THE INTERNAL DEFENSE MECHANISMS OF THE

OYSTER, CRASSOSTREA VIRGINICA, IN RESPONSE

TO THE CESTODE TYLOCEPHALUM SP.

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By

Erik Rifkin

Dissertation Committee:

Thomas C. Cheng, Chairman
Albert A. Benedict
Andrew J. Berger
Sidney J. Townsley
Mendel Herzberg

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ABSTRACT

The capsule formed in the American oyster, Crassostrea virginica, in response to the larval cestode, Tylocephalum sp., is composed of fibrous and cellular elements. The completely formed encapsulation complex surrounds the metacestode. The initial stage of capsule development occurs when the invading parasite compresses the surrounding Leydig cells. This is followed by a thickening of the intercellular material between Leydig cells and infiltration by leucocytes. Finally, fibroblastlike cells are seen in the innermost region of the capsule, and the intercellular material becomes noticeably fibrous. These reticular fibers, and the matrix within which they are embedded, include glycoproteins and/or mucoproteins and neutral mucopolysaccharides.

Electron microscope studies on the constituents of the capsule have revealed three types of cells and two types of extracellular fibers. The fibroblastlike cells include mitochondria, rough and smooth endoplasmic reticula (ER), intracytoplasmic fibrils, and cytoplasm of varying densities. Leucocytes include primarily smooth ER, lysosome-like bodies, mitochondria, dictyosomes, and occasionally, myelin-like bodies. At times the ER found in both fibroblastlike cells and leucocytes have greatly distended cisternae. The area between the cisternae is generally electron lucid. The fine structural similarities between leucocytes and fibroblastlike cells support earlier evidences that the latter have differentiated from leucocytes. Brown cells comprise the third cell type. These are situated

along the periphery of each cyst. Each brown cell includes globules of different textures and electron densities. The denser globules include protein crystals while those of moderate electron density include fibrous elements.

The major type of extracellular fiber is of medium electron density and is nonperiodic. The second type of extracellular fiber occurs adjacent to brown cells and is similar to the matrix in its electron density.

Electron micrographs have revealed the fine structure of the host-parasite interface. The tegument of the tapeworm is comprised of an external and an internal level which are partially separated by a basal lamina and two layers of muscles. Arising from the external level are microvilli, each terminating as a spherical vesicle. Attached to the unit membrane surrounding the stem of each micro-villus are extracellular fibers of the encapsulation complex. The microvilli are commonly intertwined and can be observed at the light microscope level as periodic acid-Schiff positive fibril-like projections from the parasite's body surface. This mechanism may function in preventing intimate contact between the fibers of host origin and the cestode's body surface.

The third phase of this study involved the determination of qualitative and quantitative changes in the hemolymph protein fractions of parasitized oysters by using acrilamide gel disc electrophoresis. None of the fractions from infected oysters were significantly increased when compared with those occurring in the control. In the experimental groups, four of the fractions showed a significant

decrease. In addition, oysters revealed a significant decrease in hemolymph protein concentration when placed within the environmental confines of the parasitized animals, over an extended period of time.

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CHAPTER I

INTRODUCTION

Information pertaining to invertebrate internal defense mechanisms was first reviewed by Cantacuzène in 1922, and more recently by Huff (1940). Stauber (1961), Cheng and Sanders (1962), Tripp (1963), Feng (1967) and Cheng (1967) have published most recent comprehensive articles in this area.

It is not surprising to find that most of the available information on invertebrate internal defense mechanisms, including immunity, has been obtained from studies on the economically important species. More specifically, the vast majority of the available data has been compiled from information on the Insecta, which has been, and continues to be, the most economically important group of invertebrates to man. This tendency to concentrate on certain groups because of the possibility of arriving at immediately applicable results, is one of the reasons why many invertebrates suited for basic research in this area have been neglected.

Following the classic work of Stauber (1950), biologists with diverse interests have turned their attention to the humoral and cellular reactions of invertebrates in response to natural and experimental infections. The relative simplicity of the cellular systems found in these organisms lends itself to both morphological and physiological studies.

Because of its abundance in Hawaiian waters, high rates of infection with zooparasites, and proximity to a readily accessable shoreline, the American oyster, Crassostrea virginica, was chosen as an experimental

animal to pursue further the cellular and humoral responses of an invertebrate, in this case a mollusc, to parasitic infection. The parasite involved is the larval lecanicephalid cestode *Tylocephalum* sp.

There are conflicting reports as to when *C. virginica* was first successfully introduced to the Hawaiian Islands. Brock (1960) maintains that in 1893 and 1895, a private individual was successful in transplanting the American oyster to Pearl Harbor. Edmondson and Wilson (1940), however, state that this species was first established in Hawaii when the Territorial Division of Fish and Game imported specimens to Oahu in 1921. *C. virginica*, both from the mainland and from established beds in Pearl Harbor, have been subsequently transplanted to Kauai, Molokai, Maui and Hawaii.

Parasitism, which is one type of symbiosis, is ubiquitous in the animal kingdom. It is hoped that the information derived from the studies reported herein will lead to a better understanding of host-parasite relationships in general, and in particular of certain functional aspects of the host-parasite interface.

CHAPTER II

REVIEW OF THE LITERATURE

A. Cellular Reactions in Molluscs

Stauber (1961) has classified cellular resistance in invertebrates as occurring in three ways: phagocytosis, leucocytosis and encapsulation. These processes, as well as a fourth type of cellular reaction, nacrezation, will be examined in relation to their roles as defense mechanisms in response to invasion by a parasitic helminth.

1. Phagocytosis

Phagocytosis is a well-known type of internal defense mechanism in invertebrates as well as vertebrates. It involves the uptake of foreign materials by certain types of host cells and thus prevents direct contact of such materials, biotic or abiotic, with the host's tissues. The fate of phagocytized materials may differ. Commencing with Stauber (1950), who traced the ultimate disposition of India ink particles experimentally introduced into C. virginica, a number of studies have been carried out to determine the fates of a variety of experimentally introduced foreign materials which are phagocytized by C. virginica leucocytes. It is now known that digestible particles and macromolecules are degraded intracellularly within oyster leucocytes (Tripp, 1958a, b, 1960; Feng, 1959, 1965) while indigestible particles and macromolecules are voided via the migration of foreign material-laden phagocytes across certain epithelial borders (Stauber, 1950; Tripp, 1960; Feng, 1965). Sufficient data are now available to indicate that identical mechanisms occur in all molluscs, marine, freshwater, and

terrestrial (Tripp, 1961; Arcadi, 1968; Cheng et al., 1969).

Not all phagocytized foreign materials are eliminated by the mechanisms mentioned above. A number of microorganisms (certain bacteria, Labyrinthomyxa marina), can grow and multiply within the cytoplasm of host cells (Prytherch, 1940; Mackin, 1951; Michelson, 1961). Some of these intracellularly sustained microorganisms have, through evolutionary adaptation, become mutualists of their molluscan hosts. An example of this is the occurrence of intracellular zoo-xanthellae in the marine bivalves Hippopus and Tridacna (Yonge, 1936) and in the nudibranch Aeolidiella (Naville, 1926).

Not all invading organisms are phagocytized. The presence of the sporozoan Minchinia nelsoni and the injection of the flagellate Hexamita nelsoni and Staphylococcus aureus phage 80 into Crassostrea virginica induce little or no phagocytosis (Feng, 1966; Canzonier, quoted in Feng, 1967; Feng and Stauber, 1968). Similarly, Goetsch and Scheuring (1926), Yonge and Nicholas (1940), and Buchner (1965) have reported that the mutualistic zoochlorellae in Lymnaea, Anodonta, and Unio are seldom found within host cells and are presumed to lead an extra-cellular existance and are not phagocytized by their hosts' leucocytes.

Phagocytosis alone is not a primary line of defense against helminths since these multicellular parasites are physically too large to be engulfed by molluscan leucocytes. However, since cell types involved in the encapsulation process are thought to be capable of phagocytizing, this cellular response is indirectly involved in re-actions to helminth parasites.

2. Encapsulation

Encapsulation involves the enveloping of an invading organism or experimentally introduced tissue too large to be phagocytized by cells and/or fibers of host origin. Although much is known about the nature of such encapsulating cysts as the result of descriptive studies, relatively little is known about the dynamics of the process. Encapsulation in the Mollusca due to trematode, cestode, and nematode parasites are considered at this point.

Trematodes. Encapsulation represents the primary line of defense in molluscs against helminth parasites. There are numerous instances where little or no cellular reaction occurs when the asexually reproducing larval stages of digenetic trematodes (sporocysts and rediae) are found within their natural habitats within their intramolluscan microenvironments. To select from some known examples among marine molluscs, it is known that very little reaction occurs in Crassostrea virginica when parasitized by the dendritic sporocysts of Bucephalus sp. (Cheng and Burton, 1965) nor has an encapsulating cyst been reported surrounding the sporocysts of Bucephalus cuculus, the common species in C. virginica along the Gulf and mid-Atlantic coasts of the United States (McCrady, 1873; Tennent, 1905, 1906, 1909; Hopkins, 1954). Similarly, no encapsulating cyst has been reported surrounding the sporocyst of the common European species of Bucephalus, B. haimeanus, found in the oyster, Ostrea edulis, and the cockle, Cardium tuberculatum (=C. rusticum) (Lacaze-Duthiers, 1854; Huet, 1888, 1893; Johnstone, 1904; Lebour, 1911; James et al., 1966; James and Bowers, 1967; and others). Canzonier (pers. comm.) has noticed that

encapsulation does occur around moribund and disintegrating Bucephalus
sporocysts in C. virginica. Similarly, Franz and Feng (in Feng, 1967)
have found that cellular response in the mussel Mytilus edulis para-
sitized by the sporocysts of Proctoeces maculatus only occurs around
degenerating sporocysts. It appears that the cellular reactions
observed were not directed at the parasites per se but most probably at
some yet unidentified autolytic product(s) of degenerating parasites.

Cellular reactions could also occur in response to the host's own
degenerating tissues. Howell (1967), who examined the New Zealand
mudoyster, Ostrea lutaria, infected with the sporocysts of Bucephalus
longicornutus, has found phagocytic infiltration in response to the
degenerating Leydig cells of the host which the phagocytes engulfed.
Howell has described the normally discrete Leydig cells to have become
fused as a syncytium. This condition probably reflects a cytopatho-
logical condition resulting from pressure exerted by the parasite rather
than true encapsulation, although it is known that the fusion of
adjacent cells does contribute to true encapsulation in certain in-
vertebrates such as sponges (Cheng et al., 1968). If the formation of
a syncytium by Leydig cells surrounding B. longicornutus sporocysts
does represent true encapsulation rather than the result of physical
compression due to the parasite, it is an unusually subtle reaction.
If this is a true instance of encapsulation, the occurrence of this
reaction could be reflective of the fact that B. longicornutus is
engaged in a comparatively less well adapted relationship with Ostrea
lutaria. This condition is indicated by the reports of Millar (1963)
and Howell (1967) that parasitized O. lutaria are eventually killed by

B. longicornutus. As stated, in other known instances of parasitism by Bucephalus sporocysts, encapsulation does not occur, but then, the other species which have been studied are not lethal and are assumed to be engaged in more compatible relationships.

From the examples cited, it should be apparent that, as a rule, the asexually reproducing larval stages of digenetic trematodes do not become encapsulated when found in their natural intramolluscan habitats. This suggests that a condition comparable to the "recognition of self" exists (see Sprent, 1963). On the other hand, information gained from studies on freshwater molluscs has revealed that slight encapsulation may occur if these larvae for some reason become lodged or are temporarily in transcient through other regions in their natural hosts (Cheng and Cooperman, 1964; Probert and Erasmus, 1965; Schell, 1961, 1962a, b). In addition, motile, mature cercariae passing through certain tissues within their molluscan hosts enroute to the exterior may elicit encapsulation (Cheng and Cooperman, 1964; Pan, 1965). These examples suggest that the condition of "recognition of self" is restricted to the parasites' normal microhabitats. In other words, the ability to "recognize self from nonself" as related to cellular immunity may be operative at the cellular level, with histocompatibility or incompatibility being properties of specific types of cells (or tissues) within the same organism.

Cellular reactions in molluscs are quite different when the invading parasite is the metacercarial stage of a digenetic trematode. The double nature of the so-called metacercarial cysts of several species of trematodes occurring in invertebrates are known (Ameel, 1934;

Miyazaki, 1939a, b, c, d, 1947; Cheng, 1957; Yokogawa et al., 1960; Cheng et al., 1966a). The origin of the inner wall is generally considered to be of parasite origin but the outer wall may be either of parasite origin as in the case of _Paragonimus westermani_ metacercariae in crabs (Yokogawa et al., 1960), _P. kellicotti_ metacercariae in crayfish (Ameel, 1934), and _P. ohirai_ metacercariae in brackish water crabs (Miyazaki, 1939a, b, c, d, 1947) or represent an encapsulation capsule laid down by the host as in the case of _Crepidostomum cornutum_ metacercariae in crayfish (Cheng, 1957) and _Himasthla quissetensis_ metacercariae in a number of species of marine pelecypods (Cheng et al., 1966a).

In order to ascertain the susceptibility and cellular reactions of different species of marine pelecypods to _H. quissetensis_ metacercariae, these investigators experimentally introduced 150 cercariae into specimens of _Crassostrea virginica_, _C. gigas_, _Mytilus edulis_, _Modiolus demissus_, _Ensis directus_, _Mya arenaria_, _Mercenaria mercenaria_, and _Tapes philippinarum_. In each instance, except in the case of _T. philippinarum_, _C. virginica_, and _C. gigas_, each metacercaria secreted a non-cellular inner cyst wall around itself. It was stimulated to do so by some component in the molluscan hosts' hemolymph (Cheng et al., 1966b). An outer cyst wall comprised of host leucocytes and either myofibers or connective tissue fibers, depending on the location of the parasite, occurs. Furthermore, variations occurred between the species of pelecypods tested and with the location of the parasite within the host. Complete outer walls, hence complete encapsulation, only were found surrounding metacercariae in _E. directus_, in the gills

and palps of M. arenaria, in the foot of M. edulis, and in the connective (Leydig) tissues of M. demissus. The composition of the outer cyst wall differed at these sites. In the foot of E. directus and in the gills of M. arenaria, it was composed of connective tissue fibers and leucocytes. In the case of C. virginica, encysted metacercariae were only found in the lumina of blood vessels. The noncellular, parasite-secreted inner cyst wall was present but the outer "wall" was comprised solely of leucocytes. No metacercariae were found in C. gigas. This is believed to be due to an immobilizing factor which prevented penetration (Cheng et al., 1966b). In T. philippinarum, those metacercariae found in the tissues were not enveloped by either an inner or an outer cyst wall except those located in the clam's gonads. When found at this site, an inner cyst wall was present as was an outer "wall" composed of only leucocytes.

Cheng et al., (1966b) found that Himasthla quissetensis cercariae, induced to encyst in vitro in the whole blood of 7 species of marine pelecypods (M. mercenaria, M. arenaria, C. virginica, C. gigas, T. philippinarum, M. edulis, and M. demissus), attracted leucocytes which formed an envelope peripheral to the parasite-secreted inner wall. A similar phenomenon does not occur in the case of unencysted cercariae. It is thus apparent that the parasite-secreted wall serves as an attractant to leucocytes, possibly by chemotaxis. The chemical nature of the attractant, however, remains undetermined.

The metacercariae situated in the foot of E. directus and the palps of M. arenaria are encapsulated in leucocytes and myofibers, the latter being already present at these sites, indicates that the fibrous

constituents of the encapsulation complex are from pre-existing fibers in the immediate vicinity. Similarly, the fact that metacercariae located in the gills of M. arenaria are encapsulated by leucocytes and connective tissue fibers, the latter being already present at this site, indicates that the fibers comprising this encapsulation complex are also from pre-existing fibers in the immediate vicinity. Thus, in the case of H. quissetensis-marine pelecypod relationships, the nature and quantity of the fibrous constituents of the encapsulation complex are dependent upon the nature and availability of fibers in the surrounding host tissues.

Cestodes. It appears that molluscs react considerably more severely to cestodes than to either trematodes or nematodes. Although several species of larval tetraphyllid cestodes have been reported from cephalopods (Linton, 1922; Dollfus, 1927; Stevenson, 1933; Riser, 1951, 1956a, b; Aldrich, 1964; Fields, 1965; MacGinitie and MacGinitie, 1966), the various investigators have been more interested in the morphology and taxonomy of these larval tapeworms than in the host reactions. However, considerably more is known about the reactions of marine pelecypods to larval cestodes. At the present time, four species of larval cestodes are known from marine pelecypods: the lecanicephalid Tylocephalum sp. (or spp.) from the pearl oyster, Margaritifera vulgaris, and other pelecypods in Ceylonese waters (Herdman and Hornell, 1906; Shipley and Hornell, 1906; Jameson, 1912; Southwell, 1924), from the black-lipped pearl oyster, Margaritifera margaritifera var. cumingii collected from the Gambian Archipelago (Seurat, 1904), from Meleagrina occa and M. irradians collected at Nossi-Be, Madagascar (Dollfus, 1923),

from _Crassostrea virginica_ collected in Pearl Harbor, Oahu, Hawaii
(Sparks, 1963; Cheng, 1966a; Rifkin and Cheng, 1968), from _Tapes
semidecussata_ collected in Kaneohe Bay, Oahu, Hawaii (Cheng and Rifkin,
1968), and from _Margaritifera vulgaris_ collected from Eniwetok; the
tetraphyllid _Echeneibothrium_ sp. from a "clam" collected in Puget Sound,
Washington (Hyman, 1951) and from the littleneck clam, _Venerupis
staminea_, collected in Humbolt Bay, California (Sparks and Chew, 1966);
the trypanorhynch _Eutetrarhynchus ruficolle_ in "oysters" from the
Belgium oyster beds (Fujita, 1943); and the tetraphyllid tentatively
identified as _Anthobothrium_ sp. from the clams _Tresus nuttalli, Macoma
nasuta_, and _Venerupus staminae_ collected at Elkhorn Slough, California,
and from _T. nuttalli_ collected in Humboldt Bay and Newport Bay,
California (MacGinitie and MacGinitie, 1968). The last species may be
identical with the tetraphyllid identified by Sparks and Chew (1966) as
Echeneibothrium sp. In the case of all four (perhaps three) species,
the various investigators have reported them to be completely encapsula-
ted. A detailed account of the cellular defense mechanisms of _C.
virginica_, in response to _Tylocephalum_ metacestodes is given in
Chapters II and III.

Nematodes. Only a few nematode parasites have been described from
marine molluscs (see Cheng, 1967). Practically no information is
presently available on the cellular reactions of molluscan hosts to
this category of helminths. However, Burton (in Cheng, 1967) has found
unidentified nematodes in _Crassostrea virginica_ collected in St. Mary's
County, Maryland. In all instances, these nematodes were tightly coil-
ed in the region of the hosts' digestive glands. The reports by Cobb

(1930), Gutshell (1930), and Hutton (1964), who have found the larval stage of *Porrocaecum pectinis* in *Aequipecten gibbus* at Beaufort, North Carolina, and off the east coast of Florida, would give the impression that true encapsulation does not occur in this relationship. However, Millemann (1951), in describing the larvae of the gnathostomatid nematode *Echinocephalus pseudouncinatus* from the pink abalone, *Haliotis corrugata*, from Sam Clemente Island in southern California, has mentioned that these larvae burrow into the host's foot where they encyst in the ventral portion, producing a blister-like effect on the exterior.

Cheng (1966b, c), by experimentally introducing the first stage larvae of *Angiostrongylus cantonensis* into *Crassostrea virginica* and following the fate of the intramolluscan stages at time intervals, has found that there is conspicuous leucocytic response in the host 10 to 14 days post-infection. Moreover, intraluminal larvae in blood vessels attract extraluminal leucocytes after the 10th day so that heavy perivascular capsules of leucocytes can be consistently found. There is also an increase in the number of leucocytes within the blood vessels of oysters examined on the 10th and 14th days post-infection. The leucocyte-attracting substances (LAS) is believed to be the nematode's molting fluid. In his discussion, Cheng (1966c) has pointed out and illustrated true encapsulation of *A. cantonensis* in the giant African snail, *Achatina fulica*, and the terrestrial slug, *Deroceras laeve*.

3. Leucocytosis

Leucocytosis, which is defined as an increase in the number of leucocytes, has been considered a type of cellular internal defense mechanism (Stauber, 1961; Cheng and Sanders, 1962; Feng, 1967; Cheng,

1967) although these authors recognize that leucocytosis serves as a forerunner of phagocytosis and/or encapsulation since the increased number of cells contributes to these active processes. Instances where leucocytosis has been reported in response to helminth parasitism have been mentioned under Encapsulation.

4. Nacrezation

The term nacrezation is used to describe a unique and rather specialized defense mechanism found only in those organisms capable of producing nacrous-like secretions for the purpose of walling off invading materials. Broadly speaking, it is a type of encapsulation, but due to the striking differences in the composition of the capsule, the mechanisms involved, and the area in which it occurs, it is usually considered to be a separate phenomenon.

In lamellibranch molluscs, specialized epidermal cells of the mantle secrete nacre to wall off any invading organic or inorganic substance which may come to lie between the shell and the mantle. The result is pearl formation. Pearl formation within certain pelecypods has been known for over 1,000 years. According to Tsuji (1960), pearl culture was recorded in the Gokanjo by Hanyo, a Chinese, in about 80 A.D. The cause and mechanisms involved, however, were not understood until the 19th century although it has been recognized as a type of defense mechanism against zooparasites since the report in 1655 by Worm that pearly formations occur in the mantle of Mytilus edulis collected in Sweden. Since this original report, several 20th century biologists have investigated this phenomenon (Dubois, 1901, 1907; Perrier, 1903; Jameson, 1902; Giard, 1907; and others). Trematode metacercariae,

especially those of <u>Meiogymnophallus</u> <u>minutus</u> (=<u>Gymnophallus</u> <u>mar-</u>
<u>garitarum</u>), when found between the inner surface of the shell and the
mantle of marine pelecypods, will stimulate the mantle to secrete nacre
which becomes deposited around the parasites. The process has been
reviewed by Alverdes (1913) and Tsuji (1960). The presence of a para-
site at this site will cause the formation of a pearl sac. Histolo-
gically, the pearl sac appears as an invagination of the mantle
epithelium which envelops the parasite or an artificial nucleus, and
layers of nacre are gradually deposited around it. Although fibrosis
may occur concurrently, according to Southwell (1924), nacrezation and
fibrotic response are mutually exclusive. The earlier contention that
<u>Tylocephalum</u> larvae can bring about nacrezation (Herdman and Hornell,
1906; Shipley and Hornell, 1906; Southwell, 1924) has been discredited
by Jameson (1912) and Hyman (1951).

The stimulus provided by the parasite or some other foreign
material, living or nonliving, to produce nacrezation apparently is in
the form of physical pressure. It is known that sand grains can in-
duce nacrezation. This would corroborate the evidence provided by
Nishikawa (1917) and Mikimoto (1918) that this process is not "immuno-
logic." They have reported that homograft implants of a strip of the
outer mantle epithelium into the subepithelial region of the mantle
will induce pearl formation. Normally, molluscan homografts do not
elicit any or only a minimum of cellular response (Cushing, 1957;
Tsuji, 1960; Tripp, 1961; Canzonier, in Feng, 1967). The only known
exception is the report by Drew and de Morgan (1910) that homologous
gill grafts implanted in the adductor muscle of <u>Pecten</u> <u>maximus</u> were

eventually isolated by encapsulation. But, as Feng (1967) has pointed

out, the results of Drew and de Morgan are most probably due to their

technique which caused severe damage to the grafts. Thus, the cellular

responses were most likely due to autolytic products of the graft rather

than to the homologous tissue per se.

Cheng (1967), in his review, has listed those helminths known to

provoke nacrezation in marine molluscs. Only a brief summary is

being presented here.

The metacercaria of Meiogymnophallus minutus (=Gymnophallus

margaritarum) is known to cause nacrezation in Mytilus edulis, M.

galloprovincialis, and Cardium edule (Dubois, 1901, 1907; Jameson,

1902, 1912; Giard, 1907; Lebour, 1911; Palombi, 1924). Recently,

Bowers and James (1967) have elucidated the life cycle of this parasite.

The adult is a parasite of the oystercatcher, Haematopus ostralegus

occidentalis. Bowers and James are of the opinion that Lecithoden-

dendrium somateriae (non Levinson, 1881) Jameson, 1902 in partim,

Gymnophallus oedemiae (Jameson and Nicoll, 1913) James, 1964, Cercaria

cambrensis Cole, 1938, and Gymnophallus nereicola Rebecq and Prevot,

1962, are synonyms of M. minutus.

Lebour (1911) has reported that M. minutus is alive when it first

makes contact with the host but it is eventually killed if it becomes

the nucleus of a pearl. Lebour's report and an earlier similar report

by Jameson (1902) that the metacercariae forming pearl nuclei were dead

have been erroneously interpreted by Wright (1966) to mean that only

dead metacercariae induce nacrezation.

Bowers and James (1967) did not report the nacrezation of M.

minutus metacercariae within well-developed pearl sacs. The possible examination that their metacercariae were recently established and had not had sufficient time to induce nacrezation is unacceptable since they examined over 500 specimens of Cardium edule collected from several different sites over a period of 22 months and found that "metacercariae occurred in every cockle over 1 year old and in most spat over 6 months old." Furthermore, as they have reported: "In most infections all the metacercariae are fully formed."

Metacercaria (Gymnophallus) megalocoela, another fellodistomatid trematode, is known to elicit nacrezation in its molluscan host, Tapes decussatus (=Amygdala decussata) although the resulting cyst is different from that associated with Meiogymnophallus minutus. M. megalocoela was described from the Gulf of Naples by Palombi (1934) who reported that this parasite is covered by a thick ellipsoid gelatinous coat of host origin impregnated with calcium particles. Lebour (1911) has reported that M. minutus metacercariae are sometimes enveloped by a "jelly-like mass." This condition may well represent the initial stage of nacrezation.

Recently, Obrebski (1968) has reported in an abstract that two species of Parvatrema metacercariae form "pits" on the inner surface of pelecypod shells. He has found P. obscurus to be very common in the Pacific coast clam, Transenella tentilla, and P. borealis common in the Atlantic clam, Gemma gemma. In addition, a yet unidentified species of Parvatrema has been found in Gemma collected in San Francisco Bay. Although true nacrezation apparently does not occur around the Parvatrema metacercariae examined by Obrebski, he observed

a gelatinous matrix surrounding each metacercaria. Since such a

matrix has been reported to also surround the metacercariae of

Meiogymnophallus minutus and Metacercaria (Gymnophallus) megalocoela,

the question may be raised as to whether such a gelatinous tunic

represents an earlier stage of nacrezation. This appears to be

reasonable since Palombi (1934) has found calcium particles deposited

therein. The gelatinous tunic may serve as a matrix for the deposition

of calcium carbonate. The process of nacrezation, as one form of

cellular internal defense mechanism, is in need of more detailed

examination.

B. Humoral Immunity

Humoral immunity can be classified into two categories: natural

(or innate) and acquired. Generally speaking, natural humoral immunity

refers to genetically controlled factors which are responsible for

non-specific resistance mechanisms, while acquired humoral immunity

refers to the production of demonstrable antibodies, brought about by

the introduction of a specific antigenic agent.

The first studies on humoral immunity in the Mollusca were in-

itiated during the early part of the twentieth century. Dugern (1903)

and Cantacuzène (1923) have reported their inability to detect natural

precipitins or a complement in Eledone. Drew (1911) has not been able

to demonstrate the synthesis of cytolysins and precipitins in four

pelecypods, namely, Pectin opercularis, P. disclocatus, P. maximus,

and Arca ponderosa. Cantacuzène (1915) has reported that natural

antibodies, in the form of agglutinins, precipitins, and hemolysins,

are absent in the blood of Helix pomatia.

