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Changes in hemocytes and hemolymph proteins of Aedes aegypti following encapsulation of a nematode parasite Neoaplectana carpocapsae.

Theodore G. Andreadis
University of Massachusetts Amherst

CHANGES IN HEMOCYTES AND HEMOLYMPH PROTEINS OF *AEDES AEGYPTI* FOLLOWING ENCAPSULATION OF A NEMATODE PARASITE *NEOAPLECTANA CARPOCAPSAE*

A Thesis Presented

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Theodore G. Andreadis

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Approved as to style and content by:

Donald W. Hall
(Chairman of Committee)

T M Peters
(Head of Department)

John C. Scaffidi Jr.
(Member)

Henry H. Gordon
(Member)

December, 1974

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TABLE OF CONTENTS

INTRODUCTION

Insect defense mechanisms to various foreign organisms do not appear to be homologous to their counterparts in the vertebrate system. They are far less specific since antibodies are not formed and no immunological memory is seen as measured by an increased responsiveness to foreign substances as a result of prior exposure (Good and Papermaster 1964; Saunders 1970).

Nevertheless insects are clearly capable of protecting themselves from parasitic organisms. Many insects exhibit phagocytosis against various microorganisms, such as viruses, bacteria, fungi and protozoans, wherein a single host hemocyte engulfs these foreign particles (Salt 1970). But against foreign bodies too large to be phagocytized by a single cell, such as metazoan parasites, the primary mechanism of defense is encapsulation (Salt 1970).

Encapsulation may be defined as the accumulation of host cells (usually hemocytes) around a parasite resulting in the formation of a capsule (Salt 1970). Often encapsulation is accompanied by the deposition of a black pigment, generally referred to as melanin, on or near the surface of the parasite. This response is commonly referred to as melanotic encapsulation (Salt 1963, 1970; Shapiro 1969; Poinar 1969, 1974). If encapsulation is successful, the parasite is killed or at least restricted in its movements (Salt 1970).

The study of insect defense mechanisms to various parasites is not purely academic. We are in an age where we can no longer depend solely upon chemical agents for control of insect pests. Integrated control is

becoming widely accepted and the use of parasitic organisms has great potential. Many field studies have already been conducted utilizing nematode parasites as potential biological control agents (Jaques 1967; Israel *et al.* 1969; Nash and Fox 1969; Harlan *et al.* 1971; Benham and Poinar 1973). But before we can go any farther we must develop a better understanding of the mechanisms involved in insect defense reactions. It is vital to future research to determine why certain parasites are encapsulated and others are not. For example, certain species of mosquitoes have been shown to exhibit varying degrees of resistance to parasitism by encapsulating the nematode *Reesimermis nielseni* (Peterson *et al.* 1968; Peterson and Willis 1972; Peterson 1973), while on the other hand these same species of mosquitoes readily encapsulate the nematode *Neoaplectana carpocapsae*.

Biological control programs which seek to utilize entomophilic parasites must have a better understanding of insect defense reactions. This will enable them to choose the right parasite for a particular insect pest and hopefully lead to a more effective manipulation of insect pest populations.

This thesis consists of a literature review which provides information on encapsulation and melanization of internal metazoan parasites and the changes that occur in host hemolymph proteins during these reactions. The remainder of the thesis contains all experimental work, written in manuscript form which will be submitted for publication.

LITERATURE REVIEW

Defense reactions of insects to metazoan parasites

The primary mechanism of defense in insects to metazoan parasites is encapsulation. It has been observed in a few hundred species of insects representing fifteen orders, and as more insects are investigated the list will undoubtedly increase (Salt 1970).

There appear to be two distinct methods of capsule formation: cellular and humoral, the latter occurring without the direct participation of hemocytes.

Cellular encapsulation. Cellular capsules formed in different insects are similar in basic structure but show a good deal of variety in detail that is characteristic of the insect forming the capsule and the parasite or foreign object being encapsulated (Salt 1970). In an attempt to classify capsules surrounding parasites, Salt (1970) tentatively differentiated between two kinds, the cellular and sheath type. Cellular capsules are composed of hemocytes which adhere close to the parasite and form a consolidated tissue fifty cells or more thick. Melanization may follow but is often partial. Sheath capsules, on the other hand, are much thinner and consist of a tough brown inner sheath generally referred to as melanin, overlain by relatively few layers of cells. Melanization is complete. Most evidence indicates that capsules of this nature are formed principally, if not exclusively, by hemocytes (Salt 1970). Occasionally fat body, tracheoles, muscle and even malpighian tubules are attached to the capsule, but this is believed to be due to hemocytes aggregating in such a way as to include them, rather than to their own

active participation (Salt 1970; Stoffolano and Streams 1971).

Considerable literature has been written on the structure and development of cellular capsules. Early studies at the light microscope level described spherical hemocytes aggregating around a foreign object or parasite and gradually becoming flattened over its surface (Salt 1963). It has been suggested by Nappi (1974) that the flattening of hemocytes during capsule formation may represent an unsuccessful attempt by individual cells to engulf the parasite and contributes to their ability to adhere to the parasite and cohere to each other.

As the reaction continues more hemocytes are continuously added and after a few hours to a few days, depending on the host, the capsule can be seen to be divided into two distinct layers: a relatively transparent layer next to the parasite and an outer less transparent layer which is composed largely but not exclusively of flattened hemocytes (Meyer 1926; Lartschenko 1933; Salt 1963). Many hemocytes associated with the outer layer of the capsule retain their original spherical shape and may eventually separate from the mass and disperse into the hemolymph resulting in a decrease in the size of the capsule after maximum development (Salt 1955, 1956, 1963).

While many early investigators interpreted the inner structure of the capsule as being syncytial, a disagreement existed whether this was the case (Lazarenko 1925; Meyer 1926; Chen 1934).

Recent electron microscope studies of capsule ultrastructure in some insects have shown that cells retain their integrity with no breakdown of cell membranes (Grimestone et al. 1967; Poinar et al. 1968).

Grimestone et al. (1967) studied the ultrastructure of hemocytic capsules formed around small pieces of Araldite which were implanted into the hemocoel of the Mediterranean flour moth, Ephestia kuehniella. Thick compact cellular capsules 40-50 m thick were completed by 72 hours and were comprised of three regions. The cells in all three layers retained their integrity and provided no evidence for the formation of a syncytium. However, the possibility that some fusion of cells may have occurred could not be excluded.

Poinar et al. (1968) studied the ultrastructure and formation of melanotic cellular capsules in Diabrotica sp. in response to parasitism by the nematode Filipjevivermis leipsandra. Initial hemocytes making contact with the parasite underwent autolysis releasing cytoplasm over the nematodes cuticle. Within a few hours after cell lysis, the liberated cytoplasm entered the cuticular folds of the nematode and was transformed into an electron dense layer, identified as melanin. However, the majority of cells that formed the capsule did not lyse, but displayed various degrees of necrosis and formed dense inclusions which were suggested to represent intracellular stages in the production of melanin.

By 72 hours, encapsulation was complete and definitive capsules could be seen to be comprised of four distinct regions: (1) an inner noncellular region composed of melanin, (2) a single irregular layer of entirely necrotic, partially melanized cells, (3) a third region consisting of three to four layers of closely packed flattened cells, (4) and outer region of loosely attached cells which closely resembled normal circulating hemocytes.

With the exception of those cells involved in the formation of the layer of melanin, most hemocytes retained their integrity and did not form a syncytium (Poinar et al. 1968).

Humoral encapsulation. Encapsulation as defined by Salt (1970) is carried out exclusively by hemocytes; but, recent studies suggest that in insects such as chironomids and culicids, where circulating hemocytes are rare, encapsulation and melanization can be accomplished without the direct participation of hemocytes (Gotz 1969; Poinar and Leutenegger 1971; Gotz and Vey 1974). In this case the deposition of melanin is attributed to components in the noncellular portion of the hemolymph which precipitate or coagulate out on the surface of the nematode (Poinar and Leutenegger 1971).

