HISTOLOGICAL AND HISTOCHEMICAL STUDIES ON THE EMBRYONIC MEMBRANES ENCLOSING THE ACANTHOR OF ACANTHOCEPHALUS JACKSONI AND ECHINORHYNCHUS GADI

ARTHUR JAMES WEST II.

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ON THE EMBRYONIC MEMBRANES ENCLOSING
THE ACANTHOR OF ACANTHOCEPHALUS JACKSONI AND ECHINORHYNCHUS GADI.

University of New Hampshire, Ph.D., 1964
Zoology
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HISTOLOGICAL AND HISTOCHEMICAL STUDIES ON
THE EMBRYONIC MEMBRANES ENCLOSING THE ACANTHOR
OF ACANTHOCEPHALUS JACKSONI AND ECHINORHYNCHUS GADI

BY
ARTHUR JAMES WEST, II

B.S., Suffolk University, 1951
M.A. in Ed., Suffolk University, 1956
M.S., University of New Hampshire, 1962

A DISSERTATION
Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy

Graduate School
Department of Zoology
September, 1963
This dissertation has been examined and approved.

[Signatures]

October 5, 1963.

Date
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ACKNOWLEDGEMENTS

Deep appreciation is expressed to Professor Wilbur L. Bullock for his generosity of time and patience in guiding me in the pursuit of this problem. Grateful acknowledgement is made of Dr. George M. Moore who selected me for a National Defense Educational Act Doctoral Fellowship. To the Graduate Students, Faculty of the University and particularly to the members of my Committee I would like to express my thanks. I would especially like to thank Miss M. Patricia Morse for her assistance in the preparation of the illustrations.

I am indebted to Mr. Roswald Greene, Superintendent of the New Hampton State Fish Hatchery, New Hampton, New Hampshire and to Mr. Michael Linquata of the Progressive Fish Wharf Inc., Gloucester, Massachusetts for the privileges of collecting that were extended to me. I wish, also, to acknowledge the worthy advice that was extended to me through personal communication with Dr. T. von Brand, Dr. F. L. Campbell, Dr. J. A. Clegg, Dr. J. G. Mackin, Dr. L. Monné, Dr. N. W. Runham, and Dr. T. N. Salthouse. To the Trustees and Administrators of Suffolk University I express my gratitude for the support and encouragement that they have extended to me.

Finally, I would like to dedicate the effort of this work to Dr. Robert S. Friedman, Chairman of the Biology Department of Suffolk University, who has been a good friend and inspiration throughout my entire collegiate training.
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SECTION I

INTRODUCTION

The Acanthocephala are a group of endoparasites that occupy an uncertain phylogenetic and systematic position (Hyman, 1951; Baer, 1961; Rauther, 1935). Adults of all known species are found as parasites in the intestine of vertebrates. Typically, the intermediate host is an arthropod such as an insect, amphipod, or isopod.

The adults are dioecious with fertilization and early development occurring within the ligament sacs or pseudocoel of the female body. Cleavage is of an atypical, spiral, determinate type (Meyer, 1928) in which the pattern has a bilateral nature and is not very synchronous.

The stages in the development of the parasite have been designated as the acanthor, acanthella and adult (Van Cleave, 1947). The acanthor is a larval form that has developed in the pseudocoel of the female acanthocephalan. This stage has often been referred to, erroneously, as an "egg." As Hyman (1951) has stated, "the so-called eggs of the Acanthocephala are really acanthors enclosed in a heavy elliptical shell." It remains an acanthor until it has penetrated the intestinal wall of an arthropod host and has undergone a metamorphosis in the hemocoel resulting in the formation of a second larval stage, the acanthella. The adult stage is reached when infected arthropods are ingested by the proper definitive host and sexual maturity is at-
The problem of the number and nature of the membranes that enclose the acanthor of various acanthocephalans has not been satisfactorily resolved. There have been conflicting reports over the number of such envelopes and only a partial evaluation of their chemistry.

One purpose of this study was to determine, by histological methods, the number of such coverings that surround the acanthor of *Acanthocephalus jacksoni* Bullock, 1962 and *Echinorhynchus gadi* (Zoega) Müller, 1776. *A. jacksoni* occurs in the intestine of a variety of fresh-water fishes in the New England Area (Bullock, 1962) and *E. gadi* in numerous marine fishes (Meyer, 1932). Bullock (1962) indicated the intermediate host for *A. jacksoni* as *Asellus* sp. Specimens sent to Mackin (1962, personal correspondence) were identified as being *Asellus communis* Say. The intermediate host for *E. gadi* has been established as various species of marine amphipods (Nybelin, 1923).

A second purpose of this study was to evaluate, through microchemical and histochemical methods, the constituents of each acanthor covering described. Wherever practicable, polarizing light microscopy and phase contrast microscopy have been utilized.

The third object of the study has been to evaluate the development of each acanthor covering. The earliest stages of development have been found within the germ-balls of the adult female. The germ-balls are fragments of the
original ovarian tissue which was present during the late acanthella stages.

Most of the previous investigations into this problem have been carried on with *Macracanthorhynchus hirudinaceus* (von Brand, 1940; Burnham, 1957; Baer, 1951; Kaiser, 1913; Leuckart, 1876; Manter, 1928; and, Meyer, 1928; and 1932/1933). Recently, many papers have been coming from the laboratory of Monné with reference to various histochemical properties of the embryonic coatings surrounding the "eggs" of parasitic helminths (1954a, c, d; 1955a, b; 1957; 1959; 1960, and 1962) and coccidia (1954b). Monné's acanthocephalan work has been almost exclusively with two species of *Polymorphus*. The only other detailed account of the developing envelopes is that of Meyer (1931) on *Neoechino-rhynchus rutili*.

This problem was approached from the aspect of its potential value in understanding and following embryological processes taking place in the transition from acanthur to acanthella stages.
SECTION II

REVIEW OF LITERATURE

The first critical investigation of "eggs" of an acanthocephalan was that made by Burow in 1836. Working with Corynosoma strumosum (Rudolfi, 1802), Lühe, 1904 (= Echinorhynchus strumosus Rudolfi, 1802) he observed that "the eggs have been strongly depressed to look like globules of blood." Three surrounding membranes were described as being plainly visible and were illustrated in "Fig. 4." of his publication. The outer membrane was indicated as "α"; the middle membrane as "β"; and the innermost membrane as "γ." The innermost membrane was also designated as the "amnion."

Burow did not attempt to describe the development of the "eggs" from ovarian tissue. This aspect of the problem was pursued by von Siebold (1837). What we now recognize as the germ-balls were described by him as "ovaries." As translated, he described these structures in the following manner:

This body consists of a granular, sharply defined mass, in which large and small vesicles lie buried. In many of these ovaries one also notices larger oblong-oval particles, whose form, size and contents correspond entirely to the wandering small eggs within the body cavity. In these small eggs, which are further surrounded by a covering, I never could notice the germinal vesicle, its contents always consisting of only individual dispersed vesicles and granules, which are probably surrounded by a water-clear humor. In the further growth of the free eggs soon a second and finally a third covering appears, and presents also a constriction at its both ends in Echinorhynchus
Further observations were made on the "outermost membrane" of *Echinorhynchus strumosus, hystrix, angustatus, and proteus* in which von Siebold makes reference to the unravelling of the membrane into very thin elastic filaments when subjected to crushing between two panes of glass. The two inner membranes remained uninjured with this treatment.

A detailed account of the acanthor, with its surrounding membranes, was undertaken by Wagener (1857). He made a rather extensive analysis of the spine and hook pattern in the acanthor stage of various species. Wagener proposed that the various species investigated formed four natural groups based upon the nature and distribution of the larval hooks and spines. The first group possesses a pair of large hooks on either side of the "Kopfporus" and includes the species *Polymorphus minutus* (Gozea, 1782, Løhe, 1911 (*Echinorhynchus polymorphus* Bresmer, 1824) and *Macracanthorhynchus hirudinaceus* (Pallas, 1781), Travassos, 1917 (*Echinorhynchus gigas* Bloch, 1782). The second, exemplified by *Echinorhynchus gadi* Müller, 1776 (*Echinorhynchus acus* Rudolphi, 1802), possesses a single large hook on either side of the "Kopfporus." Both of these groups have, in addition, several rows of larger hooks in the region of the head. The third group consists of those species that have head spine rows without the
large hooks, e. g. *Filicollis anatis* (Shrank, 1788), Lähe, 1911 (= *Echinorhynchus filicollis* Rudolphi, 1819). Finally, the fourth group consists of those species which show no separately distinguishable hooks or spines at the head and are exemplified by *Neoechinorhynchus rutili* (Müller, 1780) Hamann, 1892 (= *Echinorhynchus tuberosus* Zeder, 1803) and *Prosthorhynchus transversus* Rudolphi, 1819).

Wagener demonstrated four membranes surrounding the acanthor of *Polymorphus minutus* (Taf. VI, Fig. 13) and three such membranes for *Filicollis anatis* (Taf. VI, Fig. 16). He also confirmed the observations made by von Siebold that the "outer membrane," when crushed, gives rise to many fine elastic threads.

Wagener (1857) had made comments on the presence of rudimentary adult organ systems in the acanthors that he had observed. He did not know that the acanthor underwent a metamorphosis in which there was a complete reorganization of this larval form into an acanthella. It was Leuckart (1862) who drew attention to the erroneous deductions of Wagener. By successfully infecting "*Gammarus pulex*" with the acanthor of "*Echinorhynchus Proteus*," he was able to follow the metamorphosis of the acanthor into the acanthella stage. Leuckart, in describing the "ova" (acanthor) of this species, stated: "They are of a fusiform shape, and surrounded with two membranes, an external, of a more albuminous nature, and an internal, chitinous."
Further consideration was given to the anatomy of "Echinorhynchus proteus" by Pagenstecher (1863) with particular attention directed toward the development and nature of the acanthor. His work included a description of "the so-called ovary or placentula" which he portrays as developing from the wall of the suspensory ligament. Many detailed drawings were offered in support of this contention. A translation of his description of the completed acanthor is here included:

The entirely completed egg of Echinorhynchus proteus has a three-fold covering. The inner is an entirely simple elastic shell (Chitin?); the second is spindleform, much longer than the inner, the ends drawn out to a somewhat knobby constriction and at the points each is provided with a long threadlike extension. The third is a jellylike covering or albuminous clear layer, whose fibers remain fastened around the middle shell (Taf. XXIII, Fig. 15-24).

It is of interest that throughout the literature there are no observations made about the extension of this membrane into filamentous elongations. It is also noteworthy that in Kaiser's (1893) historical review of the subject he has credited Pagenstecher with describing a "vierfache Hülle" when in actuality Pagenstecher describes a "dreifache Hülle."