Couvreur (1923) has reported that a natural humoral antibody, in the form of an antitoxin, was present in the gastropod *Helix pomatia*, and Chahovitch (1921) has reported the presence of a natural hemagglutinin in *Sepia*. Tyler (1946) has reported the presence of natural hemagglutinating activity in the body fluids of six species of molluscs. He has shown that the body fluids of the gastropods *Acmea digitalis*, *Lattia gigantia*, *Tegula galena*, *Astraea undosa*, and *Megathura crenulata*, and the pelecypod *Mytilus californianus*, will agglutinate blood cells of the polychaete *Chaetopterus variopedatus*. Blood cells of the amphineuran *Mopalia mucosa*, the asteroid *Patiria miniata*, and the ascidians *Ciona intestinalis* and *Styela barnharti* have been found to be also agglutinated by the body fluids of certain of the above mentioned molluscs. Tyler also has demonstrated that the body fluids of these molluscs will agglutinate the spermatozoa of certain other animals.

More recently, Feng (1959) has found that heteragglutinins in *C. virginica* can be inactivated by heating or removed by absorption. He has shown the plasma to be bacteriocidal in certain instances, but could not demonstrate the presence of a specific antibody. Cushing et al. (1963) have shown that the hemolymph of *Octopus bimaculatus* will not agglutinate various human erthrocytes known to be carrying the A, B, O, M, and N antigens. Octopus hemolymph was, therefore, tested for its ability to inhibit commercial typing sera reactive with these antigens. As the result, no inhibition was observed with respect to anti-B, anti-M, anti-N, and anti-H, but anti-A was partially inhibited with respect to reactions with type A human cells. Johnson (1964)

has reported that saline extracts of the butter clam Saxidomus
giganteus will specifically agglutinate human red blood cells of
phenotypes A_1 and A_1B. The agglutinin can be completely adsorbed by
A_1 and A_1B cells, partially adsorbed by A_2 cells, but is unaffected by
B and O cells. The clam agglutinin is non-dialyzable and is thus
probably a large molecule. Boyd and Brown (1965) have reported that
expressed body fluids of the land snail, Otola lactea contain a power-
ful and specific anti-A agglutinin. This agglutinin, even when un-
diluted, does not agglutinate O or B cells. Cheng and Sanders (1962)
have shown a naturally occurring serum (hemolymph) hemagglutinin in
Viviporus malleatus which is specific for rabbit erythrocytes among
the various blood cells tested. The electrophoretic pattern of normal
hemolymph from V. malleatus revealed five protein fractions. Electro-
phoretic analysis of normal and adsorbed hemolymph has revealed that
the agglutinating property of the hemolymph is due to proteins which
are included in all of the five fractions.

Tripp (1958) could not detect the presence of specific agglutinins
or precipitins in C. virginica. However, more recently (Tripp, 1968),
he has shown that a protein fraction in the blood of nonparasitized
oysters (C. virginica) will agglutinate red blood cells of several
vertebrate species. Adsorption tests have indicated that this material
has a moderate degree of specificity. Furthermore, it exerts an
opsonic effect on rabbit red blood cells in vitro. McDade and Tripp
(1967b) have found a lysozyme that occurs in C. virginica hemolymph
which is capable of lysing gram-positive bacteria.

Acquired immunity in molluscs is a subject about which very little

is known. Chahovitch (1921) and Drew (1911) have reported that ac-
quired agglutinins, precipitins, and hemolysins could not be demonstrat-
ed in the blood cells of Sepia, but Cantacuzene (1915) has reported
acquired agglutinins and precipitins in Helix pomatia. Although this
has been reported confirmed by Erber (1923), further confirmation
appears desirable in light of current knowledge.

Winfield (1932) and Nolf and Cort (1933) have suggested that
acquired immunity may be present in gastropod intermediate hosts of
certain larval trematodes, and the studies of Michelson (1963) are
suggestive, although not conclusively, that a miracidia-immobilizing
substance occurs in Biomphalaria glabrata (=Australorbis glabratus)
parasitized by Schistosoma mansoni. Kagen and Geiger (1964), however,
have shown that B. glabrata may be reinfected with miracidia after a
patent infection with S. mansoni. Likewise, Barbosa and Coelho (1965)
have not been able to demonstrate protection against re-infection in
snails cured of S. mansoni infection. They did, however, observe
tissue responses in snails which had been cured and then re-exposed to
S. mansoni. Feng and Stauber (1968) have demonstrated the precipitous
reduction in the numbers of the flagellate Hexamita sp. in oysters.
They have concluded that this may be indicative of the presence of a
true acquired immune mechanism. It is evident from this study that
future experiments on acquired resistance in invertebrates should take
into account the course of infection as an index for this phenomenon.
For many years, certain antibodies in mammalian systems, such as
ablastin in Trypanosoma lewisi infection in rats (Taliaferro, 1932),
could only be demonstrated by examining the course of infection and

the parasites' morphological changes. Specifically, there is an inhibition of trypanosome reproduction by the synthesized ablastin.

CHAPTER III

ON THE STRUCTURE, FORMATION, AND HISTOCHEMICAL
CHARACTERIZATION OF THE ENCAPSULATING CYSTS IN
CRASSOSTREA VIRGINICA PARASITIZED BY
TYLOCEPHALUM METACESTODES

A. Introduction

A review of the literature pertaining to cellular defense mecha-
nisms in molluscs has revealed that practically nothing is known about
the origin and chemical composition of the fibrous cysts commonly
found encapsulating helminth parasites. As Cheng (1967) has pointed
out, the findings of Drew and de Morgan (1910) relative to the origin
of fibrous capsules in Pectin (=Aequipectin) maximus, as a reaction
toward implanted gill and digestive gland tissues, are in need of
critical re-examination. The only information pertaining to the
chemical nature of such a fibrous capsule has been contributed by
Sparks and Chew (1966), Katkansky and Warner (1968), and Warner and
Katkansky (1968). They have reported that the encapsulation cyst in
the littleneck clam, Venerupis (=Protothaca) staminea, surrounding
plerocercoid larvae of the cestode Echeneibothrium sp. is collagenous.
As will be pointed out in a later section, their assumption is question-
able.

There have been some studies made on the composition of comparable
cysts in vertebrates surrounding trematode metacercariae (Hunter and
Dalton, 1939; Herber, 1950; Cheng, 1957; Singh and Lewert, 1959;
Bogitsh, 1962; Lynch and Bogitsh, 1962; Thakur and Cheng, 1968). It
is evident from these studies that if an outer cyst wall occurs, it
is usually of host origin but its chemical composition may well vary

with the species involved (review by Stirewalt, 1963).

Since the American oyster, <u>Crassostrea virginica</u>, is known to form a fibrous capsule around the metacestode of the lecanicephalid cestode <u>Tylocephalum</u> sp. (Sparks, 1963; Cheng, 1966) and since, as stated, oysters parasitized by this larval cestode are readily available in Hawaiian waters, a study was carried out to elucidate the origin, manner of formation, and to partially characterize chemically the capsule by employing histochemistry.

The complete life history of <u>Tylocephalum</u> sp. is yet unknown. Recently, Cheng (1966) has described the coracidium and routes by which this ciliated larva can invade <u>C. virginica</u>. Since the developmental stages of this parasite beyond the intramolluscan form are yet unknown and since the morphology of the intramolluscan larva does not permit definite identification as to whether it is a procercoid or plerocercoid, in following the reasons given by Cheng (1966), it is being referred to as a metacestode.

B. Materials and Methods

The oysters used during these studies were taken from West Loch, Pearl Harbor, Oahu, Hawaii. These were maintained in a recirculating sea water tank in the laboratory until fixation. A total of 153 oysters were collected on different occasions during the period extending from January, 1965, through June, 1966. Subsequent histological examinations revealed that 52 were parasitized by <u>Tylocephalum</u> metacestodes. The findings relative to the encapsulating cysts reported herein were based on the examination of the tissues of 44 of the infected oysters. Sections of uninfected oysters served as controls.

Of the 44 infected oysters, 22 were fixed in 10% sea water –
formalin, 11 in Carnoy's (6:1:1) fixative, and 11 in Davidson's fixa-
tive. After fixation, 39 were each divided into three cross-sectional
pieces, embedded in "tissue-mat," and each segment was cut in cross
section at 10μ. The remaining five, which were all formalin-fixed,
were cut as frozen sections at 10u on a cryostat.

Representative sections of the paraffin-embedded tissues fixed in
each of the fixatives were stained with hematoxylin and eosin (H & E)
or Mallory's triple connective tissue stain for histological studies.
In addition, the following histochemical tests were carried out: (1)
the periodic acid–Schiff (PAS) reaction (McManus, 1946a); (2) the
alcian blue method for acid mucopolysaccharides (Steedman, 1950); (3)
the toluidine blue method for metachromasia (Kramer and Windrum, 1954);
(4) the Sudan black B (McManus, 1946b) and (5) the Nile blue sulfate
(Gridley, 1957) method for lipids; (6) Gomori's (1937) modification of
Bielschowsky-Maresch's method for reticulum; (7) Taenzer-Unna's orcein
stain for elastic fibers as given by Edwards (1950), and (8) Van
Gieson's picrofuchsin stain for collagen and reticulum as given by
Edwards (1950).

As controls for the PAS reaction, additional sections, which had
been pre-exposed in a 0.6% malt diastase suspension for 2 hr. at 37°C,
were subsequently exposed to the reaction. In addition, concurrent
with subjecting sections to both the alcian blue and toluidine blue
tests, additional sections, pretreated with a buffered bovine testi-
cular hyaluronidase solution as recommended by McManus and Mowrey
(1960), were also similarly stained. These were employed for the

characterization of chondroitin sulfates and as controls for the identification of hyaluronic acid.

As controls for the histochemical determination of lipids, sections pretreated with xylene were employed.

C. Results

Capsule Formation

As Cheng (1966) has pointed out, occasionally metacestodes have been observed in C. virginica which were not encapsulated. Such larvae were also encountered during this study. Furthermore, since what are believed to be progressive stages of capsule formation were also encountered, it appeared to be of interest to describe the progressive morphological changes which occurred during this process (Fig. 1).

Shortly after metacestodes penetrated the host's gut wall and underlying basement membrane, the majority became trapped by the initiation of encapsulation. A few, however, did migrate further through the underlying Leydig tissue to the area of the digestive gland. In either location, the encapsulation process was initiated when each motile metacestode mechanically pushed back the surrounding Leydig cells (Figs. 1A, 2). This resulted in the formation of a layer of compressed cells around it. Shortly thereafter, a thickening of the intercellular substance between the compressed Leydig cells, which was most evident in Mallory's stained sections, was observed (Fig. 1B). The expansion of this intercellular material contributed further to the compression of the cells. It was at this stage that a small number of leucocytes, which had migrated into the region, became trapped in the dense area surrounding each metacestode (Figs. 1C, 3). Shortly

thereafter, a few fibroblastlike cells were observed lining the inner-most region of the cyst wall (Figs. 1D, 4).

With the onset of the formation of the structures mentioned above, the parasite appeared to be completely immobilized. This was followed by the laying down of fibers, originating from the thickened intercellular material between the surrounding Leydig cells, as concentric lamellae, commencing at the interior (Figs. 1D, 5). As additional fibers were laid down around the periphery, an increase of intercellular fibrous material between the peripheral Leydig cells occurred concurrently. In this manner, the fibrous capsule was increased in thickness until what appeared to be a definitive diameter was reached. It should be noted that in nonparasitized oysters and in areas of parasitized oysters devoid of parasites, the substance situated between Leydig cells was sparse and rather homogeneous in appearance (Fig. 6).

Morphology of Definitive Cyst Wall

As Sparks (1963) and Cheng (1966) have pointed out, the metacestode of Tylocephalum sp. in C. virginica is usually encapsulated within a thick fibrous cyst wall. The observations made during this study verify Cheng's (1966) earlier finding that by far the majority of the metacestodes were encapsulated in the zone surrounding the posterior portion of the digestive tract, immediately beneath the basement membrane, beginning at the level of the stomach. Occasionally, metacestodes were also found between the digestive diverticula and in gill matrices. Those found encysted in the region surrounding the gut were encapsulated by a conspicuously thicker fibrous capsule

than those in the region of the digestive gland. Based on 10 measurements, the capsule surrounding parasites in the first location averaged 0.076 mm thick while that surrounding parasites in the second location averaged 0.021 mm thick. The composition of these capsules, irrespective of their location, was essentially the same. Each was composed of more or less concentric lamellae of fibrous elements with a few leucocytes intermingled between the fibers. It is of interest to note that in addition to leucocytes, a number of spindle-shaped nuclei, resembling fibroblast nuclei, were also found comprising the wall but these were almost exclusively limited to the inner portion (Figs. 1D, 4). These nuclei were most conspicuous in sections stained with toluidine blue. Attempts to trace the cell boundary surrounding each spindle-shaped nucleus were not always successful but from the successful attempts, there is little doubt that the outline of each cell of this type was elongate spindle-shaped and with long drawn out cytoplasmic processes at each end which may or may not be branched. Although the morphology of these cells suggest that they are fibroblasts, it cannot be stated with certainty that they are, especially since Tripp et al. (1966) have reported similar cells in blood cell preparations of C. virginica. It may be noted that Tripp et al. referred to these cells as fibroblastlike cells. His designation is being followed herein. The cytoplasmic processes of these fibroblastlike cells contributed to the fibrous nature of the innermost portion of the capsule but by no means were the bulk of the fibers of this origin.

Relative to the staining affinities of each capsule, in H & E

stained sections, the nuclei of the leucocytes and fibroblastlike cells were hematoxyphilic while the cytoplasmic extensions of the latter stained pink. The majority of the fibers, however, were either chromophobic or were only slightly pink. In sections stained with Mallory's, the nuclei were reddish while the fibers were stained deeply blue. This blue color was so intense that it was not possible to differentiate between the cytoplasmic extensions of internally situated fibroblastlike cells and the more peripherally situated fibers, which, as stated, are not believed to be of fibroblastic origin but represented thickened intercellular material which became fibrous and were laid down peripherally as the capsule increased in diameter.

In addition to the fibers which comprised each capsule per se, the conspicuously thickened intercellular material between the peripheral Leydig cells also stained intensely blue with Mallory's. Moreover, it gave the appearance of being coarsely fibrous (Fig. 5). This alteration of intercellular material is quite extensive topographically. For example, it may affect an area equal to or five to six times the width of the capsule itself (Fig. 7).

Histochemical Findings

In PAS treated sections, not only were the fibers comprising each capsule strongly PAS-positive but the thickened intercellular material between the surrounding Leydig cells was also strongly PAS-positive (Fig. 8). In diastase-treated control sections, no appreciable decrease of the typical positive reaction occurred. This could not have resulted from ineffective diastase treatment since the PAS-positive material within the digestive diverticular cells, which is known to

represent stored glycogen, was completely eliminated in diastase-treated sections.

An interesting feature of PAS-treated sections was that fine PAS-positive, diastase-resistant strands were consistently observed connecting the surface of each metacestode with the inner surface of the fibrous capsule (Fig. 9).

In both paraffin and frozen sections, application of the Sudan black B test revealed the absence of lipids in the cyst wall. However, there were considerable lipoid droplets deposited in the cytoplasm of adjacent Leydig cells (Fig. 10). It is of interest to note that by comparison, the frozen-section technique was far superior to the paraffin technique for demonstrating lipids.

From sections treated with toluidine blue, it was determined that the zone occupied by the very thin innermost layer of each capsule was gamma metachromatic (Fig. 4). This metachromatic zone was that occupied by the fibroblastic layer of the capsule.

From sections treated with alcian blue, it was determined that the same gamma metachromatic zone was also positive for acid muco-polysaccharides (Fig. 11). The remainder of the capsule was not.

No appreciable differences were noticed in sections treated with hyaluronidase prior to staining with toluidine blue or alcian blue.

Sections stained with orcein did not reveal the presence of elastic fibers associated with the capsule, parasite, or surrounding Leydig tissues (Fig. 12). Similarly, sections stained with van Gieson's picrofuchsin stain failed to reveal the occurrence of a bright pink which is characteristic of collagen; but, the fibers comprising each

capsule did take on a pale pink color which is characteristic of reticulum. The reticular nature of these fibers was confirmed in sections stained with Gomori's reticulum stain (Fig. 13). In addition to the fibers comprising each capsule, the intercellular material between Leydig cells, in both parasitized and nonparasitized oysters, gave a strong positive reaction for reticulum (Fig. 13).

D. Discussion and Conclusions

From the results, it is evident that the fibers comprising capsules around Tylocephalum metacestodes in C. virginica are not totally of fibroblastic origin. In fact, using the number and distribution of fibroblastlike cells comprising each capsule as an indicator, it is evident that only a small fraction of the fibers are of this nature and the majority, if not all, of these are limited to the innermost layer of each capsule. Histochemically, results with toluidine blue indicate that the area occupied by fibroblastlike cells is gamma metachromatic. In fact, metachromasia within the entire capsule is restricted to the innermost zone. This metachromasia is most probably due to the occurrence of acid mucopolysaccharides which have been shown to occur at the same sites by use of the alcian blue test. It is of interest to note that there was no appreciable change in control sections pretreated with bovine testicular hyaluronidase. Since it is known that bovine testicular hyaluronidase will hydrolyze chondroitin sulfates A and C in addition to hyaluronic acid (McManus and Mowrey, 1963), it may be tentatively concluded that the positive acid mucopolysaccharide reaction associated with the innermost layer is due to the occurrence of chondroitin sulfate B.

By far the majority of the fibers comprising each cyst have their origin from the intercellular material between the surrounding Leydig cells. This conclusion is based on two major observations. (1) Morphological evidence indicates that the thickened intercellular material is fibrous and the fibers are contiguous with the peripheral fibers of each capsule. (2) Histochemically, the reactions of the intercellular material are identical with those of the fibers comprising the capsule except for the innermost fibroblastic layer. Both are strongly PAS-positive and diastase-resistant and both are devoid of acid mucopolysaccharides, lipids, collagen, and elastic fibers. Furthermore, both are positive for reticulum.

Relative to the chemical nature of the bulk of the fibrous capsule as well as the intercellular material between the surrounding Leydig cells, the fact that these are strongly PAS-positive and diastase-resistant eliminates the possibility that they include glycogen. The fact that they do not include lipids, as indicated by the Sudan black B and Nile blue sulfate tests, eliminates the possible occurrence of glycolipids and phospholipids. Furthermore, the fact that no reaction of any type occurred with Nile blue sulfate indicates the absence of unsaturated lipids. According to Pearse (1961), the only other categories of compounds which are PAS-positive are neutral mucopolysaccharides and muco- and glycoproteins. Histochemical techniques which would permit differentiation between these compounds, however, are not presently available.

The negative results obtained with the elastic fiber stain eliminate this category of fibrous connective tissue. Furthermore,

the fact that the fibers are strongly PAS-positive and nearly color-
less when stained with van Gieson's picro-fuchsin strongly suggests
that they are not collagenous.

It is my opinion that the fibers are reticular. The reasons for
this are (1) the fibers are argyrophilic as indicated by their black
color after staining with Gomori's silver stain; (2) the fibers take
on a light pink color when stained with van Gieson's picro-fuchsin
which is characteristic of reticular fibers, and (3) the fibers are
strongly PAS-positive. Relative to the strong PAS-positive reaction
of these fibers, it is of interest to point out that Glegg et al.
(1953), by employing chromatographic techniques, have demonstrated
that reticulum contains galactose, glucose, mannose, fucose, and
ribose in large quantities while collagen contains very small amounts
of these sugars. Earlier, Glegg et al. (1952) had indicated that the
reactive groups of PAS-positive compounds, i.e., those groups which
are oxidized by periodic acid and later combine with Schiff's reagent
to give the characteristic color, are the hexose sugars glucose,
galactose, and mannose, and the methylpentose sugar, fucose. It
would thus appear that the strongly PAS-positive reaction observed is
the result of the occurrence of reticulum rather than collagen. It
may be noted that reticulum is a glycoprotein which is one of the
categories of compounds our histochemical tests have revealed as
comprising the fibers.

Sparks and Chew (1966) have reported that the fibrous capsule
surrounding the larva of Echeneibothrium sp. in Venerupis staminea is
collagenous. Unless this capsule is drastically different, and there
is no reason to believe that it is, their postulation is subject to

question. The possibility exists, however, that the reticulum identi-
fied by my histochemical studies could serve as the precursor of
collagen, especially if one accepts the hypothesis of Foot (1925,
1927) and Dublin (1946) which states that reticulum acts as a
"scaffold" upon which collagen is formed. This hypothesis, however,
has not gone unchallenged (see Pearse, 1961). It would appear that
the encapsulating fibers in Crassostrea, and perhaps other lamelli-
branchs, could serve as an ideal model for further studies of this
nature. It should be pointed out that in their study, Sparks and
Chew only examined sections stained with Harris' hematoxylin and
eosin and hence their conclusion that the fibers are collagenous
cannot be substantiated.

Recently, Katkansky and Warner (1968) and Warner and Katkansky
(1968) claim to have demonstrated the presence of collagen fibers in
the cyst wall surrounding two species of Echeneibothrium found in
Protothaca stamina by using Mallory's aniline blue collagen stain.
This method was also employed by Pauley (1967) to determine collagenous
material in oyster tissue. Since this conventional histological stain
causes collagen, reticulum, ground substance of cartilage and bone,
mucin, and amyloid to turn various shades of blue (McClung, 1964), it
is difficult to see how these authors can definitively differentiate
collagen from the other above mentioned materials.

Relative to the fibroblastlike cells lining the inner margin of
each cyst wall, direct evidence indicating their origin is wanting.
However, the fact that they first appeared after leucocytes had
infiltrated the capsular region suggests that these spindle-shaped

cells had differentiated from leucocytes, a phenomenon suggested by

Drew and de Morgan (1910). But, unlike Drew and de Morgan, I do not

believe that all of the fibers comprising each capsule have originated

in this manner.

No information is yet available as to what stimulates the inter-

cellular material between surrounding Leydig cells to thicken and be-

come fibrous or what stimulates the migration of leucocytes into the

area of the fibrous capsule. It may be recalled that in the case of

Himasthla quissetensis metacercariae encysted in various marine pele-

cypods, Cheng et al. (1966) have shown that it is the inner cyst wall

secreted by the parasite which attracts the hosts' leucocytes which

in turn contribute to the formation of the outer wall and Bogitsh

(1962) has shown that the parasite-secreted cyst of Posthodiplostomum

minimum is PAS-positive. The PAS-positive strands projecting into

the host-parasite interface from the surface of the parasite, appear

to be intimately associated with the intercellular cytogenous material.

Finally, it is of interest to call attention to the fact that the

capsules around metacestodes situated in the zone surrounding the

alimentary tract are consistently thicker than those surrounding meta-

cestodes located in the interdiverticular spaces of the digestive gland.

Since it has been shown that most of the fibers forming each capsule

originate within intercellular material composed of glycoproteins but

may also include neutral polysaccharides and/or mucoproteins, one

possible explanation for this difference in capsule thickness is that

there is less Leydig tissue in the digestive gland than in the area

surrounding the alimentary tract, and hence less intercellular material.

Again, it may be recalled that Cheng and Cooperman (1964) and Cheng

et al. (1966) have proposed that the presence of absence of fibers in

encapsulating cysts in molluscs is dependent on the availability and

nature of the surrounding host tissues. The explanation being offered

to account for the difference in the thicknesses of the capsules

surrounding Tylocephalum metacestodes in the two regions supports

this concept.

Figure 1

Diagrammatic drawings showing steps involved during
the formation of fibrous capsule surrounding
Tylocephalum metacestode in Crassostrea virginica.

A. Appearance of compressed Leydig cells around
   metacestode.

B. Appearance of thickened intercellular material
   between Leydig cells.

C. Infiltration of leucocytes into the area.

D. Deposition of more or less concentric lamellae
   of fibers, originating as thickened intercellular
   material, around metacestode and the appearance
   of innermost layer of fibroblastlike cells.
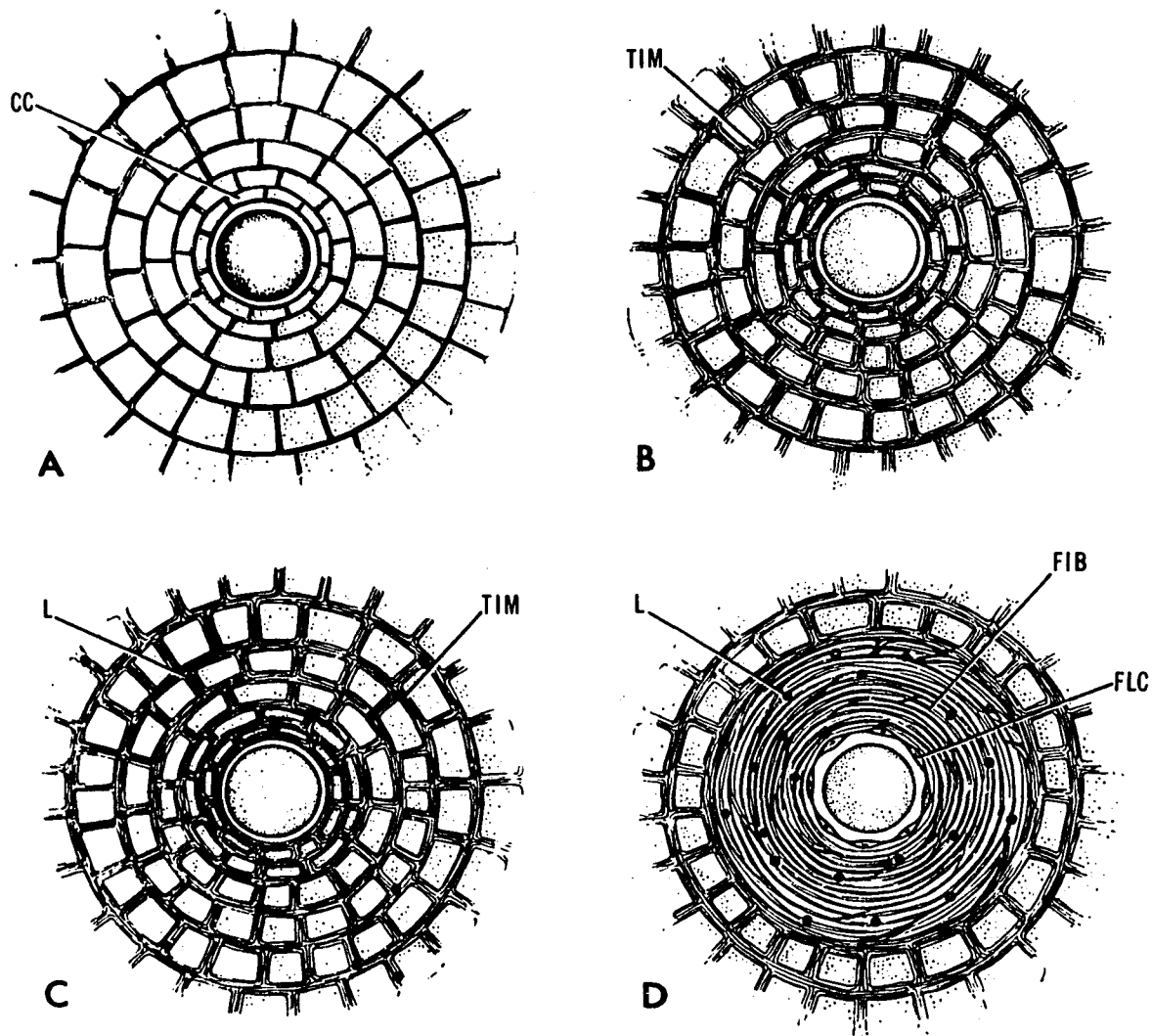
Figure 1

Figure 2

Metacestode in Leydig tissue surrounding host's gut.
Notice compressed host cells surrounding parasite and
beginning of thickening of intercellular material.
(H & E; 10x ocul., 40x obj.). (After Cheng, 1966).

Figure 3

Metacestode in Leydig tissue surrounding host's gut.
Notice number of leucocytes in dense area surrounding
parasite. (H & E; 10x ocul., 40x obj.).

Figure 4

Occurrence of fibroblastlike cells forming the inner-
most layer of the fibrous capsule surrounding a meta-
cestode (Toluidine blue; 10x ocul., 90x obj.).

Figure 5

Occurrence of thickened fibrous intercellular material
between Leydig cells situated on periphery of encap-
sulated metacestode. This material forms the bulk
of the fibers comprising the capsule. (Mallory's;
10x ocul., 90x obj.).

Figure 6

Leydig tissue of nonparasitized oyster in region
surrounding gut. Notice the sparcity of intercellular
material (Mallory's; 10x ocul., 90x obj.).