Kartman (1956) reported that in Aedes aegypti an encapsulating pigment was deposited about microfiliariae of Dirofilaria immitis as a fine coating showing cuticular striations of the nematode.

Esslinger (1962) described a case of "pigmental encapsulation" in Anopheles quadrimaculatus in response to parasitism by microfilariae of Brugia pahangi. Brown homogeneous plaques, intermingled with an acellular fibrous material, could be observed to adhere to the surface of the microfilariae in the abdominal hemocoel of the mosquito. There was no indication that this response was directly associated with any specific type of cell.

Bronskill (1962) studied the formation of capsules in larval A. aegypti parasitized by juveniles of the rhabditoid nematode Neoaplectana carpocapsae (formally known as DD-136). Shortly after invasion of the hemocoel of the host, extremely thin, minute particles of

melanin began to be deposited in a continuous layer over the surface of the nematode. The deposition of melanin continued until the nematode was completely enveloped in a thinly melanized sheath. Coincident with melanin deposition, host hemocytes, containing cytoplasmic melanin particles, accumulated about the partially melanized sheath. The proximal cells soon became completely melanized and were deposited as chunks of melanin over the nematode.

In histological section the capsule appeared as a two layered structure consisting of an inner layer of melanin clumps, deposited on the initially melanized cuticular sheath and an outer layer of host hemocytes, many of which contained melanin particles in their cytoplasm.

Similar capsule formation was also observed in larvae of Aedes stimulans and Aedes trichurus parasitized by the same nematode (Bronskill 1962).

In an ultrastructural investigation of encapsulation of N. carpocapsae in larval Culex pipiens, Poinar and Leutenegger (1971) reported the formation of a melanized sheath about the nematode without the direct participation of hemocytes. Soon after entry into the host's hemocoel an initial homogeneous matrix was deposited around the nematode. Within the matrix minute pigment granules began to form which eventually enlarged and coalesced resulting in a uniform layer of melanin around the parasite. Electron micrographs revealed a sheath composed of two regions: (1) an inner, melanized homogeneous layer adjacent to the nematode which failed to demonstrate any definite structures or cell organelles and (2) a non-melanized or lightly melanized homogeneous region about the edge of the sheath. Occasionally a third

layer containing cellular debris and tracheole elements was observed along with cell organelles, which probably arose from the lysis of tracheoles and hemocytes (Poinar and Leutenegger 1971).

They hypothesized three possibilities for the origin of this capsular matrix: (1) it could be connective tissue which adheres to the nematode as it brushes past the internal organs during its movement in the host, (2) it could arise from hemocytes liberating a deposit on contact with the parasite, (3) it could consist of protein coagulating out of the non-cellular portion of the hosts hemolymph. Based on, (1) the uniformity of the deposit, which would be unlikely to occur by chance contact with host tissue, (2) the absence of any cellular inclusions, revealed from electron micrographs and (3) the relatively few circulating hemocytes in C. pipiens, support was given to the third hypothesis.

In working with Chironomus larvae Götz (1969) reported humoral encapsulation against juveniles of the mermithid, Hydromermis contorta. Within two to three minutes after penetration of the host's hemocoel, deposition of a capsular substance appeared as droplets upon the nematode cuticle which increased in number and size until a complete envelope was formed. This was soon followed by melanization and thickening of the capsule.

The same response was elicited when nematodes were placed in a drop of isolated hemolymph in vitro, and electron micrographs showed no difference in the ultrastructure of capsules formed in vivo and in vitro (Gotz 1969). The capsular substance was not secreted by hemocytes attached to the parasite but developed from precursors dissolved in the

hemolymph (Gotz 1969).

Knowledge of this type of humoral encapsulation has not been restricted to parasitic nematodes. It occurs in Chironomus larvae in response to other nematodes, Turbatrix aceti and Rhabditis sp. (Götz 1969, 1973), and against certain pathogenic fungi, Aspergillus niger and Mucor hiemalis (Götz and Vey 1974). Humoral encapsulation does not occur in response to inanimate substances such as glass fibers, nylon, cellulose, or iron particles (Götz 1969).

Although humoral encapsulation is rare and does not usually occur against parasites in their "natural hosts," it may be a fairly common response when parasites invade "foreign hosts" (Poinar 1974).

Effects of encapsulation on the parasite. The effects of encapsulation on invading parasites will vary depending on the insect forming the capsule and the parasite being encapsulated.

Those effects, can be categorized as follows:

1. No perceivable effect--Trematode metacercariae are frequently encapsulated but their development is normal and unrestricted (Poinar 1969).

2. Suppression of parasite activity--Capsules may provide a barrier which will restrain the movements of an active parasite through the hemocoel of the host (Salt 1970).

3. Retard growth--Retardation of growth is common in parasites that are partially encapsulated and therefore not killed (Miller 1943).

4. Inhibition of development--Encapsulation may provide a physical effect by permanently imprisoning a parasite and preventing its growth but allowing it to remain alive for a considerable time (Muldrew 1953;

Bronskill 1960; Welch and Bronskill 1962; Nappi and Stoffolano 1971; Stoffolano and Streams 1971). It has been suggested that inhibition of further parasite development may be due to the capsule interfering with oxygen supply to the parasite (Bronskill 1960, 1962).

5. Death--Death due to encapsulation may result from a number of factors, none of which has proved conclusive. A good deal of evidence has shown oxygen tension to be low inside a capsule (Salt 1963) and many investigators believe that encapsulated parasites die due to asphyxiation (Muldrew 1953; Wigglesworth 1959; Bronskill 1960; Salt 1970). This could explain why encapsulation must be complete to be effective (Van den Bosch 1964).

The efficiency of encapsulation as a defense mechanism. Encapsulation in insects has been shown to be an effective method of dealing with many foreign organisms. Healthy insects, in general, will encapsulate any parasite that invades their hemocoel unless the parasite has and uses some specific means of avoidance (Salt 1970).

Habitual parasites have developed a variety of mechanisms which enable them to avoid or suppress encapsulation in their "natural hosts" (Salt 1965, 1966, 1968; Lewis and Vinson 1968; Nappi and Streams 1969; Vinson 1971).

Two general means of avoiding host encapsulation on the part of the parasite have been proposed (Nappi and Streams 1969). One theory suggests that the parasite actively suppresses the host's encapsulation reaction (active resistance) and the second theory suggests that the parasite avoids recognition by the host as a foreign object (passive resistance).

Encapsulation does not act exclusively against alien parasites. The reaction is also initiated against habitual parasites if they fail to use the means of resistance they have evolved (Salt 1966; Poinar et al. 1968).

Hemocytes and encapsulation

Changes in hemocytes as a result of encapsulation. Unfortunately, very few studies have been conducted on the changes that occur in types and numbers of hemocytes during encapsulation reactions. This is complicated by the fact that qualitative and quantitative changes may occur in response to injury and would repair as a result of oviposition by parasitic wasps or by the artificial implantation of objects or by activity of the parasite (Salt 1963, 1970). Therefore, caution must be exercised in distinguishing hemocytic changes due to wound repair and actual parasitism (Shapiro 1969).

Muldrew (1953) observed that when he implanted rose thorns into the body cavity of the larch sawfly Pristiphora erichsonii, hemocytes increased fivefold during a nine-day period followed by a decline to the normal number on the nineteenth day.