Working with Acanthocephalus anthuris (Dujardin, 1845), Løhe, 1911 (= Echinorhynchus anthuris Dujardin, 1845), Cobbold (1864) speculated further on the nature of the membranes that surround the acanthor. His paper states that:

By further development after their escape from the
so-called free ovaries, or egg-bearing masses, these vesicular bodies come to represent, as it were, the several egg-envelopes, so that in a much more advanced egg we have, at length (as in figs 6, 7 & 8), an outer envelop, a middle covering (which is coloured in at least one species of Echinorhynchus, and probably forms the true shell or chorion), and an internal or third layer. The middle layer is usually constricted at either pole, somewhat after the fashion observed in the egg-shells of Trichocephalus. The third membrane is well shown in the two highly-magnified ova drawn by Mr. Busk (Fig. 13). These are referable to Echinorhynchus angustatus. In the advanced eggs of E. anthuris I have particularly noticed the lateral disposition of a finely granular mass (figs. 7 and 8), which appears to lie between the true egg-shell and the yolk itself, but a formative granular mass, similar to that which we find in the nascent ovum of Taenia solium. It is, in point of fact, the matrix, part of which is specialised or set aside to form the yolk, whilst the remainder shows itself as a superfluous heap of granules lodged between the outer primitive envelope and the chorion. Probably the latter may subserve a nutrient purpose.

A similar account is given of the acanthor by Greeff (1864) in which he cites two enclosing membranes for Polymorphus minutus in the first section of his treatise and three membranes for "Echinorhynchus proteus" in the second section.

Lespes (1864) working undoubtedly without the knowledge of Leuckart's (1862) findings, attempted to confirm the observation of Wagener (1857). He used a species of acanthocephalan, Neoechinorhynchus rutili, whose acanthor stage he described as being "hookless." Pertaining to the membranes he has very little to say and offers nothing new. He makes reference to a shell and an inner membrane that is evident upon the crushing of the outer shell. He did feel that the "cocoons" were laid down by a secretion from a posterior gland of the uterine bell.
Two significant pieces of work were written by Leuckart, on the embryological development of the Acanthocephala, in the years 1873 and 1876. In these papers he followed the early embryology of several species of Acanthocephala and indicated that the eggs may have three or four membranes. The fourth membrane he indicates is a result of a doubling of the innermost membrane. This is the case as described for *Macracanthorhynchus hirudinaceus*. The other eggs considered, which were eggs of the Palaeacanthocephala, were described as having three membranes. Subsequent observations will show that Leuckart was probably equating the third covering with the outermost membrane.

One of the most significant and detailed monographs on the anatomy and development of the Acanthocephala is that of Hamann (1819). In this work he gives some details of stains that were utilized in studying the details of dividing cells. His descriptive work was done on *Echinorhynchus gadi*, *Acanthocephalus ranae*, and *Polymorphus minutus*; with *E. gadi* being described as much better suited for the investigation of the fertilization process.

Hamann gives detailed descriptions of the development from the germ-balls through to the adult and takes particular notice of the membranes and their mode of development. He refers to the acanthor as "Das Gastrulastadium." The sequence of formation of the membranes is such that he indicates that of the three membranes the first,
which surrounds the egg, soon becomes an external membrane with the formation of a second thicker membrane under it. A space is formed between these two membranes which later becomes filled with a third covering of interwoven, "felt-like" fibers. No mention of the inner membrane is made in the description, although in the illustration a fourth inner membrane is shown for E. gadi. For A. ranae no fibrillar shell is described but rather it is stated that a fluid may fill the space between the outer membrane and the fertilization membrane. An inner membrane is described as being tightly adherent to the embryo, except at the poles.

A very useful historical summary of the earlier works on embryonic development of the Acanthocephala is to be found in Kaiser's 1893 paper. This paper also gives comparative descriptions of the membranes for M. hirudinaceus, Moniliformis dubius, A. lucii, and A. ranae. For these forms he describes but three membranes. His description of membrane formation indicates that the outer membrane is formed by the lifting off of a membrane that surrounded the egg. This is followed by a deposition of a colorless, gelatinous mass on its inner surface. In M. hirudinaceus and M. dubius such deposition precedes the formation of the hard shell and in the other forms it precedes the formation of the fertilization membrane and accumulates primarily at the egg-poles. A second broad, compact shell, which loses its strong refractive power and the sharp contours of the outer covering, forms later.
Finally a third membrane develops and becomes closely adherent to the acanthor itself.

Kaiser claims that in all of his investigated species he finds only three coverings surrounding the acanthor. The outer covering for the terrestrial forms has earned what he calls the full right of the given name "Schale." The shell remains in its original gelatinous form in the aquatic forms.

The status of the membrane problem, before the turn of the century, was reflected in the treatment of the embryology of the Acanthocephala by Korschelt and Heider (1895). They stated the following:

The eggs of the Acanthocephali are detached from the ovarium as membraneless, usually spindle-shaped cells, and then come to lie in the interior of the body of the female. Here they are fertilized, after which each egg is surrounded by a delicate transparent membrane, and then begins to cleave. When this (in *E. gigas*) has advanced as far as the formation of twelve blastomeres, a second membrane is formed under the first, which has separated some distance from the egg, and to which are added in the course of the development two more protective envelopes, so that finally four of them are present. This applies to *E. gigas* (Fig. 113A). Ordinarily three such embryonal membranes are formed, the middle one of which acquires a considerable thickness and firmness by the deposition of concretions of a brownish colour. These structures are particularly noteworthy, for the reason that they first make their appearance during cleavage, and therefore are not to be looked upon as egg-membranes, but as a kind of embryonal membrane; still they do not appear to have any cellular structure. They recall the embryonal membranes occurring in the Taeniidae, which may also acquire a considerable firmness.

In Kaiser's 1913 paper a single sentence is devoted to the membranes: "Bevor der mit fünf derben Häufen umhüllte Embryo den mütterlichen Leib verlässt, erfahren die ihn
bildenden Zellenaggregate noch eine tiefeingreifende Metamorphose."

Notice was taken by Manter (1928) of the textbook discrepancies dealing with the "egg" membranes of *M. hirudinaceus*. He states that:

Textbooks are incorrect in ascribing three egg shells to the eggs of *M. hirudinaceus*. Four are clearly present as stated by Leuckart. The outer membrane, thin and delicate, is evanescent and may be lost in the older eggs. Beneath it is the heavy brown, irregularly grooved, or ridged shell followed by a thin elastic membrane, and then by the inner thick, transparent shell. Kaiser describes five shells or membranes, the fifth being another thin membrane close to the embryo.

A significant milestone was reached with the publications of Anton Meyer (1928, 1931, 1932-33, 1938) which deal with the early embryology of *Neoechinorhynchus rutili* and *Macracanthorhynchus hirudinaceus*. Meyer (1928), working on *M. hirudinaceus* described the development of the membranes as being fourfold. The larva was described as covered with a shell "H3" which is found between the outermost membrane "H1" and the fertilization membrane "H2" with another membrane being formed within the fertilization membrane "H4." He expressed uncertainty as to whether this fourth membrane arose from a splitting of the fertilization membrane or whether it arose anew.

His 1938(?) observations and conclusions on the nature and number of membranes include the following:

---

1 Date uncertain as noted in the Index-Catalogue of Medical and Veterinary Zoology.
Die Eier (Abb. 4 u. 5) werden im mütterlichen Korper befruchtet (oft schon im Ovarialballen), und entwickeln sich bis zu einer embryonalen Larve, die vorn symmetrische Häckehen, im übrigen Stacheln trägt und im Inneren vier Gruppen dicht gedrängter Kerne enthält ("Embryonalkern"). Das Ei ist von einer weit abstehenden, dünnen äusseren Hüllmembran (1) umgeben, unter der eine etwas stärkere, bei den Palaeacanthocephala charakteristisch geformte Befruchtungsmembran (2) liegt. Im Laufe der Entwicklung entsteht durch Verfestigung von Sekret meistens noch eine dritte Hüle (oder eine dieser homologe Bildung) von charakteristischer Struktur zwischen den beiden erstgebildeten Hüllen. Das die dritte Hüle (Schale) bildende Sekret tritt wahrscheinlich ungeformt aus dem Keim aus, durchwandert die 2. Hüle und wird durch chemische Bedingungen ausserhalb derselben (innerhalb der 1. Hüle) verfestigt. Eigröße und Form der 2. und 3. Hüle sind systematisch-diagnostisch wichtig.

From the foregoing description it is evident that Meyer recognized only three true membranes for the Acanthocephala. However, it is interesting to note that four membranes have been demonstrated by him for several species. For example, in the same work, on page X, 19, he displays the acanthor of Gordiorhynchus clitorideus as having four membranes but makes the notation that "H4 wird systematisch nicht als eigene Hüle gezahlt."

After Meyer’s extensive works on the development of the Acanthocephala further descriptive work on the acanthor membranes was directed to a chemical consideration by von Brand (1940). Working with the eggs of M. hirudinaceus, he determined that the eggs contained chitin. In describing the results of his experimental work he stated "there can be hardly a doubt that this membrane consists of chitin."

Some doubt exists however, since he only refers to three membranes. It is believed that he was calling the shell
the outer membrane and this would then justify his use of the term "inner-membrane." "Casual observations" were made on the other membrane components which indicates that the granular shell dissolves in hot KOH but seemed to be resistant to peptic and tryptic digestion. Also, the "middle membrane" swells greatly under the influence of weak alkali but dissolves with hot alkali. Its staining properties might be indicative of another carbohydrate "like cellulose or tunicine."

Working with Moniliformis dubius, D.V. Moore (1946a) described "two distinct shells plus two adhering membranes." He went on to indicate that:

The clear thick outer shell is covered by a very thin wrinkled membrane which is easily lost. The outer shell is smooth in outline, and in spite of its thickness is not particularly rigid in structure. Just beneath the outer shell lies the inner shell or middle embryonic envelope, which is rigid in structure and highly refractive. Between the middle embryonic envelope and the acanthor is a thin elastic membrane which is usually closely adherent to the acanthor, which has its rostellar hooks invaginated.

He cites the discrepancy in the observations of Yamaguti and Miyata (1938) in which they refer to an inner shell as well as outer and middle ones with no supporting photographs to show such structures. It is Moore's opinion that they were working with preserved eggs in which the acanthor had shrunk away from the thin, closely adherent inner membrane present in the living condition.

Moore (1946b) also described the egg of Macracanthorhynchus ingens as "provided with heavy, dark-brown outer
shell, irregularly grooved or ridged. Inner shell thick and highly refractive. Between outer and inner shells a thin elastic membrane."

Inconsistencies still appear in the literature dealing with the membranes of the acanthor. A review of the more recent literature follows which should point-up the present status of this problem.

Working with the eggs of Leptorhynchoides thecatus, DeGiusti (1949) describes them as a "spindle-shaped object made up of two distinct shell membranes surrounding a thin, delicate embryonic membrane containing an embryo." Contrast this with the description of Polymorphus botulus and minutus as observed by Monné and Honig (1954), in which they state that "the embryonic envelopes of Polymorphus botulus and minutus are composed of the outermost membrane, the fibrillar coat, the shell, and the innermost membrane." Strangest of all is the description of the membranes surrounding the acanthor of Neoechinorhynchus emydis as observed by Hopp (1954). He states that "there are three egg membranes: the outer shell, the inner fertilization membrane, and between them a middle shell membrane which is the most conspicuous and distinctive feature of the egg." Indeed, this is a most phenomenal case in which the middle membrane is described as occupying only a portion of the space between the other two membranes and resembling a vacuole more than anything else. Commenting further upon this phenomenon he stated that "the structure of the middle shell membrane
suggests that were it enlarged to surround the embryo, its edges would meet to form a raphe extending the length of the egg as described for certain other acanthocephala."