Figure 7

Extensive thickened intercellular material on periphery
of fibrous capsule surrounding metacestode. (Mallory's;
10x ocul., 10x obj.).

FIB=capsular fibers; FN=nucleus of fibroblastlike cell; GE=gut
epithelium; L=leucocyte nucleus; LEY=Leydig cell; MET=metacestode;
TIM=thickened intercellular material between Leydig cells.
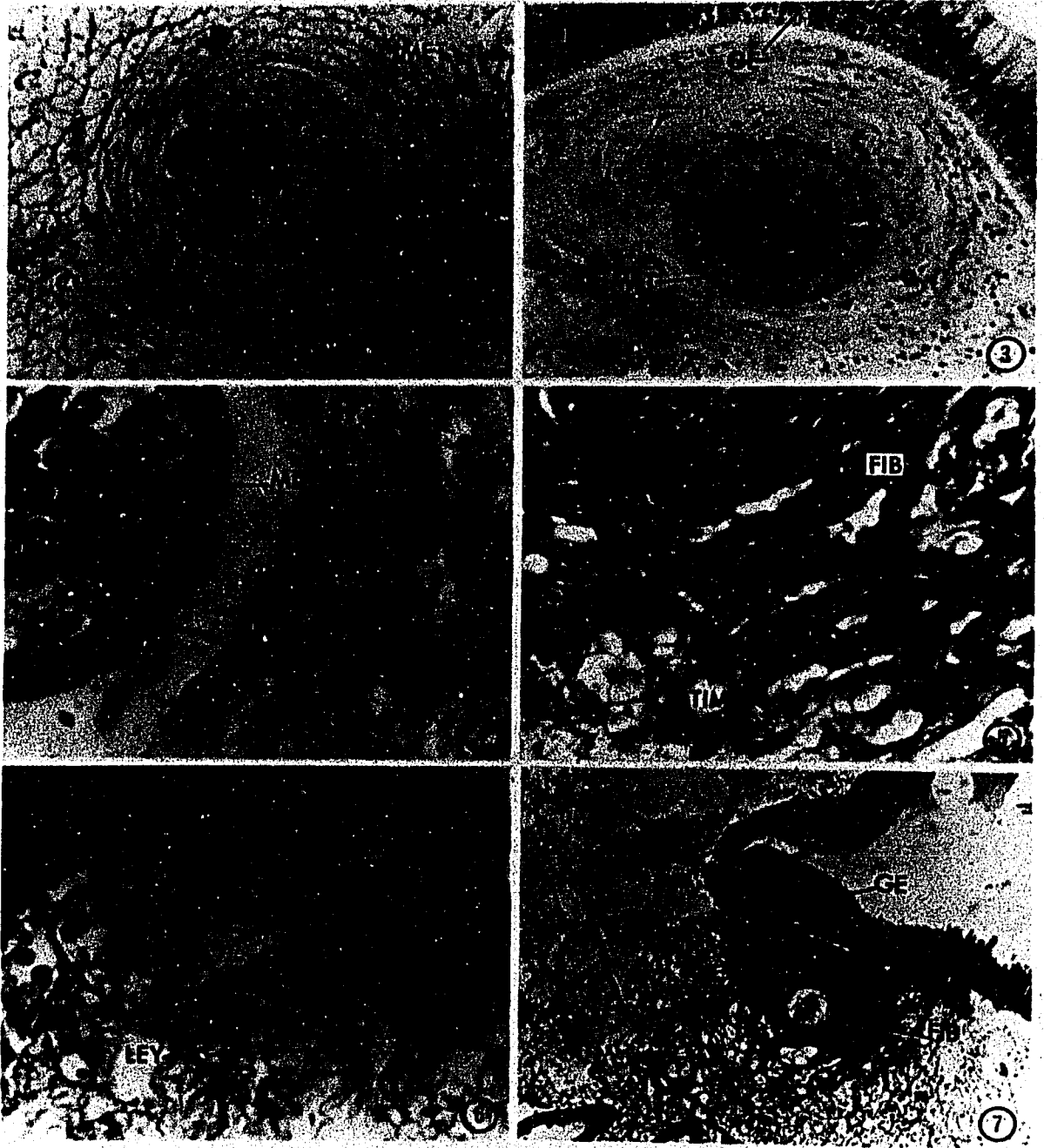
Figure 8

Strong PAS-positive reaction of capsular fibers and
thickened intercellular material between surrounding
Leydig cells. (PAS reaction; 10x ocul., 10x obj.).

Figure 9

Fine PAS-positive, diastase-resistant strands
connecting surface of metacestode with inner surface
of capsule. (PAS reaction; 10x ocul., 40x obj.).

Figure 10

Occurrence of lipoid deposits in surrounding Leydig
cells but not in fibrous capsule. (Cryostat section,
Sudan black B; 10x ocul., 10x obj.).

Figure 11

Occurrence of acid mucopolysaccharides in innermost
stratum of capsule. (Alcian blue; 10x ocul., 10x obj.).

Figure 12

Absence of elastic fibers associated with capsule and
surrounding Leydig tissue. (Orcein; 10x ocul., 10x
obj.).

Figure 13

Strong positive reaction for reticulum of capsular
fibers and intercellular material between surrounding
Leydig cells. (Gomori's; 10x ocul., 90x obj.).

AMP=acid mucopolysaccharides; FIB=capsular fibers; MET=metacestode;
TIM=thickened intercellular material between Leydig cells.
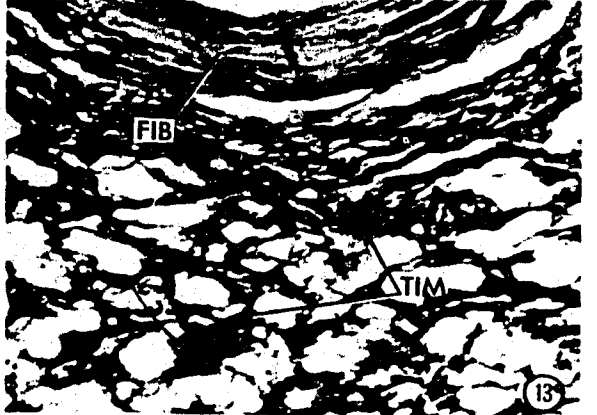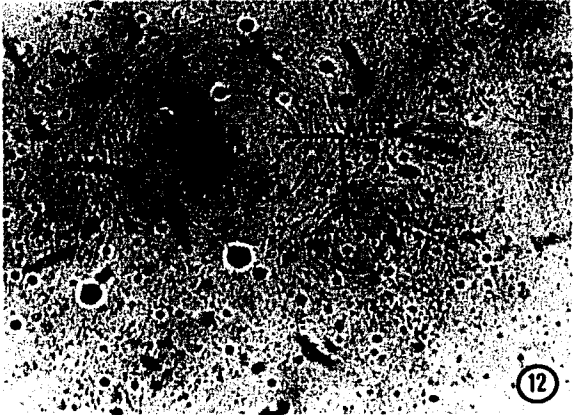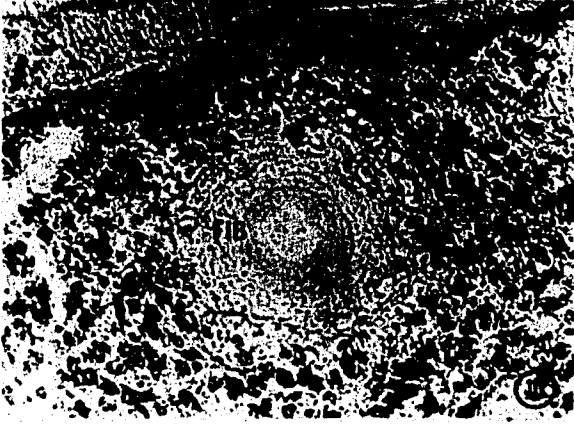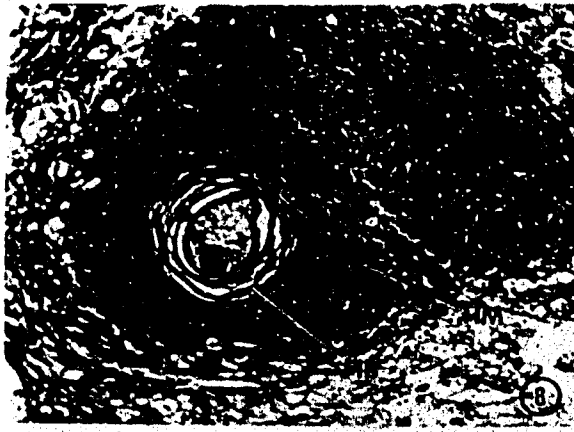
CHAPTER IV

AN ELECTRON MICROSCOPE STUDY OF THE CONSTITUENTS OF
ENCAPSULATING CYSTS IN CRASSOSTREA VIRGINICA FORMED
IN RESPONSE TO TYLOCEPHALUM METACESTODES

A. Introduction

Although the origin, formation, development, and structure of
encapsulating cysts have been studied with the light microscope, many
fundamental aspects of the problem, especially at the cellular level,
remain unresolved due to limitations in resolution. In order to more
clearly elucidate this fundamental aspect of invertebrate pathology,
an electron microscopel study of the structure of encapsulating cysts
in the American oyster, C. virginica, formed in response to Tylo-
cephalum metacestodes has been carried out.

It is known that each encapsulating cyst in C. virginica is com-
posed primarily of more or less concentric layers of fibrous elements
with leucocytes and fibroblastlike cells intermingled in between.
Furthermore, the majority of the fibers appears to originate from the
intercellular material situated between the surrounding Leydig cells.
Histochemical tests have revealed that the fibers include glyco-
proteins and/or mucoproteins and neutral mucopolysaccharides. Addi-
tional tests have indicated that these fibers are reticular rather
than collagenous. Results of the electron microscope studies reported
herein serve to confirm these findings as well as provide additional
information.

Presently, any comparison of the fine structure of the cells and
fibers comprising an encapsulation cyst in C. virginica will have to be

made with vertebrate connective tissue fibers and cells associated with wound healing as there are no published electron microscope studies of the encapsulation complex in invertebrates. The only exception lies with the contribution by Grimstone et al. (1967) who have contributed an electron microscope study of the cyst formed around Araldite spheres in the moth *Ephestia kuehniella*. This cyst, however, is entirely cellular in nature and hence is fundamentally different from that developed in *C. virginica* in response to *Tylocephalum*.

It is of interest to note that the phenomenon of encapsulation in many vertebrates and invertebrates share such common denominators as the proliferation and differentiation of cells and the formation of inter- and intracellular fibers and amorphous substances. Consequently, the study of this phenomenon in invertebrates may well lead to a better understanding of the phylogeny of this process.

B. Materials and Methods

The oysters (*Crassostrea virginica*) used during this study were taken from West Loch, Pearl Harbor, Oahu, Hawaii, and placed in a running seawater system (salinity: 32 o/oo, temperature: 25° C) until their subsequent use.

After the animals were removed from their shells, smears of thin cross-sectional pieces were examined under a low power (10x) light microscope to determine if the animals were parasitized by *Tylo-cephalum* metacestodes. If an oyster was infected, an adjacent section was removed, placed over ice, and minced with a pair of iridectomy scissors into blocks approximately 1 cu. mm. Each piece was then placed on a glass slide under slight pressure from a coverglass and

the occurrence of Tylocephalum larvae was verified under the light microscope. If the block contained parasites, the coverglass was re- moved and the tissue was subsequently fixed. This procedure did not appear to distort any of the intra- and intercellular details of either the oyster or cestode tissues. Other methods employed for locating parasites in situ, such as vital staining, were not success- ful.

The fixative employed was cold (3° C) 2.5% Glad's glutaraldehyde in oyster basic salt solution (BSS) (Tripp et al., 1966) maintained at pH 7.5. After fixation for 2 hr, the tissues were washed for 2 hr in oyster BSS and postfixed in an oyster BSS solution of 1% osmium tetro- xide, dehydrated via an ethanol series, and embedded in Epon.

One micron thick sections were cut in a Porter Blum MT-1 ultra- microtome and stained with toluidine blue in order to locate the para- sites. After staining, ultra-thin sections, approximately 700-1000 $\overset{\circ}{A}$ (gold and silver interference colors), cut on the ultra-microtome, were stained with lead citrate (Reynolds, 1963), picked up on carbon-coated copper grids, and examined on the Hatachi HU-11A electron microscope operated at 50 Kv.

In addition to sections of encapsulated parasites, sections of tissues from comparable areas in nonparasitized oysters were similarly prepared and examined.

C. Results

The results of the electron microscope studies have verified the fact that the encapsulating cyst formed by C. virginica in response to Tylocephalum metacestodes includes cellular as well as extracellular

components. The fine structures of both of these are presented at this point.

Fine Structure of Cells. Three types of cells are found intermingled with the fibrous elements of each cyst. The most frequently encountered cell type in the innermost region of the cyst if fibroblastlike. These cells are generally intimately apposed to the tegumentary spines and microvillar extensions of the cestode's body surface (Fig. 14) but in some cases they may be separated from the parasite's surface by intercellular material comprised of fibers embedded in a matrix (Fig. 15). The cells are elongate and their cytoplasm extends on each side of the nucleus (Figs. 14, 15). Each cell is approximately 2 μ wide but the length varies considerably between cells, with some extending for a considerable distance along the body surface of the parasite. The nucleus is elipsoidal and contains electron-dense material which most probably represents chromatin. This material is frequently located along the inner surface of the nuclear membrane in addition to being dispersed throughout the nucleus (Fig. 15). The mitochondria present are round or elongate and are randomly scattered throughout the cytoplasm. Their profiles measure approximately 0.5 μ in length. The inner membrane of each mitochondrion is folded to form long, tubular cristae which extend toward the center of the organelle (Figs. 15, 18). Characteristic of this type of cell is the presence of an intricate network of rough endoplasmic reticulum (ER) (Figs. 15, 18). At times, smooth ER may also be observed.

The cisternae of the ER are at times greatly dilated by an accumulation of cell products; however, these distentions are generally in

the form of electron-lucid vesicles which may or may not include a
material of medium electron density. The dilated cisternae are connect-
ed with the adjacent normal ER (Fig. 18).

Another striking characteristic of the fibroblastlike cells is the
occurrence of regions of varying cytoplasmic densities. Portions or
all of the cytoplasm of a single cell may be relatively dense while
other areas of the cytoplasm of the same or an adjacent cell may be
leached out and include relatively few organelles (Fig. 18).

The main cell type found throughout the cyst, intermingled with
the fibers, is the leucocyte. It measures approximately 7 μ in diameter
(Fig. 19). Unlike the fibroblastlike cells, the leucocyte is generally
ovoid but the distribution of its nuclear chromatin, mitochrondria,
and its extensive ER system bears a striking similarity to that of
their counterparts found in fibroblastlike cells. Lysosome-like bodies
found in some leucocytes are in the form of membrane-lined vacuoles
containing numerous small vesicles (Figs. 20, 21). These cells also
contain dictyosomes, each consisting of a series of stacked cisternae
(Fig. 20). As in the case of the fibroblastlike cells, the cisternae
of the ER in leucocytes may be distended (Figs. 21, 22) and areas of
the cytoplasm of the same or adjacent cells may vary greatly in density
(Fig. 22). Very occasionally, myelin-like bodies have been observed in
the cytoplasm of leucocytes (Fig. 22).

Intracytoplasmic fibrils are found in both fibroblastlike cells
and leucocytes associated with the encapsulation cyst. They are not
found in similar cells located elsewhere in the oyster or in uninfected
oysters. These fibrils are situated toward the periphery of each cell,

with their longitudinal axes being parallel to the plasma membrane

(Fig. 18). Each fibril is approximately 500 Å in diameter, of in-

determinate length, nonperiodic, and of uniform, medium electron

density. When observed in cross-section, thin branches can be found

emanating from the main fibrils and these branches, together with the

primary fibrils, form an anastomosing network (Figs. 16, 17). These

intracytoplasmic fibrils generally occur in the peripheral regions of

cells where the cytoplasm appears leached (Fig. 18).

The third type of cell occurring in each cyst is found towards the

peripheral zone and corresponds to the so-called "brown cell"

(Takatsuki, 1934). Its size is extremely variable but averages approxi-

mately 6 x 10 μ. Its most characteristic inclusions are in the form of

large, ovoid globules of different textures and electron densities

(Fig. 23). Many of the globules of moderate density contain units of

a short, irregular, fibrous material while the more dense globules

include well-defined crystals (Figs. 23, 23A). The fibrous inclusions

are of greater electron density than the intraglobular matrix in which

they are embedded and each unit seldom, if ever, measures more than

0.5 μ in length. The crystals found in the denser globules appear as

squares or rectangles in sections and measure approximately 0.5 μ or

less along each edge. They portray a distinct periodicity of 200 Å

(Fig. 23A). The cytoplasm of each brown cell appears leached and in-

cludes mitochondria, extended rough ER, and the globules of varying

sizes and densities already described. A well-defined nucleus occurs

in close proximity to the limiting membrane of each cell (Fig. 24).

Peripheral to the capsule proper are found the large cells

characteristic of oyster connective tissue, the so-called Leydig cells.
Each of these measures approximately 35-40 μ in diameter and includes
highly leached cytoplasm similar to that occurring in brown cells
(Figs. 25, 26). The nucleus contains randomly arranged chromatin
patches throughout the nucleoplasm (Fig. 26). An intricate network of
rough ER is characteristically found close to the outer surface of the
nuclear membrane (Fig. 26). Although the majority of the mitochondria
are also found in this region, some also occur in the zone mediad to the
cell surface (Figs. 25, 26). The most conspicuous structures in the
cytoplasm are the small, clustered, electron-dense granules which most
probably represent glycogen deposits, and larger vesicles, each
measuring about 0.8 μ in diameter, enclosing a homogeneous material of
moderate electron density (Figs. 25, 26). Both types of inclusions
are quite numerous and neither show any particular distribution within
the cell.

Fine Structure of Fibers. The three types of cells associated with the
cyst proper are embedded in an amorphous, homogeneous matrix of moderate
electron density. Also embedded in this matrix are two types of fibers.
The more prominent type is convoluted, approximately 70 $\overset{\text{o}}{\text{A}}$ thick, and
has an electron-dense periphery (Figs. 27, 27A, 28).

The amount of fibers varies with their proximity of the cestode.
The closer the area is to the parasite, the more heavily the matrix is
packed with fibers except in the immediate area of the parasites body
surface where there is a noticeable decrease in the number of fibers.
Moreover, the matrix is also less dense in this area (Fig. 29). The
matrix also permeates the spaces between the microvilli projecting from

the cestode's tegument and many of the fibers embedded in the matrix in this region are attached to the outer surface of the unit membrane surrounding each microvillus (Figs. 29, 30).

In areas where the fibers are most densely packed, the shorter elements give the appearance of coalescing to form a large anatomosing network (Fig. 28). These densely packed areas are usually in close proximity to leucocytes and fibroblastlike cells.

The second type of fibers found embedded in the matrix is far less conspicuous and abundant. These fibers are situated primarily in the proximity of brown cells although on rare occasions some have been found near leucocytes and fibroblastlike cells. Each fiber is wavy, measures about 100 Å in diameter, of indeterminate but greater length than the first type, and of an electron density similar to that of the homogeneous matrix. The longitudinal axis of each fiber lies parallel to the plasma membrane of the adjacent brown cell (Fig. 23).

D. Discussion

The encapsulation complex in molluscs has not been studied at the ultrastructural level. As has been stated, the only published account of an electron microscope study of encapsulation cysts in an invertebrate is that by Grimstone et al. (1967). These authors have examined the nature of encapsulation cysts in the flour moth, Ephestia kuehnella, induced by implanting Adraldite spheres. The resulting cysts are fundamentally different from those in Crassostrea virginica in being entirely cellular. Those produced in C. virginica include both fibers and cells, primarily the former (Sparks, 1963; Cheng, 1966; Rifkin and Cheng, 1968).

Since the cysts in oysters are primarily comprised of fibers, it is of considerable interest to understand their origin and the processes by which they are formed. Unfortunately, the origins of both types of extracellular fibers described herein still remain ill-defined. Nevertheless, my findings suggest that many of the problems related to the formation of this reticular complex are analogous to those related to the formation of vertebrate fibers, particularly pertaining to collagen. For this, Ross and Benditt (1961) have proposed three possible mechanisms which may be responsible for the origin of extracellular fibers: (1) the fibers are formed intracytoplasmically and are subsequently deposited extracellularly, (2) the fibers are formed extracellularly independent of cells, and (3) they are formed extracellularly but the process is dependent on some factor(s) contributed by a cell. I think that the fibers comprising the cyst in _C. virginica_ are not formed intracellularly despite the finding of intracellular fibrils at the cytoplasmic periphery of leucocytes, fibroblastlike cells, and within certain cytoplasmic globules within brown cells. This opinion is based on the fact that inspite of critical examination of several hundred sections, I have found nothing that would suggest a transfer of fibrous material across the plasma membrane to the exterior. Furthermore, as noted, there are gross morphological differences between intra- and extracellular fibrous elements.

It is also doubtful whether the development of the extracellular fibers is independent of any type of cell. In molluscs, the abundance of fibrous cytogenous material appears to be directly related to the types of cells and/or fibers in the area of cyst formation. It has

been demonstrated that the ability to mobilize the largest concentration
of extracellular fibers appears to be greatest in regions rich in
Leydig cells (Cheng and Cooperman, 1964; Cheng et al., 1966). It is
also in such regions that the largest number of extravascular leuco-
cytes, fibroblastlike cells, and brown cells are found. My observation
that the major type of extracellular fibers are always found in juxta-
position to either a fibroblastlike cell or leucocyte suggest that
these cells may play a significant, though not yet determined, role in
the formation of the fibers. Thus, the third hypothesis as proposed
by Ross and Benditt (1961), i.e., extracellular fibrillogenesis is
dependent upon some type of cellular function, seems to be the most
applicable to this particular situation. Possibly, both leucocytes
and fibroblastlike cells are capable of secreting some precursor sub-
stance or stimulate some precursor present outside of the cell to
transform into fibers. It has already been suggested that the fibers
originate from a thickened intercellular material which transforms into
fibrous elements.

The lack of periodicity in the extracellular fibers confirms the
finding that these are not collagenous. The histochemical results have
indicated that these fibers are reticular.

Morphologically, the fusiform fibroblastlike cells consistently
found in the area of the cestode's tegumentary microvilli are similar
in a number of ways to vertebrate fibroblasts found in areas of wound
repair and pathological conditions in general (Ross and Benditt, 1961;
Chapman, 1961; Movat and Fernando, 1962; Goldberg and Green, 1964).
The most conspicuous of these is the extensive dilatation of the rough

ER. It has been found by the authors cited above that the electron density in distended cisternae of vertebrate fibroblasts varies from a relatively dense floccular appearance to an almost clear electron-lucid one. It has been noted that distended cisternae also occur in both the fibroblastlike cells and leucocytes of parasitized *C. virginica*. These dilations, when present in large numbers, give the cell a vacuolated appearance similar to that described in vertebrate fibro-blasts.

The intracellular fibrils occurring in both leucocytes and fibro-blastlike cells of parasitized *C. virginica* have counterparts in a variety of types of vertebrate cells (dePetris et al., 1962; Buckley and Porter, 1967), including fibroblasts (Ross and Benditt, 1961; Chapman, 1961; Movat and Fernando, 1962; Goldberg and Green, 1964). All of these investigators have reported that in vertebrates, as in the oyster, the majority of the fibrils found in fibroblasts and other types of cells are located toward the periphery of the cytoplasm. Buckley and Porter (1967) have suggested that these fibrils serve to stabilize areas of cellular attachment and regulate intracellular move-ments by influencing cytoplasmic viscosity. Thus, if indeed the fibrils found in oyster leucocytes and fibroblastlike cells are invertebrate counterparts of those found in vertebrate cells, they could serve the same hypothetical functions. Despite these similarities in topography, morphological differences, however, do exist. The fibrils in oyster cells measure approximately 500 Å in diameter and are branched while those of vertebrate cells measure from 50-70 Å in diameter and are unbranched (Chapman, 1961; Ross and Benditt, 1961; Goldberg and Green,

1964).

It is of considerable interest to note that similarities exist between oyster leucocytes and fibroblastlike cells at the fine structural level. Although the latter are fusiform while leucocytes are most commonly oval, both include (1) extensive dilatations of the rough ER, (2) similar intracytoplasmic fibrils, and (3) areas of leached cytoplasm. On the other hand, differences also exist, e.g. fibroblastlike cells contain primarily rough ER while leucocytes contain primarily smooth ER. In addition, leucocytes also contain conspicuous lysosome-like bodies which have not been found in fibroblastlike cells. These differences, however, may be explained by variations in function. Protein synthesis in fibroblastlike cells, for example, would explain the abundance of rough ER and the well-known phagocytic role of leucocytes could explain the presence of the lysosomes.

The basic fine structural similarities between leucocytes and fibroblastlike cells suggest that these two types of cells, when associated with an encapsulating cyst in C. virginica, may have differentiated from an immediate common precursor cell or, more likely, that the fibroblastlike cells have differentiated from leucocytes. The latter hypothesis is supported by the finding of Tripp et al. (1966) that some mature oyster leucocytes maintained in vitro do become fibroblastlike and by our earlier report (Rifkin and Cheng, 1968) that the innermost layer of the encapsulating cyst, which is comprised of fibroblastlike cells, appears only after leucocytes infiltrate into the area.

The presence of crystal- and fibril-containing globules of varying densities is being reported for the first time in brown cells. The

crystals occurring in the denser globules most probably represent proteins (Fawcett, 1966) and may be large aggregates of enzymes and sphingomyelins which have been reported from these cells (Cheng and Burton, 1966; Cheng, 1967). It has been suggested that brown cells may play a role in internal defense since they are most abundant in parasitized oysters (Mackin, 1951; Stein and Mackin, 1955; Cheng and Burton, 1965); however, this study has not clarified the nature of their functional role.

Finally, an interesting aspect of host-parasite relationship revealed by the present study deserves mention. The electronmicrographic evidences suggest that the long, filamentous microvilli projecting from the cestode's tegument are capable of undulatory movements and during this process, many of the fibers of host origin become attached to the unit membrane surrounding each microvillus (Fig. 30). It is difficult to speculate at this time on the physiological and/or immunological implications associated with this phenomenon. The host material adhering to the microvilli may serve as a nutrient source for the parasite or this may reflect a defense mechanism on the part of the cestode in preventing the accumulation of possible deleterious macromolecules on its surface.

Figure 14

Electronmicrograph of oyster-Tylocephalum interface showing

spatial relationship between fibroblastlike cell and microvilli

and spines of the cestode's tegument. (x 23,100).

Figure 15

Electronmicrograph of oyster-Tylocephalum interface showing

extracellular fibers between fibroblastlike cell and cestode

surface. (x 24,500).

