AN EXAMINATION OF TAPEWORM HISTOLOGICAL AND TOTO-MOUNT TECHNIQUE

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by

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ABSTRACT

Comparative tests to determine suitable methods of relaxing, fixing, staining, destaining, and clearing tapeworms for toto-mount examination were carried out. A new triple stain technique for histological sections of tapeworms was established and described.

Four relaxing agents were utilized to determine their effectiveness in preventing tapeworms from contracting in formalin solution. Visual observations with the aid of a binocular microscope were made to determine the compatibilites of seventeen stains with Dibothriocephalus latus (Linnaeus, 1758) specimens fixed with sixteen different fixatives. Photography was used to the greatest possible extent in illustrating the effects of the fixative-stain combinations. Four groups of clearing agents involving the essential oils, synthetic clearers, higher alcohols, and mixtures were investigated for possible use with tapeworms. Coelestin blue B, eosin B, and light green were the essential components of the histological triple stain for tapeworms.

The investigation showed that menthol, as an anesthetizing agent, permitted the least contraction of tapeworms in formalin solution. Most of the haematoxylin stains and one of the aniline stains gave superior results with tapeworms especially after mercuric chloride fixatives.

Methyl salicylate produced the greatest optical clearing in tapeworms. The use of the triple stain provided a rapid and effective method of preparing tapeworm sections.

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CHAPTER I

INTRODUCTION

One of the problems encountered by helminthologists in tapeworm study is the microtechnical preparation of specimens for observations. Trematodes present relatively few difficulties in preparation as compared with cestodes where one must often contend with thick cuticular and muscular layers.

It has been the purpose of this investigation to determine suitable methods of relaxing, fixing, staining, and clearing tapeworms for toto-mount examination. In addition, a limited number of special techniques have been included in the investigation along with a detailed description of a triple stain developed for histological sections of tapeworms. Most phases of the problem were studied by a comparison of techniques, the evaluation of which indicated the most suitable methods of tapeworm preparation.

Despite the number and complexity of microscopic techniques now found in the literature, very little has been written on tapeworm techniques. This lack of information indicated the necessity of a detailed inquiry into the subject. Often a tapeworm species must be identified by the use of but a few specimens. It becomes obvious that important material cannot be wasted in trial and error preparation. This can be obviated by handling the material

in the proper way the first time. There can be no doubt that incorrect descriptions have been made because of inadequacies in technique. Thus, by determining ideal combinations of fixatives and staining compounds, greater accuracy can be attained.

The investigation has been discussed in a logical manner beginning with a comparison of four methods of relaxing tapeworms. This was followed by a presentation of the results of using seventeen staining compounds on specimens of the tapeworm, Dibothriocephalus latus, which had been placed in sixteen fixing solutions. The effects of a wide range of clearing agents on tapeworms were then discussed. A section on special techniques completed the toto-mount experimentation. Finally, a detailed description of the use of a triple stain for tapeworm sections terminated the write-up of the experimental work.

CHAPTER II

HISTORICAL

Looss (1901) dealt extensively with a method of preliminary handling of helminths. This technique came to be known as Looss' shake method and involved the use of sodium chloride and corrosive sublimate for relaxing and fixing specimens. His method has been widely used but several workers have indicated that his use of salt solution was superfluous. Newer methods have generally replaced his technique.

Baylis (1922), in his paper on helminthological techniques, included a section on tapeworm proceedure. He recommended several mechanical methods of preventing contraction during fixation, such as stretching on a glass plate, dipping suspended specimens, and winding long specimens around a glass jar, then immersing the whole into the fixative. These methods are still in use but are not fully satisfactory. Worms possessing powerful longitudinal musculature invariably contract on contact with irritating fixing fluids. His dipping method proves time consuming when large amounts of material must be prepared. The writer concurs with Baylis' opinions on types of fixing solution to use. He maintained that Bouin's fluid was superior to Zenker's fluid for cytological purposes only.

He recommended a solution roughly akin to Schaudinn's fluid for tapeworm wholemounts. In addition, he concluded that, "Formalin may be used in case of necessity, but is not satisfactory, as specimens fixed in it are often difficult to stain."

Meggitt (1924) furthered the work of Baylis (1922) by describing detailed methods of removing cestodes from the host intestine and of the use of lactophenol for rapid examination of the scolex. He disagreed with Looss (1901) stating that a salt solution and cold water should never be used in the preliminary preparation of helminths. In the use of fixatives he stated:

> All fixatives with the exception of alcohol should be used cold. Zenker's fluid is undoubtedly the best but must not be allowed to act for more than 24 hours and subsequent prolonged washing (24 hours) in running water is necessary.

Meggitt rejected the popular belief that metallic instruments cannot be used with corrosive sublimate. The writer found no ill effects imparted to specimens, provided immersion time was kept to a minimum and instruments were wiped frequently. Meggitt recommended the use of haematoxylin, either Delafield's or Ehrlich's, as being a superior permanent wholemount stain.

Wardle (1932) presented one of the most complete analysis of tapeworm techniques. He supported the use of salt solution to which he added 5 per cent of egg white, pointing out that muscular contraction followed by

disintegration occurred if fresh water was used. He recommended relaxing specimens by painting them with a charged brush of hot water (60°C). His method does not allow for rapid handling of quantities of specimens. He pointed out that alum - cochineal stain worked successfully on a specific type of worm, while the same stain was unsuitable with other types. Wardle described histological staining methods, one of them including safranin, a stain which on this continent has been generally restricted to plant material.

Mendheim (1947) extensively critized Looss' (1901) shake method but added that the technique could not be dispensed with in certain cases. He pointed out that fragility of specimens resulted from corrosive sublimate fixation. The use of Lugol's solution (iodine and potassium iodide in 70 per cent alcohol) was suggested for removing the remaining traces of formalin from tissues. Mendheim staunchly supported the regressive staining method with painstaking destaining, believing it to be better than progressive staining.

Riser (1950) recommended the use of coelestin blue B for toto-mount and histological staining. With regard to fixatives, he stated that, "Bouin's fluid is not recommended for the preparation of wholemounts of flatworms at any time." This is in agreement with Baylis (1922) but at variance with Southwell (1930).

Abdel - Malek (1953) utilized menthol for relaxation of tapeworms. His technique dispensed with excessive handling and allows of treating quantities of material rapidly. He suggested that the menthol be dissolved in either salt solution or clear water according to the choice of the individual but added that the former soltion required a longer time period.