Using the electron microscope, Burnham (1957) demonstrated that there were four envelopes covering the larvae. He describes these as "the thin outermost membrane, the shell, and two inner membranes." Burnham goes on to describe the structure of each of these membranes as observed with the electron microscope.

In giving a detailed account of Mediorhynchus grandis, Moore (1962) states that the eggs are "provided with two shells and three accessory membranes." In his description of the developmental stages he goes on to say that:

The three accessory membranes associated with the egg consist of a thin elastic membrane covering the outer shell, which is seldom seen on eggs from the feces; a thin membrane between the outer and inner shells which is only observed in artificially hatched eggs; and the thin, closely adherent membrane enveloping the acanthor.

A review of several textbooks dealing with the subject of parasitology demonstrates again the state of confusion that exists over the number of membranes. Not only are there inconsistencies among members within the phylum, but there exist many inconsistencies in the descriptions of species within the phylum.

In the textbook of Faust (1949), dealing with human helminthology, an all encompassing statement is made which more or less indicates the problem of inconsistency.
He refers to "the eggs, which are provided with three (at times probably four) enveloping membranes." Ward and Whipple (1918) in discussing parasitic organisms that are found in fresh-water, indicate that the eggs are "surrounded by a heavy covering of three distinct membranes." These would not appear to be important inconsistencies when one realizes that the fibrillar coat is very much reduced in at least some forms that occupy an aquatic habitat.

In their textbook dealing with veterinary helminthology, Morgan and Hawkins (1953) describe the eggs as being "thick-shelled consisting of three embryonic membranes." On the other hand Cameron (1956) says that the eggs are "surrounded by three or four membranes."

One of the most widely used textbooks in parasitology and certainly one that is held in high regard by many in the field is that of Chandler and Read (1961). Their statement on the subject indicates that the acanthor is "surrounded by three envelopes, the outer of which often has shapes or markings useful in identification." Obviously what they are making reference to is the pattern that is often evident in the shell of such forms as M. hirudinaceus and Neoechinorhynchus prolixoides, (Bullock, 1963). This is the third membrane of other described species and is further covered by the outer thin fourth membrane.

As recently as 1958 Belding described the acanthor of M. hirudinaceus as being "encased in 3 embryonic envelopes," as well as stating that Moniliformis moniliformis
"have 3 envelopes." Rogers (1962) has more or less stated the case quite adequately about the membrane problem as it stands to date:

The eggs of the Acanthocephala have not been examined in detail. It appears that they are formed largely as a result of the activity of the egg itself and consist of three or four layers. Monnê and Hönlig (1955b)[sic] observed the effects of solvents and histochemical reagents on the egg-shells of two species of Polymorphus. They found four layers which in order from the inside were (a) the innermost membrane chiefly of chitin but with some keratin-like protein, (b) the "shell" similar to (a) but with much less chitin, (c) the fibrillar coat of keratin-like protein without chitin, and (d) the outermost coat of non-keratinous protein. Von Brand (1940) found chitin to be present in the innermost membrane of Macracanthorhynchus hirudinaceus. There has been no suggestion that quinone-tanned protein is present, nor has a layer of lipid been reported. In spite of this, however, the egg-shells of the Acanthocephala are clearly more like those of the Nematoda than those of the parasitic platyhelminths.
SECTION III

MATERIALS AND METHODS

Parasite and Host Collecting

**Definitive hosts:** Mature and gravid females of *Acanthocephalus jacksoni* were collected either in natural bodies of fresh-water in and around the Durham area or through the cooperation of the Superintendent at the New Hampton State Fish Hatchery at New Hampton, New Hampshire. The natural bodies of water were all tributaries of the Oyster River.

The collecting periods were confined primarily to the months of June, July, August and September based upon previous reports of maturation for this species (Bullock, 1962). The fishes used were either brook trout, *Salvelinus fontinalis* or rainbow trout, *Salmo gairdneri*.

The mature acanthocephalans were generally found in the posterior portion of the alimentary canal. However, in heavy infections, they were often found extending more anteriorly to the regions of the small intestine. The females were obtained with the aid of dissecting forceps by grasping the body of the parasite and quickly jerking them free from the intestinal wall of the host. Specimens thus obtained were either relaxed in distilled water and prepared for subsequent fixation or were opened immediately with the pseudocoelomic contents of the worm being smeared directly onto a clean glass slide.
Relaxed and fixed specimens were prepared for paraffin sectioning; smears were examined either in the fresh condition or were fixed and stained for examination under various types of microscopy. Some chemical tests were run that did not necessitate the handling of the worms other than to immerse them in hot, concentrated KOH.

Definitive hosts for Echinorhynchus gadi were obtained through the courtesy of the Progressive Fish Wharf, Inc., at Gloucester, Massachusetts. Codfish, Gadus morhua, and pollack, Pollachius virens, intestines were obtained either directly from the fish that had been recently caught and brought to Gloucester or by delivery from the fishing banks where the fish were processed. The same techniques that were described for obtaining and utilizing adult female A. jacksoni were used for this species. Echinorhynchus gadi was collected on a year round basis since adult forms can be found at all seasons of the year.

Intermediate hosts: The intermediate host for A. jacksoni has been cited as a fresh-water isopod of the genus Asellus. Mackin (1962, personal correspondence) has confirmed the opinion of this author that the species involved is Asellus communis. These were collected at various times in the spring, summer, and fall from the Oyster River and Old Reservoir in Durham and at the New Hampton Hatchery. Collections were made with the use of a collecting net that was dragged across the bottom along the shore. Leaves and other debris were pulled up in this manner and
most of the isopods could be found among this material.

The isopods were examined for the determination of the acanthella stages in the hemocoel. Those found to be infected were kept either in the cold room at Spaulding Life Science Building or in one of the laboratories for subsequent infecting procedures. In each instance they were maintained on leaves collected from the trees prevalent along the banks of the collecting areas and were constantly aerated.

Experimental Infection

Attempts at experimental infection were carried out in the following manner. Four to six isopods were isolated into separate stender dishes and were deprived of food for several hours. The purpose of this procedure was to clear the intestinal tract for easier viewing under the microscope after they had been fed shelled acanthors. This was followed by a change of fresh water and the introduction of either acanthocephalan eggs or whole female acanthocephalans.

Investigations of the alimentary tract were made at several hourly intervals for the purpose of observing liberated acanthors. The alimentary tract was obtained by pulling off the head segment from the rest of the body segments. In this way the alimentary tract remained attached to the posterior segment and permitted optimum microscopic examination.

Various species of Gammarus have been described as
being the intermediate host for *E. gadi* (Nybelin, 1923). No attempt was made to collect gammarids and infect them with the acanthors of *E. gadi*. However, a student at Suffolk University has been working on a special problem dealing with this aspect of the study.

**Chemical Methods**

In an attempt to determine whether or not chitin was present in the membranes of the acanthor of the two species, as reported by von Brand (1940) for *Macracanthorhynchus hirudinaceus* and Monné and Hö nig (1954) for *Polymorphus botulus* and *Polymorphus minutus*, initial attention was directed to the techniques of demonstrating the presence of this substance. According to Richards (1951)

"chitin does not occur naturally as a distinct and separate chemical entity . . . chitin is no more a 'natural' compound than are the various degradation products than can be prepared from it." In defining chitin he states that it "is now known to be a high molecular weight polymer of anhydro-N-acetylglucosamaine residues joined by ether linkages of the $\alpha$-glycosidic type between carbon atoms nos. 1 and 4 of adjacent residues."

Chemically, chitin can be isolated as chitosan by treating it with saturated potassium hydroxide at a temperature of $160^\circ$ centigrade for a period of fifteen to twenty minutes. The techniques used for isolating chitin was that used by Campbell (1929) for insect chitin. Several whole acanthocephalans were placed in a shell vial, measuring 1.9
centimeters by 7.6 centimeters, with about three cubic centimeters of saturated potassium hydroxide solution added. The vial was closed with a one-hole rubber stopper into which was inserted a Bunsen valve. The shell vial was then immersed into a beaker of glycerine such that the level of the alkali was at the level of the glycerine (Fig. 1). The sides of the vial were kept from contact with the beaker by supporting the vial with a clamp. The glycerine was then heated to 160° centigrade and held there for fifteen to twenty minutes. What remained was the alkali-resistant materials which were presumed to be chitosan, as previously suggested by von Brand (1940).

The alkali-resistant materials remain suspended in the saturated alkali, undoubtedly due to the specific gravity of chitin being 1.40 and the specific gravity of saturated potassium hydroxide being 1.54. To recover the alkali-resistant membranes, the suspension was diluted, centrifuged and the solid material washed several times with distilled water.

Once isolated, this alkali-resistant material was treated with a 3% solution of acetic acid. This reagent will dissolve any chitosan present. Further treatment of the solution with concentrated sulphuric acid forms a white precipitate which is chitosan sulphate.

The solubility of chitin was tested by application of known chitin solvents such as concentrated hydrochloric acid, sulphuric acid, nitric acid and sodium hypochlorite.
Suspected keratin-like proteins were investigated by the use of a keratin solvent. This was a sodium sulphide solution as employed by Monné and Höög (1954) and described by Goddard and Michaelis (1934).

**Histological and Histochemical Methods**

In order to determine the number of membranes present various methods were utilized. In conjunction with standard histological procedures, various histochemical methods were employed to ascertain the chemical constituents of each membrane. The details of the procedures used will be found in the Appendix.

Whole acanthocephalans or smears of "eggs" were fixed in a variety of fixatives; those most commonly used were Bouin's and neutral buffered formalin. Other fixatives that were used included Demke's, Helly's and Zenker's. Some procedures were carried out on freshly prepared and unfixed smears.

Many histological and histochemical procedures were employed following appropriate fixation. Hematoxylin and eosin were used to show general histological details. The Feulgen reaction was employed to show more specific distribution of nuclear material at various stages of development. This technique was also utilized to follow the fate of the polar bodies.

The periodic acid-Schiff technique was used in combination with various controls to determine the presence and distribution of aldehyde groups generally associated
with polysaccharides. Various blocking reactions, as outlined by Pearse (1961) on pages 832-833, were used to determine the carbohydrate nature of the PAS positive materials. This technique was also used with various deacetylating techniques in the hope of better demonstrating the presence of chitin.

Several staining techniques were followed because of their described effects with chitin or cellulose. These included the tests of Zander and Schulze, acidified alcian blue as recommended by Salthouse (1962, personal correspondence), Bethe's stain, chlorazol black E, picro-nigrosin and the Hale-Muller reaction.

As an indicator for the general distribution of protein material, the Millon reaction and the Bromphenol blue tests were run. For a more specific indication of the presence of particular proteins a variety of procedures were followed. These included the Gram-Weigert reaction, Mallory's phosphotungstic acid hematoxylin, Toluidine blue 0 and the "azan" method of Heidenhain for the demonstration of fibrin-like proteins.

Fontana's ammoniacal silver nitrate stain in combination with the catechol reaction was run to determine whether or not polyphenols or polyphenol oxidases were present. These substances, when present, are generally considered as an indication of quinone-tanning of proteins.