Nappi and Streams (1969) investigated the changes in hemocytes associated with the immune reaction of Drosophila melanogaster when parasitized by Pseudeucoila bochei. Differential hemocyte counts taken during the early stages of capsule formation showed an increase in the percentage of lamellocytes and a decrease in the percentage of crystal cells. The increase in lamellocytes was attributed to the early transformation of large numbers of plasmatocytes, while the decrease in crystal cells was believed to have resulted from the lysis of these cells and the release of phenolic substances respon-

sible for the melanization of the capsule. The presence of abnormally large numbers of lamellocytes before there was any visible evidence of encapsulation suggested that these cells were stimulated to react without making direct contact with the parasite (Nappi 1973a). This view was earlier rejected by Salt (1970) who proposed that cellular reactions were initiated by accidental contact of host hemocytes with foreign organisms rather than the specific attraction of these cells from a distance.

In a different host species Drosophila euronotus, eggs and larvae of the same parasite are melanized but not encapsulated (Nappi 1970). Differential hemocyte counts showed a decrease in the percent of oenocytoids (believed to be involved in phenoloxidase activity), while there was no significant change in the percent of lamellocytes.

In many insects which do not elicit a host response when parasitized either because the reaction is suppressed or the parasite avoids recognition, there are no significant changes in the total or differential hemocyte counts (Nappi and Streams 1969; Vinson 1971).

Nappi and Stoffolano (1971, 1972a, 1972b) investigated the hemocytic changes that occurred in Musca domestica and Orthellia caesarion in response to parasitism by the nematode Heterotylenchus autumnalis. In nonparasitized larvae the majority of hemocytes were concentrated in the last two segments of the body, and only in larvae ready to pupate could hemocytes be seen to circulate throughout the hemocoel. However, when larvae were parasitized, large numbers of hemocytes left this region, entered circulation and encapsulated parasites in various regions of the body. The initial reaction, which was characterized by the aggregation and fusion of oenocytoids and the deposition of a pigmented

layer of melanin, was followed by the subsequent aggregation of other hemocytes to form a complete capsule.

Based on these findings, Nappi and Stoffolano (1972b) suggested that some stimulus in infected larvae, either coming from the parasite or the initial hemocytes making contact with the parasite and/or certain biochemical changes due to parasitism, caused hemocytes to move out of the posterior areas prematurely and encapsulate the nematodes.

Maier (1973) found that when larvae of Chironomus thummi were parasitized by a mermithid nematode, there was a decrease in the number of granular transitional hemocytes and an increase in the number of plasmatocytes. He suggested the lysosome rich granular hemocytes underwent autolysis releasing a substance which may have been responsible for the activation of a phenoloxidase.

The hemocyte complex in mosquitoes. Examinations of hemocytes in mosquitoes have shown circulating hemocytes to be extremely rare or nonexistent (Jones 1953).

In an examination of living specimens, Jones (1953) reported that larvae of C. pipiens contained a variable number of ovoid cells which circulated freely in the hemocoel in the region of the perivisceral sinus. Anopheles quadrimaculatus larvae were shown to possess very few cells which rarely circulated or when they did, only for very short distances. They were normally found lying against the inner wall of the epidermis. Larvae of A. aegypti contained no free hemocytes when examined but were observed to have few to numerous sessile hemocytes lying within the anal papillae (Jones 1958).

In lieu of these observations it would appear to be advantageous

for insects with few hemocytes to shift the encapsulation reaction from a function of hemocytes to that of a function of free hemolymph (Gotz and Vey 1974).

Melanization

Encapsulation is often accompanied by the deposition of a dark pigment, generally referred to as melanin. The incidence of melanization is variable. It may or may not accompany encapsulation, and when it does it may occur promptly or be delayed, depending on the insect host (Muldrew 1953; Nappi and Streams 1969; Salt 1970).

Melanization may also occur in the absence of cellular encapsulation, either as a hemocytic response (Nappi 1970) or as a purely humoral reaction without the apparent intervention of hemocytes (Bronskill 1962; Poinar and Leutenegger 1971; Götz and Vey 1974).

Where melanization accompanies encapsulation, melanization of the capsule is presumably caused by the disintegration of the innermost hemocytes. This results in the deposition of melanin particles over various parts of the body, which eventually cover the entire parasite (Salt 1970).

On the other hand, melanization in humoral reactions is attributed to the non-cellular components of the hemolymph and is characterized by the deposition of minute melanin particles which coalesce to form a continuous layer (Bronskill 1962; Poinar and Leutenegger 1971).

Inert objects implanted in insects usually do not induce a melanin reaction, presumably because they do not cause disorganization of cells by movement or by lysis. However, if an inert object is continuously moved, cells may be broken and melanin deposited (Salt 1956).

Evidence for melanin in the capsular matrix. Evidence to determine the nature of the brownish black pigment in capsules has for the most part been circumstantial with only a few authors actually demonstrating the presence of melanin. Salt (1956) showed that the deposition of pigment in capsules of Carausius could be inhibited by the injection of phenylthiourea (PTU), a known inhibitor of melanin formation. Nappi (1973b) demonstrated that by feeding host larvae of Drosophila algonquin on a diet containing PTU, the deposition of melanin on eggs of the parasitoid P. bochei could be blocked.

Chemical tests have been conducted for melanin in cellular capsules formed in response to nematode parasitism (Poinar et al. 1968) and in humoral capsules formed in response to fungi, nematodes and implanted latex particles (Poinar and Leutenegger 1971; Gotz and Vey 1974). Solubility tests showed the pigmented material was soluble in concentrated sodium hydroxide, was bleached in hydrogen peroxide and gave positive results for melanin with ammoniacal silver nitrate (Mason-Fontana test). These results strongly suggested that the pigmented material surrounding these foreign objects was melanin.

Enzyme substrate localization and mode of activation. Melanin formation in insects has frequently been investigated, but primarily in connection with hardening and darkening of the cuticle (Dennell 1958; Cottrell 1964; Hackman 1964). The actual formation of melanin results from the enzymatic action of phenoloxidase (also referred to as tyrosinase, phenolase, polyphenoloxidase, dopa oxidase and catechol oxidase) on tyrosine which is hydroxylated to form 3,4-dihydroxyphenylalanine (DOPA). DOPA is oxidized to dopa-quinone which undergoes

spontaneous ring closure and decarboxylation to form 5,6-dihydroxyindole which is polymerized to form melanin (Lerner and Fitzpatrick 1950; Gilmour 1965).

The substrates, tyrosine and DOPA are present in insect hemolymph (Florkin and Jeuniaux 1974) and in several tissues including hemocytes (Rizki and Rizki 1959) and fat body (Price 1972).

The enzyme, tryrosinase is also present in insect hemolymph (Sussman 1949) but exists as an inactive proenzyme which requires a specific protein activator for activation (Ohnishi 1959; Evans 1967; Hackman and Goldberg 1967). In addition certain insect hemocytes normally synthesize and/or concentrate various amounts of phenols and enzymes associated with tyrosine metablism (Dennell 1958; Rizki and Rizki 1959; Chadwick 1966; Preston and Taylor 1970; Pye and Yendol 1972).

Presumably melanization is normally controlled by the structural isolation of either enzyme and substrate or inactive proenzyme and activator (Ohnishi 1959; Rizki and Rizki 1959; Hackman and Goldberg 1967; Evans 1967). Crystal cells in D. melanogaster contain both substrate (tyrosine) in crystal inclusions and enzyme (tyrosinase) in the surrounding cytoplasm. Any treatment which may disrupt the structural integrity of the cell will allow the enzyme and substrate to come together resulting in the production of melanin (Rizki and Rizki 1959). It has been suggested that this mechanism is involved in the melanization of certain parasites. Hemocytes, upon coming in contact with the parasite, may release substances which initiate the localized synthesis of melanin (Poinar et al. 1968; Nappi and Streams 1969).

Where hemocytes are not involved (humoral melanization), the parasite or foreign object may upset the mechanism which allows tyrosine and inactive tyrosinase to co-exist in the hemolymph thus resulting in melanin formation (Götz 1969; Poinar and Leutenegger 1971; Götz and Vey 1974).