Smyth (1951) demonstrated the affinity of eggshell material, an orthodihydroxyphenol - protein complex, for malachite and methyl green - pyronin, thus providing a histological stain for the vitellarian system and ova of certain helminth groups. These stains only showed effective staining of ova in wholemount preparations. Smyth (1954) delved further into the histochemistry of the internal structures of helminths by using a catechol solution on wholemounts to show by tanning, the presence of an enzyme, polyphenol oxidase, in quinone - tanning female genitalia systems. The writer maintains a comparable method, via chemical affinites, can be used to demonstrate testicular sites of tapeworms.

Additional workers as Mayhew (1925), Hunter (1927), Becker and Roudabush (1935), and Demke (1951) added only minor innovations to tapeworm technique.

CHAPTER III

MATERIALS AND METHODS

All tapeworm material used was obtained locally. Triaenophorus nodulosus (Pallus 1760) was removed from the intestinal tract of Northern pike, Esox lucius, in a living state. The intestinal tract was slit lengthwise with dull-pointed scissors and the worms removed in small groups to a beaker containing a minimum of lukewarm water in which was dissolved a small amount of detergent. By directing a heavy stream of water down the side of the beaker intestinal debris and mucous was separated from the worms and decanted off. The process was repeated if necessary. Within three minutes of removal from the host gut, worms could be placed in physiological saline for future treatment.

Dibothriocephalus latus (Linnaeus 1758) was collected from two young mongrel dogs. Thirteen worms totaling over sixty feet were fixed in 16 different solutions for comparative staining tests. Six haematoxylin, six carmine, and five synthetic nuclear stains were compounded. A total of 272 fixative - stain combinations was tried. Syracuse dishes were used as dehydrating and staining receptacles. A small portable timing clock with attached bell greatly facilitated the task of recording time lapses of the various staining stages. The majority of the specimens were

dehydrated in ethyl alcohol, cleared in Beechwood creosote, and mounted in xylol - based Permount. The photography was done with a 35 mm. reflex camera. Unfortunately, many photographs were made useless due to the limitations of black and white prints.

CHAPTER IV

RELAXATION OF TAPEWORMS

Two chemical relaxing agents, menthol and chloretone, and two physical relaxing agents, cold and hot water were used for comparative tests on T. nodulosus. The worm was well suited to the tests in view of its extensive musculature.

Abdel - Malek (1953) recommended using one - half gram of menthol crystals to one hundred cc. of warm water. Another method suggested was 24 grams of menthol dissolved in ten cc. of 95 per cent ethyl alcohol. One drop of this solution saturates one hundred cc. of water. The writer used the latter method but raised the number of drops to 3 since fifty worms were being anaesthetized at a time. The menthol solution was allowed to act for 45 to 75 minutes while the worms were being individually measured. A 5 per cent formalin solution was used as the control fixative into which the worms were transferred after measurement to remain a minimum of twelve hours. Measurements were again taken and a percentage figure for the average contraction was computed. Data recorded on menthol relaxation appear in TABLE I.

Chloretone was proposed by Hargis (1953) as an anaesthetizing agent for helminths. He stated that both the quantity of chloretone and time of actioncouldbe varied

according to the size of the specimen. The writer used two grams of chloretone to five hundred cc. of water. The specimens were immersed in this solution for 45 to 75 minutes with intermittent shaking. The worms were measured, fixed in 5 per cent formalin for twelve hours and remeasured. Average contraction was computed and recorded in TABLE II.

Becker and Roudabush (1935) and Chandler (1955) recommended the use of cold water (not salt solution) for relaxing tapeworms. Water is absorbed by the worm thus preventing contraction during fixation. Fifty specimens were placed in a refrigerator for two hours until muscular contractions ceased. Measurements were taken of specimens while they lay on a glass plate wet with water. They were then fixed in 5 per cent formalin for a minimum of twelve hours and remeasured. Results of cold water relaxation are contained in TABLE III.

Fifty tapeworms were immersed in 45°C tapwater for 25 minutes. Intermittent shaking was done over this time period. Measuring, fixing in 5 per cent formalin, and remeasuring were done as before, the results being tabulated in TABLE IV.

In carrying out the relaxation tests, the fixing solution was used after cessation of muscular contraction of the worms was determined by irritating them with a brush.

The results of the tests showed that the smallest

average per cent contraction--3.8 per cent--was obtained by the use of the menthol method. Average per cent contraction after chloretone, using a fifty worm sample was 6.7 per cent. Cold and hot water methods followed at 9.4 per cent contraction and 11.7 per cent contraction respectively.

The writer considered the use of menthol superior to the other three methods. No ill effects were observed on staining processes by the use of menthol for relaxation.

TABLE I

LENGTH MEASUREMENTS IN CM. AND PER CENT CONTRACTION OF *T. nodulosus* AFTER MENTHOL RELAXATION

After relax.	After 5% form.	Diff.	%	After relax.	After 5% form.	Diff.	%
40.0	30.0	10.0*	25.0	30.6	29.5	1.1	3.4
44.3	42.0	2.3	5.2	32.1	31.7	0.4	1.2
30.0	29.0	1.0	3.3	31.0	30.8	0.2	0.6
24.4	21.6	2.8	11.5	30.8	30.6	0.2	0.6
28.1	27.5	0.6	2.1	28.4	27.5	0.9	3.2
24.2	23.3	0.9	3.7	48.2	44.8	3.4	7.0
20.8	20.0	0.8	3.8	18.3	17.8	0.5	2.7
25.0	22.7	2.3	9.2	47.8	46.2	1.6	3.3
32.8	32.2	0.6	1.8	40.8	40.6	0.2	0.5
27.7	27.0	0.7	2.5	36.2	35.6	0.6	1.7
27.0	25.8	1.2	4.4	33.6	32.6	1.0	3.0
20.0	19.2	0.8	4.0	29.3	28.1	1.2	4.1
26.8	25.8	1.0	3.7	24.5	23.5	1.0	4.1
31.5	31.3	0.2	0.6	22.8	21.8	1.0	4.4
21.0	20.4	0.6	2.9	27.6	27.4	0.2	0.7
31.8	29.6	2.2	6.9	32.3	31.7	0.6	1.9
23.8	22.6	1.2	5.0	21.7	20.7	1.0	4.6
13.8	12.8	1.0	7.2	28.6	28.2	0.4	1.4
20.0	19.8	0.2	1.0	29.6	27.7	1.9	6.4
35.6	34.9	0.7	2.0	23.5	23.0	0.5	2.1
42.8	42.0	0.8	1.9	16.4	15.5	0.9	5.5
20.6	19.6	1.0	4.9	27.5	26.9	0.6	2.2
25.2	24.5	0.7	2.8	20.6	20.0	0.6	2.9
28.3	27.4	0.9	3.2	32.5	31.9	0.6	1.8
29.2	28.7	0.5	1.7	12.5	12.4	0.1	0.8