Elastic substances were investigated by using the Taenzer-Unna orcein stain and the Aldehyde Fuchsin methods.
The only other specific routine run in conjunction with protein analysis was the performic acid-Schiff for demonstrating keratin-like proteins.

The investigation for lipid material was carried out with sudan black B and oil red O stains. Suitable controls were run in each instance to determine the comparative effects of the stains utilized.

**Microscopic Methods**

Due to the difficulties of viewing the membranes under the compound microscope in cases where the membranes were either reduced or restricted, as was often the case in studying the inner membrane or the fibrillar coat of *A. jacksoni*, use was made of the phase-contrast microscope. Phase microscopy made it easier to differentiate the four coverings and relate what was observed under these conditions to observations made with the compound microscope with ordinary lighting conditions.

Since Monné and Hö nig (1954) reported various birefringence phenomena associated with various parts of the acanthor and its membranes, attention was given to this aspect of the problem. The Geology Department at the University of New Hampshire, through the courtesy of Professor Glenn W. Stewart, was kind enough to loan me a polarizing microscope.

The form of birefringence that is observable with such a tool is often an indication of the material that is present. Birefringence may be due to form or intrinsic
birefringence. Form birefringence is due to the shape of the molecule whereas intrinsic birefringence is due to the orientation of molecules. Pyridine was used on fresh eggs to remove form birefringence. After application of this material, the birefringence that is in evidence is considered to be due to the crystalline nature of the substances present and therefore an indication of intrinsic birefringence.
SECTION IV
OBSERVATIONS

General Observations

The acanthor: During the initial stages of this work, considerable attention was devoted to observing the acanthor of *A. jacksoni* (Figs. 2 and 4). Experimental infections were accomplished using the intermediate host, *Asellus communis* Say, in order to evaluate the nature of the acanthor when it was devoid of its enclosing membranes.

After many attempts at trying to observe an acanthor, free in the alimentary tract of the isopod, a successful result was achieved. Several hours were spent in observing the larva moving about the intestine of the isopod. Its method of locomotion was unique in that only the anterior part of the acanthor showed any contractile activity.

At the anterior end of the larva there was an obvious groove that was bordered by several large hooks. The action of the anterior portion seemed to be associated with a probing action of the hooks. By moving the surrounding tissues of the body, the hooks were manipulated in such a way that they were directed anteriorly with their pointed ends forward, and then withdrawn backward. This is most likely the procedure that is used in penetrating the wall of the intestine of the intermediate host during the migration into the hemocoel.
larval spines were in evidence all over the body of the larva. Highly refractive nuclei were observed in the central portion of the body. Under polarizing microscopy, the retractor muscles (Fig. 12) were seen. These appear to be the major muscles of the acanthor associated with the contraction and relaxation of the anterior apparatus.

**Acanthor membranes:** The actual emergence of the larva from its embryonic coverings was not observed. There have been reports (Leuckart, 1876) that the removal of the outer coats precedes the emergence of the acanthor from the inner coat. It has also been stated by Manter (1928) that in observing the process of artificially hatching *M. hirudinaceus* larva that "hatching seemed an entirely passive process on the part of the larva."

After histological, histochemical, chemical and microscopical properties were analyzed, it was determined that the acanthors of each species observed were enclosed within four membranes. The greatest difficulty was experienced in evaluating the fibrillar layer of the *A. jacksoni* acanthor (Fig. 4). This layer is very much reduced in this species and observations with stained specimens demonstrate that the fibers are independent of the outer membrane. Much clearer observations were made on fresh smears of the "eggs" of both species.

Equally difficult was the determination of the limits of the inner membrane in both species. Since this was so closely associated with the acanthor it was very diffic-
cult to determine whether or not it was present or whether
the surface of the acanthor was creating a refractive arti-
fact. It was only after concentrated viewing with the
phase-contrast and the polarizing microscopes, and the em-
ployment of various histochemical stains, that this mem-
brane became clearly evident. It was more readily identi-
fiable in fresh smears of *E. gadi* than *A. jacksoni*.

The origin and development of the various membranes
were followed from their appearance in the germ-balls (Figs.
5 and 7) to the point of full maturation of the acanthor.
In the case of *E. gadi* the fertilized eggs emerged from the
germ-ball soon after the initial formation of the fertili-
zation membrane. In this condition the fertilized eggs
emerged surrounded by two membranes; the original membrane,
that enclosed the unfertilized egg, and the fertilization
membrane that forms after the penetration of the egg by the
sperm. In *A. jacksoni* advanced larvae have often been ob-
served retained in the germ-ball (Fig. 8).

As the acanthor proceeds in its development, the
second membrane, the fertilization membrane, begins to
thicken. During the period of membrane thickening, fibrils
of the third covering become very much in evidence between
the outer membrane and the fertilization membrane in the
eggs of *E. gadi* (Fig. 9). It would appear that the fibers
of the third membrane are fairly well developed before the
fertilization membrane completes its development. In both
species, the fertilization membrane elongates eventually in
the region of the axial poles. This membrane develops into the characteristic "shell" of the palaeacanthocephalan "egg."

The inner membrane was the last to appear but at just what point it made its appearance was somewhat in doubt. The difficulty in making this determination was due to the closeness of the fertilization membrane to the acanthor during the early stages of development. It was in evidence at the time that the fertilization membrane elongated at the poles. At first it was an extremely thin membrane but became more evident in the more advanced stages. It would appear that this membrane took a long time to develop even though never becoming very thick.

Associated with the observed retention of more advanced larvae in the germ-balls of *A. jacksoni* was the scarcity of "eggs" with only two membranes in the pseudocoel of the adult female. There were many more younger stages of an acanthor seen in the pseudocoel of *E. gadi* females.

Motile sperm have been observed in smears of *E. gadi* that were made after the females had been kept in the refrigerator for several days in Ringer's solution. They were seen to be long, filamentous structures with little differentiation of head from tail. At no time were these observed within the eggs of either species. Fertilization was assumed to have occurred before the appearance of the second membrane.
Specific Observations

**Chitin and cellulose:** Following the technique of Campbell (1929), alkali-resistant membranes (Fig. 10) were recovered for each of the species investigated. These membranes were dissolved upon the addition of a 3% solution of acetic acid. However, no precipitate was formed upon the addition of a 1% solution of sulphuric acid. This was most likely due to the small quantity of available material.

Upon the addition of a 10% solution of sodium hydroxide to freshly prepared and unfixed eggs, the inner membrane was found to be tightly adherent to the surface of the acanthor. Richards (1951) and von Brand (1940) indicate that chitin undergoes shrinkage in the presence of weak alkali. This shrinkage did not occur in the fertilization membrane as might be anticipated with this procedure if chitin were present. Chitin has been reported in this membrane for species of *Polymorphus* (Monné and Höngig, 1954).

With the addition of concentrated solutions of hydrochloric, sulphuric and nitric acids drastic changes were observed in the various membranes. Specifically, it was noted that the inner membrane seemed to be little affected, and the fertilization membrane was still intact although somewhat thinner and shrunken. All of the membranes were removed upon the addition of stock (4-6%) sodium hypochlorite solution.

Using the methods of Schulze and Zander, it was
determined that the inner membrane gave typical reactions
descriptive of chitin whereas the fertilization membrane
reacted more like cellulose. The periodic acid-Schiff pro-
cedure, as outlined by Pearse (1961), for the demonstration
of carbohydrate substances, clearly indicated the presence
of mucopolysaccharide materials in the inner membrane and
fertilization membrane. Various attempts were made to
alter the chemical nature of chitin to a form that would
react with mucopolysaccharide stains. Techniques of dea-
cetylation (Lillie, 1954; Glick, 1949) were performed on
fixed smears and paraffin sections in the hope that the
removal of the acetyl group from the N-acetyl glucose amine
polymer of chitin would be converted to a histochemically
reactive material for mucopolysaccharide staining. No ap-
preciable changes in staining results were noted after the
application of deacetylating techniques.

The periodic acid-Schiff technique was also run in
combination with the acidified alcian blue technique. This
procedure gave a color modification somewhere between the
typical red and blue of each polysaccharide stain when used
individually. With the acidified alcian blue technique, as
recommended by Salthouse (1962, personal correspondence),
the inner membrane and the fertilization membrane showed
typical and positive reactions for mucopolysaccharide
staining.

The Hale-Muller reaction for mucopolysaccharides
gave positive results for both the inner membrane and the
fertilization membrane. This result was also obtained after salivary controls were run on smears to remove the possibility of glycogen.

Using the polarizing microscope in conjunction with fresh smears (Figs. 12 and 13) and smears treated with pyridine (Figs. 14 and 15) there was further evidence for the presence of chitin in the inner membrane and cellulose in the fertilization membrane. After the removal of form birefringence with pyridine, there was evidence of positive birefringence in the fertilization membrane. The inner membrane showed less birefringence indicating that its birefringence may be negative and indicative of chitin. All available evidence seemed to indicate that there was less cellulose in the fertilization membrane of *A. jacksoni* than *E. gadi*.

In an attempt to remove chitin from the inner membrane and thus have a control for the histochemical demonstration of chitin with various stains, G. B. I. (General Biochemicals Incorporated) Chitinase was used. However, since the enzyme showed no digestive qualities on G. B. I. chitin, the tests were not considered to be valid. Further work would have to be carried out to ascertain the effectiveness of this enzyme before any decisive comments could be made.

**Glycogen:** There was glycogen in evidence throughout the entire body of the acanthor with the periodic acid-Schiff routine run with salivary controls. There was peri-
odic acid-Schiff positive material still in evidence in the region of the embryonalkern even after salivary controls. There was also a rather significant localization of stained material in the embryonalkern with the acidified alcian blue stain. Von Brand (1939a, 1939b and 1940) has mentioned a polysaccharide material that was more resistant to salivary digestion than glycogen in the acanthor of *M. hirudinaceus*, *Leptorhynchoides thecatus* and *Echinorhynchus coregoni*. Bullock (1949) also made a similar observation for the region in the acanthors of *Echinorhynchus coregoni*, *E. gadi*, *Pomphorhynchus bulboccoli*, *Neoechinorhynchus cylindratus* and *N. emydis*. Von Brand has speculated that this may be due to a galactose bearing polysaccharide.

**Lipids**: Neither the oil red O technique nor the sudan black B technique showed any evidence of lipid material in the membranes. There were globules of lipid material in evidence within the body of the acanthor.

**Proteins**: With the Millon test for tyrosine all but the fibrillar coat gave positive results. The mercury bromphenol blue test for proteins showed positive results for all four membranes.

**Fibrin**: Gram-Weigert positive sphericals were found both within the body of the acanthor and at various places within the membranes. These were still in evidence after treatment of sections with hot trichloracetic acid prior to staining. Gram-Weigert positive results were obtained for both the inner membrane and the fertilization membrane.
Both these membranes still gave positive results when smears were treated with hot trichloracetic acid. With Mallory's phosphotungstic acid hematoxylin stain, which will demonstrate the presence of fibrin-like materials as blue and collagen as red, the inner membrane and the fertilization membrane gave positive indications of fibrin. With the "azan" method of Heidenhain the results also indicated the presence of fibrin in the inner membrane and the fertilization membrane.

