as generic specificity. Egg antiserum might pick up the more specific proteins present in extracts of younger stages which are either changed, completely removed, or more likely, masked during the ontogeny of later stages. This concept is supported by the observations that very few of the antigens from the more highly differentiated stages stimulated antisera capable of detecting the presence of egg antigens.

A point of interest was the unexpected lack of general reactivity from female adult antisera as compared to that of male adult antisera. This might be due to the production of proteinaceous materials in the female associated with ovarian development and with the salivary glands which are not found in any of the other stages in the life cycle or in the adult males.

In spite of apparent advantages, even the egg system does not fully satisfy the requirements for specific serological reagents. Although unaltered extracts of whole animals of various stages do give some indications of specificity and can be standardized and kept in a stable form, they are not entirely satisfactory as serological reagents for taxonomic or physiological purposes.

In view of the many cross-reactions observed with antigens and antisera prepared from whole animal extracts, it seems that studies with simple organ proteins might yield reagents more specific than those described here. Other workers have reported greater specificity with organ proteins (Downe, 1962).

Absorption and fractionation of the antigenic extracts should provide a means toward the preparation of reagents with greater serological specificity. Preliminary studies with immunoelectrophoretic techniques have revealed that some of the precipitin lines observed with the Ouchterlony method contain as many as five and six components. Separations of these components by chromatographic methods and the use of other serological tests, e.g. hemagglutination, appears to offer promising lines of research.

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