Total per cent $\frac{189.5}{50}$ = 3.8 per cent
Total sample

*Note extent of contraction due to too hasty removal from anaesthetizing solution

TABLE II

LENGTH MEASUREMENTS IN CM. AND PER CENT CONTRACTION OF *T. nodulosus* AFTER CHLORETONE RELAXATION

After relax.	After 5% form.	Diff.	%	After relax.	After 5% form.	Diff.	%
34.6	31.7	2.9	8.4	27.4	26.1	1.3	4.7
28.6	27.7	0.9	3.1	29.1	27.8	1.3	4.5
21.2	18.3	2.9	13.7	25.7	24.0	1.7	6.6
21.4	19.6	1.8	8.4	26.5	25.4	1.1	4.2
44.8	43.5	1.3	2.9	37.9	35.4	2.5	6.6
32.4	30.5	1.9	5.9	20.0	18.7	1.3	6.5
35.9	34.2	1.7	4.7	31.3	30.4	0.9	2.9
20.2	19.7	0.5	2.5	26.4	25.0	1.4	5.3
27.3	25.4	1.9	7.0	23.6	22.0	1.6	6.8
32.0	30.4	1.6	5.0	23.1	21.4	1.7	7.4
29.2	27.8	1.4	4.8	29.4	28.0	1.4	4.8
35.8	33.3	2.5	7.0	21.9	20.5	1.4	6.4
27.0	25.5	1.5	6.0	19.0	17.2	1.8	9.5
29.9	21.8	8.1	27.1	32.1	30.6	1.5	4.7
42.5	42.0	0.5	1.2	26.0	24.8	1.2	4.6
25.3	23.9	1.4	5.5	19.5	18.0	1.5	7.7
22.3	20.7	1.6	7.2	18.6	17.3	1.3	7.0
28.0	19.3	8.7	31.1	25.3	23.4	1.9	7.5
30.3	29.2	1.1	3.6	26.6	24.4	2.2	8.3
26.4	24.5	1.9	7.2	25.0	24.6	0.4	1.6
24.5	22.9	1.6	6.5	20.6	19.4	1.2	5.8
26.2	24.7	1.5	5.7	23.0	21.5	1.5	6.5
28.2	27.2	1.0	3.5	21.8	20.8	1.0	4.6
29.3	27.0	2.3	7.8	21.7	20.5	1.2	5.5
26.6	24.9	1.7	6.4	23.6	22.6	1.0	4.2

Total per cent $\frac{336.4}{50}$ = 6.7 per cent
Total sample

TABLE III

LENGTH MEASUREMENTS IN CM. AND PER CENT CONTRACTION OF *T. nodulosus* AFTER COLD WATER RELAXATION

After relax.	After 5% form.	Diff.	%	After relax.	After 5% form.	Diff.	%
24.8	24.5	0.3	1.2	10.0	8.9	1.1	11.0
22.2	20.8	1.4	6.3	11.4	10.3	1.1	9.6
13.4	12.2	1.2	9.0	14.8	13.5	1.3	8.8
17.6	16.4	1.2	6.8	16.0	14.5	1.5	9.4
16.4	15.2	1.2	7.3	23.0	18.2	4.8	20.9
16.7	15.8	0.9	5.4	15.4	14.2	1.2	7.8
19.0	17.8	1.2	6.3	14.8	13.5	1.3	8.8
20.3	18.9	1.4	6.9	12.2	11.1	1.1	9.0
15.0	14.1	0.9	6.0	14.5	13.1	1.4	9.7
26.2	23.9	2.3	8.8	19.1	17.9	1.2	6.3
15.0	13.6	1.4	9.3	12.2	10.5	1.7	13.9
21.0	17.8	3.2	15.2	13.1	12.0	1.1	8.4
15.7	14.4	1.3	8.3	16.1	15.0	1.1	6.8
21.8	20.2	1.6	7.3	14.1	12.7	1.4	9.9
13.2	12.0	1.2	9.1	17.2	15.5	1.7	9.9
16.8	15.3	1.5	8.9	14.9	12.9	2.0	13.4
15.8	14.0	1.8	11.4	27.4	24.8	2.6	9.5
13.9	12.7	1.2	8.6	9.3	7.8	1.5	16.1
27.0	24.3	2.7	10.0	20.8	18.5	2.3	11.1
19.3	17.6	1.7	8.8	21.5	19.7	1.8	9.1
17.1	15.5	1.6	9.4	13.6	11.7	1.9	13.8
14.9	13.0	1.9	12.8	11.0	10.2	0.8	7.3
14.1	12.4	1.7	12.1	13.4	11.8	1.6	11.9
20.1	17.5	2.6	12.9	15.0	14.3	0.7	4.7
15.1	14.2	0.9	6.0	12.0	10.7	1.3	10.8

Total per cent $\frac{472.1}{50}$ = 9.4 per cent
Total sample

TABLE IV

LENGTH MEASUREMENTS IN CM. AND PER CENT CONTRACTION OF *T. nodulosus* AFTER HOT WATER RELAXATION

After relax.	After 5% form.	Diff.	%	After relax.	After 5% form.	Diff.	%
25.7	23.3	2.4	9.3	20.3	17.4	2.9	14.3
18.2	15.8	2.4	13.2	17.2	14.5	2.7	15.7
20.0	18.6	1.4	7.0	21.5	18.7	2.8	13.0
17.5	15.7	1.8	10.3	26.2	23.7	2.5	9.5
13.4	11.4	2.0	14.9	14.3	12.6	1.7	11.9
19.2	17.4	1.8	9.4	15.2	13.8	1.4	9.2
27.9	25.6	2.3	8.2	12.1	11.4	0.7	5.8
20.7	17.9	2.8	13.5	24.5	22.3	2.2	9.0
21.1	18.5	2.6	12.3	36.3	31.0	5.3	14.6
14.0	13.5	0.5	3.6	23.3	21.2	2.1	9.0
20.4	17.6	2.8	13.7	27.0	22.2	4.8	17.8
18.8	17.3	1.5	8.0	22.0	19.9	2.1	9.5
16.0	14.2	1.8	11.3	21.3	17.0	4.3	20.2
22.6	19.4	3.2	14.2	24.8	21.5	3.3	13.3
18.7	17.6	1.1	5.9	20.8	17.9	2.9	13.9
30.3	26.2	4.1	13.5	15.9	14.1	1.8	11.3
22.0	19.8	2.2	10.0	25.0	20.9	4.1	16.4
36.8	30.7	6.1	16.6	19.7	17.1	2.6	13.2
23.9	21.4	2.5	10.5	21.0	18.0	3.0	14.3
21.1	20.0	1.1	5.2	30.6	27.5	3.1	10.1
19.8	17.4	2.4	12.1	16.8	14.6	2.2	13.1
24.5	20.9	3.6	14.7	14.9	11.9	3.0	20.1
10.6	10.0	0.6	5.7	21.8	20.0	1.8	8.3
17.1	14.0	3.1	18.1	31.1	26.2	4.9	15.8
22.0	20.3	1.7	7.7	14.2	12.9	1.3	9.2

Total per cent $\frac{587.4}{\text{Total sample } 50} = 11.7$ per cent

CHAPTER V

FIXATIVES AND FIXATION OF TAPEWORMS

This section of the problem deals with types of fixatives used, methods of fixation, and the external effects of these solutions on the specimens.