**Elastin:** With the Taenzer-Unna orcein test the inner membrane and the fertilization membrane gave positive results. The outer membrane showed a somewhat lower reaction to the stain but it certainly gave a positive reaction. The aldehyde fuchsin reaction was positive for the inner membrane and the fertilization membrane.

**Keratin:** There was a birefringence phenomenon associated with the fibrillar coat that could be indicative of keratinous substance. This birefringence was removed upon the application of pyridine thus indicating that it was due to form birefringence rather than any crystalline order possessed by the material.

The fibrillar coat was easily removed upon the application of sodium sulphide solution. The performic acid–Schiff technique, although somewhat unsatisfactory, indicated a positive test for keratin in the fibrillar coat and the fertilization membrane. The unsatisfactory aspect of the test was in the nature of a dissolving action on the
fibrils of the fibrillar layer and a distortion of the fertilization membrane.

**Polyphenols:** There was no evidence for quinone-tanning of the membranes using the Fontana ammoniacal silver nitrate test. However, there was a positive reaction in evidence for the spines and hooks that covered the body of the acanthor of *A. jacksoni* and the larval hooks of the acanthor of *E. gadi*. There was a positive catechol reaction in the body of the acanthor which demonstrates the presence of the enzyme polyphenol oxidase. This enzyme is present wherever quinone-tanning is occurring.
TABLE I
HISTOCHEMICAL STAINING RESULTS FOR ACANTHOR MEMBRANES
OF ACANTHOCEPHALUS JACKSONI AND ECHINORHYNCHUS CADI

<table>
<thead>
<tr>
<th>Histochemical test</th>
<th>Inner</th>
<th>Fertilization</th>
<th>Fibrillar</th>
<th>Outer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Periodic acid-Schiff</td>
<td>pink</td>
<td>pink</td>
<td>pink</td>
<td>-</td>
</tr>
<tr>
<td>2. Zander</td>
<td>violet</td>
<td>violet</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. Schulze</td>
<td>brown</td>
<td>violet</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. Acid, alcian blue</td>
<td>blue</td>
<td>blue</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. Acid, alcian blue-P.A.S.</td>
<td>azure</td>
<td>azure</td>
<td>pink</td>
<td>-</td>
</tr>
<tr>
<td>6. Bethe's</td>
<td>blue</td>
<td>blue</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7. Chlorazol black E</td>
<td>green</td>
<td>green</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. Picro-nigrosin</td>
<td>green</td>
<td>green</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9. Hale-Muller</td>
<td>blue</td>
<td>blue</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10. Millon</td>
<td>purple</td>
<td>purple</td>
<td>-</td>
<td>purple</td>
</tr>
<tr>
<td>11. Hg-bromphenol blue</td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
<td>blue</td>
</tr>
<tr>
<td>12. Gram-Weigert</td>
<td>blue</td>
<td>blue</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13. Mallory's P.T.A.H.</td>
<td>blue</td>
<td>blue</td>
<td>red</td>
<td>-</td>
</tr>
<tr>
<td>14. Toluidine blue O</td>
<td>blue</td>
<td>orange-yellow</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15. Heidenhain's &quot;azan&quot;</td>
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<td>red</td>
<td>blue</td>
<td>blue</td>
</tr>
<tr>
<td>16. Fontana-Masson</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>17. Taenzer-Unna orcein</td>
<td>black</td>
<td>black</td>
<td>-</td>
<td>purple</td>
</tr>
<tr>
<td>18. Aldehyde-fuchsin</td>
<td>blue</td>
<td>blue</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19. Performic acid-Schiff</td>
<td>-</td>
<td>pink</td>
<td>pink</td>
<td>-</td>
</tr>
<tr>
<td>20. Sudan black B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21. Oil red 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- = negative reaction or no reaction.
SECTION V
DISCUSSION

There has been a controversy in the literature concerning the number of coverings that enclose the acanthors of various species of acanthocephalans. With the exception of Manter (1928), this controversy has not been acknowledged. Generally, each investigator has given only cursory consideration to these coverings and many have failed to give adequate recognition to descriptions previously made.

A most significant piece of work was done by Meyer (1928) on the membranes enclosing the acanthor of Macracanthorhynchus hirudinaceus. He clearly demonstrated the presence of four membranes for the acanthor. This work has been cited often and has been supported by the electron microscopic studies of Burnham (1957). It would seem, therefore, that this description can be used as the standard for comparison to other species. True, there are differences found among the three orders of the acanthocephalans, but it would appear that all show some modification based upon the four membranes described by Meyer.

Another problem involves the variety of names applied to each of the membranes. This is due, in part, to the use of the term "shell." The thick, outer, hard covering found around the acanthor of M. hirudinaceus has been justifiably called "shell" by most workers. It is when the term "shell" is applied to the structures surrounding the
Acanthorales of palaeacanthocephalans and eoacanthocephalans that difficulties arise. What has been referred to as the "shell" in these forms has been, in reality, the modified fertilization membrane. The layer that would correspond to the "shell" of *M. hirudinaceus* appears to have been replaced in the two species investigated by a layer of fibrous keratin-like material. In some instances this fibrous layer is so reduced, as in *A. jacksoni*, that it has led some investigators to the assumption that the few remaining strands that are in evidence are a result of the unravelling of the outer membrane.

Further confusion over the number of membranes has come about by reports that the outer membrane was absent. These reports have stated that the outer membrane was delicate and easily destroyed upon the application of various chemical reagents. Still others have overlooked or failed to describe the presence of the outer membrane when it represented a closely adherent structure to the thick "shell."

Confusion might be avoided in the future if investigators would compare their findings to the classical description of four coverings as depicted by Meyer. Particular attention should be directed to the covering that would correspond to the "shell" of *M. hirudinaceus*. The properties of this covering would appear to have the greatest degree of variation among the various species of acanthocephalans. The shape and extent of the fertilization membrane would also appear to warrant detailed
description as this is the structure that has been so often misnamed the "shell" in species that have a reduced third covering. It is this layer that gives the characteristic shape and form to the acanthors of the two species investigated in this work.

The multiple layers of material that are found surrounding the acanthor have been of interest for many years. Leuckart (1876) stated that "the obvious power of resistance of eggs is due to the unusually compact and numerous membranes." The variation that is seen among acanthocephalan "eggs" has been explained as being related to the conditions under which the "eggs" may be called upon to exist in the environment (Meyer, 1933). Others have suggested that in addition to this the "eggs" may show variation in form related to the ease of ingestion by the intermediate host.

The coverings around the acanthor have been reported to protect the larva in its environment quite adequately. They have been described as being quite resistant to many unfavorable conditions that might be encountered in the environment such as desiccation, bacterial destruction and chemical disintegration. In the case of terrestrial acanthocephalans the thickened membranes would appear to protect the acanthor from desiccation and assure its retention as a discrete particle within or on the soil where it could be ingested by terrestrial arthropods. Those acanthocephalans using an aquatic arthropod as the intermediate
host would have less restrictions placed upon the acanthor, so far as dessication is concerned, and therefore the acanthor would show a better adaptation if the development of the coverings were directed toward the problem of facilitating ingestion.

The experimental evidence of other workers (Manter, 1928; Kates, 1942) points to the fact that the outer three membranes are easily discarded when the acanthor reaches the intestine of the intermediate host. Whether or not this is due to some digestive quality of the intermediate host or whether this is related to some osmotic reaction on the part of the acanthor itself is still not decided. The evidence does indicate, however, that the inner membrane is cast off by the enlargement and activity of the acanthor. This would seem to indicate that when the acanthor is stimulated by the proper environmental conditions the acanthor will force itself out of its closely restricting membrane.

Without the advantage of precise chemical analysis for all of the materials encountered in the membranes of the acanthor, it is difficult to assign specific notations to the materials identified. It is for this reason that the suffix "-like" has been appended to so many of the materials. They have given typical histochemical results that have been observed by other investigators where they have been able to supplement their histochemical findings with chemical analysis.
Another reason for caution in using histochemical results for describing specific chemical materials is that most of the literature for such techniques is based upon observations made on vertebrate or, more specifically, on mammalian tissue. There is comparatively little in the way of precise histochemical knowledge about many of these chemical constituents of invertebrate tissues.

The presence of cellulose or similar reacting substance in the fertilization membrane of the two acanthors investigated might tend to support the contention that there is a modification of the acanthor providing structural rigidity. In this form it would be more easily ingested by the intermediate host. The occurrence of this material in the fertilization membrane of the acanthor has been suggested previously by von Brand (1940) for *M. hirudinaceus*. It is interesting to note the possible presence of this material since tunicin, a cellulose-like material, is only known to exist in the animal kingdom among the tunicates. However, the histochemical staining reactions, response to 10% sodium hydroxide and the birefringence activity after pyridine leaves little else to consider in the selection of a polysaccharide other than cellulose. It would, however, require a greater quantity of material than was available in this study to do a precise chemical analysis on the material to ascertain definitely that the material was truly cellulose.

The presence of elastin-like substances in the
fertilization membrane and the inner membrane might well serve the acanthor under conditions of stress and strain. Without the presence of some material with elastic properties the acanthor might rupture prematurely. This substance would certainly furnish the acanthor with a consistent form when found in association with other materials.

Observations made upon developing oocytes in the germ-balls of both investigated species had shown that they are surrounded by a thin proteinaceous membrane. Therefore, it was no surprise to find that the same histochemical results were obtained for the outer membrane of the acanthor since this is in reality the persistent membrane that covered the unfertilized egg. This membrane may provide the developing egg and acanthor with some degree of resistance to unfavorable environmental conditions as indicated by its general resistance to most reagents and conditions under which the eggs and acanthors were observed.

The dissolving of the fibrillar coat in sodium sulphide, a keratin solvent, coupled with the birefringence seen in this coat and the positive performic acid-Schiff reaction suggests that this layer is made up of keratin-like substance. Keratins are known to be proteinaceous substances that characteristically are high in disulphide bonds. It is this property which is utilized in dissolving the keratins. Sodium sulphide has the property of breaking the disulphide linkages that are characteristic of keratin
and keratin-like substances.

The performic acid-Schiff test was chosen (Pearse, 1961) because of its described ability to demonstrate the presence of disulphide bonding. There are a number of other histochemical tests that might be employed to demonstrate the presence of cystine, a sulphur containing amino acid that is present in great quantity in keratin.

The birefringence, which is form birefringence, is a result of the shape of the keratin-like molecules that are found in the fibrillar coat. The fact that the form birefringence could be removed upon the application of pyridine indicated that this regularity of form can be removed upon the application of a material that is capable of interposing itself between the molecular structure of keratin fibrils, a condition that would not exist if the fibrils were in a state of molecular bonding that would prohibit the intervention of a substance like pyridine.

There is reason to believe that this material is a rather unique form of keratin-like substance. The areas of the acanthor that gave positive results for keratin staining also gave some contradictory results with other histochemical routines. For instance, the fibrillar layer gave a positive periodic acid-Schiff reaction as well as a blue color with the "azan" method of Heidenhain. These results might lead one to believe that the material present was collagen. However, collagen should give a Gram-positive reaction and show some reaction to silver staining.
techniques. Neither of these results was obtained for the fibrillar coat. This layer needs a great deal more analysis both chemically and histochemically before the true nature of the material can be ascertained.