Injury or wounding would also upset this mechanism as melanin is formed when an insect's body is opened and tissues exposed (Rizki and Rizki 1959).

Melanization as a mechanism of defense and the role of the polyphenol-polyphenoloxidase system. The role of melanization in defense reactions is unclear. In many cases it is secondary to cellular encapsulation and appears to provide no additional protection (Salt 1970). However, in a few hosts, melanization is known to be essential for the death of the parasite which can survive cellular encapsulation but not subsequent melanization (Salt 1970). Melanization always occurs promptly in humoral encapsulation (Götz and Vey 1974).

Taylor (1969) believes the polyphenol-phenoloxidase system is directly involved in invertebrate immunity and suggests that it may be one of the most primitive, nonspecific defense systems of all living forms, capable of killing microorganisms and parasites, isolating foreign objects and sealing and repairing wounds. Brewer and Vinson (1971) have reported that injections of PTU and glutathione (known inhibitors of phenoloxidase) into experimental animals will inhibit not only melanization but also encapsulation of a parasite.

Effects of parasitism on hemolymph proteins

Several studies have been conducted on the changes that occur in

hemolymph proteins of insects when parasitized by habitual parasites that are not encapsulated.

Vinson and Barras (1970) demonstrated several changes in the protein pattern of Heliothis virescens following parasitism by Cardiochiles nigriceps. Results of disc electrophoresis of hemolymph removed from parasitized larvae ten days after initial parasitism revealed the loss of one major band, the addition of one minor band and an overall shifting of banding patterns. They suggested the parasite utilized or destroyed certain hemolymph proteins while causing a shift in free amino acids to the bound state or the secretion of other proteins into the hemolymph. Barras et al. (1972) demonstrated changes in hemolymph proteins of Heliothis zea parasitized by Microplitis croceips. Electropherograms revealed one additional major and minor band, significant changes in banding patterns, and major changes in band intensity. They suggested the parasitoid broke down or utilized certain host proteins for its own development and caused the release or synthesis of other proteins by the host.

Unfortunately, only one study has been conducted to date which has examined the changes in hemolymph protein when a parasite is actually encapsulated. Brewer et al. (1973) conducted a comparative study of hemolymph proteins from H. zea when parasitized by C. nigriceps. Electropherograms of hemolymph from superparasitized individuals revealed a reduction in the total number of protein fractions and contained four bands which were not detectable in control larvae. They suggested these additional bands might play a significant role in the encapsulating capacity of H. zea. It appeared that H. zea possessed a mechanism to

overcome the defensive coatings or secretions of the parasite and thus enable it to encapsulate. This mechanism may well have been represented by one or more of these protein bands.

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ABSTRACT

Changes that occur in hemocytes and hemolymph protein of larval Aedes aegypti during encapsulation and melanization of a nematode parasite Neoaplectana carpocapsae were investigated. Following parasitism, there were significant decreases in both the total hemocyte count and in the number of DOPA-oxidase positive hemocytes within the anal papillae. Ligation experiments indicate these hemocytes are not necessary for successful capsule formation. The possibility of these changes resulting from a pathological condition created by the parasite are discussed.

Disc electrophoresis revealed several changes in the protein migration pattern of A. aegypti hemolymph following encapsulation of N. carpocapsae. Gels stained with amido schwartz showed a shift in certain bands, a reduction in intensity of another and the presence of an additional protein fraction. These changes appear to be specific for parasitism and/or encapsulation. Incubation of gels in DOPA solution revealed an increased intensity of one protein fraction which is not specific for encapsulation but may be the result of a wound response. It appears that some protein is released by the host or by the parasite in response to parasitism. While the function of this protein is unknown, it may play a role in the defense reactions of the host.

INDEX DESCRIPTORS: Aedes aegypti; Neoaplectana carpocapsae; encapsulation; melanization; hemocytes; hemolymph proteins; DOPA-oxidase.

INTRODUCTION

The primary mechanisms of defense in insects to invasion by nematode parasites are encapsulation and melanization. These responses are typically characterized by the aggregation of host cells (usually hemocytes) around the parasite and the disintegration of the innermost cells which results in the deposition of melanin and the formation of a capsule (Poinar et al. 1968; Salt 1970). In insects with few circulating hemocytes encapsulation and melanization can occur without the direct participation of hemocytes (Götz 1969; Poinar and Leutenegger 1971; Götz and Vey 1974). In this case the deposition of melanin and the capsular matrix are attributed to components in the non-cellular portion of the hemolymph which precipitate out on the surface of the nematode parasite (Poinar and Leutenegger 1971).

Encapsulation and melanization reactions in larval Aedes aegypti in response to parasitism by the nematode Neoaplectana carpocapsae have been reported to occur without the apparent initial intervention of host hemocytes (Bronskill 1962). This would appear to be advantageous as these insects have been reported to contain no free circulating hemocytes but rather have sessile hemocytes within the anal papillae (Jones 1953, 1958).

However, the situation in A. aegypti may be analogous to that which occurs in Musca domestica and Orthellia caesarion (Nappi and Stoffolano 1971, 1972a, 1972b). In nonparasitized larvae the majority of hemocytes are concentrated in the last two segments of the body. When parasitized, large numbers of hemocytes leave this region, begin to circulate, and

encapsulate parasites in various regions of the body.

Unfortunately, very few studies have been conducted to demonstrate the changes that occur in hemocytes and hemolymph proteins during encapsulation and melanization reactions. Nappi and Streams (1969) reported an increase in lamellocytes and a decrease in crystal cells in Drosophila melanogaster following parasitism by Pseudeucoila bochei. The increase in lamellocytes was attributed to the early transformation of plasmatocytes. The decrease in crystal cells, which contain both substrate and enzyme necessary for melanin formation (Rizki and Rizki 1959), was believed to have resulted from the lysis of these cells and the subsequent release of phenolic substances responsible for melanization of the capsule.

Brewer et al. (1973) demonstrated several changes in hemolymph proteins of Heliothis zea when parasitized by Cardiochiles nigriceps. Electropherograms revealed the presence of several protein fractions which were not present in hemolymph from nonparasitized individuals. They suggested these additional protein bands may have resulted from the action of parasitism or the reaction of melanization and may play a role in the encapsulating capacity and defense of the host to the parasite.

The present study was undertaken to demonstrate the changes that occur in hemocytes and hemolymph proteins of larval A. aegypti during encapsulation and melanization reactions and to further investigate the possible distribution of DOPA-oxidase within hemocytes.

MATERIALS AND METHODS

Experimental animals

Aedes aegypti used in this study were a University of Massachusetts strain originally obtained from Rutgers University. A standard rearing technique was used (Peters *et al.* 1969) which yielded individuals of uniform size, age and presumably similar physiological condition.

Neoaplectana carpocapsae nematodes were originally obtained from Dr. Donald Harlan of the Bioenvironmental Insect Control Laboratory, Stoneville, Mississippi and propagated in larvae of the wax moth *Galleria mellonella* which yielded large numbers of infective stage juveniles.

Infection of mosquito larvae

To parasitize *A. aegypti*, 40 fourth instar larvae were placed in 60 x 15mm petri dishes containing approximately 500 infective stage juvenile nematodes per ml of distilled water. Larvae were exposed for three hours at 25-27°C and then examined for capsules under a dissecting microscope. This procedure was used in all experiments.

Total hemocyte counts

To characterize the change in hemocytes of the anal papillae, total hemocyte counts (THC) were made on parasitized mosquito larvae containing encapsulated nematodes. Controls consisted of larvae of the same age that were not exposed to nematodes. All larvae were fixed in 4% formaldehyde (buffered at pH 7.4 with 0.1M cacodylate buffer, made hypertonic with 0.44M sucrose)(Rodriquez and McGavran 1969) and examined

by phase contrast microscopy. Earlier investigations showed that treatment with fixative did not destroy these cells and its use facilitated observations on individual mosquito larvae at approximately the same stage of the encapsulation procedure. Spindle shaped plasmatocytes and round or ovoid oenocytoids were observed but not differentiated, as oenocytoids comprised an insignificant percentage of the THC in both treatments. Total counts were tabulated per mosquito and statistically analyzed.