Dibothriocephalus latus was chosen for the comparative fixing - staining tests for three reasons; (1) ease of observation of internal structures; (2) advantages in handling large tapeworm segments; and (3) availability of a large quantity of material. The fixing solutions were chosen not only to include those in common use and those recommended, but also those having widely different chemical compositions. The writer felt it pertinent to include limited descriptions of most of the fixing solutions. All solutions were freshly prepared and used cold except for Allen's B-15. D. latus material was relaxed by the cold water method to eliminate any possibility of menthol affecting the results. Specimens were placed in at least fifty times their volume of fixative.

Alcohol (70 per cent ethyl). Tapeworm specimens were immersed in alcohol which was changed once after 24 hours and replaced with afresh solution as a preservative. No contraction or shrivelling of specimens resulted.

Allen's B-15 Fixative. The solution was made by

adding chromic acid crystals and urea crystals to Bouin's solution. Fixation of material was done at 37°C for 22 hours. Specimens were transferred to 70 per cent alcohol at 35°C for washing. No lithium carbonate was added to the wash fluid. Storage was in 70 per cent ethyl alcohol. Specimens showed a minimum of picric acid color and no rippling.

Bouin's Fixative. Specimens were fixed for 22 hours and treated as after Allen's B-15. Washing alcohol was changed daily until no yellow color was imparted to the solution. Baylis (1922) reported that Bouin's "does not give such straight and extended specimens" but no indication of this was observed.

Cleverdon's Fixative (Cleverdon 1943). The fixative was made by using picric acid, 70 per cent isopropyl alcohol, acetone, glacial acetic acid, and 40 per cent formaldehyde. The fixative was allowed to act for 19 hours on the material then exchanged for 70 per cent isopropyl alcohol at 35°C until the picric acid was removed. Storage was in 70 per cent isopropyl alcohol. The material contracted considerably and became translucent.

Demke's Fixative (Demke 1951). This fixative was originally described by Becker and Roudabush (1935) but was redescribed by Demke under whose name it is referred to in the literature and in this work. Components of the

fixative were 95 per cent ethyl alcohol, 40 per cent formaldehyde, glacial acetic acid, glycerol, and distilled water. The material was fixed for 21 hours. A fresh solution was used for preservation. Material appeared white and unrippled. No contraction was observed.

Formalin (5 per cent). In view of its wide use, tapeworm material was fixed and preserved in this solution to compare its usefulness against other fixatives. Specimens remained white and firm in this solution.

Gilson's Fixative (mercuric-acetic-nitric mixture) Specimens were fixed for 19 hours, transferred to 70 per cent alcohol, and treated with Lugol's iodine to remove mercuric chloride precipitates. Preservation of material was in 70 per cent alcohol. The material remained white and unshrivelled but ultimately became fragile to the point of making handling difficult.

Helly's Fixative. The fixative required the following chemicals: potassium dichromate, mercuric chloride, sodium sulphate, 40 per cent formaldehyde, and distilled water. Material was fixed for 20 hours, washed for 18 hours, and dehydrated to 70 per cent alcohol for Lugol's iodine treatment. Storage was in 70 per cent alcohol. The material darkened and wrinkled slightly during fixation. Some hardening was also noted.

Kleinenberg's Fixative. The solution was made by

adding 100 cc. of 1 per cent sulphuric acid to 49 cc. of saturated aqueous picric acid. The material was washed in 70 per cent alcohol at 35°C. Storage was in 70 per cent alcohol. Some contraction occurred during fixation.

Lavdowsky's Fixative. The solution was made with formalin, alcohol, glacial acetic acid, and distilled water. Specimens were fixed for 19 hours and transferred directly to 70 per cent alcohol. The material remained white and unwrinkled.

Lewitsky's Fixative. This solution required 100 cc. each of 40 per cent formaldehyde, 5 per cent aqueous chromic acid, and distilled water. Specimens were fixed for 19 hours and washed in daylight in running water for an equal length of time. Storage was in 70 per cent alcohol. Slight darkening and hardening occurred during fixation but the specimens remained pliable.

Petrunkevitch's Fixative. The solution was made with 60 per cent alcohol, nitric acid, ether, cupric nitrate, and parnitrophenol crystals. The material was fixed for 12 hours and placed directly into 70 per cent alcohol. The cupric nitrate color was removed in alcohol. Specimens were soft and pliable after fixation but complete disintegration occurred after extensive handling.

Schaudinn's Fixative. The solution was made with mercuric chloride, alcohol, and glacial acetic acid. Specimens were fixed for 21 hours then placed directly

into 70 per cent alcohol for Lugol's iodine treatment and storage. Fixation resulted in white, pliable but firm specimens.

Susa (Heidenhain's) Fixative. The solution was made with mercuric chloride, glacial acetic acid, sodium chloride, trichloroacetic acid, formalin, and distilled water. Specimens were fixed for 20 hours, washed in 70 per cent alcohol and treated with iodine. Storage was in 70 per cent alcohol. Slight shrinkage was noted after fixation, otherwise the material appeared white and unwrinkled.

Tellyesniczky's Fixative. Potassium dichromate, glacial acetic acid, and distilled water were used in this solution. Specimens were fixed for 24 hours and washed in running water for an equal time. Storage was in 70 per cent alcohol. Fixative caused slight darkening and considerable contraction of material.

Zenker's Fixative. The specimens were fixed for 20 hours and washed in running water for 18 hours. Iodine treatment and storage was done at the 70 per cent alcohol stage. Specimens appeared firm and smooth with slight darkening. No contraction resulted from the fixation.

CHAPTER VI

RESULTS OF FIXATIVE - STAIN TESTS

In recording the results of 272 different fixative - stain combinations, it was felt that graphic representations, as for example tables, would limit the descriptions. Accordingly, individual descriptions were made but the results were condensed as much as possible. Descriptions of unsatisfactory fixative - stain combinations were omitted unless particular results warranted their inclusion. Omitted specimens showed one or both of the following inadequacies; (1) some or all of the internal structures were not made visible by the technique; (2) parenchymal musculature showed poor destaining qualities. Regressive staining was done with all toto - mount specimens. The best staining times were determined for D. latus material by trial tests with the haematoxylin stains and most of the carmine and aniline stains. Acid alcohol solutions were made with concentrated HCl and 70 per cent ethyl alcohol. Hunter's (1927) alkaline alcohol solution, for controlling the destaining action, was used wherever necessary. An appropriately graded series of isopropyl alcohol was used with specimens fixed by Cleverdon's solution. Formalin and Demke - fixed specimens were washed for at least two hours in running water prior to being stained.