C = chromatin, EF = extracellular fibers, M = cestode microvilli,

MA = matrix, MI = mitochondria, NFC = nucleus of fibroblastlike

cell, RE = rough endoplasmic reticulum, S = tegumentary spine

of cestode.
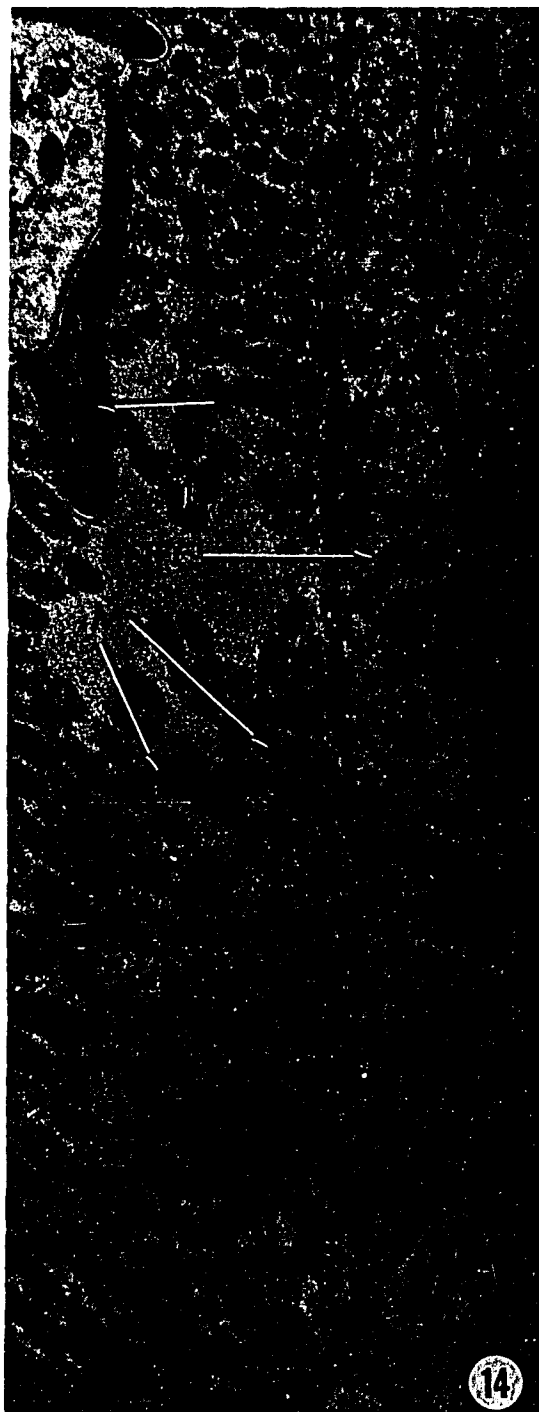
Figures 16, 17

Electronmicrographs showing portions of leached cytoplasm of fibroblastlike cell enclosing cross-sections of primary and secondary intracytoplasmic fibrils at two different magnifications. (x 17,500, x 40,000).

Figure 18

Electronmicrograph showing longitudinal sections of fibroblastlike cells with associated organelles and structures. (x 13,200).

BF = intracytoplasmic fibrillar branches, DC = dilated cisterna,

IF = intracytoplasmic fibrils, LC = leached cytoplasm,

MI = mitochondrion, NFC = nucleus of fibroblastlike cell,

PF = primary intracytoplasmic fibril, RE = rough endoplasmic reticulum.
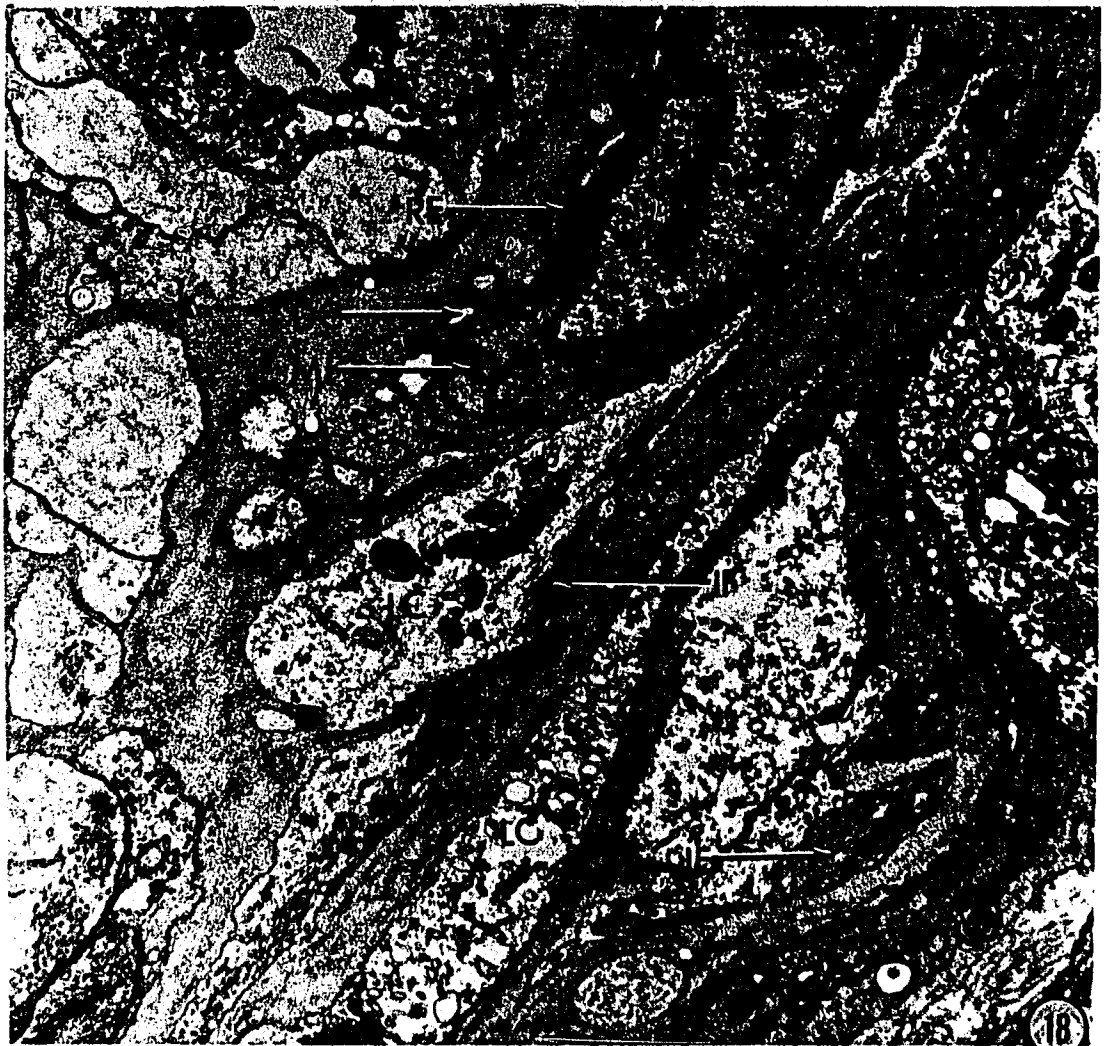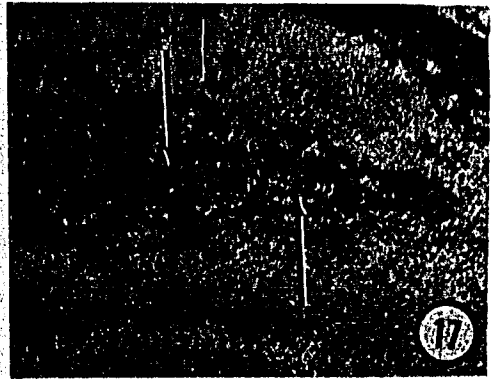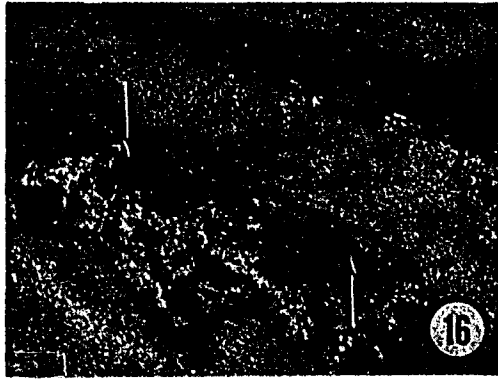
Figure 19

Electronmicrograph showing section of oyster leucocyte
associated with encapsulation complex. (x 17,100).

Figure 20

Electronmicrograph showing portion of oyster leucocyte
associated with encapsulation complex. Notice presence
of dictyosome and lysosomes. (x 18,200).

C = chromatin, DI = dictyosome, LN = nucleus of leucocyte,
LY = lysosome-like body, MI = mitochondrion, SE = smooth
endoplasmic reticulum.
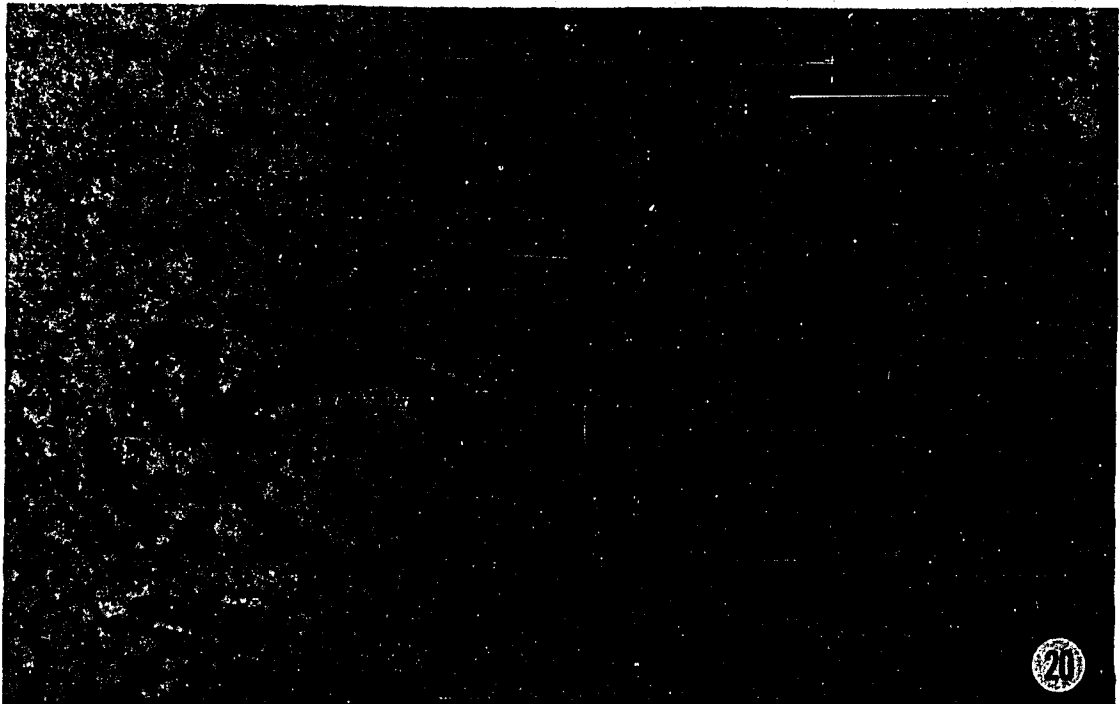
Figure 21

Electronmicrograph showing portion of section of elongate

oyster leucocyte associated with encapsulation complex.

Notice presence of conspicuous distended cisternae. (x 17,100).

Figure 22

Electronmicrograph showing portion of section of oyster leucocyte

associated with encapsulation complex. Notice leached cytoplasm

of adjacent (left) leucocyte and myelin-like body. (x 20,300).

DC = dilated cisternae, LC = leached cytoplasm, LN = nucleus of

leucocyte, LY = lysosome-like body, MI = mitochondria, MY =

myelin-like body. (x 20,300).

Figure 23

Electronmicrograph showing portion of section of brown cell.
Notice presence of globules of varying densities and second
type of extracellular fibers on right. (x 16,500). Fig.
23A (insert). Electronmicrograph of section of crystal found
in dense globule within brown cell. (x 34,850).

Figure 24

Electronmicrograph showing portion of section of brown cell
enclosing conspicuous globules. Notice peripherally situated
nucleus. (x 13,300).

CR = intraglobular crystal in brown cell, DG = electron-dense
globule, F = intraglobular fibrous material in brown cell,
LC = leached cytoplasm, MG = moderately dense globule, MI =
mitochondrion, NB = nucleus of brown cell, RE = rough endo-
plasmic reticulum, SF = second type of extracellular fibers.
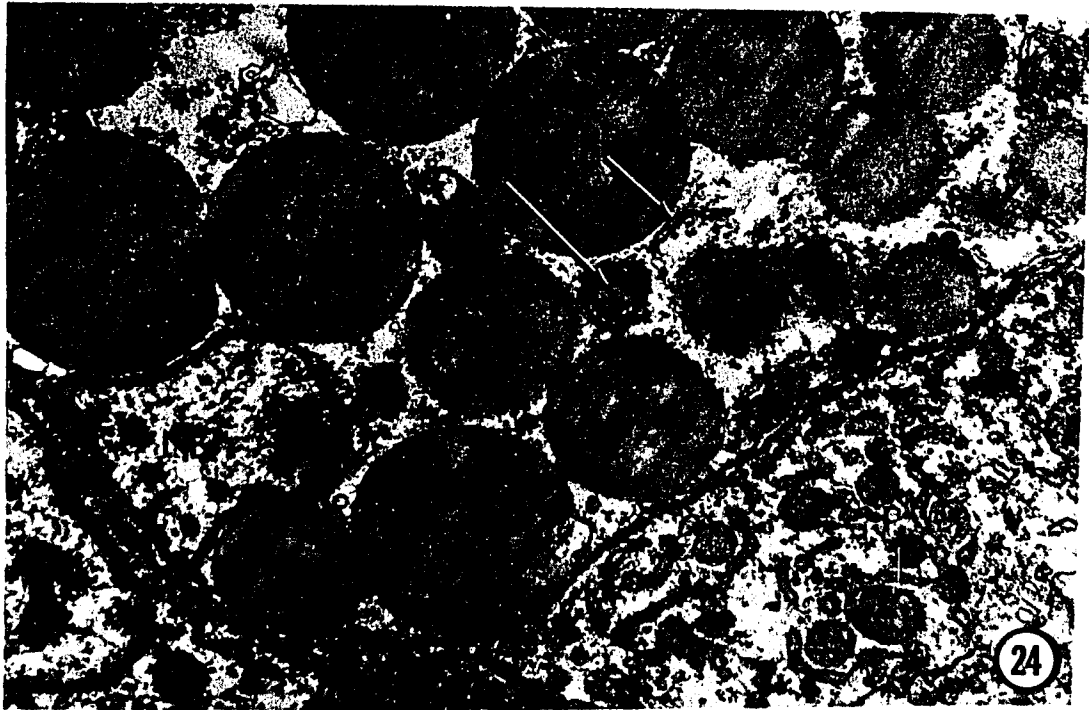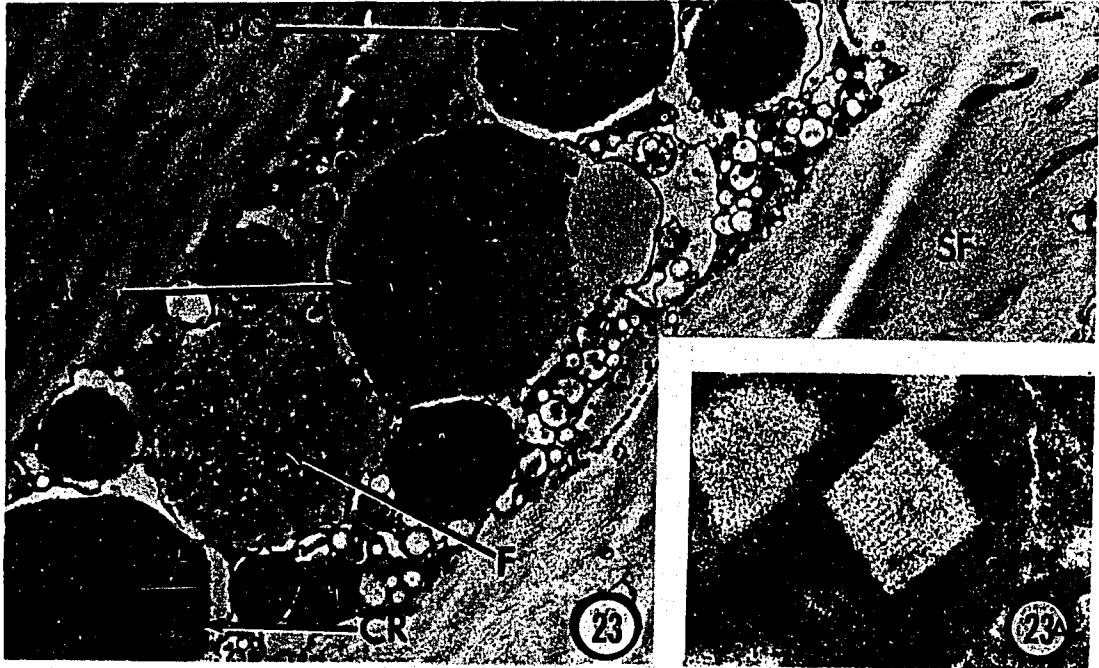
Figures 25, 26

Electronmicrographs showing portions of sections of Leydig

cells of oyster.   (x 9,000, x 10,000).

G = glycogen granules, LC = leached cytoplasm, MI = mito-

chondria, NL = nucleus of Leydig cell, RE = rough endoplasmic

reticulum, V = vesicles in brown cell.
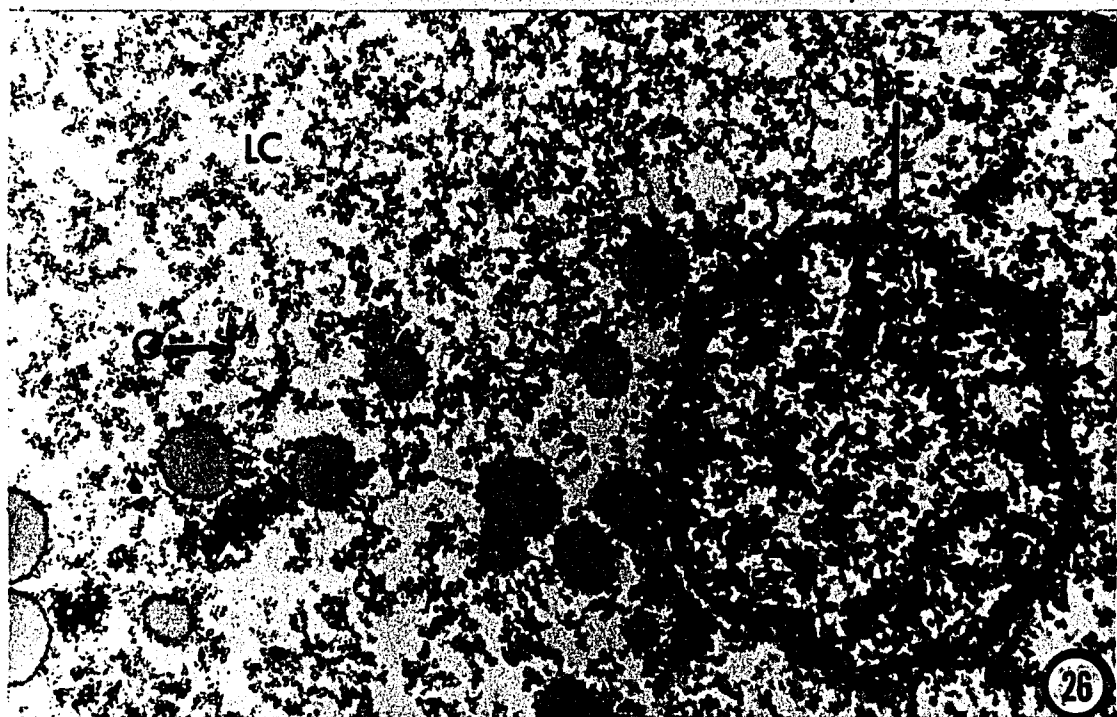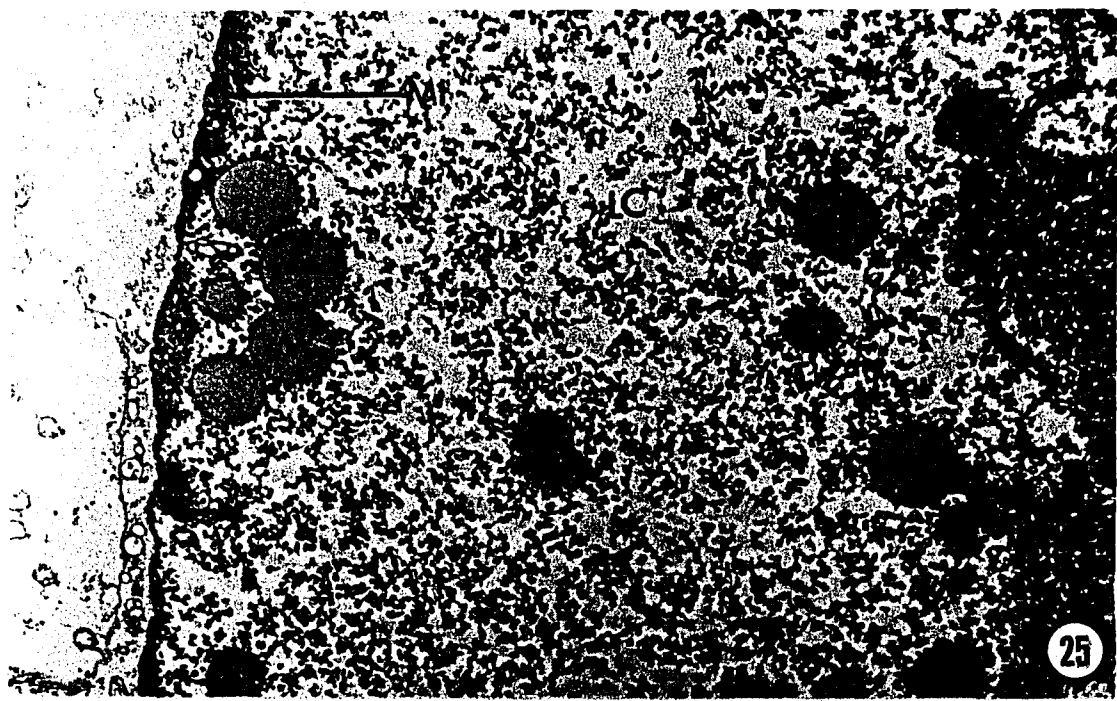
Figure 27

Electronmicrograph of portion of extracellular matrix with

embedded fibers. (x 49,000). Fig. 27A (insert). Electron-

micrograph of extracellular fibers at greater magnification.

(x 89,100).

Figure 28

Electronmicrograph of anastomosing extracellular fibers

embedded in matrix. (x 53,650).

EF = extracellular fibers.
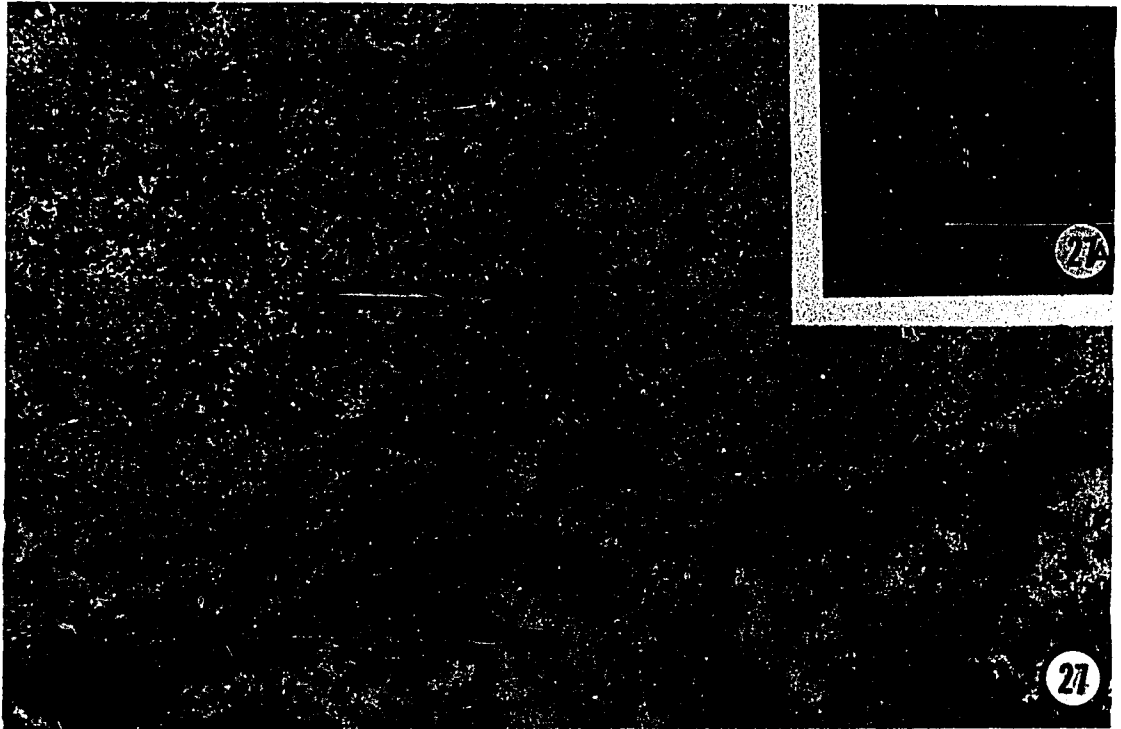
Figure 29

Electronmicrograph of oyster-_Tylocephalum_ interface showing
extracellular fibers adhering to surfaces of parasite's
microvilli. (x 28,750).

Figure 30

Electronmicrograph of longitudinal sections of tegumentary
microvilli of _Tylocephalum_ metacestode showing extracellular
fibers of host adhering to outer unit membrane. (x 84,525).

EF = extracellular fibers, M = tegumentary microvillus of
cestode, S = tegumentary spine.
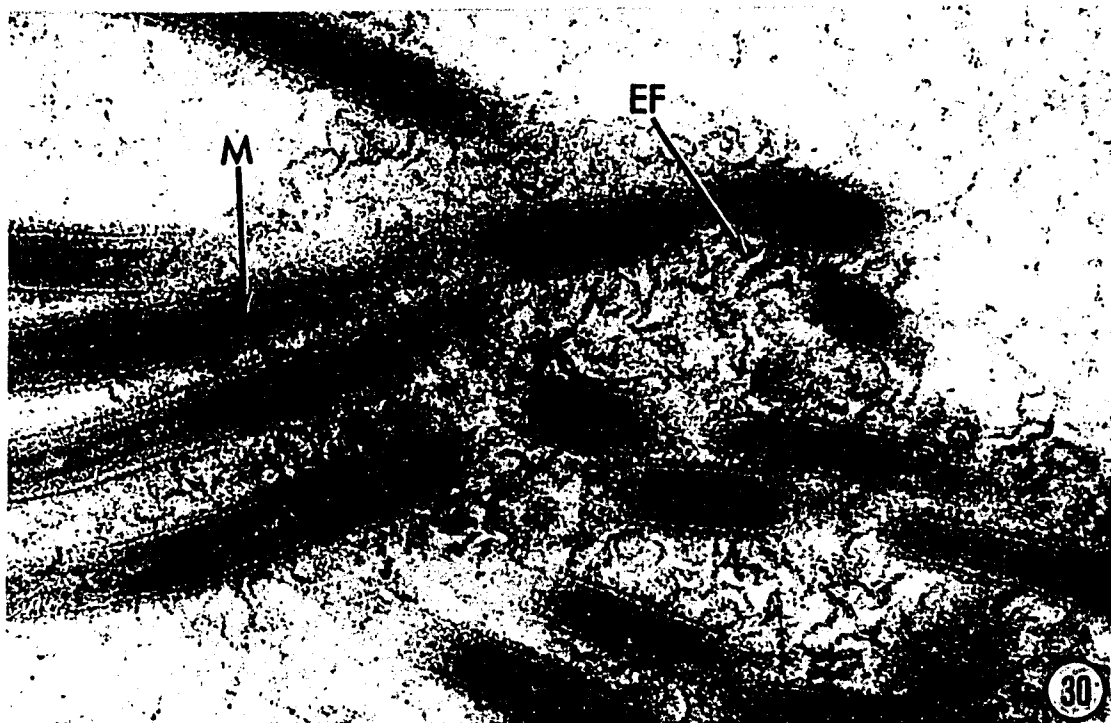
CHAPTER V

THE FINE STRUCTURE OF THE TEGUMENT OF TYLOCEPHALUM METACESTODES: WITH EMPHASIS ON A NEW TYPE OF MICROVILLUS

A. Introduction

The coracidium of Tylocephalum is ingested by the oyster during feeding. It then burrows through the lining of the alimentary canal and the underlying basement membrane and becomes encapsulated in the connective tissue (Leydig) region surrounding the alimentary canal and less frequently in the digestive gland, between the diverticula (Cheng, 1966). At this stage, the parasite's body surface is in close proximity to the encapsulation complex.

The origin, structure, and histochemistry of the encapsulating cyst surrounding Tylocephalum metacestodes in C. virginica has been reported as has the fine structure of its constituent parts. During the course of examining the host's reaction cells and fibers at the host-parasite interface with the light microscope, a series of fibril-like projections were observed extending from the parasite's body surface. Due to limitations in resolution, the structure and possible function of these fibrils could not be determined. Consequently, this electron microscope study was carried out to clarify the nature of these structures.