DOPA-oxidase activity in hemocytes

Hemocytes were examined for DOPA-oxidase activity according to the method of Rodriquez and McGavran (1969). Whole mosquito larvae were prefixed in Karnovsky's fixative (Karnovsky 1965) for 30 min. in an ice bath. Anal papillae were then excised and fixed for an additional 1½ hrs. at ice bath temperature. Controls consisted of anal papillae from nonparasitized mosquitoes with: (1) no additional treatment, (2) heat treated to 95°C for 30 min., and (3) pre-incubated in 0.1% phenylthiourea (PTU) before incubation. All papillae (from parasitized and control larvae) were incubated at 37°C for six hrs. in 0.1% DL-dihydroxyphenylalanine (DOPA) in 0.1M cacodylate buffer (pH 7.4) with the exception of those previously treated with PTU. They were incubated in a solution of 0.1% PTU and 0.1% DOPA. Solutions were changed every two hrs. The deposition of a brown-black pigment within the hemocytes was considered to be a positive response for DOPA-oxidase activity.

To observe the effect of encapsulation on those hemocytes that elicited a positive response for DOPA-oxidase activity, THCs were made on DOPA incubated anal papillae from parasitized and control mosquitoes.

Melanized and nonmelanized cells were differentiated and counts were analyzed for statistical differences due to treatments.

Ligation experiments

Ligations were performed on larvae to isolate hemocytes in the anal papillae from the rest of the body and prevent their possible circulation in response to nematode parasitism. This procedure involved tying a small piece of nylon thread around the anal segment. Larvae were then exposed to nematodes and examined for the presence of encapsulated and nonencapsulated nematodes. Capsules, when found, were dissected out and compared with those from mosquitoes which were not ligated.

Electrophoresis of hemolymph proteins

Hemolymph samples were obtained from parasitized, nonparasitized, and wounded mosquito larvae and separated using disc gel electrophoresis as described by Davis (1964). Hemolymph was extracted by making a small incision in the side of the thorax with a fine minuten pin and collecting the exuding droplets with a 5 µl pipette. Thirty µl of hemolymph were obtained from a total of 200 mosquito larvae. Extreme care was exercised so as not to puncture the gut. Wounding entailed making a small tear in the cuticle 30 min. before extraction of hemolymph samples. Samples were immediately transferred into small vials containing a few crystals of PTU and maintained at 0°C. Ten µl samples were mixed with an equal amount of 40% sucrose solution with bromphenol blue tracking dye added. Protein separation was accomplished using a Buchlar disc electrophoresis apparatus. The electrophoresis was conducted through a

7.5% separating gel (pH 8.8-9.0) at a current of 3.0 mA per gel until the tracking dye was approximately one cm from the bottom of the tube. Gels were immediately transferred to amido schwartz stain for two hrs. or incubated in 0.1% DOPA (pH 7.4) for three hrs. Controls for DOPA incubated gels consisted of gels incubated in a solution of 0.1% PTU and 0.1% DOPA. Gels stained with amido schwartz were electrophoretically destained in 7% acetic acid at 10 mA per gel. All gels were stored in 7% acetic acid. Densities of the stained bands were analyzed on a Gilford 240 spectrophotometer with a gel scanning adapter. Relative mobility (Rm) values for each fraction were determined from gel scanning graphs by measuring the distance from the origin each band migrated and dividing this value by the total distance the tracking dye migrated from the origin. The mean (N=9) and standard deviation were calculated for each band.

RESULTS

Hemocytes were clearly visible through the transparent cuticle of the anal papillae when examined by phase contrast microscopy. Spindle shaped plasmatocytes appeared most numerous while a few ovoid cells were also observed (Figs. 1, 3).

THCs taken from the anal papillae of 25 parasitized and control mosquito larvae revealed a marked change due to parasitism. Nonparasitized larvae averaged 29.8±10.0 hemocytes per mosquito while parasitized individuals averaged only 4.2±6.4. This difference was found to be highly significant (Table I). While there appeared to be a wide range in the number of hemocytes from parasitized larvae, the statistical treatment showed no significant differences among individual mosquito larvae within each treatment.

An examination of hemocytes from anal papillae incubated in DOPA solution showed a darkening of the cytoplasm of certain hemocytes while others appeared colorless (Fig. 2). The intense melanosis was attributed to the presence of DOPA-oxidase within the cytoplasm of these hemocytes. Whole mount examinations revealed the presence of many cytoplasmic extensions radiating outward from these melanized hemocytes which in some cases appeared to link various groups of cells together (Fig. 4). Heat and PTU treated controls showed an absence of any pigment within cells.

The effect of parasitism on those hemocytes which contain DOPA-oxidase was determined by THCs. Fifty individual anal papillae each from parasitized and control mosquito larvae were examined. Melanized

and nonmelanized hemocytes were differentiated. Nonparasitized mosquito larvae averaged 22.5±16.3 nonmelanized and 8.7±5.8 melanized hemocytes per papilla. There was a highly significant difference in the combined total hemocytes (melanized and nonmelanized) between parasitized and control mosquito larvae (Table II). Split plot analysis (Steel and Torrie 1960) showed the average number of melanized hemocytes from parasitized mosquito larvae to be significantly lower than that from nonparasitized mosquito larvae. Nonmelanized hemocytes showed a highly significant decrease due to parasitism when compared in a similar manner (Table II).

These hemocytes from the anal papillae did not appear to be necessary for successful capsule formation. Ligation of mosquitoes did not inhibit their ability to encapsulate nematodes; in all cases where nematodes were found they were encapsulated. An examination of these capsules revealed no difference in structure or pigment deposition when compared to those which were not ligated.

The results of electrophoretic separation of hemolymph proteins stained with amido schwartz are shown in Figs. 5 and 6. Seven migrating protein bands were observed from the hemolymph of nonparasitized mosquito larvae (Fig. 5). Hemolymph from parasitized mosquitoes (Fig. 6) showed an apparent shift in bands I and II and a significant reduction in band VI due to parasitism. An additional band (VII) was also detected from mosquito hemolymph following parasitism and appeared to be specific for parasitism or encapsulation. Electropherograms of hemolymph from wounded mosquito larvae were identical to those from nonparasitized mosquitoes with the only major differences being those of intensity of

staining.

The results of electrophoretic separation of the hemolymph proteins incubated in DOPA are shown in Figs. 7 and 8. Electropherograms of hemolymph from nonparasitized and parasitized mosquito larvae appeared as four migrating protein bands. The only major difference due to parasitism appeared to be the increased intensity of band C. This was also observed in electropherograms of hemolymph from wounded mosquito larvae and thus does not appear to be specific for parasitism but may represent a general wounding response. The pattern of band B was quite diffuse and most likely due to diffusion of product prior to polymerization. Unfortunately we were unable to correlate the bands of DOPA incubated gels with those stained in amido schwartz.

DISCUSSION

Certain hemocytes from the anal papillae of A. aegypti show a darkening of the cytoplasm when incubated in DOPA solution. This observation leads to the conclusion that DOPA-oxidase is present within the cytoplasm of these cells. However, the role of these and other hemocytes of the anal papillae, in encapsulation and melanization reactions is unclear. While THCs revealed a significant decrease in both types of hemocytes following parasitism, results from ligation experiments indicate that hemocytes in the anal papillae are not necessary for successful capsule formation. However, this does not rule out the possible involvement of hemocytes in other parts of the body. The situation in A. aegypti does not appear to be analogous to that which occurs in M. domestica and O. caesarion which release a large number of encapsulating hemocytes when invaded by a nematode parasite (Nappi and Stoffolano 1971, 1972a, 1972b). Hemocytes in the anal papillae may circulate when the host is parasitized but their involvement in the encapsulation reaction is not essential. Furthermore, the decrease in DOPA-oxidase containing hemocytes cannot be directly attributed to the release of phenolic substances responsible for melanization of the capsule as is the case with crystal cells in D. melanogaster (Nappi and Streams 1969). Melanization of the capsule in A. aegypti occurs when DOPA-oxidase containing hemocytes are isolated in the anal papillae.