DELAFIELD'S HAEMATOXYLIN

Tapeworm material was stained for 45 minutes and destained with 5 per cent acid alcohol for 3 to 10 minutes. Following are the results with the various fixatives.

Alcohol- results were unsatisfactory; ovary was indistinct; testes faintly stained; uterus well stained; Mehlis' gland was evident; see FIGURE 1.

Allen's B-15- light staining action; good fixation of testes; ovary very light; vitellaria finely fixed but lightly stained; vaginal canal partly stained; see FIGURE 2.

Bouin's- internal structures showed exceptionally fine fixation but stain did not remain in tissues; ovary and testes were understained; see FIGURE 3.

Demke's- good fixation and staining resulted; reproductive organs were clearly stained.

Formalin- results were poor; ovary was lightly stained; testes not evident; uterus well stained; see FIGURE 4.

Gilson's- genitalia were well stained; see FIGURE 5.

Kleinenberg's- internal structures were stained; destaining action was poor.

Lavdowsky's- good results; ovary was dark; testes were stained but obscured by vitellaria; see FIGURE 6.

Petrunkevitch's- fixation was good; testes stained but ovary was only lightly stained.

Schaudinn's- stain was compatible with fixative; see FIGURE 7.

Zenker's- testes only lacked stain; see FIGURE 8.

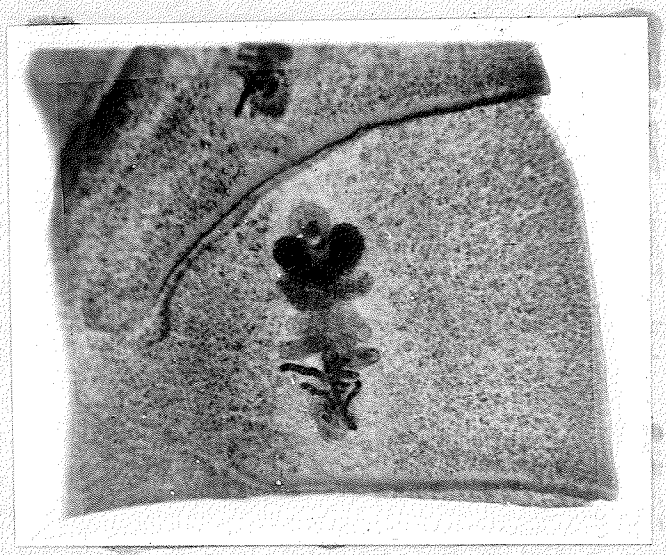

FIGURE 1.

D. latus ALCOHOL FIXATIVE-DELAFIELD'S HAEMATOXYLIN STAIN

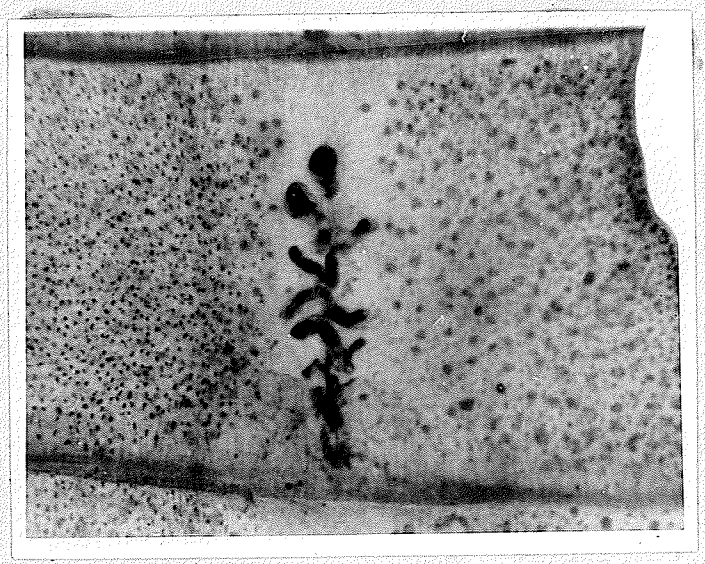

FIGURE 2.

D. latus ALLEN'S B-15 FIXATIVE - DELAFIELD'S HAEMATOXYLIN STAIN

FIGURE 3.

D. latus BOUIN'S FIXATIVE - DELAFIELD'S HAEMATOXYLIN STAIN

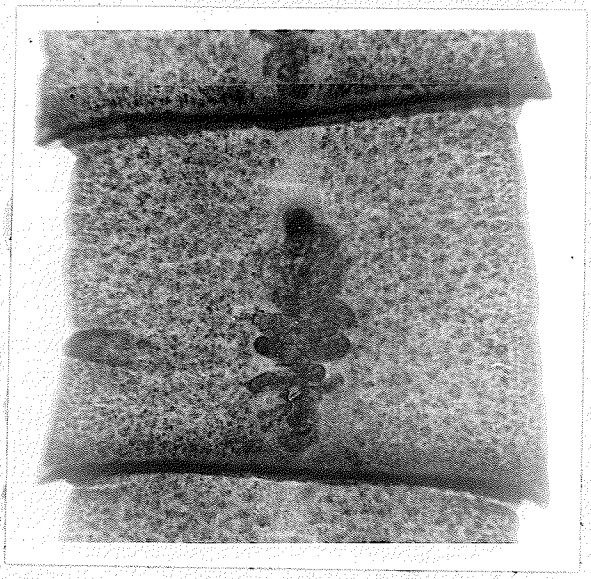

FIGURE 4.

D. latus FORMALIN FIXATIVE- DELAFIELD'S HAEMATOXYLIN STAIN

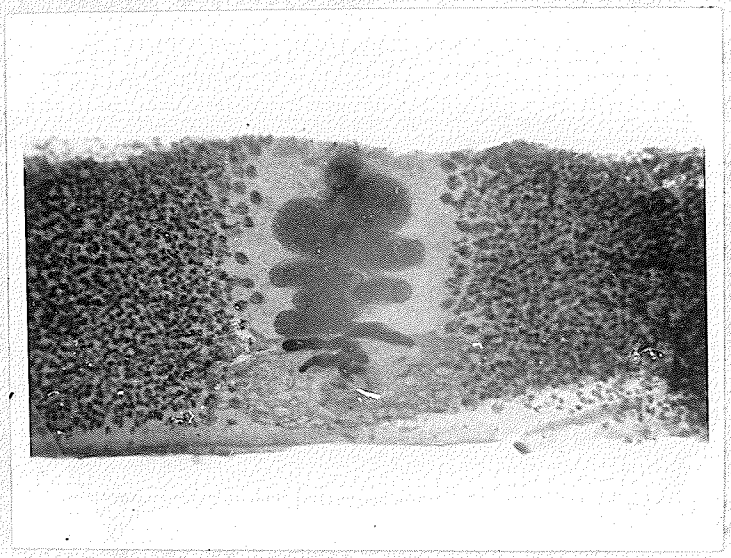

FIGURE 5.