The degree of keratin-like material found in the fibrillar layer of both species has raised a question about its significance. Smyth and Clegg (1959) have made the suggestion that the keratin-like substances found in the cyclophyllideans may be responsible for retarding the desiccation of the onchosphere, the larval stage of a cyclophyllidean cestode. This may be the role that the fibrillar layer is performing in the two species investigated. However, in these cases, the desiccation may be associated with the degree of salinity in the environment in which the acanthor of *E. gadi* would find itself as opposed to the fresh-water environment for *A. jacksoni*. If the fibrillar coat imparts a protection against desiccation for the acanthor then there would be a greater advantage to having a more extensive keratin-like coating in the case of *E. gadi* than *A. jacksoni*. This may be, in part, the explanation for the differences noted between the two species.

The indication of a fibrin-like substance in the membranes of the acanthor is in agreement with the explanation of this material in the exoplasm of various platyhelminths and aschelminths by Monné (1955a). He has correlated protoplasmic clotting with its similarity to blood clotting and has related the similarity of fibrin-
like proteins in their histochemistry to the fibrinogen-fibrin staining reactions demonstrated in blood clotting. He has stated: "Thus, there is no doubt that the Gram-positive substances of the above-mentioned worms are structural proteins similar to fibrinogen-fibrin."

The development of the membranes has been demonstrated as coming from the condensation of Gram-positive sphericals. These sphericals were still demonstrated after treatment of the "eggs" with hot trichloracetic acid; a reagent that will remove nucleic acids from tissue sections (Monné, 1957). Trichloracetic acid was described by Monné as having an effect on the staining qualities of the exoplasts of various parasitic helminths. That is, the exoplasts that display a blue color with the "azan" method for collagen may become strongly Gram-positive after the application of hot trichloracetic acid. The reagent, however, does not alter the histochemical reaction for fibrin-like proteins.

After treatment with hot trichloracetic acid, typical Gram-positive results were obtained from the inner membrane and the fertilization membrane. Coupled with the results of the Mallory's phosphotungstic acid hematoxylin test for demonstrating fibrin it seems evident that these two membranes contain fibrin-like proteins similar to that described by Monné (1955a) for several platyhelminths and aschelminths as well as the acanthocephalans Polymorphus botulus.
The presence and significance of fibrin in the membranes might have its explanation in the manner in which the material is produced. As has been mentioned in the observations on the inner membrane and the fertilization membrane these membranes undergo a long period of development. As the acanthor proceeds in its development these membranes become thicker. The explanation of the mechanism associated with the build-up of fibrin from Gram-positive granules might account for the thickening of these membranes.

Chitin has been reported as being present in the inner membrane of *M. hirudinaceus* by von Brand (1940) and by Monné and Hönig (1954c) for two species of *Polymorphus*. Chitin is a polymer of N-acetylglucosamine and may exist in varying complexities of chitobiose units. Runham (1963, personal conversation) has related that the typical acid mucopolysaccharide staining reactions that are encountered with some forms of chitin may be related to the greater number of short-chain chitobiose units found in newly synthesized chitin. This would provide many more reactive end groups for histochemical staining and thus account for the frequently encountered staining of chitin with acid mucopolysaccharide stains. It would also tend to bear out the observation that the inner membrane has not always given the same staining results with the mucopolysaccharide tests. The more advanced acanthors have, for the most part, given negative mucopolysaccharide results, indicating that there has been a greater degree of polymerization at
the more advanced stages.

Chitin has been noted for its durability (Richards, 1951). The presence of this substance in the inner membrane may aid in protecting the acanthor until conditions are conducive to hatching. It is only through the effort of the acanthor itself that the larva is ultimately released from this membrane.

The fact that chitin could not be consistently demonstrated histochemically might, in part, be due to the degree of acetylation of chitin. As Runham (1961 and 1962) and Salthouse (1962) have indicated, chitin may give typical acid mucopolysaccharide staining reactions depending upon the degree of acetylation the chitin has undergone. It has been suggested that there is a greater degree of acetylation associated with the aging of chitin. Newly formed chitin has given positive acid mucopolysaccharide tests to various histochemical reagents in the newly formed radula of the mollusc *Patella vulgata* (Runham, 1961).

This variation of reactions to acid mucopolysaccharide staining procedures was in evidence for the inner membrane of both species. It was generally found to be positive in those acanthors that had not reached the stage of complete development. The fact that the fertilization membrane remained so closely adherent to the inner membrane and to the acanthor itself for such a great deal of the time made the observation quite difficult. However, with the elongation of the fertilization membrane at the poles,
it was possible to see the inner membrane before the acan­thor had completed its development.

The relationship of the various substances found in the membranes surrounding the acanthon as well as the acanthon itself raises some interesting questions about the phylogenetic relationship of these to other helminth para­sites. Hyman (1951) in attempting to draw the relationship of acanthocephalans to other forms has stated: "The general structure is rather on the aschelminthic side, whereas the embryology presents more points of resemblance with the platyhelminths."

Unlike other helminth parasites, the eggs are either released directly into the pseudocoel of the parent female or remain free-floating in the suspensory ligament. Here, development to the acanthon stage takes place entirely outside of the confines of the female genitalia indicating that little or no influence is being exercised over the formation of the enclosing membranes by the access­sory reproductive organs of the female parent. In fact, this is even more evident when one takes into consideration the retention of the larva by the germ-ball in the case of A. jacksoni.

Smyth and Clegg (1959) have shown that egg-shell formation among various cestodes and trematodes is prima­rily a function of the vitelline cells. Even though the eggs pass through specialized portions of the female geni­talia it is a question of just how much influence, if any,
the female apparatus has on the formation of the shell. Clegg (1963, personal conversation) has, however, recently demonstrated that the Mehlis' gland of *Fasciola hepatica* is in fact secreting a lipoprotein that forms a layer of the egg-shell immediately under the enclosing outer membrane. Egg-shell formation has been related primarily to the activity of the granular secretions coming from the vitellarian cells that surround the egg. The thin outer membrane surrounding these eggs is formed from a condensation of the granules originally secreted by the vitelline cells. The secondary membrane was considered to be the result of extensive granular secretion from the vitellarian cells producing the thick shell of the egg. Clegg's recent observations do modify this concept, however, in that he now provides some evidence to indicate that there is a contribution made to the egg membranes by the Mehlis' gland. Rogers (1962) states that in addition to the regular membranes that are present, some cestodes possess a "tertiary membrane" in that a part of the uterus or the whole proglottid may serve to further enclose and protect the egg until it reaches the intermediate host. Rogers also indicates that the parasitic platyhelminths possess eggs whose membranes lack chitin and seem to be composed chiefly of a quinone-tanned protein.

Parasitic platyhelminths may have operculate or non-operculate eggs. The raphe found characteristically in the shelled acanthur of *Macracanthorhynchus hirudinaceus* where
the two halves of the thick shell meet is a site of acan­
thor hatching and might be compared, at least in function, to the opercular apparatus of operculate platyhelminths. Other than the fact that the acanthor seems to resemble the onchosphere in that it possesses a rostellum armed with hooks, there is little to support the taxonomic affinity of the acanthocephalan larva to the platyhelminth larva. The presence of chitin in the membranes of acanthors and the absence of quinone-tanned proteins in the membranes of these larva would tend to refute any affinity of the acanthocephalan larva with the platyhelminth larva.

Evidently, there has been as much confusion over the number and nature of the membranes surrounding the "eggs" of parasitic nematodes as there has been for the acanthor. Rogers (1962) has characterized the nematode "egg" as being surrounded by four layers that are formed by the activity of the egg itself. He has also indicated that layers may be missing or fused with one another. The innermost layer is described as being composed of lipid material. This layer is surrounded by two or three layers of material that are composed of substances with composition and optical properties different from those of the inner layer.

The outermost layer is the thickened fertilization membrane which may be separate and distinct in some species or simply the outermost and thickest part of the primary covering. This layer has been reported by many investi-
gators to be formed of protein.

Below the fertilization membrane is found a thickened layer which usually possesses the chitin of the "egg-shell." Between the inner lipid layer and the layer of chitin another hard layer has been observed and has been termed the "Yanagisawa's layer" (Rogers, 1962). This layer has been formed from materials that resemble the "hyalin spheres identified in Parascaris equorum" (Rogers, 1962).

In addition to the layers that have been described above for the parasitic nematode "eggs" there are secretions from the cells lining the walls of the uterus that produce a "tertiary membrane" in some nematodes. In some it may represent the thickest portion of the egg covering. Monné and Höög (1955) identified this material in Ascaris lumbricoides as consisting of "protein and a mucopolysaccharide provided with sulphuric acid residues."

It would seem that if phylogenetic affinities are to be made then the description of the materials and arrangement of the membranes of the acanthor would more nearly be related to the parasitic nematodes than to the parasitic platyhelminths. However, when Rogers (1962) makes the point that "the egg-shells of the parasitic platyhelminths are often more like those in the Insecta (Wardle and McLeod, 1952, pp. 47, 51)," this author hesitates to speculate about the phylogenetic relationships of the acanthor to any other member of the animal kingdom. In fact, it is difficult to explain the similarities and
differences that are to be found between and within the three orders of the Acanthocephala.
SECTION VI

CONCLUSIONS

The acanthors of Echinorhynchus gadi (Zoega) Müller and Acanthocephalus jacksoni Bullock possess four enclosing membranes. The outermost membrane is of a proteinaceous material and represents the persistent structure that surrounded the unfertilized egg.

The most prominent covering in both acanthors studied was the second innermost membrane, the fertilization membrane. This membrane forms the characteristic "shell" of the palaeacanthocephalan "egg" to which parasitologists so often refer. It is the second covering to appear and one of the last to complete its development. It is composed of cellulose-like, keratin-like and elastin-like substances.

Between the outer membrane and the fertilization membrane a fibrillar coat was found. This was the most distinguishing feature of the two species investigated. It was much more pronounced and obvious in E. gadi than A. jacksoni. It not only demonstrated a variation in content but also a variation in form between the two species. This coat was found to consist of a keratin-like material.

The acanthor itself is surrounded by an inner fourth membrane that appears to be the last covering to develop as well as being one of the thinnest of the coverings. This membrane consists of chitin and elastin-like
substances.

Analytical tests performed on the membranes indicated the presence of a variety of materials within the various membranes. In each instance the verification of membrane substances was attempted by utilizing several histochemical methods, chemical solvents and polarizing microscopy. Even at that, the best that can be stated is that all of these tests gave suggestions of materials that were present. The exact identification must wait upon more specific biochemical analyses. This will have to be in the nature of microbiological analysis as the amount of material available from either of these two species imposes the greatest limitations upon this type of analysis.

One of the most interesting and yet unexpected highlights of this work has been the demonstration of the larval hooks and spines with the Fontana ammoniacal silver nitrate stain. The differences noted between the two acanthors would seem to offer a possibility that future pursuit of this study would be most gratifying. There may be a significant taxonomic value to descriptions of the acanthor hook and spine pattern.