The decrease in hemocytes of the anal papillae following parasitism may be the result of a pathological condition created by the parasite. The active movement of the nematode through the hemocoel of

the host, which disrupts fat body and other tissues, may cause the release of some substance that initiates a wound repair response and thus the breakdown or circulation of the hemocytes. Hemocyte counts should be done on wounded larvae to check this possibility. Quantitative and qualitative changes in hemocytes have been reported to occur in response to injury or wound repair (Salt 1963, 1970). Cherbas (1973) has identified as "injury factor" released from injured tissues of Hyalophora cecropia which activates hemocytes causing them to increase in mobility and adhesiveness and to aggregate at sites of injury.

Disc electrophoresis revealed several changes in the protein migration pattern of A. aegypti following encapsulation of N. carpocapsae. Gels stained in amido schwartz showed a shift in bands I and II, a reduction in band VI and the presence of an additional band (VII) which were not present in hemolymph from nonparasitized or wounded mosquito larvae. While these protein changes appeared to be specific for parasitism and/or encapsulation, it is difficult to definitely state their origin or their role in the encapsulation response of A. aegypti. Brewer et al. (1973) suggested the increased number of protein fractions in H. zea following encapsulation might play a significant role in the defense mechanisms of the host and these additional bands may result from the reaction of melanization.

Several possibilities exist for the origin of the additional protein fraction. It may represent an activated phenoloxidase involved in the melanization reaction. Götz and Vey (1974) have demonstrated that in Chironomus the capsule is formed by the activity of hemolymph borne phenoloxidases. These enzymes have been shown to normally occur in the

hemolymph of insects as inactive proenzymes which require a specific activator for activation (Ohnishi 1959; Evans 1967; Hackman and Goldberg 1967). The additional band may represent some protein released by hemocytes or fat body in response to nematode parasitism. We also cannot rule out the possibility of the nematode itself as the sourse of this protein faction. However, since there was usually only one nematode present in the hemocoel of the host at the time of hemolymph extraction, it would seem unlikely that the nematode could be responsible for such a large increase in a protein in such a short amount of time (3 hrs. exposure).

The results of incubation of hemolymph proteins with DOPA were inconclusive. Banding patterns were not distinct owing to diffusion of the product during incubation prior to polymerization to form melanin. Furthermore, we were unable to correlate these bands with any of those obtained from gels stained with amido schwartz. However, band C did appear more intense in hemolymph samples obtained from both parasitized and wounded mosquitoes and thus does not appear to be specific for parasitism but may represent a wound repair response.

In conclusion these results indicate that encapsulation in A. aegypti can occur without the direct participation of hemocytes contained within the anal papillae. While certain of these cells do contain DOPA-oxidase, their role in melanization of internal nematode parasites is not essential. It appears that some protein is released by the host or by the parasite in response to parasitism. The function of this protein is unknown but it is specific for parasitism and may play a role in the defense reactions of the host.

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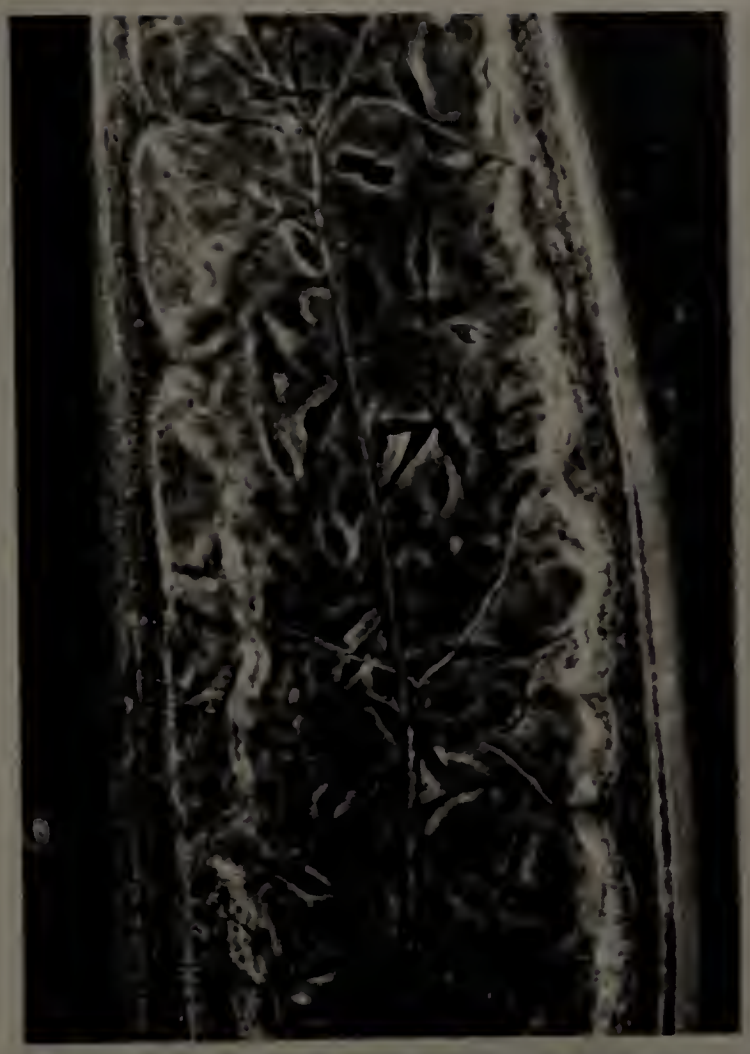
FIGURES AND TABLES

Fig. 1. Mosquito anal papilla containing numerous hemocytes (Phase contrast, X 288).

Fig. 2. DOPA incubated anal papilla with melanized and nonmelanized hemocytes (phase contrast, X 194).

Fig. 3. Typical spindle shaped plasmatocytes of the anal papillae (phase contrast, X 789).

Fig. 4. Melanized hemocytes of an anal papilla after incubation in DOPA solution. Note cytoplasmic extensions (phase contrast, X 1956).




2


4

Fig. 5. Densitometric profile curve of the electrophoretic separation of hemolymph proteins (stained with amido schwartz) from non-parasitized mosquito larvae. Relative mobility values (Rm) ±S.D. are given for each protein fraction.