D. latus GILSON'S FIXATIVE-DELAFIELD'S HAEMATOXYLIN STAIN

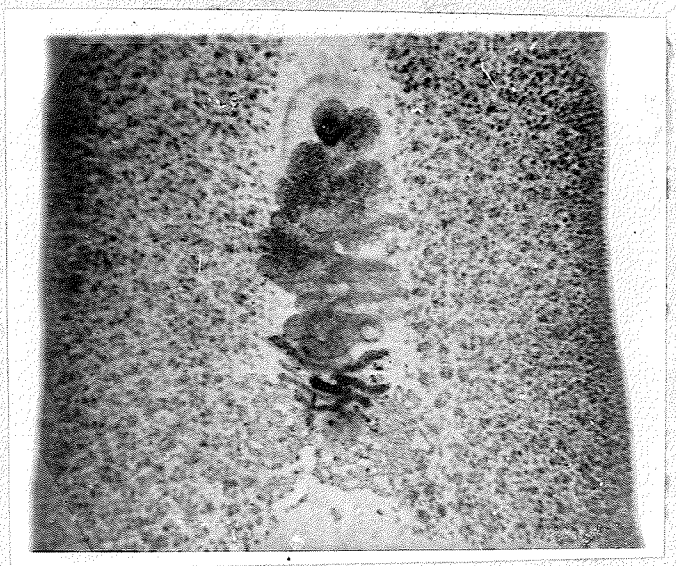

FIGURE 6.

D. latus LAVDOWSKY'S FIXATIVE-DELAFIELD'S HAEMATOXYLIN STAIN

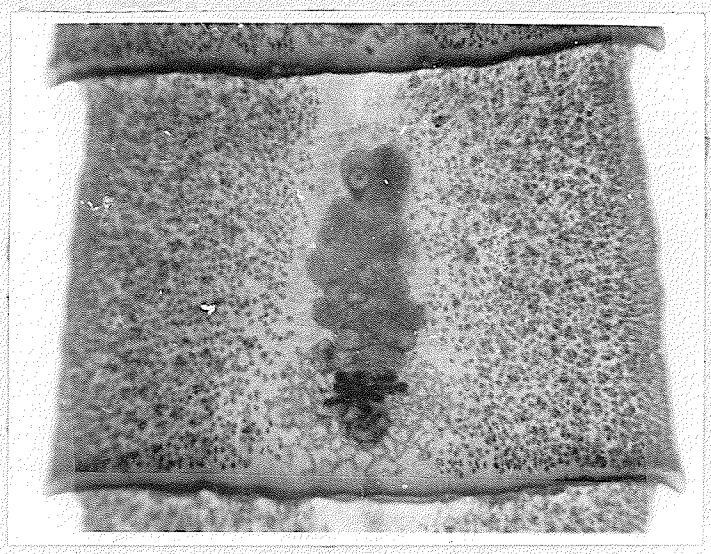

FIGURE 7.

D. latus SCHAUDINN'S FIXATIVE-DELAFIELD'S HAEMATOXYLIN STAIN

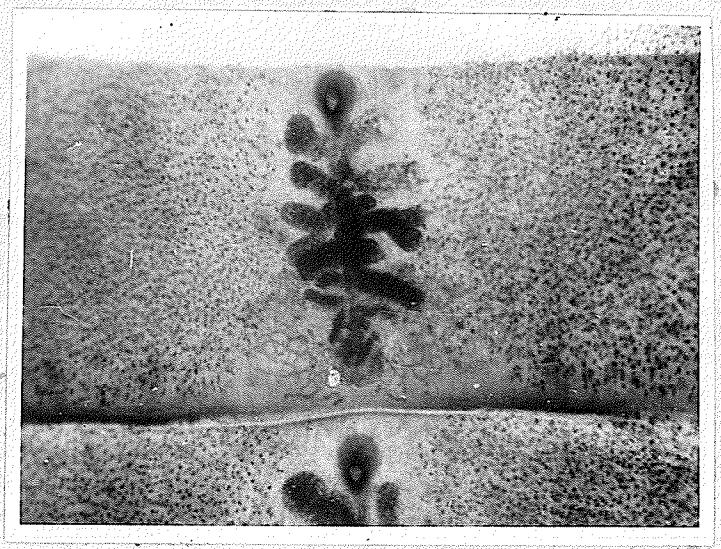

FIGURE 8.

D. latus ZENKER'S FIXATIVE-DELAFIELD'S HAEMATOXYLIN STAIN

EHRLICH'S ACID HAEMATOXYLIN

Tapeworm material was stained for 45 minutes and destained in 10 per cent acid alcohol for 0 to 10 minutes. Following are the results with the various fixatives.

Alcohol- testes and ovary were lightly stained; parenchyma showed good destaining; see FIGURE 9.

Allen's B-15- testes were stained but ovary only lightly; vaginal canal was evident; destaining was rapid.

Bouin's- stain did not take well after this fixative; stain was removed in the alcohol series without the use of acid alcohol.

Demke's- stain showed strong affinities for internal structures.

Formalin- stain showed a slight improvement over Delafield's haematoxylin.

Gilson's- genitalia were well stained; parenchyma was destained sufficiently.

Lavdowsky's- results were comparable to Delafield's haematoxylin; see FIGURE 10.

Petrunkevitch's- stain brought out testes but not ovary; vitellaria were extremely dark; deeply stained nuclei of cortical layer increased opacity of specimen.

Schaudinn's- results were satisfactory; Mehlis' gland only showed light staining.

Susa- stain was unsuitable; ova contents in all Susa - fixed specimens showed shrivelling and blackening.