B. Results

The general arrangement of the tegument of Tylocephalum metacestodes is similar to that in other cestodes, both larvae and adults, which have been studied (see Lee, 1966 for review)(Fig. 31). The tegument

is divided into an external and an internal level which are partially

separated by a basal lamina (or basement membrane) and the subsurfacial

muscles (Figs. 32-34). The two levels are connected by intermittent

cytoplasmic bridges.

The external level is comprised of an anucleate cytoplasmic

syncytium including mitochondria and numerous membrane-lined vesicles

(Fig. 34). These vesicles enclose a homogeneous material of moderate

electron density. The larger vesicles are approximately 0.3-0.7 μ in

diameter (Figs. 34-37) while the smaller ones average 0.1 μ in diameter

(Figs. 34-36). Conspicuously absent from this region are dictyosomes

and both rough and smooth endoplasmic reticula.

The outer surface of the external level is bordered by a unit

membrane 75 $\overset{o}{A}$ thick (Fig. 38) while the basal lamina, measuring 0.15-

0.25 μ thick, serves as the inner boundary of this level (Figs. 32-35).

Extensions of the basal lamina protrude into the external level at

irregular intervals (Figs. 34, 35).

The most striking difference between the external level of the

tegument of Tylocephalum and that of other larval and adult cestodes

which have been studied with the electron microscope is the appearance

and structure of its tegumentary microvilli. These projections from

the body surface are more or less evenly spaced at a distance of appro-

ximately 100 mμ and are distributed over the entire body surface of

the metacestode except on the exposed surfaces of the anteriorly

situated myzorhynchus. The morphology of the microvillus is different

from previously described ones. Each extends from the body surface as

a curved, slender, tubule of uniform diameter (90 mμ) and considerable

length (approximately 6-8 μ). It is bordered by a unit membrane which is continuous with that covering the intervillar surfaces of the external level of the tegument. This membrane, however, is slightly thicker, being about 100 Å, where it borders microvilli (Fig. 38). The main shaft of each microvillus consists of three layers enclosed by a unit membrane (Figs. 38, 40-42). The first layer, measuring 170 Å thick, is of medium electron density. The second layer, measuring 100 Å thick, is very electron-dense, and the core is electron lucid. These layers are not separated by membranes. All three layers appear to merge at their bases directly with the syncytial cytoplasm of the exterior level of the tegument (Fig. 38). Each microvillus is bent at its free end like the recurved handle of a shepherd's cane (Figs. 40, 42). In addition, the membrane on the inside of the curved "handle" is extended into a bulbous swelling (Figs. 40, 42), the lumen of which is electron transparent. Schematic diagrams of these microvilli and terminal bulbs are presented in Figure 42.

The tegument of Tylocephalum is also beset with hooks. Each hook averages 2.2 μ from the tip of the blade to the tip of the anchor and 1.2 μ from the tip of the anchor to the tip of the guard. The anchor and guard of each are embedded in the syncytial external tegumentary level while the blade or acies protrudes from the body surface (Figs. 38, 39). The blade is still covered by the plasma membrane but the membrane is slightly thicker, being approximately 150 Å thick, where it overlays the blade. Furthermore, a thin homogeneous zone of medium electron density occurs between the membrane and the surface of the hook (Fig. 39). The width of this zone equals that of the overlaying

membrane. The matrices of the anchor and guard are uniformly electron dense while the core of the blade appears to be laminated and of uneven electron density (Fig. 39).

The basal lamina is comparatively electron-lucid and fibrillar in appearance (Figs. 34, 35). In some, this layer is straight (Figs. 32, 33) while in others, the contraction of the underlaying circular muscles causes the basal lamina to be slightly folded so as to give an undulating appearance (Fig. 34). When this occurs, the corresponding body surface of the metacestode is also slightly folded.

A layer of circularly arranged muscles, measuring 0.7 μ in width, abuts the medial surface of the basal lamina and a second stratum of longitudinal muscles, measuring 0.5 μ in width, lies mediad to the circular muscles (Fig. 34). The fine structure of these muscles is similar to those of other cestodes which have been studied (see Lee, 1966 for review).

The so-called internal level of the tegument lies mediad to the subsurfacial muscles. It is comprised of a discontinuous layer of cells each of which averages 7 x 13 μ. In addition to the typical nucleus, the cytoplasm of each cell is tightly packed with large (0.3-0.7 μ) and small (0.1 μ) unit membrane-lined vesicles enclosing a finely granular material of medium electron density (Figs. 31-33, 36). Intermingled with these vesicles are mitochondria as well as rough and smooth endoplasmic reticula (Figs. 31, 36). On rare occasions, a cytoplasmic bridge can be seen joining the internal with the external level of the tegument (Fig. 35).

Mediad to the cells of the internal tegumentary level, and

sometimes interdigitating with these, are a large number of cells which comprise a second type of parenchymal cell. Each of these cells is relatively large, averaging 15 x 10 μ, and includes a prominent nucleus with uniformly distributed chromatin. Embedded in the cyto- plasm are aggregates of glycogen granules and mitochondria (Figs. 31, 32, 36). The profiles of the latter, each averaging 0.6 μ in length, are similar to those found in the vesicle-rich cells comprising the internal tegumentary level. No connection has been observed between this type of cell and the external level of the tegument although occasionally a thin cytoplasmic projection of one of these cells, sandwiched between adjacent vacuole-rich cells, does reach the level of the subsurfacial muscles (Figs. 31, 32, 36).

C. Discussion

Erasmus (1967) has stated the host-parasite interface represents that surface of the helminth parasite which is in intimate contact with the fluids and tissues of the host and through which interchange of materials of physiological and immunological importance takes place. In the case of cestodes, both larval and adult, the lack of a mouth and alimentary tract renders the tegument of the utmost importance from the standpoint of nutrient uptake. Newer attempts to elucidate the actual uptake mechanisms (see Read and Simmons, 1963 for review) have given impetus to studies on the fine structure of the tegument as adjunct information to this aspect of cestode physiology. As a result, beginning with Read (1955), the occurrence of microvilli (the microtriches of some authors) have been demonstrated on the surface of a number of adult cestodes (Kent, 1967; Rothman, 1959, 1960, 1963;

Rosario, 1962; Threadgold, 1962, 1965; Howells, 1965; McCaig and Hopkins, 1965; Lumsden, 1966; Morseth, 1966). Information on microvilli of larvae has been limited to the plerocercoid of Schistocephalus pungitius (see Timofeev, 1964) and the procercoid and plerocercoid of Diphyllobothrium latum (see Bråten, 1968a, b), both pseudophyllideans; the coenurus of Multiceps serialis (see Race et al., 1965), the hydatid cyst and protoscolex of Echinococcus granulosus (see Morseth, 1967), and the cysticercus of Taenia crassiceps (see Baron, 1968), the last three being cyclophyllidean species. Thus, the fine structure of Tylocephalum metacestodes, as reported herein, appears to be of considerable interest not only because the order Lecanicephala, of which Tylocephalum is a member, is generally considered to be one of the more primitive ones but also because of the occurrence of severe reactions in the molluscan hosts which commonly lead to the death and resorption of the parasite (Cheng, 1966; Cheng and Rifkin, 1968).

As in the case with all the cestodes which have been studied with the electron microscope, what has been considered to be a noncellular cuticle is now known to be a cytoplasmic syncytium enclosing organelles such as mitochondria and vesicles. In addition, tegumentary microvilli have been consistently found in all the species studied. Some striking differences, however, exist between the fine structure of the microvilli of Tylocephalum and those of the other species. Rothman (1963) has reported that the tip of each microvillus (he refers to these as microtriches) is electron dense, unmedullated, and highly osmiophilic and he has postulated that it may not serve an absorptive function, but rather a positioning one. The electron dense nature of

each microvillar tip has also been reported by Threadgold (1962, 1965)
in <u>Dipylidium</u> <u>caninum</u> and <u>Proteocephalus</u> <u>pollanicoli</u> adults, by Lumsden
(1966) in <u>Hymenolepis</u> <u>diminuta</u>, <u>Lacistorhynchus</u> <u>tenuis</u>, and <u>Callio-</u>
<u>bothrium</u> <u>verticillatum</u> adults, by McCaig and Hopkins (1965) in <u>Schisto-</u>
<u>cephalus</u> <u>solidus</u> adults, by Morseth (1966, 1967) in <u>Echinococcus</u>
<u>granulosus</u>, <u>Taenia</u> <u>hydatigena</u>, and <u>T.</u> <u>pisiformis</u> adults and on <u>E.</u>
<u>granulosus</u> protoscoleces, by Bråton (1968) in plerocercoids and adults
of <u>Diphyllobothrium</u> <u>latum</u>, and by Baron (1968) in the cysticercus of
<u>Taenia</u> <u>crassiceps</u>. In addition, Threadgold (1962, 1965), McCaig and
Hopkins (1965), Morseth (1966), Baron (1968), and Bråton (1968) have
reported that the electron-dense tip is separated from the proximal
portion of each microvillus by a membranous cap which is not continuous
with the external plasma membrane. In the case of <u>Tylocephalum</u> meta-
cestodes, the electron-dense tip is absent from each microvillus and
instead, the apex bears a spherical vesicle. Somewhat similar
structures have been found by Bråton (1968) in <u>D.</u> <u>latum</u> procercoids
but the microvilli of these larvae are club-shaped, much shorter, and
electron-dense.

The function(s) of the microvilli and their terminal vesicles in
<u>Tylocephalum</u> remains unknown. The fact that many of the fibrillar units
comprising the encapsulating cyst of host origin are adhered to the
microvillar surfaces may be interpreted as a mechanism to prevent in-
timate encapsulation by these fibrous elements. The probable absorptive
and secretory functions of the tegumentary microvilli of cestodes have
been repeatedly suggested (Lee, 1966; Bråton, 1968). The terminal
vesicles of the microvilli of <u>Tylocephalum</u> metacestodes may serve in

such a capacity. The main stalk of each microvillus may be concerned with absorption or secretion although its complex structure, involving three discrete layers, may possibly limit the movement of macromolecules across its wall. The fine structure of the microvillar stalks of Tylocephalum appears to be comparable to that of D. caninum (Lumsden, 1966) and S. solidus (Morseth, 1966). Their relative position and their shape suggest a continuous undulating motion. This motion could possibly act as an additional physical defense mechanism to prevent the accumulation of host-secreted cystogenous fibers from making contact with the parasite proper.

The finding of a unit membrane which borders the microvilli and is continuous with that bordering the external level of the tegument is consistent with all earlier reports. Furthermore, the finding that this membrane is thicker (100 Å) where it covers the microvilli confirms the earlier observations by Rosario (1962) and Br⅄ton (1968).

The positions of the microvilli, as revealed in sections, strongly suggest that adjacent microvilli are commonly intertwined. This phenomenon is likely to have resulted from their undulating movements. Definite strands of fibrous material have been observed in light microscope preparations of Tylocephalum metacestodes in situ. As no projections, other than microvilli, arise from the surface of these parasites, it must be concluded that the strands as seen with the light microscope represent bundles of intertwined microvilli.

The contraction of the body musculature, specifically the circular muscles, resulting in the formation of undulations by the basal lamina and body surface is of interest. Rietschel (1935) has postulated that

"fibrils" on the body surfaces of adult cestodes may serve to agitate the microhabitat. Similarly, Rothman (1963) is of the opinion that microvilli on the body surfaces of Hymenolepis diminuta adults can serve the same function and as the result, the host's intestinal fluids in the immediate vicinity, including nutrients and metabolic wastes, are kept in a state of flux. It is conceivable that the contraction and relaxation of the body surfaces of Tylocephalum metacestodes in oysters could cause the microvilli to agitate the constituents of the parasites' microhabitat and thus churn up the hemolymph and other constituents. This would keep these materials, including nutrients, in a state of flux, thus resulting in more uniform and efficient uptake by the parasites.

The report of the fine structure of the tegumentary hooks as presented herein is, to my knowledge, the first account of this type in cestodes. The fact that each hook is covered by the plasma membrane attests to its intracellular nature as has been proposed by Ogren (1958) as the result of light microscope studies on cestode embryology.

A comparison of our electronmicrographs with those of earlier investigators has revealed that there are considerably more vesicles in the tegument of Tylocephalum metacestodes, with the possible exception of the external level of the tegument of Lacistorhynchus tenuis (see Lumsden, 1966). L. tenuis is a member of the order Trypanorhyncha the adult members of which, as in the case of the Lecanicephala, are restricted to selachians. Thus, the presence of large numbers of tegumentary vesicles would appear to be characteristic of what are generally considered to be the more primitive orders of marine cestodes

(Wardle and McLeod, 1952). The function of these vesicles, at least those present in the external level, has not been unequivocally demonstrated, although Threadgold (1965) considers some to be of pinocytotic origin. It is of interest to note, however, that the cytoplasmic vesicles packed in the cells comprising the external level are morphologically identical with those embedded in the internal level, where they occur in large numbers. Possibly, therefore, some of these vesicles originate from the internal level and may have been transferred to the exterior level via the cytoplasmic bridges connecting the two levels.

The second type of subsurfacial cell described is believed to be a somatic cell which constitutes the basic cellular constitutent of the parenchyma (Cheng, 1966).

Figure 31

Semidiagrammatic drawing showing constituents of the tegument

of _Tylocephalum_ metacestode and associated structures.

Figure 31

Semidiagrammatic drawing showing constituents of the tegument

of Tylocephalum metacestode and associated structures.
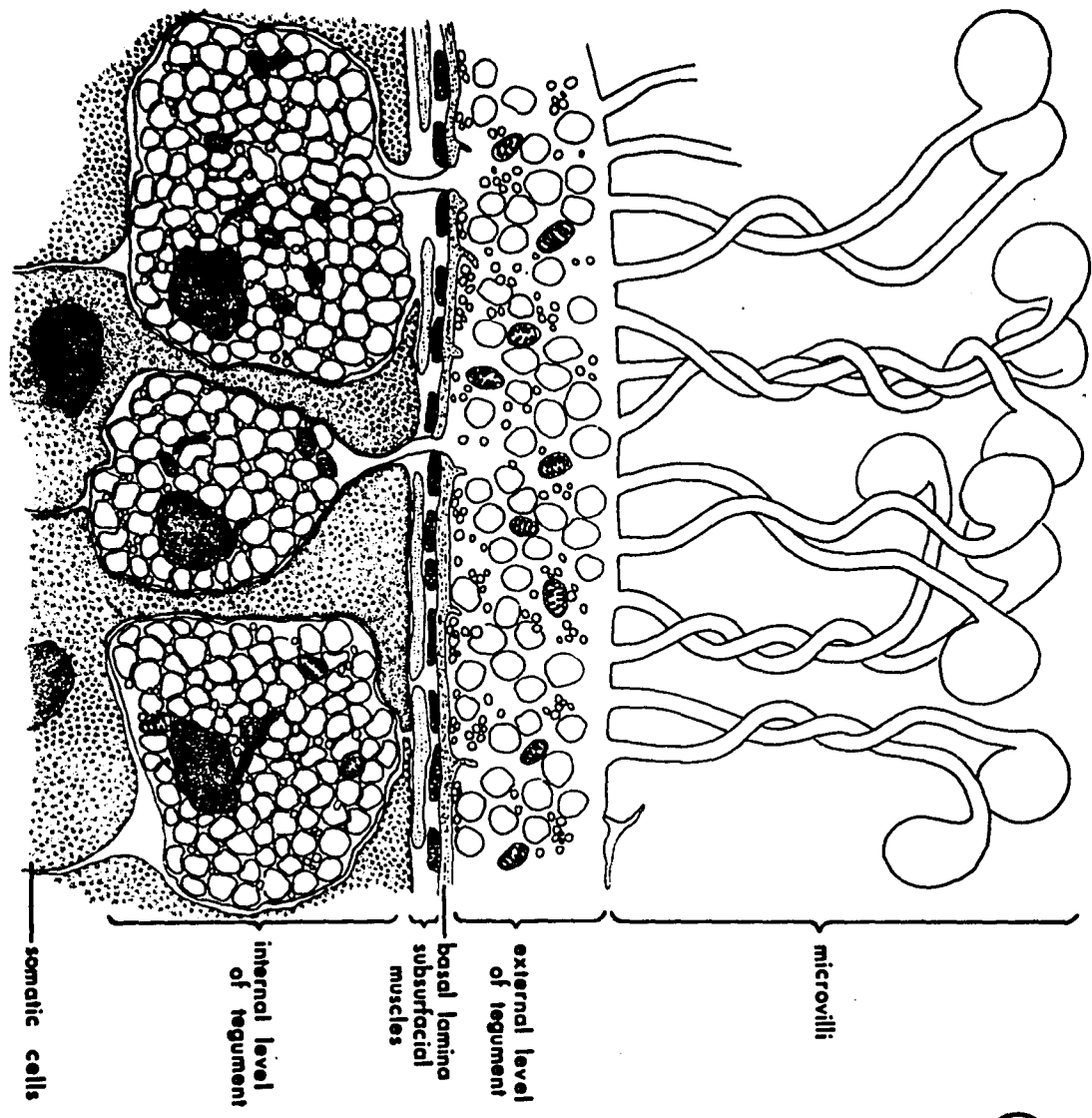
somatic cells

internal level of tegument

basal lamina subsurfacial muscles

external level of tegument

microvilli

(31)

Figure 32

Electronmicrograph showing portion of tegument of Tylocephalum metacestode. Notice nonconstricted basal lamina. (x 12,800).

Figure 33

Electronmicrograph showing portion of tegument of Tylocephalum metacestode. Notice noncontracted basal lamina. (x 8,000).

BL = basal lamina, CM = circular muscles, G = glycogen granules, LM = longitudinal muscles, LV = large vesicle, MI = mitochondrion, NS = nucleus of somatic cell, NV = nucleus of vesicle-containing cell, SV = small vesicle.
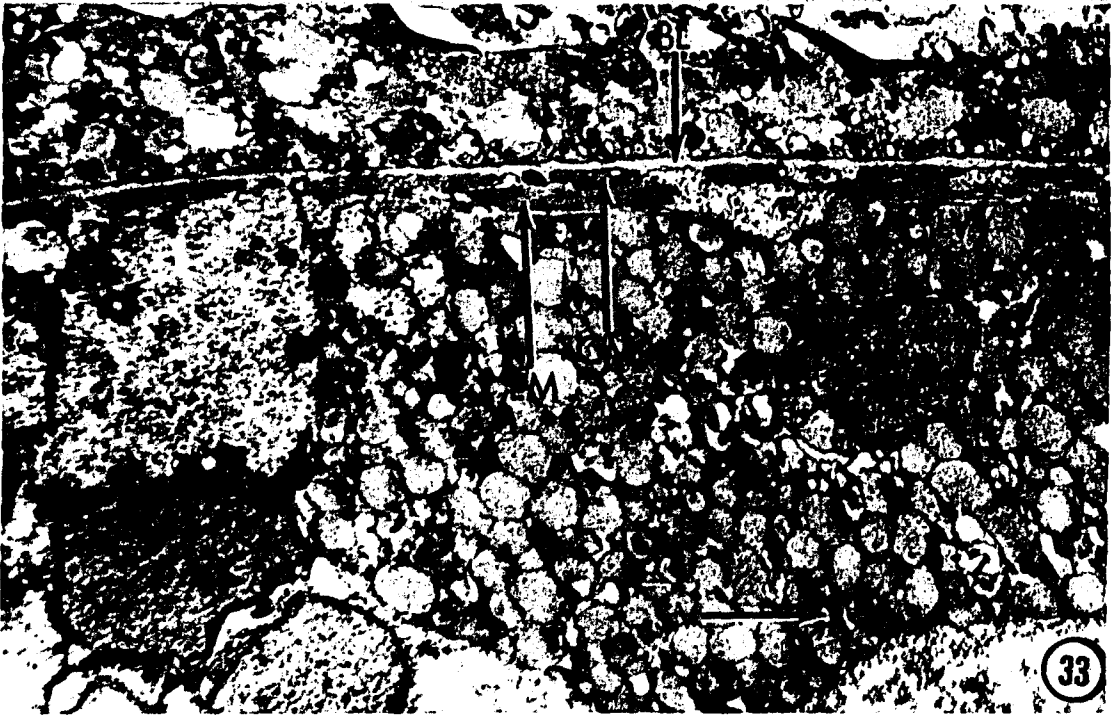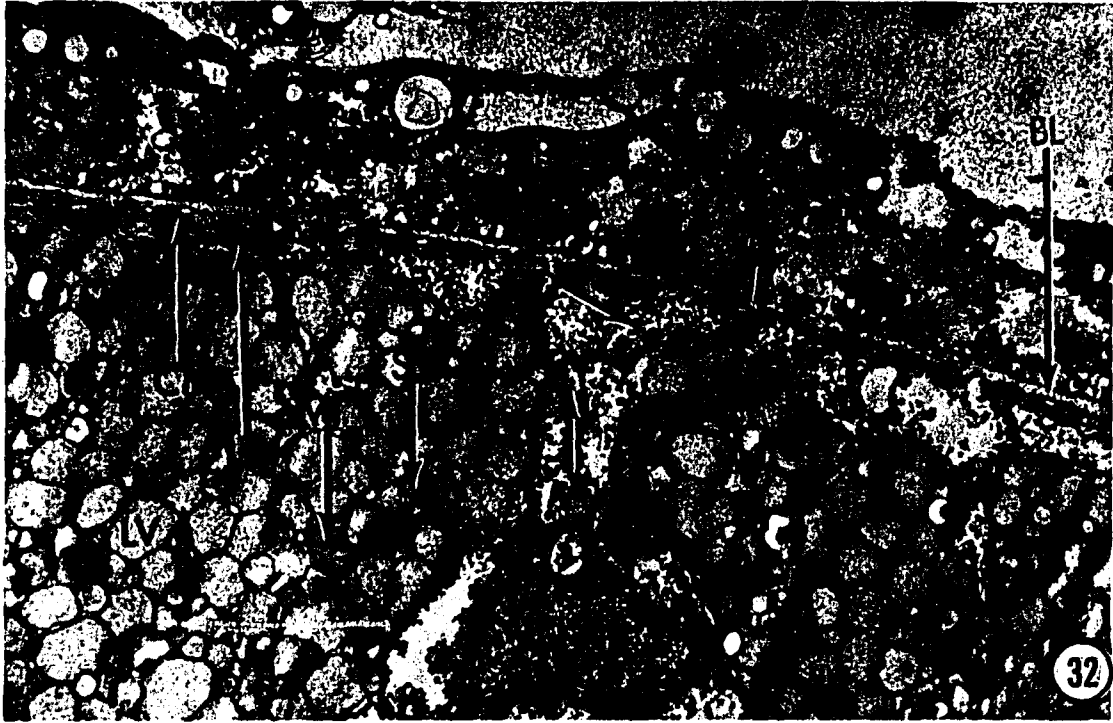
Figure 34

Electronmicrograph of portions of external level of tegument
of Tylocephalum metacestode showing folded basal lamina and
body surface. (x 15,400).

Figure 35

Electronmicrograph of portions of external and internal levels
of tegument of Tylocephalum metacestode showing interconnecting
cytoplasmic bridge and projections of basal lamina into ex-
ternal level. (x 24,500).

BL = basal lamina, CB = cytoplasmic bridge, CM = circular
muscles, EB = extensions of basal lamina, LM = longitudinal
muscles, LV = large vesicle, MI = mitochondrion, SV = small
vesicle.
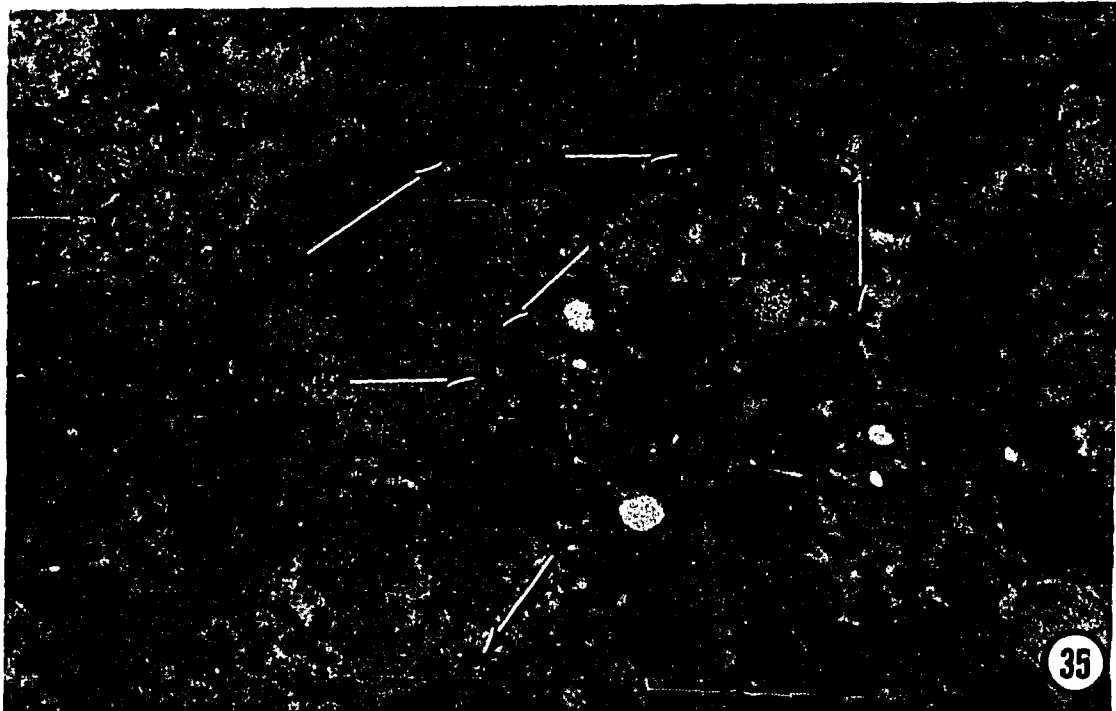
Figure 36

Electronmicrograph of portions of external and internal levels

of tegument of Tylocephalum metacestode showing large and

small vesicles in both levels and endoplasmic reticulum in

cell of internal level. (x 15,000).

Figure 37

Electronmicrograph of large vesicles found in internal level

of tegument of Tylocephalum metacestode. (x 95,000).