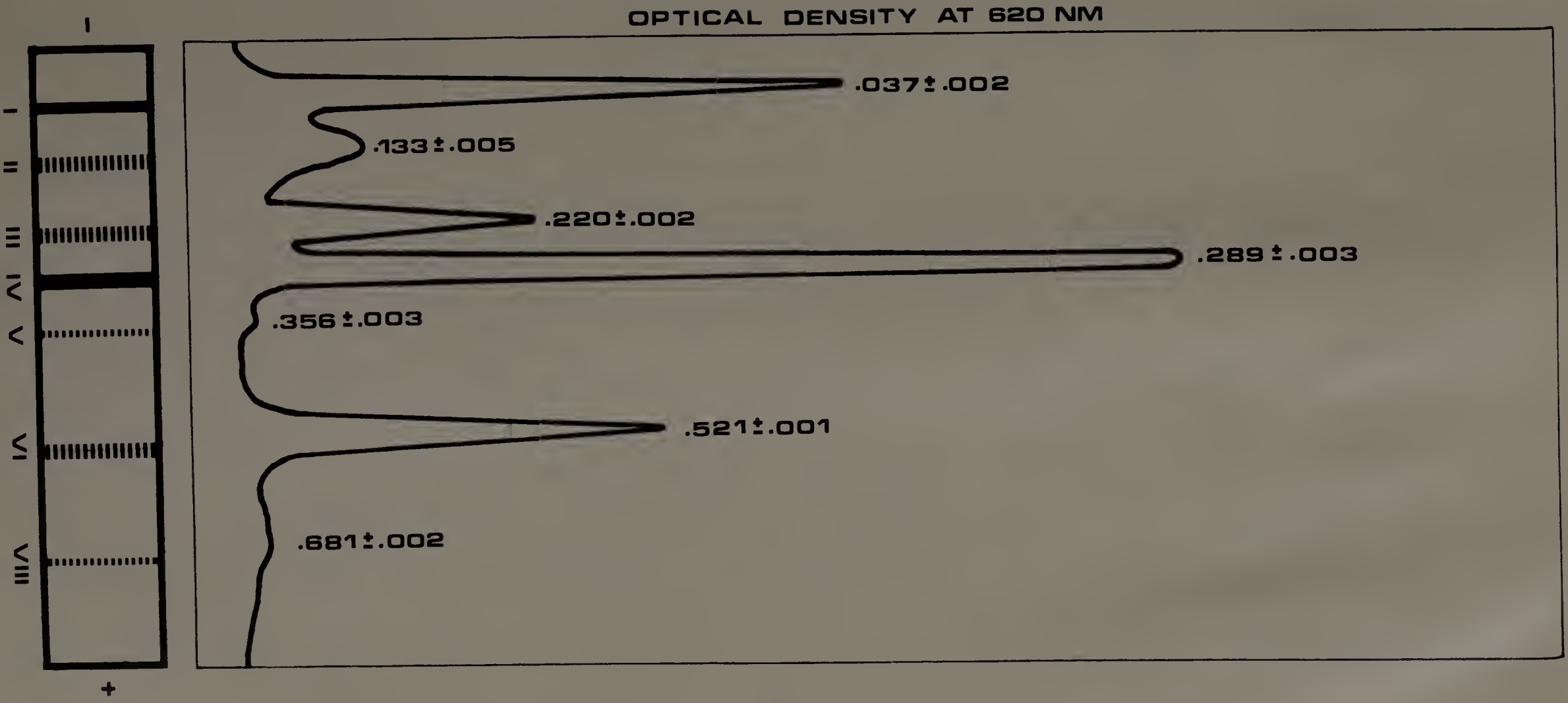

OPTICAL DENSITY AT 620 NM
I
II
III
IV
V
VI
VII
VIII
-
+
.037±.002
.133±.005
.220±.002
.289±.003
.356±.003
.521±.001
.681±.002

Fig. 6. Densiometric profile curve of the electrophoretic separation of hemolymph proteins (stained with amido schwartz) from parasitized mosquito larvae. Rm ±S.D. are given for each protein fraction and corresponding bands obtained from the electrophoretic separation of hemolymph from nonparasitized mosquitoes (Fig. 5) are labeled accordingly.

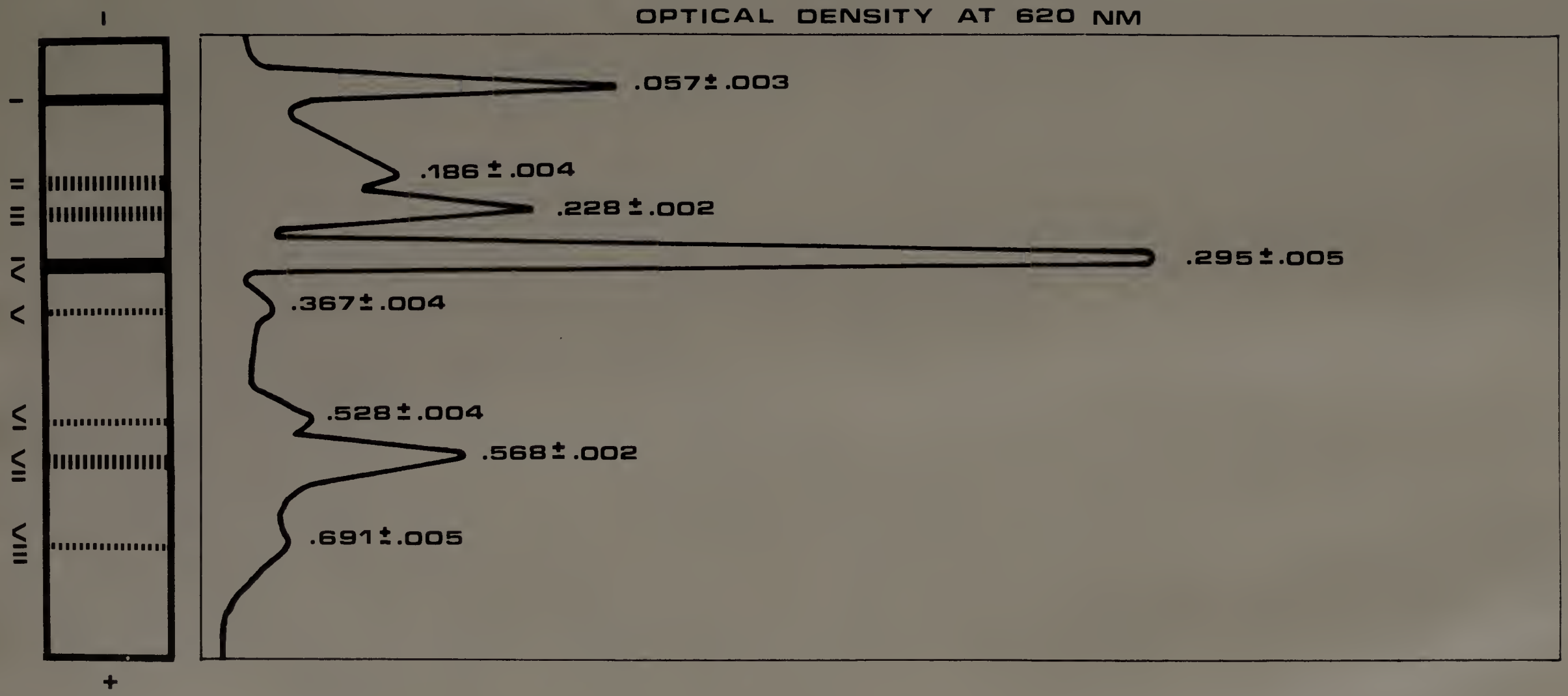

OPTICAL DENSITY AT 620 NM
I
II
III
IV
V
VI
VII
VIII
-
+
.057±.003
.186±.004
.228±.002
.295±.005
.367±.004
.528±.004
.568±.002
.691±.005

Fig. 7. Densitometric profile curve of the electrophoretic separation of hemolymph proteins (incubated in DOPA solution) from non-parasitized mosquito larvae. Rm ±S.D. are given for each protein fraction.