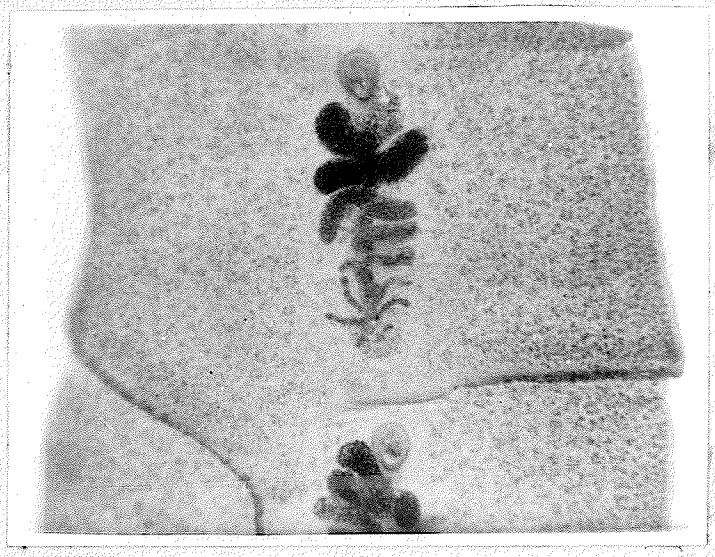

FIGURE 9.

D. latus ALCOHOL FIXATIVE-EHRLICH'S ACID HAEMATOXYLIN STAIN

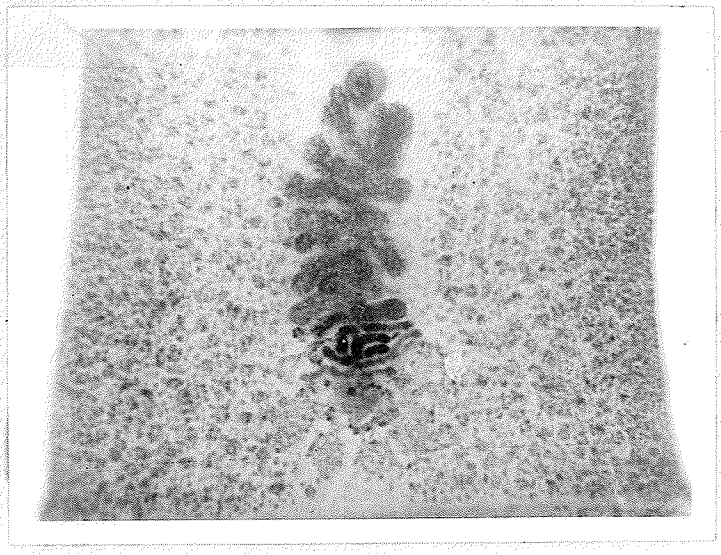

FIGURE 10.

D. latus LAVDOWSKY'S FIXATIVE-EHRLICH'S HAEMATOXYLIN STAIN

GROAT'S HAEMATOXYLIN

This stain was compounded by Groat (1949) for use with sections after Susa or Petrunkevitch's fixative. Structures became brownish - black in this stain. Stain was allowed to act for 18 1/2 hours with destaining with 10 per cent acid alcohol for 2 to 30 minutes. Following are the results after the various fixatives.

Allen's B-15- all major internal structures except the ovary were well stained; destaining was typically rapid.

Cleverdon's- stain did not take well after this fixative; see FIGURE 11.

Demke's- results were satisfactory; destaining produced suitably clear musculature.

Gilson's- this specimen required only 3/4 hour staining; internal structures including vaginal canal were deeply stained; see FIGURE 12.

Lavdowsky's- results were satisfactory;see FIGURE 13.

Lewitsky's- the lateral canals and Mehlis' gland showed well in all Lewitsky - fixed specimens; only Groat's stain brought out the genitalia to any degree; see FIGURE 14.

Schaudinn's- results were entirely satisfactory.

Susa- genitalia were suitably stained; blackening of specimen occurred 48 hours after mounting but was subsequently corrected by the introduction of calcium carbonate chips into the clearing agent and mountant.

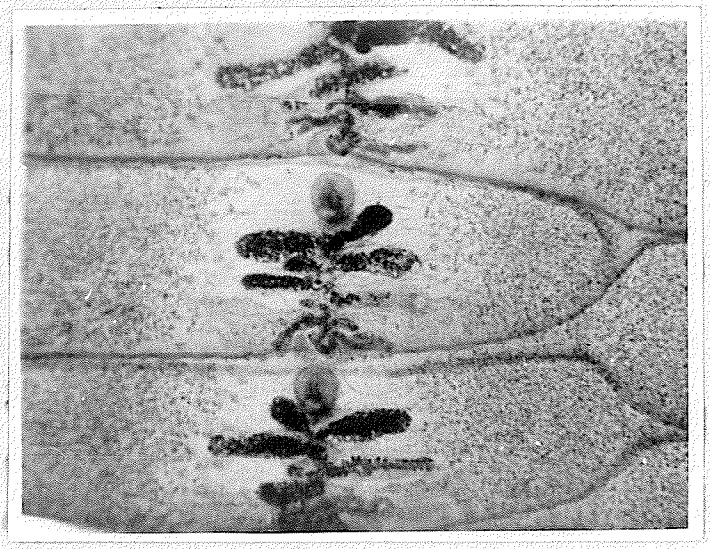

FIGURE 11.

D. latus CLEVERDON'S FIXATIVE-GROAT'S HAEMATOXYLIN STAIN

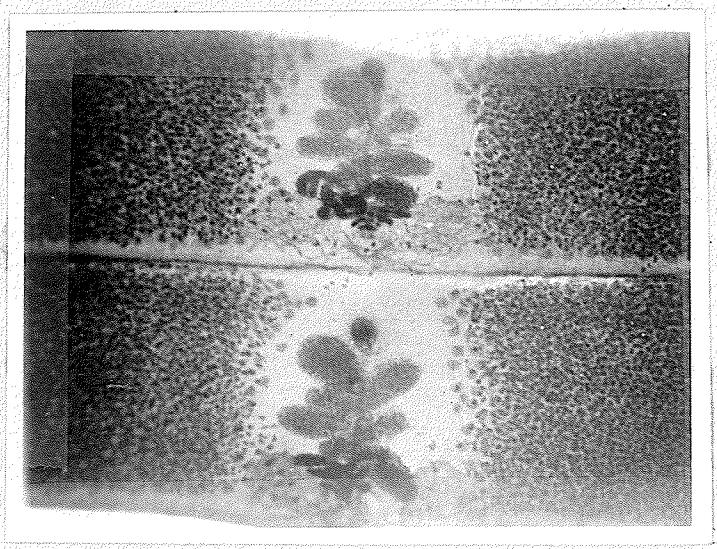

FIGURE 12.