ER = endoplasmic reticulum, G = glycogen granules, LV = large

vesicle, MI = mitochondrion, SV = small vesicle.
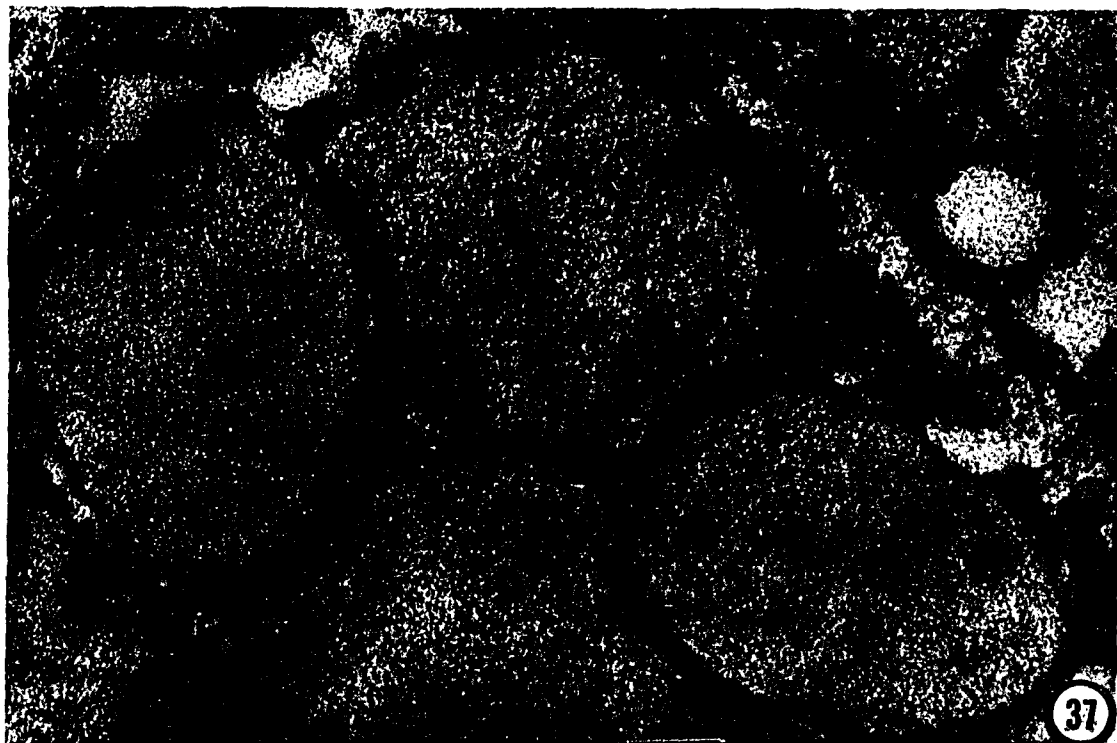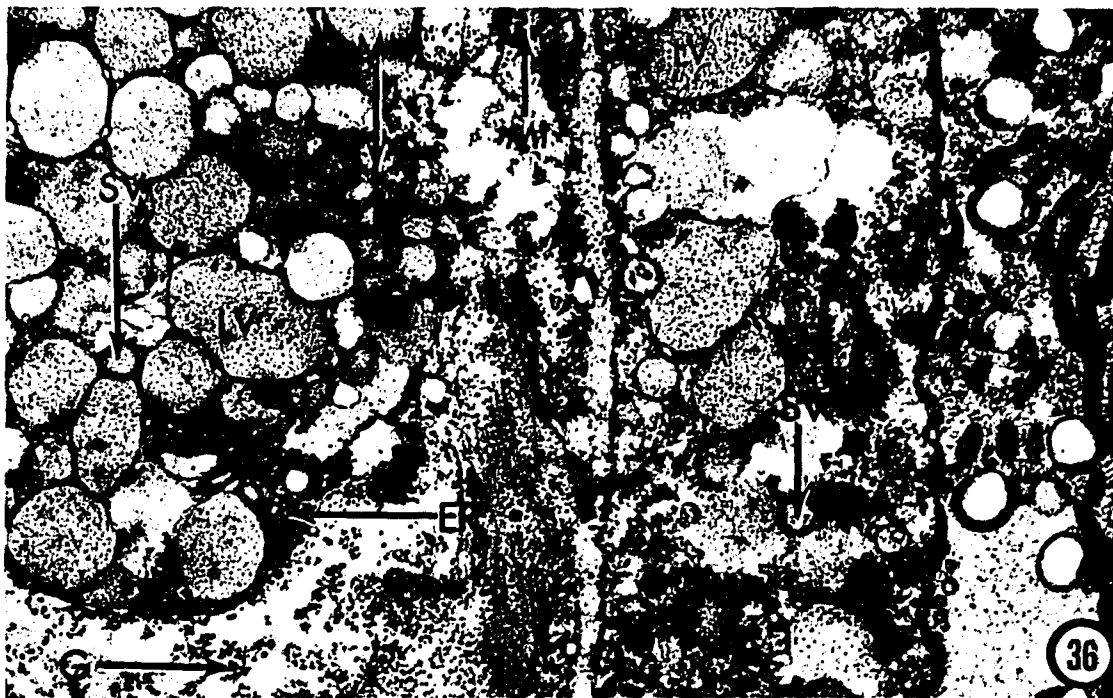
Figure 38

Electronmicrograph showing portion of outer border of external

level of tegument of Tylocephalum metacestode. Notice continuous

membrane covering microvilli and body surface. (x 62,350).

Figure 39

Electronmicrograph showing tegumentary hook of Tylocephalum

metacestode. (x 50,350).

AH = anchor of tegumentary hook, BH = blade of tegumentary

hook, GH = guard of tegumentary hook, HZ = homogeneous zone

between membrane and hook surface, IL = inner (core) layer

of microvillus, M = unit membrane, MH = membrane overlaying

hook, ML = middle layer of microvillus, MMV = unit membrane

overlaying microvillus, OL = outer layer of microvillus.
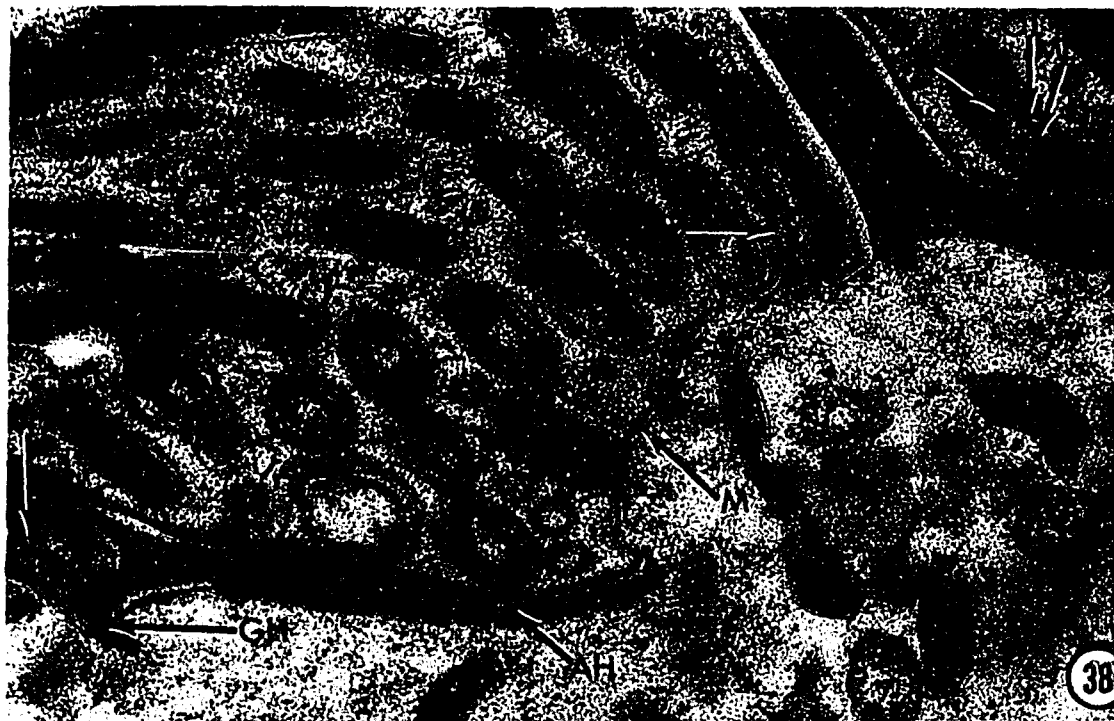
Figure 40

Electronmicrograph showing longitudinal sections of stalks
of tegumentary microvilli and longitudinal- and cross-sections
of terminal vesicles. (x 36,575).

Figure 41

Electronmicrographs showing cross-sections of tegumentary
microvilli of Tylocephalum metacestode. Notice presence of
relatively thick membrane covering each microvillus and the
three characteristic strata. (x 102,000).

IL = inner (core) layer of microvillus, ML = middle layer of
microvillus, MMV = unit membrane overlaying microvillus,
OL = outer layer of microvillus, TV = terminal vesicle of
microvillus.
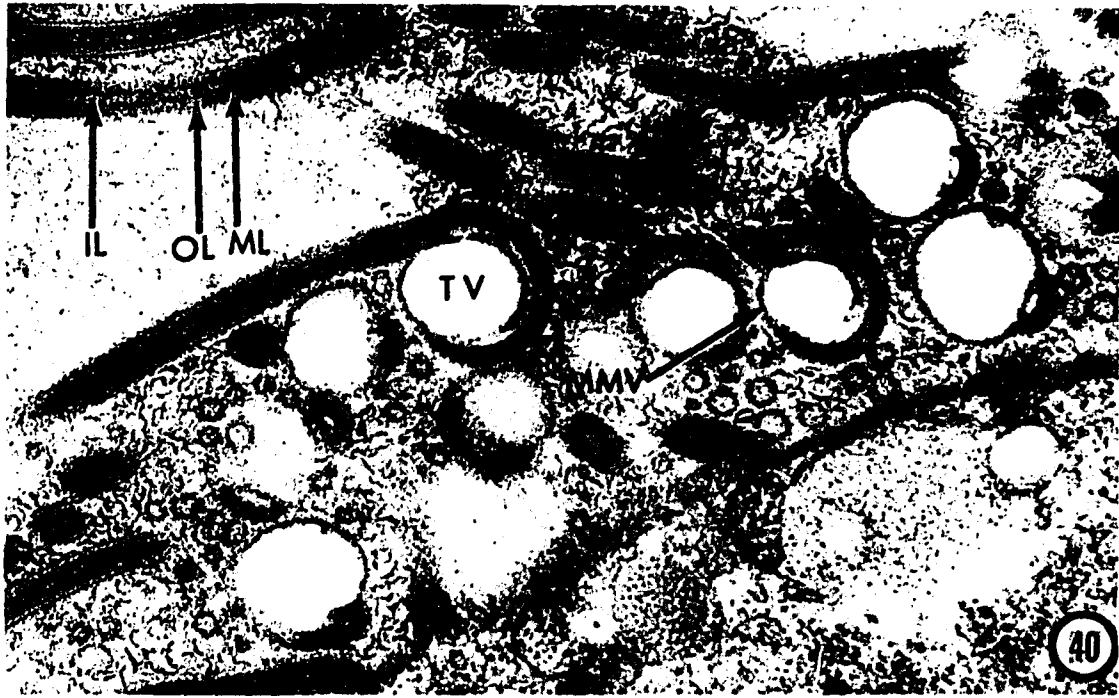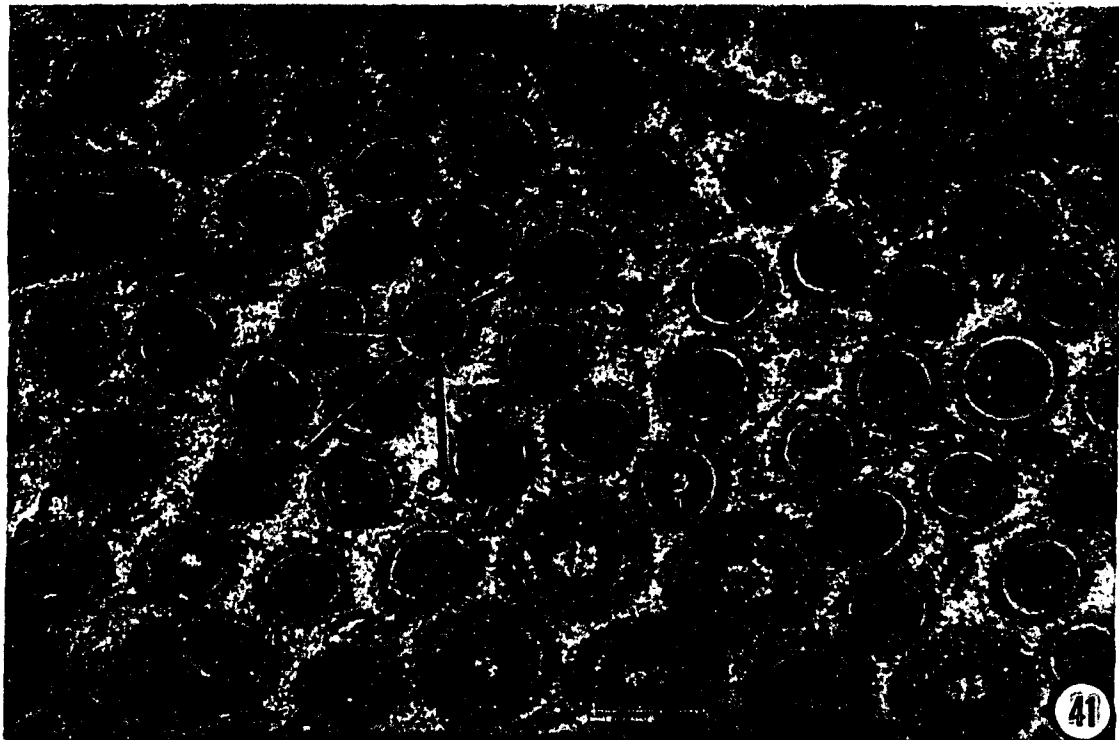
Figure 42

Schematic drawings showing ultrastructure of tegumentary

microvillus of Tylocephalum metacestode.

A. Longitudinal section of portion of microvillus showing
the overlaying unit membrane and three layers.

B. Longitudinal section of terminal vesicle showing con-
stituent parts.

C. Cross-section of terminal vesicle at level indicated
by dotted line in B showing constituent parts.

IL = inner (core) layer of microvillus, ML = middle layer

of microvillus, MMV = unit membrane overlaying microvillus,

OL = outer layer of microvillus, TV = terminal vesicle.

A

MMV
OL
ML
IL

B

TV

C

TV

42
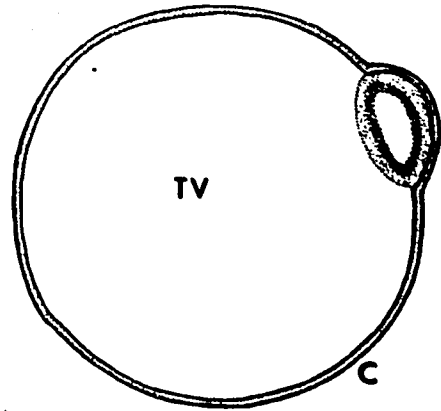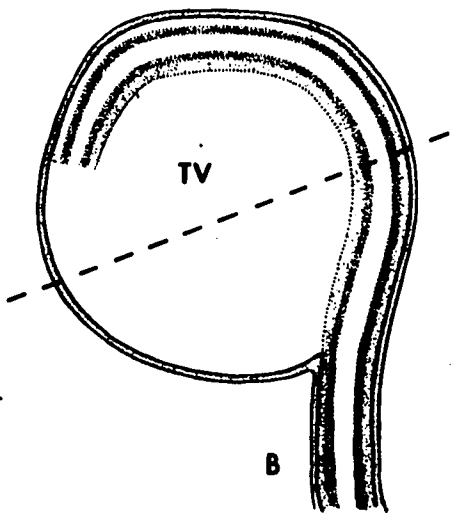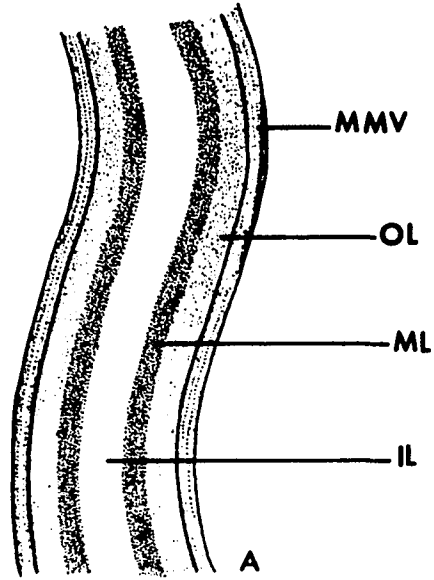
CHAPTER VI

AN ELECTROPHORETIC ANALYSIS OF HEMOLYMPH
PROTEINS OF CRASSOSTREA VIRGINICA PARASITIZED BY
TYLOCEPHALUM SP. LARVAE

A. Introduction

The fourth phase of this study involved the determination of

differences in the total hemolymph protein concentrations as well as

changes in the hemolymph protein fractions of parasitized oysters.

In addition, the influence of the Pearl Harbor environment on the

total protein concentration of oysters was also determined.

Since quantitative electrophoresis has been employed to determine

changes in the serum protein fractions of parasitized vertebrates

(Stauber, 1954; Kabat and Mayer, 1961; and many others), this technique

was applied to an invertebrate system, in this case, to Crassostrea

virginica, in an attempt to find comparable changes and to correlate

these, if feasible, with the presence of Tylocephalum. Because of the

open vascular system of marine molluscs which enables the mixing of

sea water with hemolymph in the mantle cavity and sinuses resulting

in dilutions, the application of paper, starch-gel, and cellulose ace-

tate electrophoresis was impractical. However, it was determined that

satisfactory separations of hemolymph protein fractions could be ob-

tained by the more sensitive polyacrylamide-gel disc electrophoresis

system.

B. Materials and Methods

The 25 parasitized oysters, C. virginica, used during this study

were taken from West Loch, Pearl Harbor. The uninfected oysters used

as controls and for determining the influence of the environment on

total protein concentrations were collected from Tomales Bay, San Francisco, California. These were sent via air freight to Honolulu in the frozen state.

Fifteen hemolymph samples from oysters forming the control group were obtained, by the method described below, shortly upon arrival from California.

In order to assess the influence of environmental conditions prevailing in Pearl Harbor, 50 of the California oysters were placed in a rectangular steel cage which was securely fastened to the bay bottom. These were sampled bimonthly over a period of six months; specifically, at 8, 16, and 24 weeks. At each of these periods, hemolymph samples were collected from the retrieved specimens by the method given below and each oyster was checked for the presence of Tylocephalum and other endoparasites.

Hemolymph samples used for analysis were obtained directly from the hearts of oysters in the following manner. The left valve of the shell was carefully removed by use of a short bladed "shucking" knife. It is on this side that the pericardial wall lies directly under the shell. On the right side, the promyal chamber extends over the heart and the mantle separates the pericardium wall from the shell. Sterile forceps were used to remove the pericardium, thereby exposing the heart. A 27 gauge hypodermic needle was inserted directly into the heart and approximately 0.3 ml of hemolymph was removed. The cellular constituents were removed by centrifugation, and the hemolymph was stored in the frozen state in individual vials. The quantitative total protein determinations, however, were carried out before freezing.

As stated, the cellular constituents of the hemolymph were re-
moved by centrifugation prior to the determination of both total pro-
tein concentrations and electrophoretic separations. This was
accomplished by placing each hemolymph sample in a capillary tube
(75 mm long; I.D. 1.1-1.2 mm; wall 0.2-0.02 mm thick) and centrifuged
at 11,500 r.p.m. for 10 minutes in an International Micro-Capillary
Centrifuge, Model MB.

Total Protein Concentrations

The total protein concentration of each hemolymph sample was
determined by use of the Goldberg refractometer (AO Model 10401).
This refractometer actually measures the refractive indices which
were converted to protein concentrations and recorded as grams of
protein per 100 ml of hemolymph. Because of the greater ionic con-
centrations in sea water, which, as indicated, is found in the hemo-
lymph of marine molluscs, refractometric determinations of protein
concentrations of marine molluscan hemolymph were determined to be
consistently higher than the true values. Consequently, a correction
factor had to be employed. This factor was determined by ascertaining
the total protein concentrations of selected hemolymph samples by use
of the Folin test (Lowry, et al., 1951) and compared with the values
obtained by refractometry. It was thus determined that the true total
protein concentration of oyster hemolymph was 1/6 of that determined
by refractometry, hence the correction factor was x6 (Fig. 43).

Electrophoretic Studies

The hemolymph samples were fractionated electrophoretically by

the polyacrylamide-gel disc electrophoresis system (Canalco Model 24).

A 0.05 ml sample of hemolymph was used in each instance. Each run

was carried out at 5 milliamperes supplied by a Canalco Model 300A

power source until the bromphenol blue tracking dye band reached the

surface of the lower buffer bath which usually occurred in about 35

minutes. The buffer was composed of 2-amino-2-hydroxymethel-1, 3-

propanediol (Tris), 14.4 gm of glycine, and sufficient distilled water

to make 1 liter. The pH was 8.5. The columns were stained with

aniline blue-black and destained with 7% acetic acid at 12.5 milli-

amperes for 45 minutes.

After destaining, each column was stored in 7% acetic acid and

subsequently quantified by use of a recording densitometer (Photovolt-

Densicord) coupled to an electric integrator (Photovolt). The res-

ponse selector switch of the densitometer was set at the 8 position.

The decade marker of the integrator permitted partial quantification

of the areas occupied by each protein fraction as seen in profile.

From these data, the relative proportion of each fraction of the total

protein concentration of the sample was determined.

In addition to oyster hemolymph, several categories of mammalian

serum proteins for which electrophoretic migration patterns are known

were similarly subjected to electrophoretic analysis. These included

2.5% and 5% mammalian saline solutions of bovine gamma globulin and

bovine albumin and undiluted as well as a 5% saline solution of calf

serum.

C. Results

Careful examination of the California oysters transplanted in

Pearl Harbor revealed the absence of parasites. All of the native
Pearl Harbor oysters employed in this study were heavily infected with
Tylocephalum metacestodes.

Total Protein Concentrations

The total protein concentrations of California oysters examined
shortly after arrival and native Hawaiian oysters parasitized with
Tylocephalum were ascertained to be significantly different. The
transplanted California oysters revealed a decrease in their hemolymph
total protein concentrations over an extended period of time (see
Table 1, Fig. 44) but the level was never as low as that of parasitized
oysters indiginous to Pearl Harbor. In the latter, the lowest mean
total protein concentration was 0.21 gm/100 ml. This was less than
half the mean total protein concentration of fresh California oysters.
The latter group revealed the highest total protein concentration.

Electrophoresis

Each of the protein fractions separated by polyacrylamide-gel
disc electrophoresis (Fig. 45) was expressed as a percentage of the
total protein concentration because of the variation occurring even
among oysters of the same group (Table 2).

Electrophoretic separations revealed the presence of 10 discern-
able protein fractions in the hemolymph of most of the oysters (Figs.
46-50). These have been designated as fractions 1 through 10, with
fraction 1 being the one with the least mobility. It should be noted,
however, that not all 10 fractions were apparent in every sample. For
example, among members of the control group, fractions 8, 9, and 10

tended to merge together (Fig. 46). The only explanation possible at this time is that this merging of fractions 8 through 10 was due to the greater protein concentrations in the hemolymph samples from the members of this group.

Quantitative analyses of the various protein fractions (Figs. 51-56) revealed that there was no significant increase in any fraction in parasitized Hawaiian oysters and in transplanted California oysters. On the other hand, comparison of the quantitative data revealed that fractions 2, 3, and 4 of both the parasitized group and transplanted California group were significantly decreased (Figs. 51-53). Moreover, fraction 1 was only present in the control group. Thus, in both the parasitized and transplanted groups there was a decrease in four hemolymph protein fractions, namely, 1, 2, 3, and 4.

D. Discussion

The reductions of hemolymph protein concentrations in the transplanted California group of oysters can most probably be attributed to the environmental conditions prevailing at West Loch, Pearl Harbor, and not to the presence of parasites. The validity of this conclusion is supported by the fact that not a single transplanted oyster was found to be parasitized. As to whether the lower total protein concentrations in the parasitized Hawaiian oysters is totally due to environmental stress remains a moot point since the possibility exists that the extremely low total protein concentrations found among members of this group may be caused, at least in part, by the presence of _Tylocephalum_ metacestodes.

The reductions in the total hemolymph protein concentrations in

transplanted California oysters and in parasitized Hawaiian oysters can be attributed to decreases in fractions 2, 3, and 4, and the total absence of fraction 1.

Relative to hypoproteinemia resulting from helminth parasitism, Cheng (1963) has demonstrated a decrease in the hemolymph proteins of the gastropods Physa gyrina, Helisoma trivolvis, and the pelecypod Musculium partumeium when parasitized by sporocysts of Glypthelmins quieta, the rediae of Echinoparyphium, and the sporocysts of Gorgodera, respectively. Similarly, Gilbertson et al. (1967) had shown that the total hemolymph proteins were significantly decreased in the snail Biomphalaria glabrata when parasitized by larval Schistosoma mansoni. Since hypoproteinemia is known to occur in parasitized molluscs, it should be restated that the extreme reduction of total protein concentrations in Hawaiian oysters parasitized by Tylocephalum may be due in part to their parasitized state.

TABLE 1

Mean Total Protein Concentrations of Oyster Hemolymph
Samples Determined in the Five Groups at the Specified
Time Intervals

	0 wk. (control)	8 wk.	16 wk.	24 wk.	32 wk. +
protein concen- tration gm/100 ml	0.54 (0.09)	0.47 (0.07)	0.42 (0.05)	0.37 (.05)	0.21 (.06)

*S.D. in (   )

TABLE 2

Mean Percentages of Total Protein Concentrations in Oyster Hemolymph
Fractions of Five Groups Determined at the Specified
Time Intervals

Fraction Number	(control) 0 wk.	8 wk.	16 wk.	24 wk.	32 wk. +
1	6.31(N.D.)	N.P.	N.P.	N.P.	N.P.
2	7.10(0.09)	0.86(0.08)	0.80(0.19)	1.55(0.12)	1.12(0.15)
3	8.34(0.51)	2.30(0.38)	1.20(0.21)	3.35(0.23)	2.63(0.43)
4	5.86(0.49)	2.82(0.17)	1.94(0.37)	3.60(0.45)	3.18(0.51)
5	4.60(0.96)	2.16(0.38)	6.75(1.05)	N.P.	4.17(0.60)
6	3.55(0.54)	5.82(1.29)	1.91(0.37)	1.51(0.65)	N.P.
7	3.55(0.77)	4.56(1.13)	1.96(0.37)	N.P.	N.P.
8	N.D.	N.D.	N.D.	13.56(1.62)	18.02(2.54)
9	N.D.	N.D.	N.D.	32.08(1.89)	25.82(2.48)
10	N.D.	N.D.	N.D.	14.23(1.94)	8.41(3.65)

N.P. = not present
N.D. = not determined
*S.E. in (   )