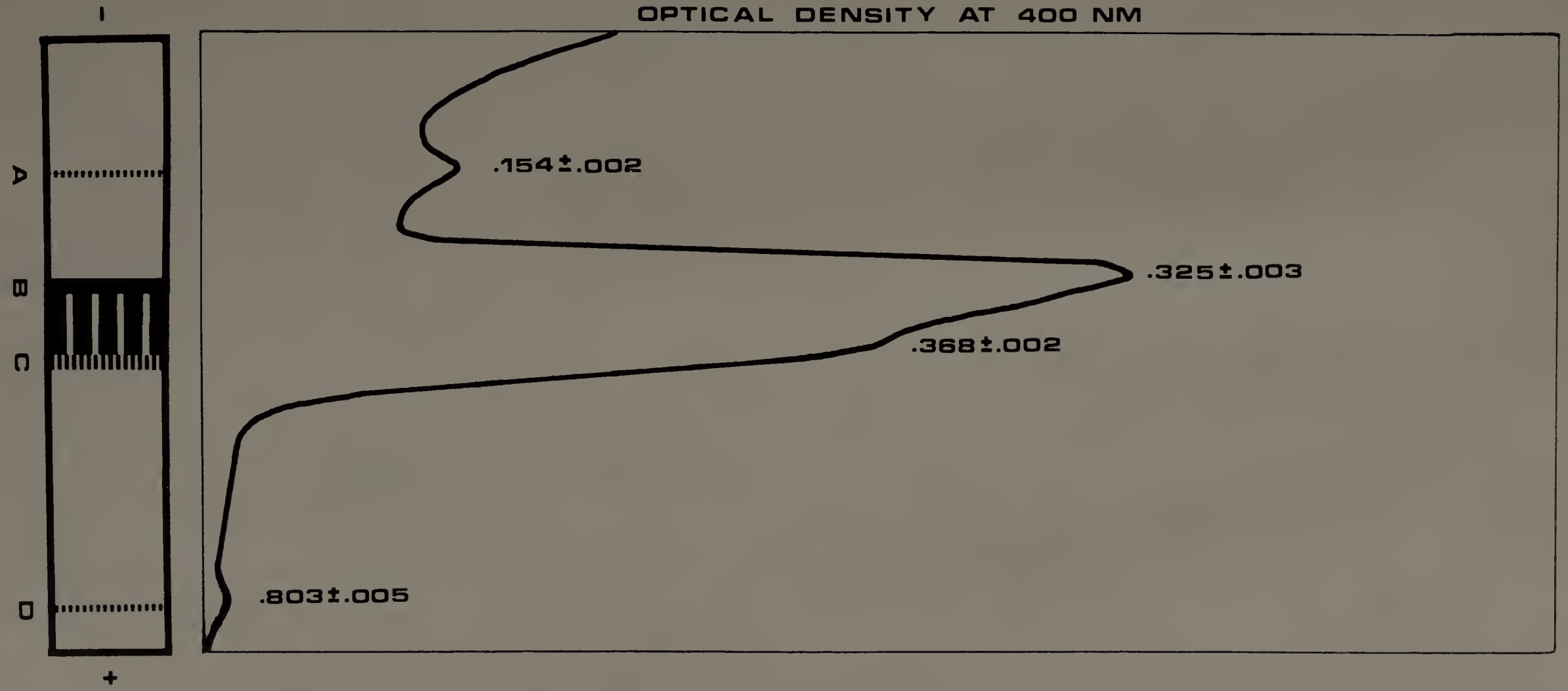

I
A
B
C
D
+
OPTICAL DENSITY AT 400 NM
.154±.002
.325±.003
.368±.002
.803±.005

Fig. 8. Densitometric profile curve of the electrophoretic separation of hemolymph proteins (incubated in DOPA solution) from parasitized mosquito larvae. Rm ±S.D. are given for each protein fraction and corresponding bands obtained from the electrophoretic separation of hemolymph from nonparasitized mosquitoes (Fig. 7) are labeled accordingly.

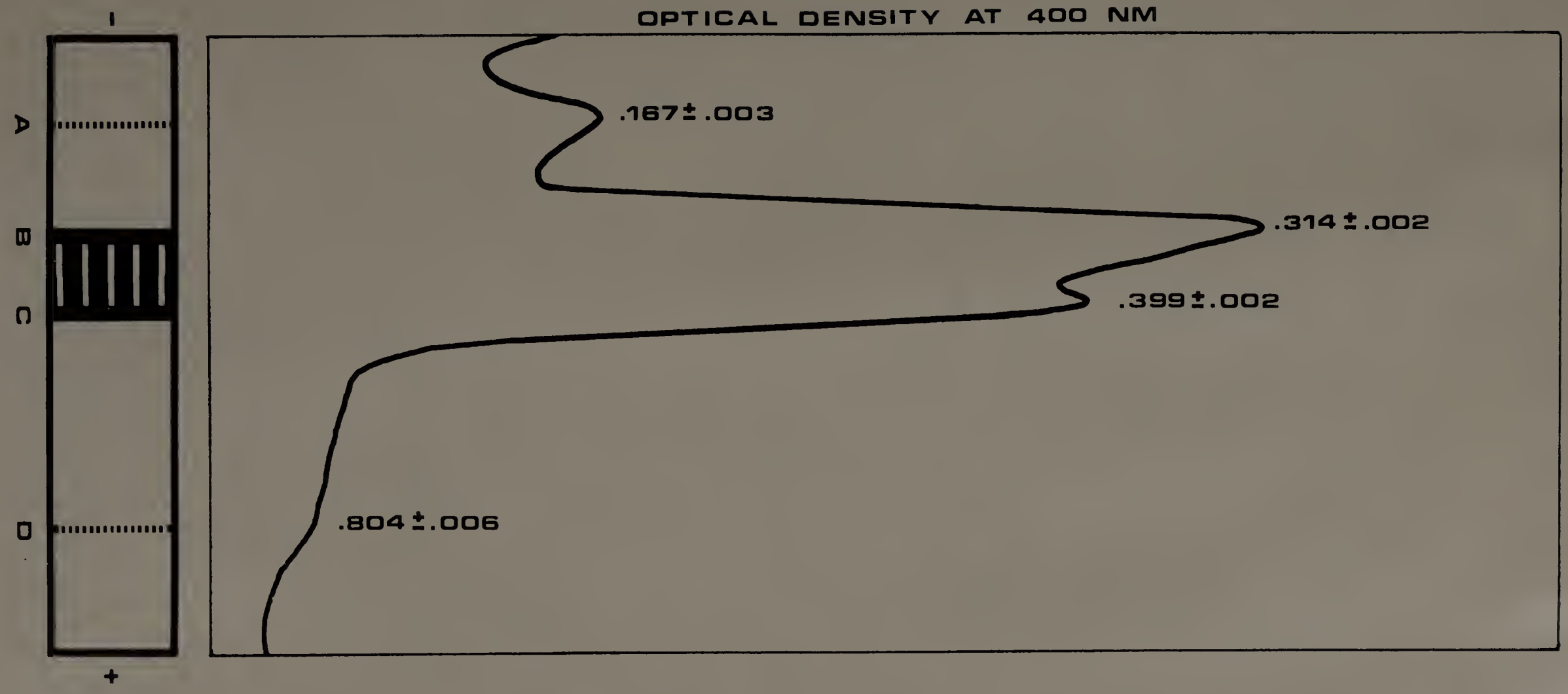

1
A
B
C
D
+
OPTICAL DENSITY AT 400 NM
.167 ± .003
.314 ± .002
.399 ± .002
.804 ± .006

TABLE I

Analysis of variance for total hemocyte counts

parasitized vs. nonparasitized

Source of variation	df	SS	MS	F
Total	199	5241.99		
Treatments	1	2067.25	2067.25	117.54**
Mosquitoes : Treatments	48	844.25	17.59	1.13
Error	150	2300.50	15.54	

**p<0.01

TABLE II

Analysis of variance (split plot design) for hemocyte counts of DOPA incubated anal papillae parasitized vs. nonparasitized

Source of variation	df	SS	MS	F
Total	199	32286.00		
Treatments (T)	1	5554.58	5554.58	35.79**
Papillae (P) : T	98	15210.92	155.21	
Hemocyte response (H)	1	2930.22	2903.22	42.50**
H X T interaction	1	1922.00	1922.00	28.13**
H X P : T	98	6695.28	68.32	

**p<0.01

Difference between	df	t
Parasitized and nonparasitized hemocytes with a (+) response for DOPA-oxidase	98	2.053*
Parasitized and nonparasitized hemocytes with a (-) response for DOPA-oxidase	98	7.919**

*t<0.05
**t<0.01