Another point that was realized as a result of this investigation was the variation in the time of expulsion of the developing acanthor from the germ-ball. It would be interesting to determine if other species of Acanthocephala have a prolonged period of retention of the acanthor as was experienced with *A. jacksoni*. 
As had been stated at the outset of this work, this problem was approached from the aspect of its potential value in understanding and following embryological processes taking place in the transition from acanthor to acanthella stages.
SECTION VII

FIGURES

Explanation of Figures

The drawing of the apparatus (Fig. 1) is a visual representation of the equipment that was assembled from a description by Campbell (1929). The drawings of the authors of *Acanthocephalus jacksoni* Bullock (Fig. 2) and *Echinorhynchus gadi* (Zoega) Müller (Fig. 3) were prepared with the aid of a camera lucida.

Figures 8 and 10 are photomicrographs taken with a Graphlex camera using 3" x 4" Kodak Panatomic X film. The remaining photomicrographs were taken with an Exakta VX 35 mm. camera using Kodak Plus-X film.
Figure 1. Apparatus for obtaining alkali-resistant materials from acanthocephalan "eggs".
Figure 2. Acanthor of *Acanthocephalus jacksoni* Bullock.
Figure 3. Acanthor of *Echinorhynchus gadi* (Zoega) Muller.
Figures 4 and 5.

Figure 4. A photomicrograph of a shelled acanthor of *Acanthocephalus jacksoni* Bullock. The surface of the acanthor shows some of its spines. The inner membrane is in close proximity to the acanthor. The fertilization membrane is the prominent structure with polar elongations. The fibrillar coat is more evident in the vicinity of the polar elongations and can be seen wrapping itself around the fertilization membrane. The thin outer membrane encloses the entire acanthor. Fresh, unfixed material. X 1,500.

Figure 5. A photomicrograph of a germ-ball of *Acanthocephalus jacksoni* Bullock. A thin enveloping membrane surrounds the entire germ-ball. Developing acanthors are retained within the germ-balls with a prevalence of them located at the periphery of the germ-ball. Each developing acanthor is enclosed in a membrane that will persist as the outer membrane of the shelled acanthor. The dark region surrounding the acanthor is the fertilization membrane. Fresh, unfixed material. X 1,500.
Figures 6 and 7.

Figure 6. A photomicrograph of a shelled acanthor of *Echinorhynchus gadi* (Zoega) Müller. Three hooks are in evidence with the body of the acanthor noticeably devoid of spines. The inner membrane appears as a thin structure. Polar elongations appear as extensions of the fertilization membrane. The fibrillar coat covers the fertilization membrane and is composed of many fibrils. The thin outer membrane encloses the entire acanthor. Fresh, unfixed material. X 1,300.

Figure 7. A photomicrograph of a germ-ball of *Echinorhynchus gadi* (Zoega) Müller. A thin enveloping membrane surrounds the entire germ-ball. The two prominent objects within the body of the germ-ball are oocytes. Each oocyte is enclosed in a thin membrane which continues as the persistent outer membrane of the acanthor. A fertilized egg can be seen emerging from the germ-ball. Fresh, unfixed material. X 650.
Figures 8 and 9.

Figure 8. A photomicrograph of an *Acanthocephalus jacksoni* Bullock germ-ball. The retention of an advanced larval form is shown within the germ-ball. All four membranes are in evidence. The elongation of the fertilization has commenced at one of the axial poles. The fibrillar coat is more pronounced in the region of the polar elongation. The thin, dark structure surrounding the entire acanthor is the outer membrane. A membrane surrounds the entire germ-ball. Fixed in Bouin's; paraffin sections of 7 micra through a transverse section of the pseudocoel of an adult female. Stained with PAS. X 1,800.

Figure 9. A photomicrograph of a developing acanthor of *Echinorhynchus gadi* (Zoega) Müller. The fertilization membrane is elongated at the axial poles. The fibrillar coat is in evidence prior to the completion of the fertilization membrane. The acanthor develops within the pseudocoel of the female parent. Fresh, unfixed material. X 1,500.
Figures 10 and 11.

Figure 10. A photomicrograph of chitosan "shells" obtained by treating adult female acanthocephalans with concentrated potassium hydroxide at a temperature of 160°C. for a period of 15 minutes. X 1,200.

Figure 11. A photomicrograph of a shelled acanthor of *Acanthocephalus jacksoni* Bullock after treatment with a 10% solution of sodium hydroxide. The fibrillar coat has been dissolved whereas the other three membranes are still in evidence. The inner membrane has become tightly adherent to the body of the acanthor. The fertilization membrane has swelled with the outer membrane in closer proximity to the fertilization membrane. Fresh, unfixed material. X 1,000.
Figures 12 and 13.

Figure 12. A photomicrograph of a shelled acanthor of *Acanthocephalus jacksoni* Bullock taken with the nicols of the polarizing microscope crossed. Birefringence is in evidence in each of the membranes. Strong birefringence is experienced in the nuclei of the embryonalkern. Fresh, unfixed material. X 1,500.

Figure 13. A photomicrograph of a shelled acanthor of *Echinorhynchus gadi* (Zoega) Müller taken with the nicols of the polarizing microscope crossed. Birefringence is in evidence in each of the membranes. The fibrillar coat shows extensive birefringence. Strong birefringence is found in the nuclei of the embryonalkern. The larval retractor muscles are birefringent. Fresh, unfixed material. X 1,500.
Figures 14 and 15.

Figure 14. A photomicrograph of a shelled acanthor of *Acanthocephalus jacksoni* Bullock after the sample was treated with pyridine and observed with the nicols of the polarizing microscope crossed. Most of the birefringence has been removed from the membranes. A thin portion of the fertilization membrane shows intrinsic birefringence. Some birefringence is also in evidence in the inner membrane. The nuclei of the embryonal-kern still retains its birefringence. Fresh, unfixed material. X 1,500.

Figure 15. A photomicrograph of a shelled acanthor of *Echinorhynchus gadi* (Zoega) Müller after the sample was treated with pyridine and observed with the nicols of the polarizing microscope crossed. Intrinsic birefringence is in evidence in the fertilization membrane and to a lesser degree in the inner membrane. The nuclei of the embryonal-kern still retains its birefringence. The fibrillar coat has been most effected by the pyridine. Fresh, unfixed material. X 1,500.


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APPENDIX

FORMULARY OF STAINS AND PROCEDURES

Bouin's Fixative

Glacial acetic acid
Saturated, aqueous picric acid
40% formaldehyde

5.0 ml.
75.0 ml.
25.0 ml.

Demke's Fixative

40% formaldehyde
Glacial acetic acid
Glycerine
Ethyl alcohol
Distilled water

5.0 ml.
5.0 ml.
10.0 ml.
24.0 ml.
46.0 ml.

Helly's Fixative

Distilled water
Mercuric chloride
Potassium dichromate
40% formaldehyde
Anhydrous sodium sulfate

100.0 ml.
5.0 g.
2.5 g.
5.0 ml.
1.0 g.

Neutral Buffered Formalin

40% formaldehyde
Distilled water
Acid sodium phosphate, monohydrate
Anhydrous disodium phosphate

100.0 ml.
900.0 ml.
4.0 g.
6.5 g.

10% Formalin

40% formaldehyde
Tap water

100.0 ml.
900.0 ml.

Ringer's Solution

Sodium chloride
Potassium chloride
Calcium chloride
Distilled water

8.5 g.
250.0 mg.
300.0 mg.
1000.0 ml.

Zenker's Fixative

Distilled water
Glacial acetic acid
Mercuric chloride
Potassium dichromate
Anhydrous sodium sulfate

100.0 ml.
5.0 ml.
5.0 g.
2.5 g.
1.0 g.
Lugol’s Solution

Iodine 4.0 g.
Potassium iodide 6.0 g.
Distilled water 100.0 ml.

Schiff Reagent (Lillie, 1954, p. 155)

Dissolve 1.0 g. of basic fuchsin in 100.0 ml. hot (90-95°C.) distilled water.
Filter at 50-60°C. as it cools.
Cool and add 2.0 g. sodium bisulfite and 20.0 ml. N HCl.
Stopper tightly and store in the dark overnight at room temperature.
Add 300.0 mg. finely powdered charcoal.
Shake one minute and then filter.
Store in the refrigerator.

Sodium Sulfide Solution (Goddard and Michaelis, 1934)

Sodium sulfide 3.9 g.
Distilled water 96.1 ml.

Ehrlich’s Acid Hematoxylin (Cavanaugh, 1956, p. 18)

Hematoxylin 2.0 g.
Ethyl alcohol, 100% 100.0 ml.
Glacial acetic acid 10.0 ml.
Glycerine 100.0 ml.
Distilled water 100.0 ml.
Aluminum ammonium sulfate 10.0 g.
Let mixture ripen in the light until dark red.

Hematoxylin and Eosin (Bullock’s Procedure)

Procedure: Various fixatives for smears and sections.

1. Bring to water; I₂ in 85% alc. if necessary
2. Ehrlich’s hematoxylin 1:9 20 min.
3. Wash in running water 20 min.
4. Dehydrate
5. Counterstain in 0.1% alcoholic eosin 15-30 sec.
6. Complete dehydration, clear and mount.

Feulgen Reaction (Bullock’s Procedure)

Procedure: Various fixatives (not Bouin’s) on smears and sections.

1. Bring to water.
2. 10% perchloric acid at room temp. 12-24 hrs.
3. Drain, put in Schiff reagent (room temp.) 20 min.
4. Wash in three two-minute baths of 0.5% Na bisulfite (7.5 ml. of 10% Na bisulfite in 150 ml. water).
5. Wash in running water. 5-10 min.
6. Dehydrate, counterstaining in alcoholic fast green at 95% (0.5 ml. of 0.5% alc. fast green in 50 ml. of 95% alcohol). 2-3 min.

Periodic Acid-Schiff (Bullock's Procedure)

Procedure: Various fixatives on smears and sections.

<table>
<thead>
<tr>
<th>Experimentals</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>1. Bring to water.</td>
<td>1. Bring to water.</td>
</tr>
<tr>
<td>2. Hydrolyze 10 min. in 1.0 g. Na periodate 0.5 ml. conc. HNO₃ 100 ml. dist. H₂O₂</td>
<td>2. Saliva, strained through cheesecloth, 1 hour.</td>
</tr>
<tr>
<td>3. Rinse in running water for 5 minutes.</td>
<td>3. Wash in running water for 5 minutes.</td>
</tr>
<tr>
<td>4. Schiff reagent, 15 minutes.</td>
<td>4. Proceed as for experimentals beginning with step 2.</td>
</tr>
<tr>
<td>5. 3 changes of sodium-bisulfite (7.5 ml. of 10% in 150 ml. total).</td>
<td></td>
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<tr>
<td>6. Running water, 10 minutes.</td>
<td></td>
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<tr>
<td>7. Ehrlich's hematoxylin 1:9 for 20 minutes.</td>
<td></td>
</tr>
<tr>
<td>8. Running water, 30 minutes.</td>
<td></td>
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<tr>
<td>9. Dehydrate, clear and mount.</td>
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</tbody>
</table>

Picro-nigrosin Stain (Lee, 1946, p. 548)

Procedure: N.B.F. fixed paraffin sections.

1. Xylol to water.
2. Stain for 6 minutes. Saturated, aqueous solution of nigrosin 1 part Saturated, aqueous solution of picric acid 9 parts
3. Wash in tap water.
4. Rinse rapidly in 70% and 90% alcohol
5. Dehydrate, clear and mount.