Figure 43

Graph showing the relationship between the refractive

index (R.I.) as determined by the Goldberg Refractometer

and the actual protein concentration as determined by

the Folin test. The vertical scale on the left indicates

the optical density (O.D.) as read on a Baush and Lomb

Spectronic 20 Colorimeter.
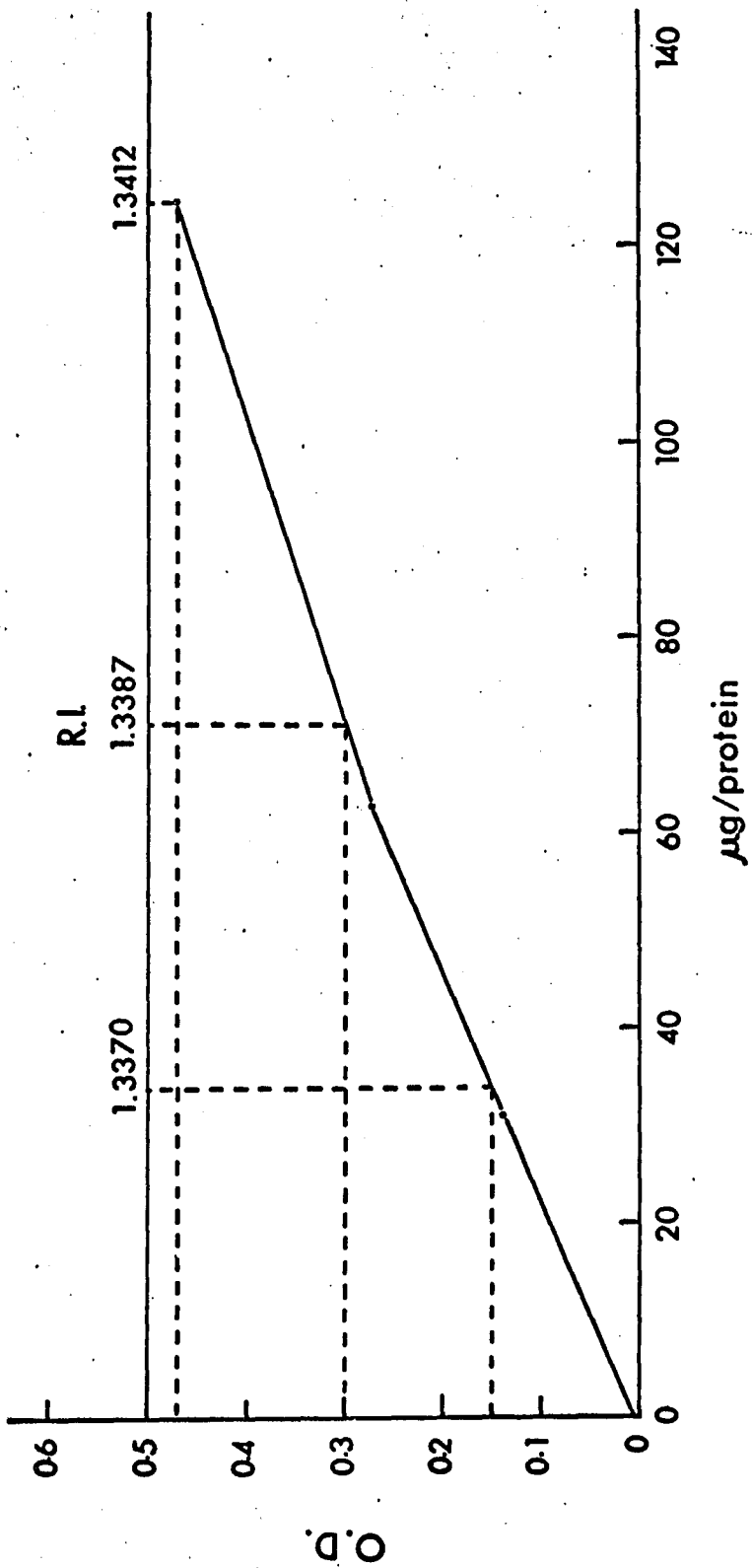
Figure 44

Graph showing a significant regression (linear regression
test; b=-.3314*) in the total protein concentration over
the time intervals specified. Individual oyster samples
are represented by the dots.
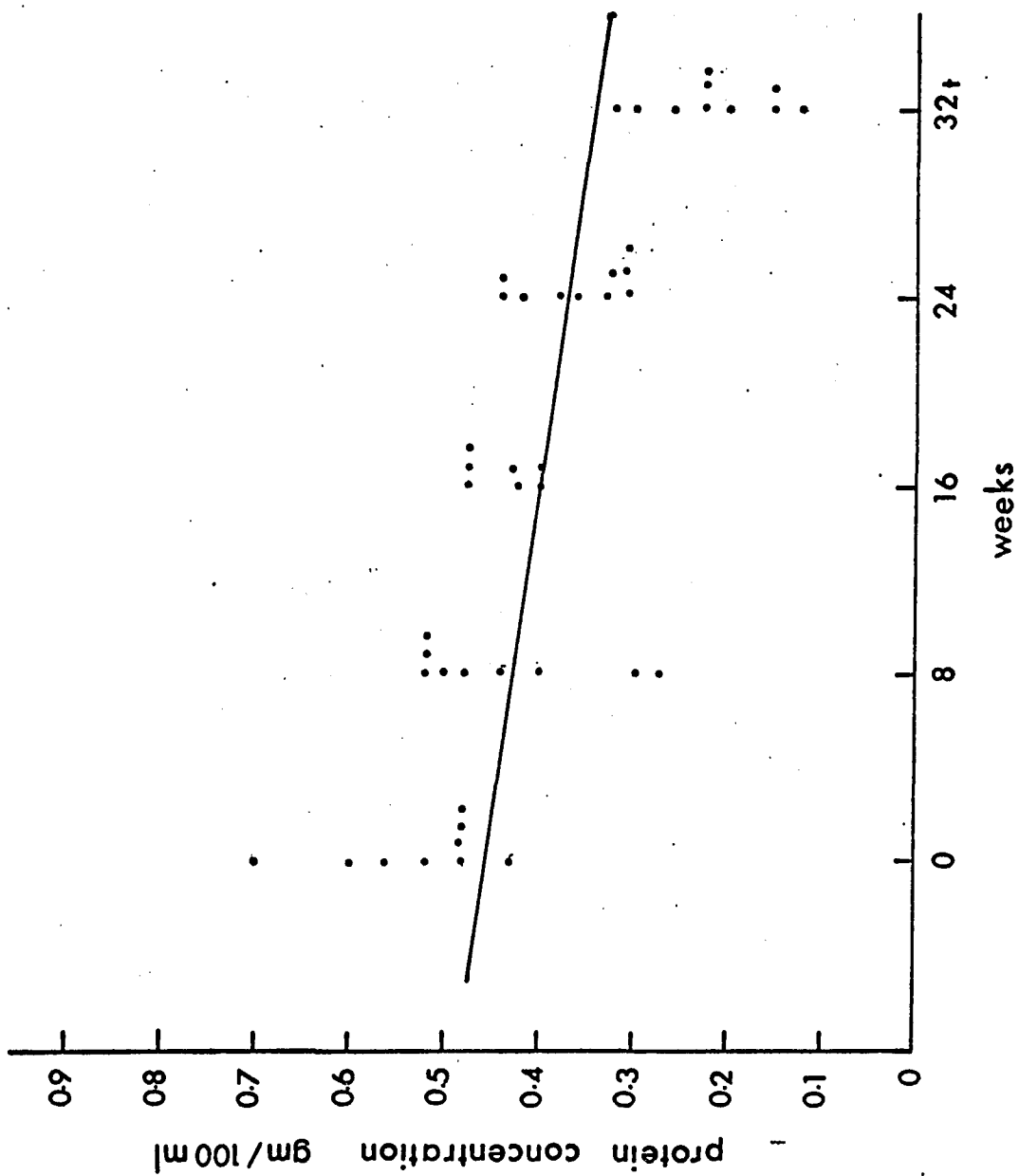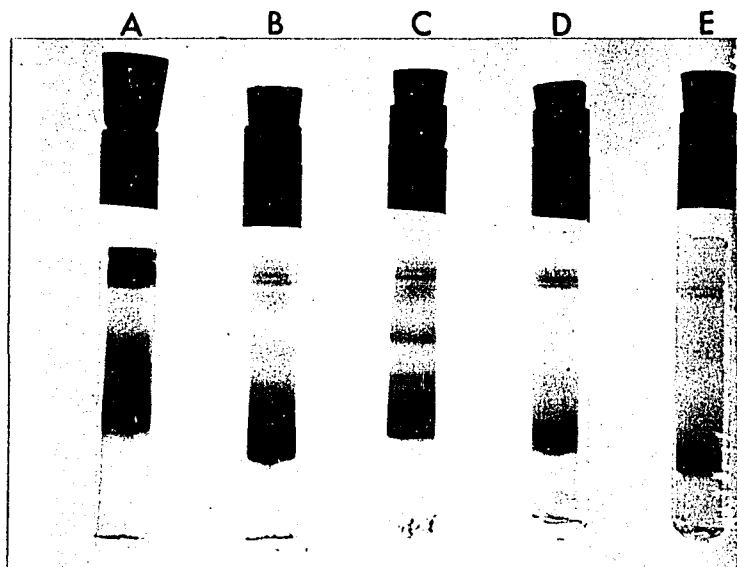
Figure 45

Photograph of polyacrilamide gel disc electrophoresis columns

showing a representative hemolymph sample of each group.

A. Hemolymph from a Tomale Bay import before exposure to
   the environment at West Loch, Pearl Harbor.

B. Hemolymph from a representative of the experimental
   group after 8 weeks in West Loch, Pearl Harbor.

C. Hemolymph from a representative of the experimental
   group after 16 weeks in West Loch, Pearl Harbor.

D. Hemolymph from a representative of the experimental
   group after 24 weeks in West Loch, Pearl Harbor.

E. Hemolymph from a representative of the parasitized
   group indiginous to West Loch, Pearl Harbor.

Figure 46

A typical electrophoretic profile of oyster hemolymph
from the Tomales Bay imports before exposure to the
environment at West Loch, Pearl Harbor. This is a
representative sample of the control group. Fraction
1 represents that fraction with the least mobility.
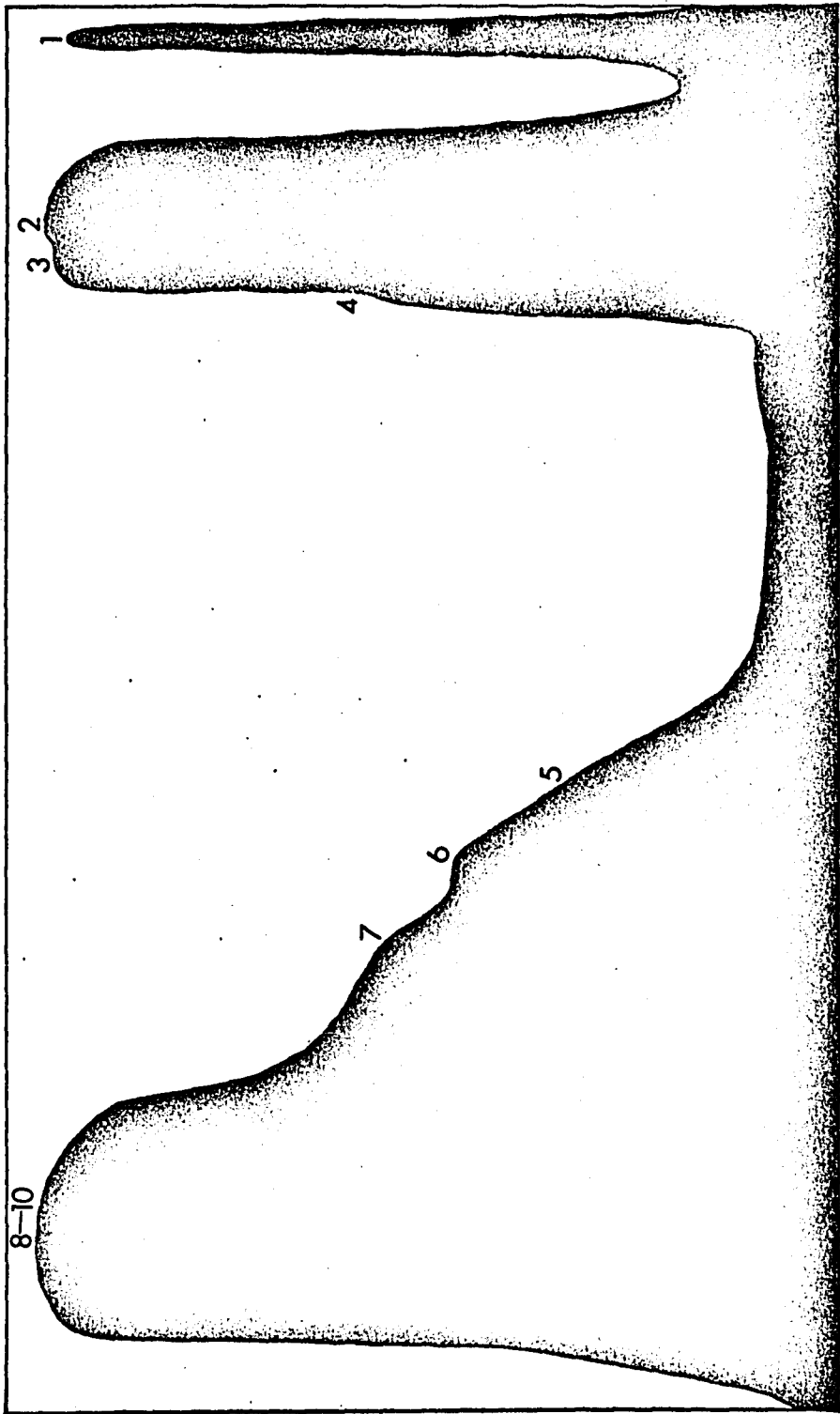
Figure 47

A typical electrophoretic profile of oyster hemolymph

from an experimental group after 8 weeks in West Loch,

Pearl Harbor. Fraction 1 represents that fraction

with the least mobility.
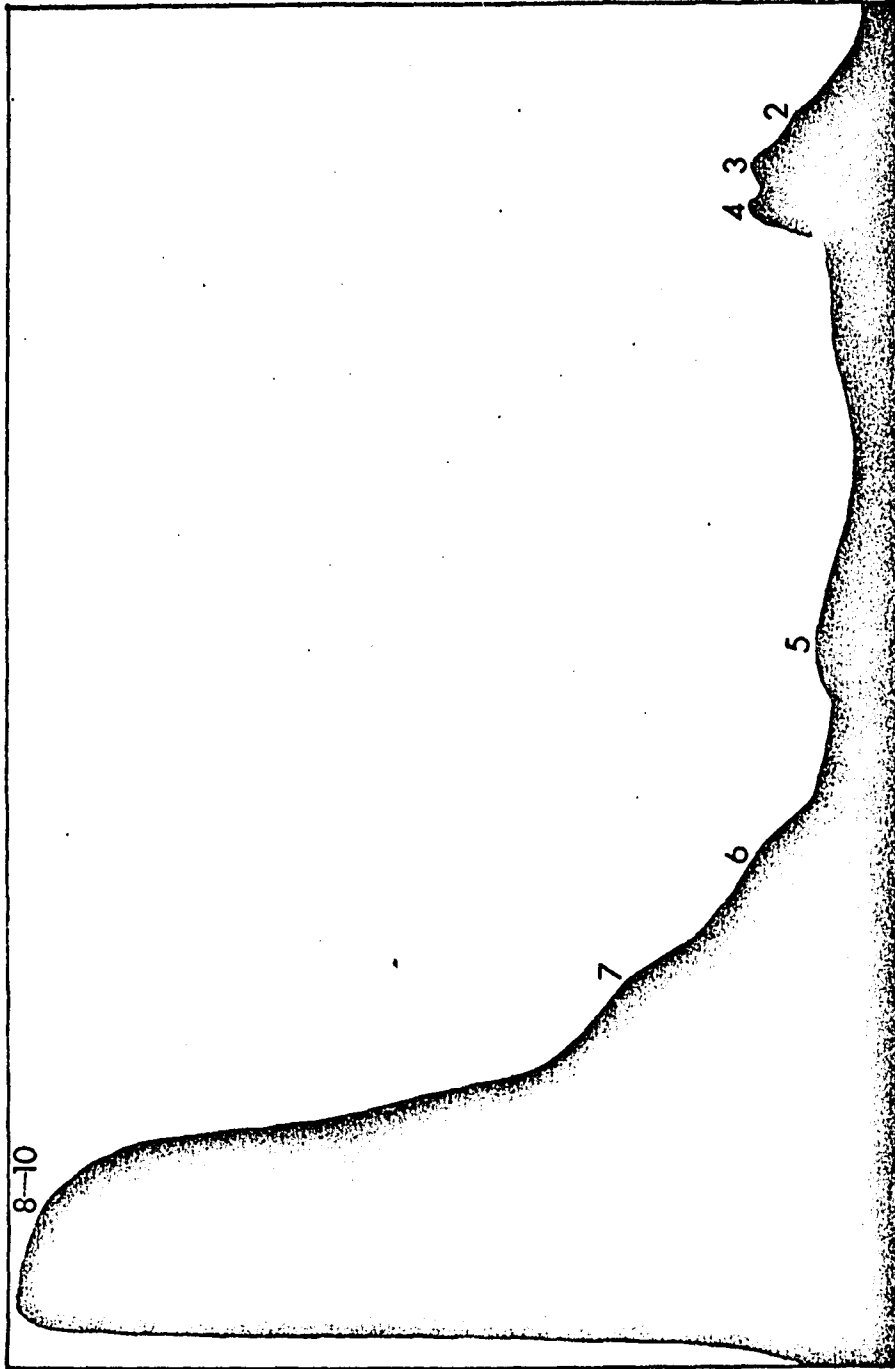
Figure 48

A typical electrophoretic profile of oyster hemolymph

from an experimental group after 16 weeks in West Loch,

Pearl Harbor. Fraction 1 represents that fraction

with the least mobility.
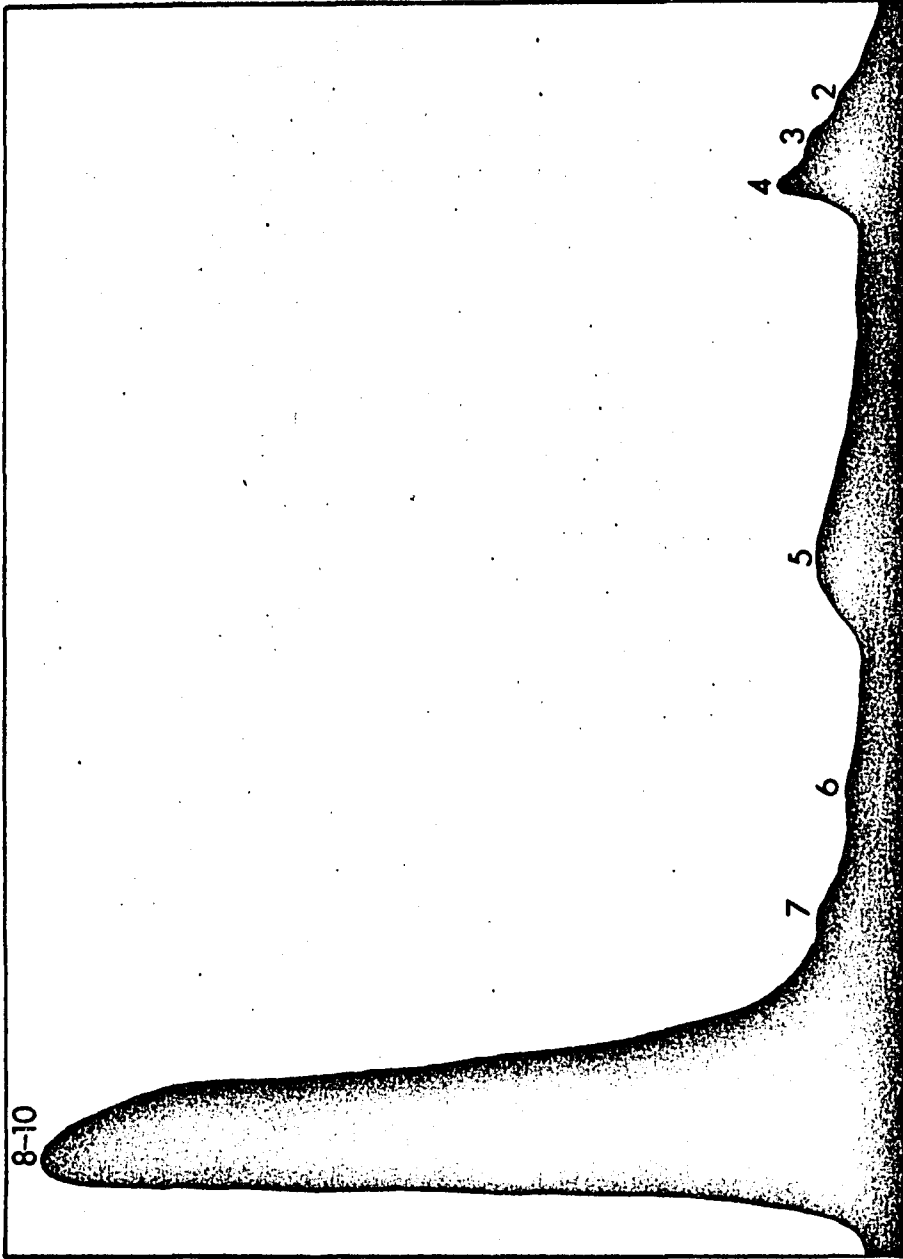
Figure 49

A typical electrophoretic profile of oyster hemolymph

from an experimental group after 24 weeks in West Loch,

Pearl Harbor. Fraction 1 represents that fraction

with the least mobility.
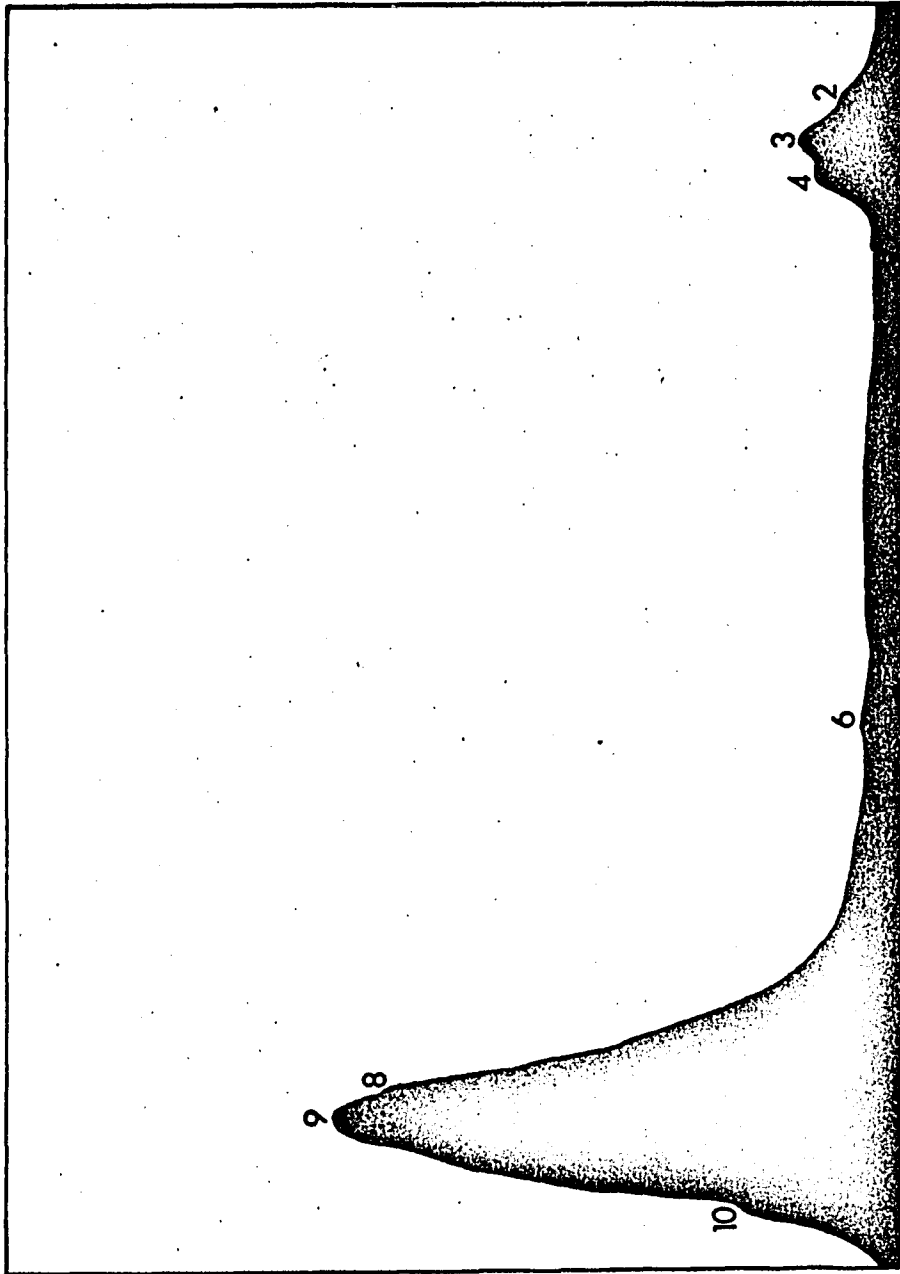
Figure 50

A typical electrophoretic profile of oyster hemolymph

from the parasitized group indiginous to West Loch,

Pearl Harbor. Fraction 1 represents that fraction

with the least mobility.
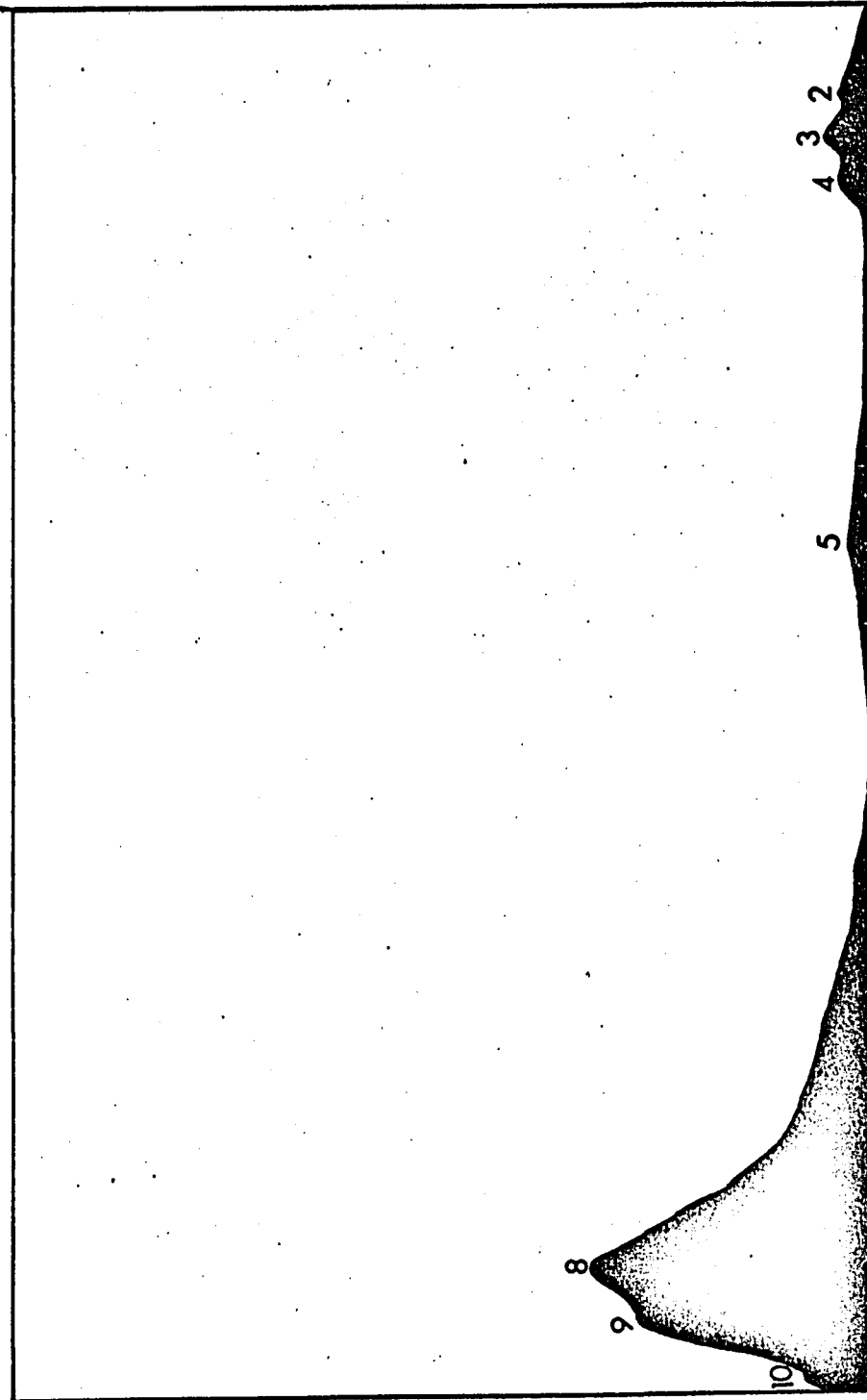
Figure 51

Graph showing percentages of the total protein concentration
of fraction 2 at the time intervals specified. The shaded
area represents the 95% confidence limits of the control.
The 95% confidence limits of the experimental group are
represented by the extended vertical lines. The mean total
protein concentration is indicated by the horizontal lines.

% protein concentration

weeks
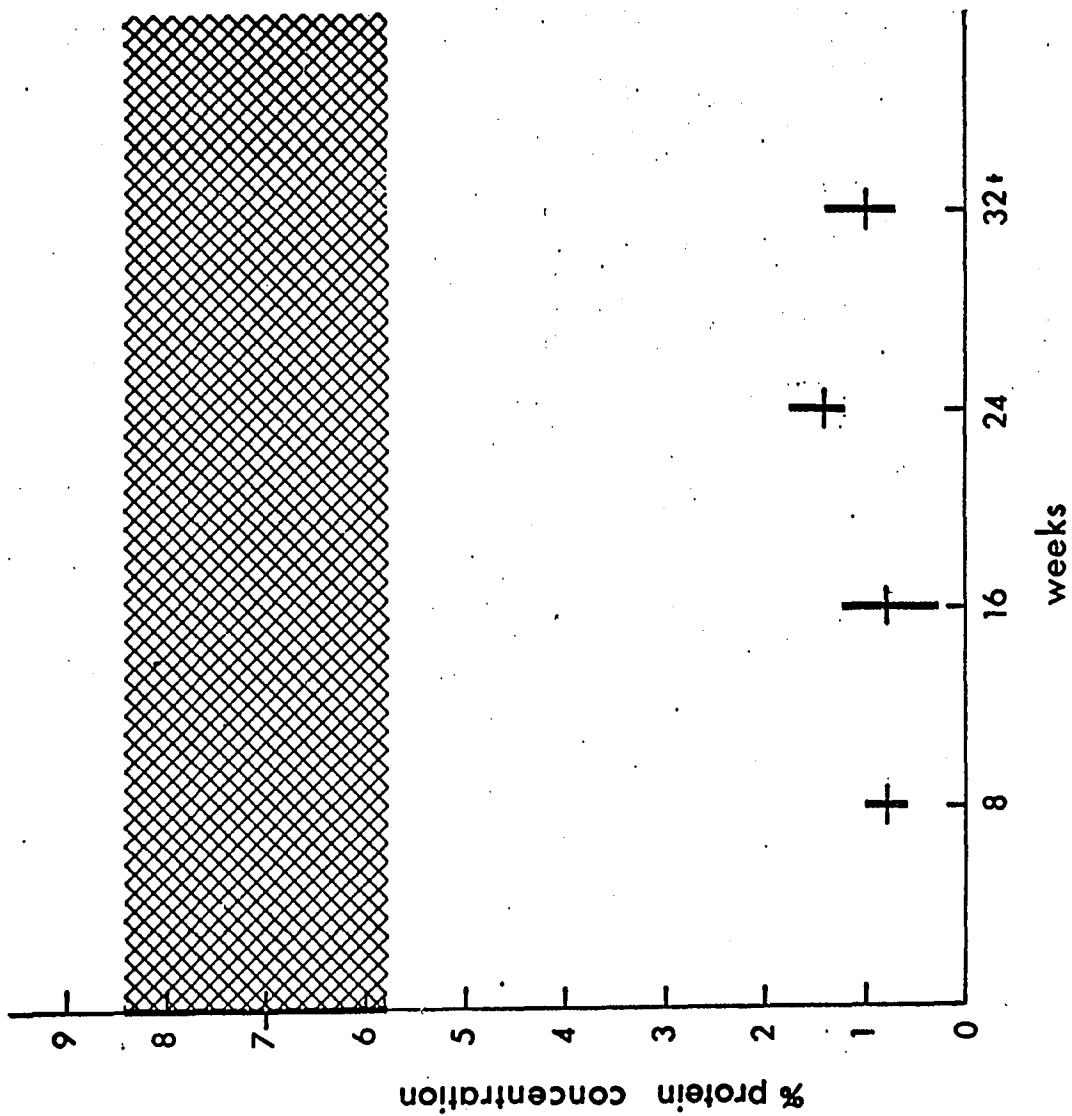
Figure 52

Graph showing percentages of the total protein concentration

of fraction 3 at the time intervals specified. The shaded

area represents the 95% confidence limits of the control.

The 95% confidence limits of the experimental groups are

represented by the extended vertical lines. The mean total

protein concentration is indicated by the horizontal lines.
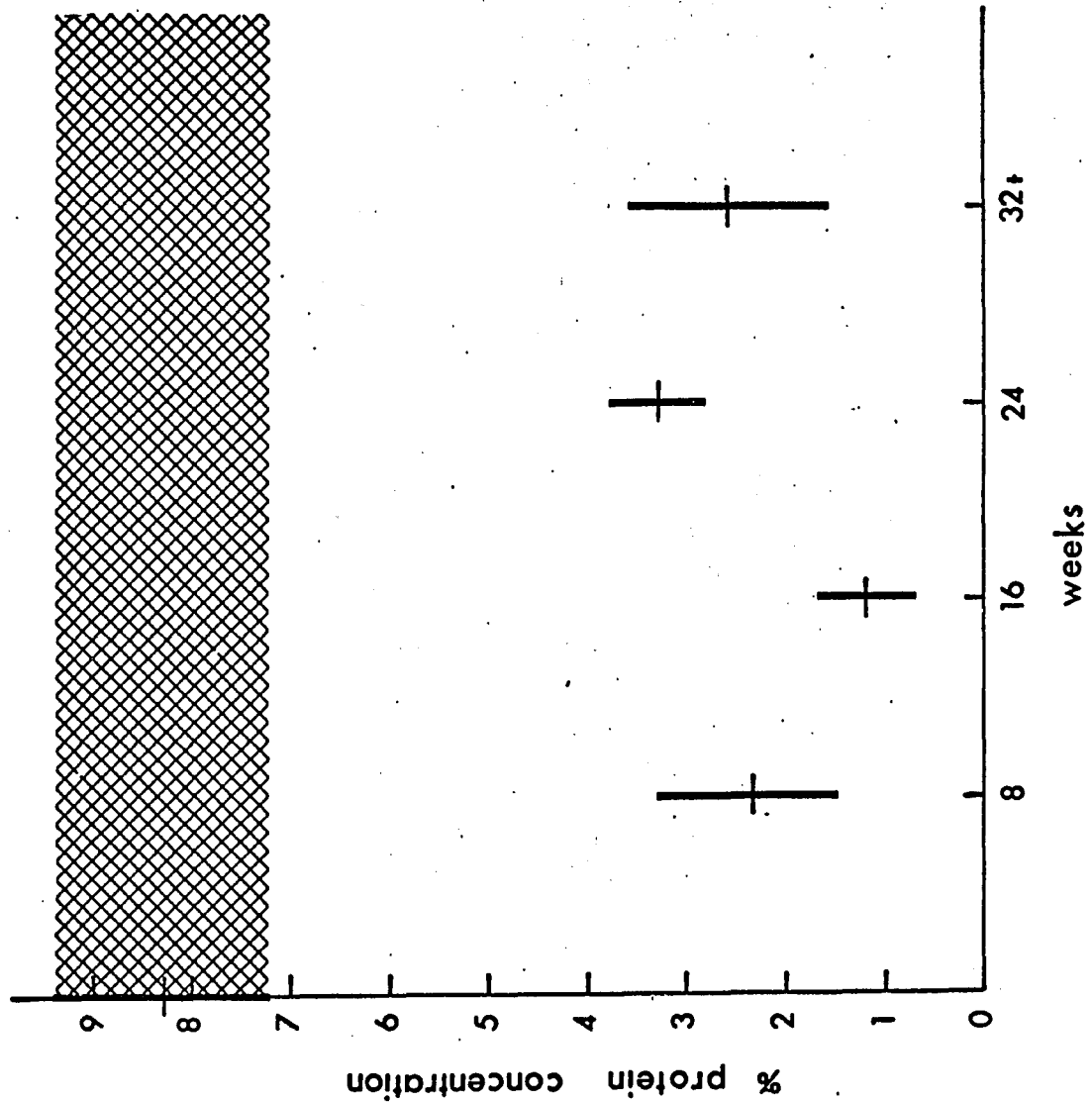
Figure 53

Graph showing percentages of the total protein concentration
of fraction 4 at the time intervals specified. The shaded
area represents the 95% confidence limits of the control.
The 95% confidence limits of the experimental groups are
represented by the extended vertical lines. The mean total
protein concentration is indicated by the horizontal lines.
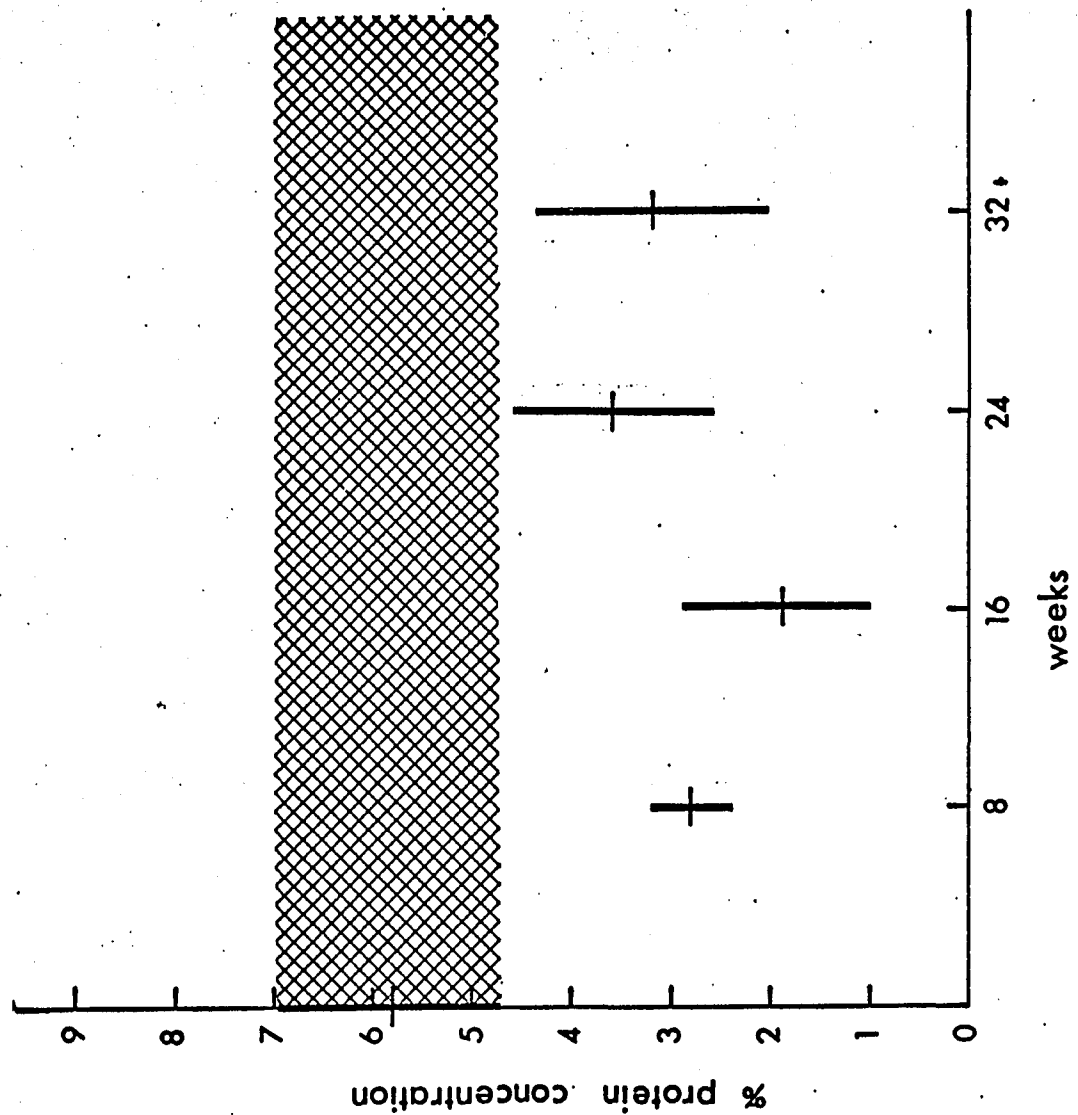
Figure 54

Graph showing percentages of the total protein concentration

of fraction 5 at the time intervals specified. The shaded

area represents the 95% confidence limits of the control.

The 95% confidence limits of the experimental groups are

represented by the extended vertical lines. The mean total

protein concentration is indicated by the horizontal lines.
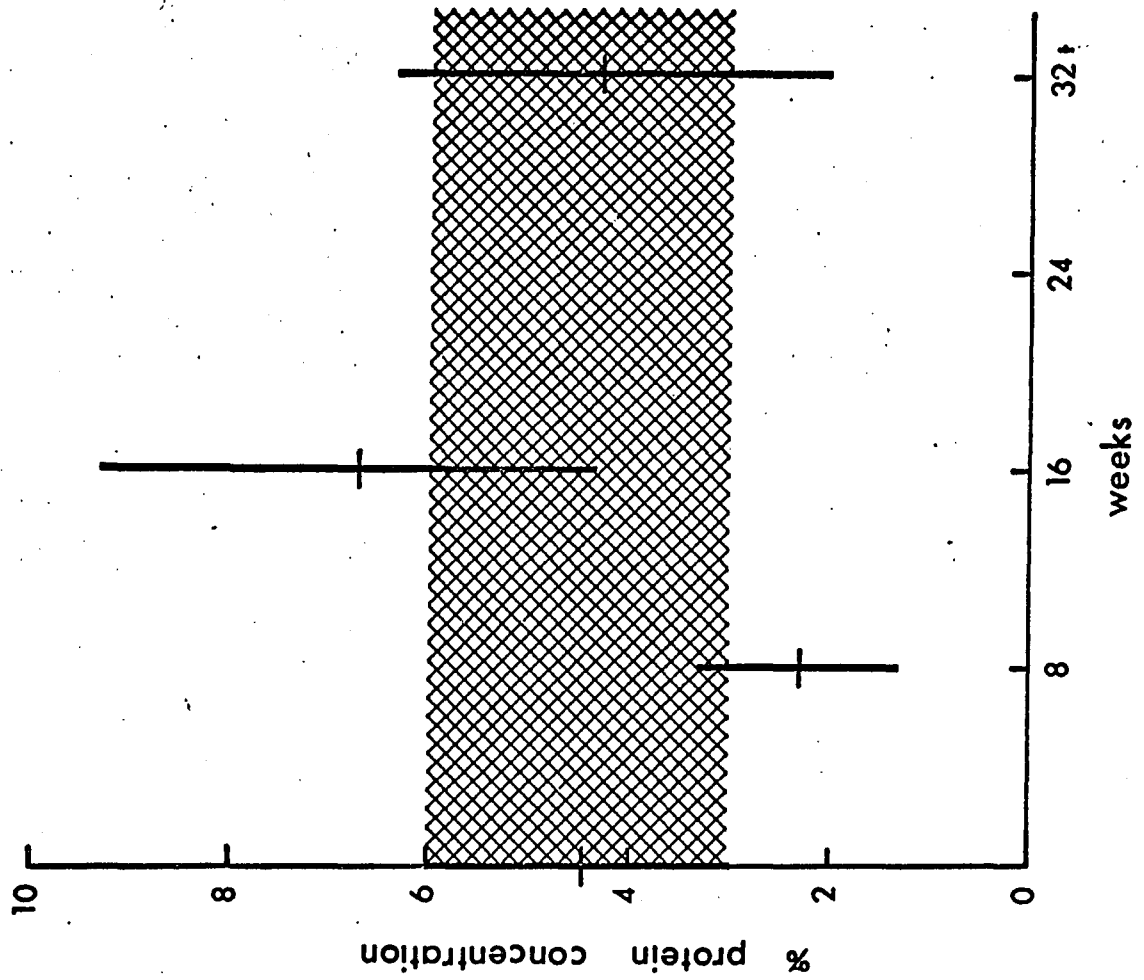
Figure 55

Graph showing percentages of the total protein concentration
of fraction 6 at the time intervals specified. The shaded
area represents the 95% confidence limits of the control.
The 95% confidence limits of the experimental groups are
represented by the extended vertical lines. The mean total
protein concentration is indicated by the horizontal lines.
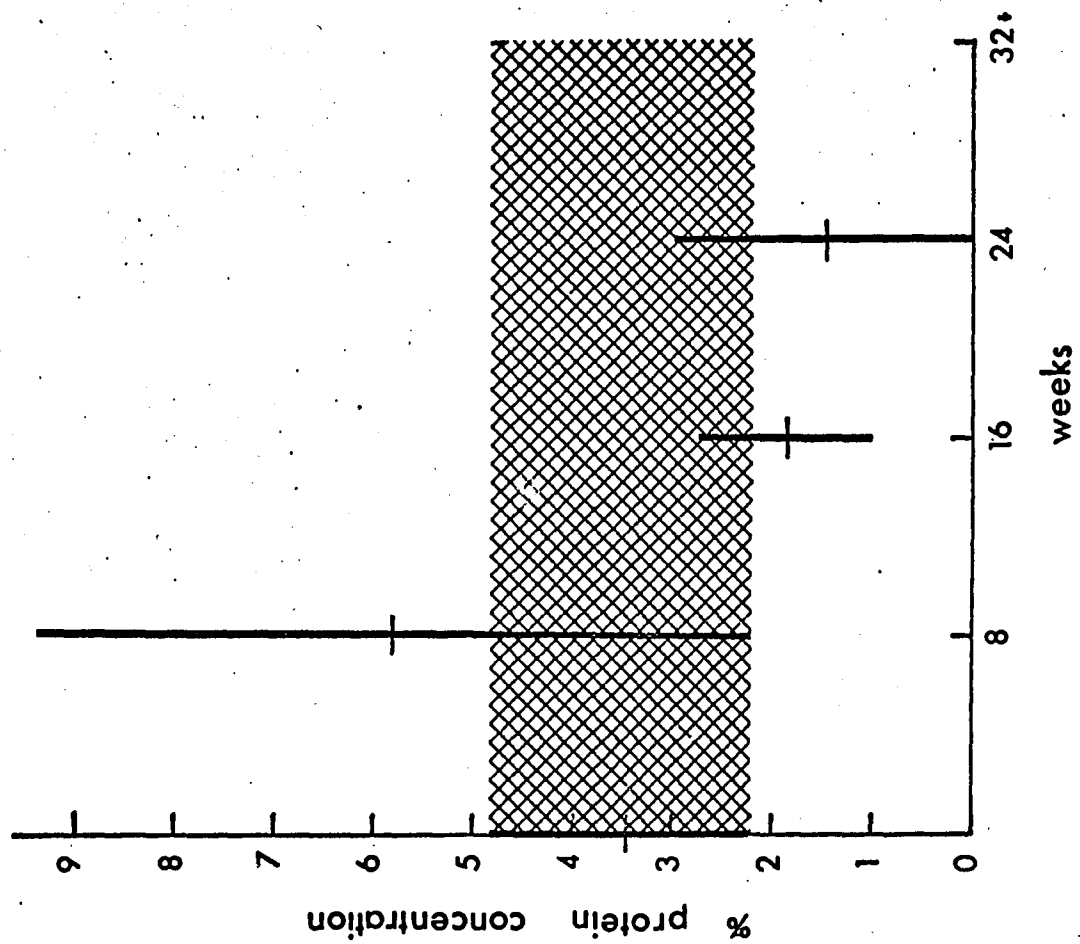
Figure 56

Graph showing percentages of the total protein concentration
of fraction 7 at the time intervals specified. The shaded
area represents the 95% confidence limits of the control.
The 95% confidence limits of the experimental groups are
represented by the extended vertical lines. The mean total
protein concentration is indicated by the horizontal lines.
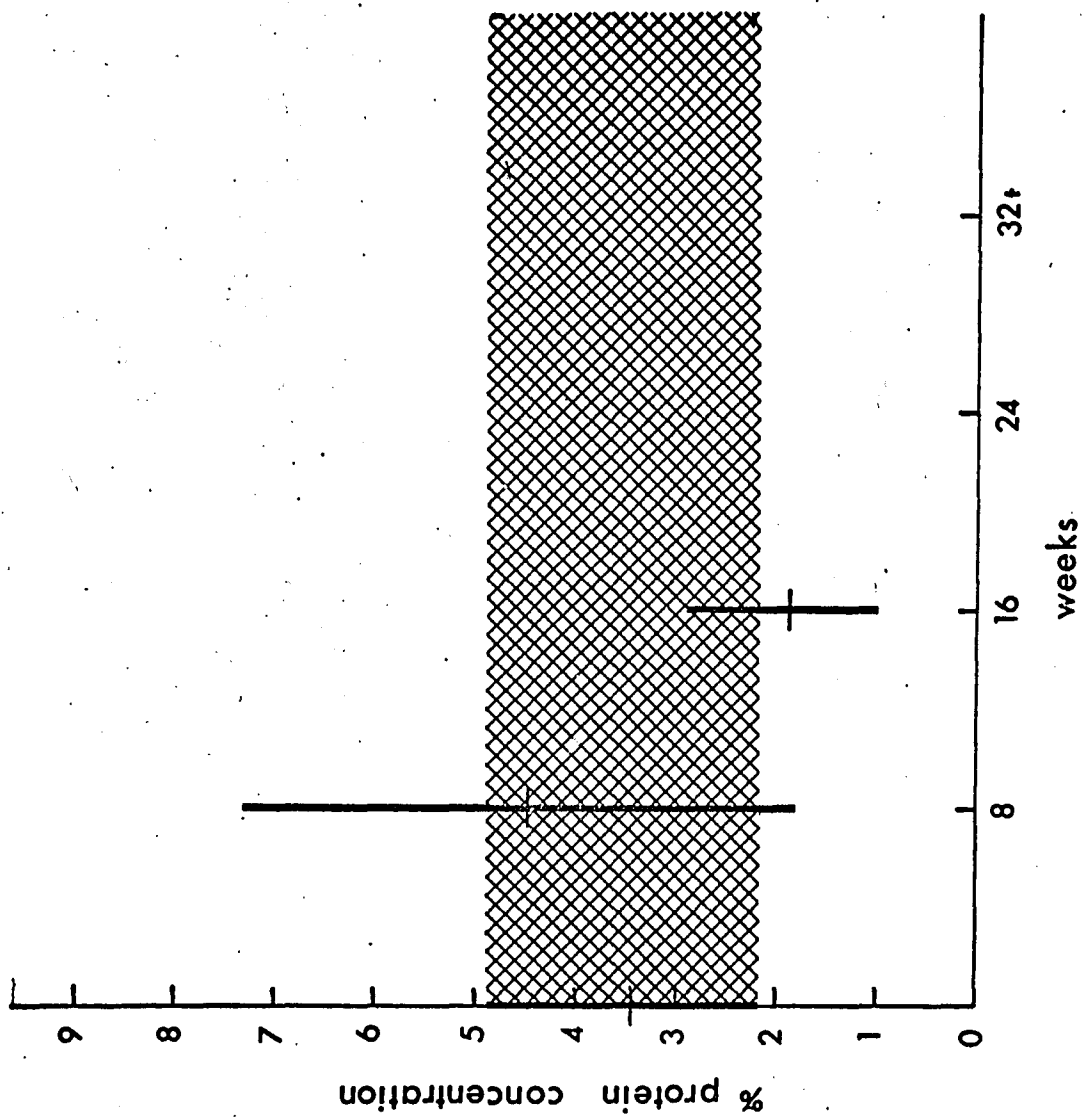
CHAPTER VII

THE ATTRACTION OF CRASSOSTREA VIRGINICA LEUCOCYTES TO
THE METACERCARIAL CYSTS OF PHILOPHTHALMUS GRALLI

A. Introduction

As has been mentioned earlier, the accumulation of leucocytes in
the region of the cyst formed by Crassostrea virginica in response to
the metacestode of Tylocephalum sp. represents one of the characteristic
features of the encapsulation process. Although the factor(s) res-
ponsible for the attraction of blood cells to the focal zone of in-
flammation has not as yet been definitively elucidated, a number of
theories have been proposed, especially in vertebrate systems. It has
been reported that a leucocytosis promoting factor (LPF) is liberated
by injured cells and leucocytes are attracted into the area by this
chemotactic substance (Menkin, 1947). Another study has shown that
the early migration of polymorphonuclear cells is caused primarily by
leucotaxine, a polypeptide to which there may be attached a prosthetic
group (Menkin, 1947).

Components of the plasma known as opsinins can enhance the cellu-
lar uptake of foreign particles (Wright and Douglas, 1903; Jenkin and
Rowley, 1961; Filkins and Smith, 1965; Norman and Benditt, 1965).
Opsinins promote the adhesion of coated particles to the surface of
phagocytes by decreasing the surface electrical potential. "Immunized"
organisms contain active opsinins in the form of antibodies; however,
naturally occurring opsinins may produce similar effects. Tripp (1966)
has found that a natural hemagglutinin occurring in the plasma of C.
virginica has opsonic effects.

It has been shown that the extracellular cystogenous material comprising the cyst formed around Tylocephalum metacestodes is laid down prior to the accumulation of leucocytes. It is possible that some of its chemical constituents act as a chemotactic stimulus to oyster leucocytes. The results of histochemical tests reported earlier (Chapter 1) have shown this extracellular material to be similar in many respects to the cystogenous material of metacercarial cysts as demonstrated by Singh and Lewart (1959), Bogitsh (1962a, b), Dixon and Mercer (1964), Dixon (1965), Erasmus (1967), Thakur and Cheng (1968). Furthermore, it has been demonstrated (Hunter and Dalton, 1939; Hunter and Hunter, 1940; Chandler, 1951; Hoffman, 1958; Bogitsh, 1962a, b; Cheng, 1967) that hosts reaction cells will form an outer cystogenous wall in response to an inner parasite secreted wall.

Since the chemical nature of the metacercarial cyst of Philophthalmus gralli has been determined (Thakur and Cheng, 1968), the metacercariae, as well as other larval stages of this trematode have been employed as a model to study the reactions of C. virginica in vitro.

B. Materials and Methods

The oysters used for this study were collected at West Loch, Pearl Harbor, Oahu, and placed in a running seawater system (salinity; 32 o/oo, temperature; 25° C) until their subsequent use.

Oyster blood was obtained in the following manner. The left valve of each shell was removed, exposing the pericardium. Sterile forceps and scissors were used to expose the pericardial membrane. A 27 gauge hypodermic needle was inserted directly into the pericardium and approximately 0.6 ml of whole blood was removed. The blood was then

placed into a Mackaness type chamber (Mackaness, 1952). The rediae, cercariae, and metacercariae of P. oralli were obtained from naturally parasitized Tarebia granifera collected from sugar-cane drainage ditches at Honouliuli, Oahu. The rediae and cercariae were inserted directly into the blood-filled chambers by use of micropipettes. Metacercariae were obtained by allowing the free swimming cercariae to encyst on the top cover slip of the chamber. This cover slip was then placed on the chamber so that the metacercariae were in contact with the blood. Finally, the stainless steel sealing cap was securely fastened in place.

The controls used for this experiment consisted of sterile glass beads, agar blocks, and agar blocks soaked in oyster hemolymph. These objects were placed in the chambers in the same manner as were the larval stages of P. gralli.

After it was determined that oyster leucocytes are attracted to larval P. gralli, an attempt was made to determine how the molluscan cells were attached in the cyst of the metacercariae. This was accomplished by removing the cover slip with attached metacercariae after 1 hr of exposure to oyster blood and mounting it on a microscope slide. The slide was then placed under a compound microscope and examined.

C. Results

There was a noticeable accumulation of leucocytes in the proximity of the metacercariae and to a lesser extent, leucocytes gathered in the vicinity of the cercariae. On the other hand, there was no observable concentration of leucocytes around rediae nor was this phenomenon associated with any of the controls.

Although the chambers were observed for two days, the majority of the host cells had reached the periphery of the metacercarial cyst within 1 hr. Leucocytes also gathered around free swimming cercariae but the accumulation was not as extensive as that described for the metacercariae, and it took approximately 5 hrs for the majority of the amoebocytes to reach the cercariae.

A microscopal study of the interaction between the metacercarial cyst and leucocytes revealed that the cells appear to adhere to the cystogenous material comprising the cyst. Cells were observed to be connected to this material by extended cytoplasm or in certain cases, the entire cell was embedded in the metacercarial cyst. In most instances the oyster amoebocytes were of the granular type and these displayed projecting pseudopodia.

D. Discussion

There have been a number of studies on the chemical composition of metacercarial cysts. Lenhoff et al. (1960) examined the hydroxyproline content of the cyst of Ascocotyle sp. and Singh and Lewart (1959) found that the three walls of Notocotylus urbanensis cysts are all PAS-positive; a mucoid-like substance was found in the outer wall, while the inner and walls included proteins in the bound form as either muco- or glycoproteins. Bogitsh (1962a, b) also found a PAS-positive reaction in both walls of Posthodiplostomum minimum cysts. The inner wall was found to contain a glycoprotein or a highly polymerized carbohydrate-protein complex, while the outer wall was noticeably rich in arginine. Fasciola hepatica cysts are divided into four layers when observed with the electron microscope (Dixon and Mercer, 1964; Dixon, 1965), the

outer and inner are totally of protein while the middle two layers
include acid mucopolysaccharides. Erasmus (1967), who studied the
cysts of *Cyathocotyle* *bushiensis*, demonstrated the presence of acid
mucopolysaccharide-rich lamellae, separated by thin layers of protein.
In a histochemical study of the cyst formed by *P.* *gralli*, Thakur and
Cheng (1968) demonstrated the presence of three walls; the outer and
middle walls are PAS-negative but the inner wall is PAS-positive and
diastase resistant. All three walls include proteins.

It would appear from the above that a glycoprotein, a neutral
mucopolysaccharide, or an acid mucopolysaccharide, the first two of
which are PAS-positive, occurs in·most of the metacercarial cysts
studied to date. Such a protein-carbohydrate complex is believed to be
the attractant molecule to host cells. This hypothesis is supported
by Bogitsh's (1962a) finding that hosts reaction cells will form the
outer cystogenous wall in response to PAS-positive inner secreted walls.

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