Bethe's Stain (Lee, 1946, p. 600)

Procedure: N.B.F. fixed paraffin sections.
1. Xylool to water
2. Freshly prepared 10% solution of aniline hydrochloride with one drop of HCl for each 10 ml. 4 min.
3. Wash in running water 5 min.
4. 10% solution of potassium bichromate 4 min.
5. Wash in running tap water 5 min.
6. Dehydrate, clear and mount.

Acidified Alcian Blue Stain (Steedman, 1950; Mowry, 1956)

Procedure: Bouin's fixed sections and smears.
1. Bring to water
2. Stain in 0.1% alcian blue in 3% acetic acid (pH 2.5-2.6) 30 min.
3. Rinse in distilled water to remove excess stain
4. Stain in hemalum 5 min.
5. Dehydrate, clear and mount.

Chlorazol Black E Stain (Lillie, 1954, p. 279)

Procedure: Bouin's fixed paraffin sections.
1. Xylool to water
2. Water to 70% alcohol
3. Stain in 1% chlorazol black E in 70% alc. 30 min.
4. Drain and into 80% alcohol
5. Dehydrate, clear and mount.

Acidified Alcian Blue-Periodic Acid Schiff (Ambrogi, 1960, p. 142)

Procedure: Formalin and Zenker fixed smears.
1. Xylool to water
2. Stain in acidified alcian blue 30 min.
3. Rinse in distilled water
4. Periodic acid-Schiff routine
5. Dehydrate, clear and mount.

Hale-Muller Reaction (Pearse, 1961, pp. 256-259)

Procedure: Unfixed smears or formalin fixed paraffin sections.
1. Bring to water
2. "Ferrihydrozydsol" 10 min.
   12 ml. 32% FeCl₃
   750 ml. boiling distilled water
3. Wash well with distilled water
4. Flood with acid ferrocyanide solution 10 min.
   Equal parts of: 0.02 M K₄Fe(CN)₆
   0.14 M HCl

5. Wash in water

6. Counterstain with 1% aqueous neutral red 1 min.

7. Dehydrate, clear and mount.

Gram-Weigert Stain for Fibrin (Monné, 1955a)

Procedure: Bouin's fixed smears and sections.

1. Bring to water

2. Aniline crystal violet 3 min.
   1.2 g. crystal violet
   12 ml. of 95% alcohol
   100 ml. 2% aniline in water

3. Drain and blot with filter paper

   1 g. iodine
   2 g. potassium iodide
   100 ml. distilled water

5. Wash in water

6. Wipe back and sides of slide and blot

7. Immerse in 50:50 aniline xylene mixture, 2-3 changes, blotting between each dip until no more purple washes out.

8. Blot and rinse in several changes of xylene


Fontana-Masson Stain (Ambrogi, 1960, p. 103)

Procedure: Formalin fixed smears and sections.

1. Bring to water

2. Immerse slide in silver nitrate solution for one hour at 56°C.
   Dissolve 10 g. silver nitrate in 100 ml. water
   To 95 ml. add ammonium hydroxide until a clear solution with no precipitate is obtained. Add, drop by drop, enough of the remaining 5 ml. of silver nitrate solution until a slightly cloudy solution is produced. Let stand overnight before using. When ready to use, dilute each 25 ml. of solution with 75 ml. of distilled water and filter.

3. Rinse in distilled water

4. Immerse in gold chloride solution 10 min.
   1% gold chloride solution, 10 ml.
   Distilled water, 40 ml.

5. Rinse in distilled water

6. Sodium thiosulfate solution 5 min.
   Sodium thiosulfate, 5 g.
   Distilled water, 100 ml.

7. Rinse in distilled water
8. No counterstain used (eosin or nuclear fast red recommended)
9. Rinse in distilled water, dehydrate, clear and mount.

Zander's Stain (Glick, 1949, p. 54)

Procedure: Fresh smears or paraffin sections with various fixatives.

1. Bring sections to water
2. Few drops of Lugol's solution 1 min.
3. Rinse in distilled water
4. Few drops of strong ZnCl₂ solution 1 min.
5. Rinse in distilled water and observe

Schulze's Stain (Glick, 1949, p. 54)

Procedure: Fresh smears or paraffin sections with various fixatives.

1. Bring sections to water
2. Few drops of Lugol's solution 1 min.
3. Rinse in distilled water
4. Few drops of conc. sulphuric acid 1 min.
5. Rinse in distilled water and observe

Catechol Method (Johri and Smyth, 1955)

Procedure: Fix preferably in 70% alcohol.

1. Wash in water 30 min.
2. Place in 0.1% catechol (freshly prepared) at 40°C. 60-90 min.
3. Wash in water 15 min.
4. Dehydrate, clear and mount.

Millon Reaction (Pearse, 1961, pp. 791-792)

Procedure: Fresh material or formalin fixed paraffin sections.

1. Bring sections through 50% alc. to water
2. Place sections in a small beaker containing
   Million reagent and boil gently
   Million reagent:
   400 ml. conc. nitric acid (sp. gr. 1.42)
   600 ml. distilled water
   Let stand for 48 hours
   Dilute 1:9 with distilled water
   Saturate with crystalline mercuric nitrate
   Filter and add 400 ml. of the filtrate 3 ml.
   of the original diluted nitric acid and 1.4 g. of sodium nitrite
3. Stop heating and allow solution to come to room temperature.
4. Remove sections and wash three times in distilled water (two minutes each wash).
5. Dehydrate, clear and mount.

Mercury-Bromphenol Blue Method (Pearse, 1961, p. 792)

Procedure: Fresh material or formalin fixed paraffin sections.

1. Bring paraffin sections to water.
2. Stain in 1% HgCl₂ and 0.05% bromphenol blue in 2% aqueous acetic acid for two hours at room temperature.
3. Rinse sections for five minutes in 0.5% acetic acid.
4. Transfer sections directly into tertiary butyl alcohol.
5. Clear in xylene and mount.

Taenzer-Unna Orcein: Romeis's Method (Lillie, 1954, pp. 360-361)

Procedure: Bouin’s fixed smears or paraffin sections.

1. Bring to 70% alcohol.
2. Stain one hour in 1% orcein, 1% HCl, 70% alcohol.
3. Wash thoroughly in two changes distilled water.
4. Stain heavily with Ehrlich’s acid hematoxylin.
5. Wash ten minutes in distilled, tap and distilled water.
6. Stain in 60 ml. 0.1% acid fuchsln in saturated aqueous picric acid solution to which is added 0.25-0.3 ml. 2% acetic acid.
7. Rinse in 60 ml. distilled water containing 2.5 ml. of the acidified picrofuchsln solution above for not more than 2-4 seconds.
8. Blot dry.
9. 95% alcohol for one minute.
10. 100% alcohol for three minutes, clear and mount.

Sudan Black B (Pearse, 1961, p. 850)

Procedure: Fresh smears.

1. Bring smears to 70% alcohol.
2. Stain for 30 minutes at room temperature in saturated sudan black B in 70% alcohol.
3. Rinse quickly in 70% alcohol to remove excess dye.
4. Wash in running water.
5. Counterstain in 1% aq. neutral red for one min.
6. Wash in water and mount in glycerine jelly.


**Procedure**: Fresh smears.

1. Fresh water to 60% isopropanol.
2. Oil red O solution for ten minutes.
   - 0.5 g. of oil red O
   - 100 ml. of 99% isopropanol
   - 6 ml. of above solution and add 4 ml.
     distilled water. Let stand for ten minutes and filter.
3. Differentiate briefly in 60% isopropanol.
4. Wash in water.
5. Stain for five minutes in Mayer's haemalum.
6. Wash in running water for at least ten minutes.
7. Mount in glycerine jelly.

**Aldehyde Fuchsin Stain: Rosa** (Pearse, 1961, pp. 815-816)

**Procedure**: Formaldin and Bouin's fixed smears and sections.

1. Bring to water.
2. Oxidize sections in Lugol's iodine for ten minutes to one hour.
3. Remove iodine with 5% thiosulfate for one min.
4. Immerse in aldehyde-fuchsin five minutes to one hour.
   - 1 ml. conc. HCl
   - 1 ml. paraldehyde
   - 100 ml. of a 0.5% basic fuchsin in 70% alcohol.
   - Allow to ripen at room temperature for three days.
   - Add 100 ml. of the mixture to 50 ml. chloroform in a separatory funnel and add 200 ml. dist.
     water.
   - Shake briefly and allow ppt. to settle.
   - Drain off ppt. and filter without suction.
   - Dry at 50°C. and store in stoppered bottle.
5. Rinse in several changes of 70% alcohol.
6. Dehydrate, clear and mount.

**Performic Acid-Schiff: Pearse** (Pearse, 1961, p.805)

**Procedure**: Helly's fixed smears.

1. Bring smears to water and remove mercury ppt.
2. Treat with performic acid solution for 10-30 min.
   - 40 ml. of 98% formic acid
   - 4 ml. of 30% (100 vol) H₂O₂
   - 0.5 ml. of conc. H₂SO₄
Allow to stand for at least one hour but use within 24 hours.

3. Wash in water for 2-5 minutes.
4. Immerse in Schiff solution for 30-60 minutes.
5. Wash in warm running water for ten minutes.
6. Dehydrate, clear and mount.

Toluidine Blue O: Kramer and Windrum (Pearse, 1961, p. 834)

Procedure: N.B.F. fixed smears.

1. Bring to water.
2. Stain in 0.1% toluidine blue in 30% ethanol for 5-20 minutes.
3. Rinse in 95% alcohol.
4. Dehydrate in absolute alcohol.
5. Clear and mount.


Procedure: Zenker fixed smears.

1. Bring to water and remove mercury salts with iodine and remove the iodine with alcohol, not thiosulfate.
2. Postchromo for 30 minutes in a mixture of three parts of 3% aqueous K$_2$Cr$_2$O$_7$ and one part of 10% HCl.
3. Wash in water.
4. Differentiate for one minute in acid permanganate.
5. Wash in water.
6. Bleach in 1% oxalic acid until white.
7. Rinse in water and transfer to P.T.A.H. for 12-24 hours.
   Hematoxylin, 0.1 g.
   Phosphotungstic acid, 2.0 g.
   Distilled water, 100 ml.
   Dissolve separately and mix.
   Leave to ripen for several months.
8. Shake off excess stain.
10. Clear and mount.

Azan Method (Lillie, 1954, p. 351)

Procedure: Bouin's fixed smears.

1. Bring to water.
2. Stain 30-60 minutes in a covered dish at 50-55°C. and then 1-2 hours at 37°C. in azo-carmine B.
   0.25-1.0 g. azo-carmine B (C.I. No. 829)
   100 ml. cold water (distilled)
   1 ml. glacial acetic acid
3. Wash in distilled water.
4. Differentiate in 0.1% aniline in 95% alcohol and rinse in 1% acetic acid in 95% alcohol.
5. Mordant 30 minutes to three hours in 5% phosphotungstic acid.
6. Rinse in distilled water.
7. Stain 1-3 hours in 25% dilution of a stock solution of aniline blue 0.5 g., orange G 2 g., glacial acetic acid 8 ml., distilled water 100 ml.
8. Rinse in water.
9. Dehydrate and differentiate in 95% alcohol; then 100% alcohol.
10. Clear and mount.