D. latus GILSON'S FIXATIVE-GROAT'S HAEMATOXYLIN STAIN

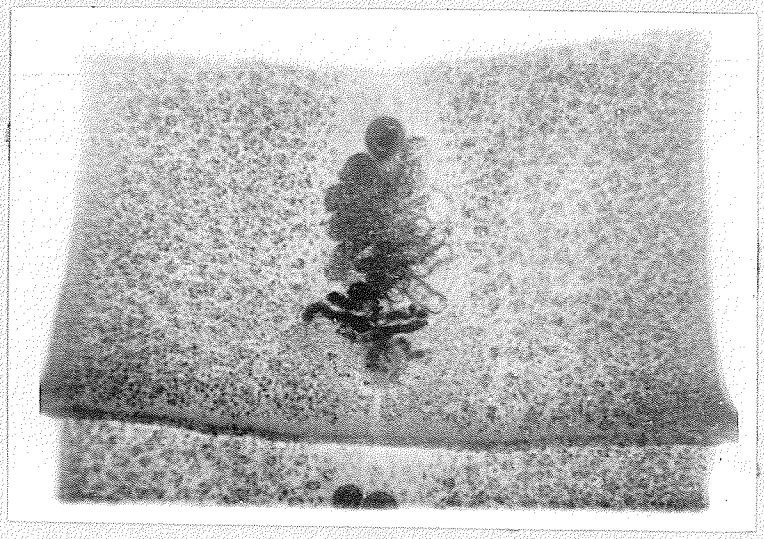

FIGURE 13.

D. latus LAVDOWSKY'S FIXATIVE-GROAT'S HAEMATOXYLIN STAIN

FIGURE 14.

D. latus LEWITSKY'S FIXATIVE-GROAT'S HAEMATOXYLIN STAIN

HARRIS' HAEMATOXYLIN MODIFICATION (ISA VARIANT)

This stain, recorded by Little (1954), was allowed to act for 45 minutes. Destaining in 5 per cent acid alcohol was necessary. Following are the results with the various fixatives.

Allen's B-15- only the ovary lacked stain in this specimen; vaginal canal showed clearly; see FIGURE 15.

Demke's- satisfactory results were obtained.

Formalin- musculature showed poor destaining action; internal structures were not finely fixed; see FIGURE 16.

Gilson's- suitable staining resulted; increased staining time of 17 hours did not improve results; see FIGURE 17.

Lavdowsky's- suitable results were obtained with this combination; see FIGURE 18.

Petrunkevitch's- the combination proved successful; uterus and cirrus pouch only lacked deep staining; see FIGURE 19.

Schaudinn's- stain was very effective after this fixative; see FIGURE 20.

Susa- stain was not taken up by the genitalia.

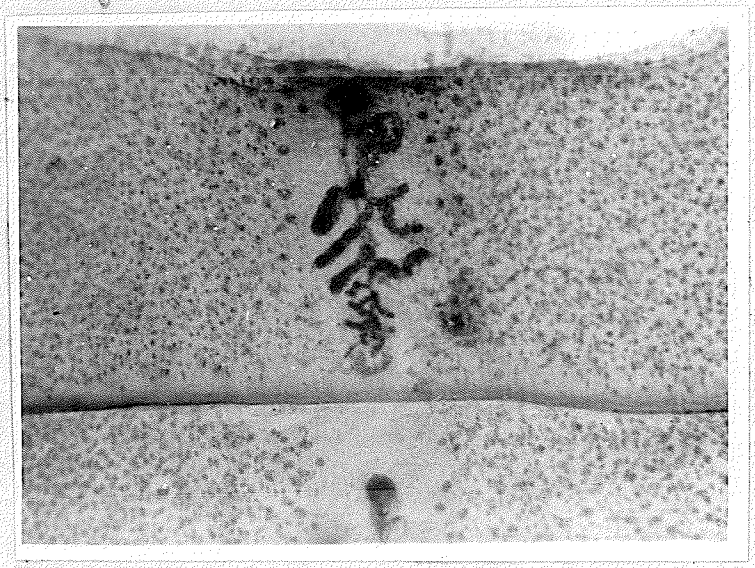

FIGURE 15.

D. latus ALLEN'S B-15 FIXATIVE-HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

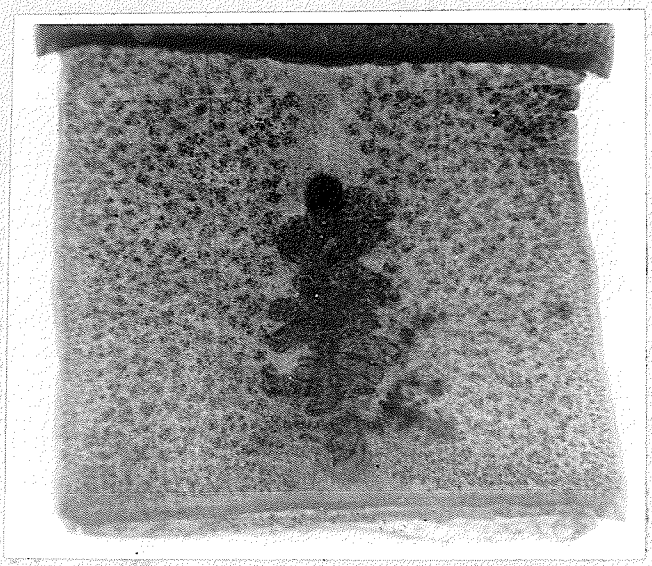

FIGURE 16.

D. latus FORMALIN FIXATIVE-HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

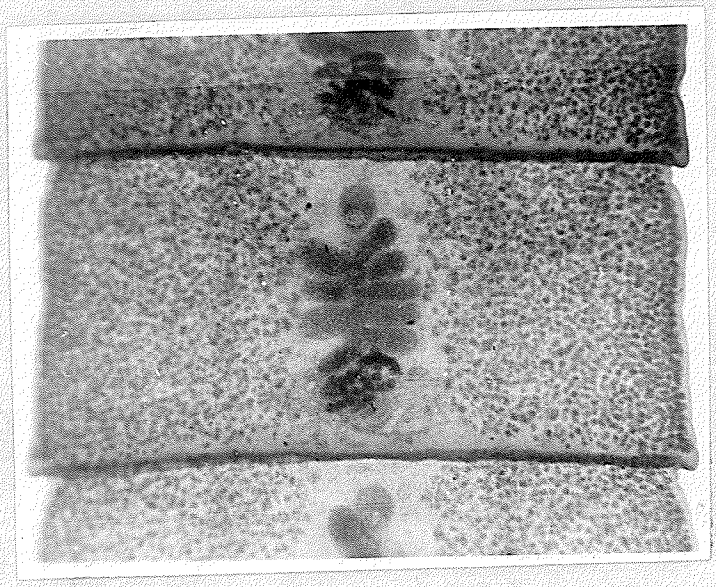

FIGURE 17.

D. latus GILSON'S FIXATIVE - HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

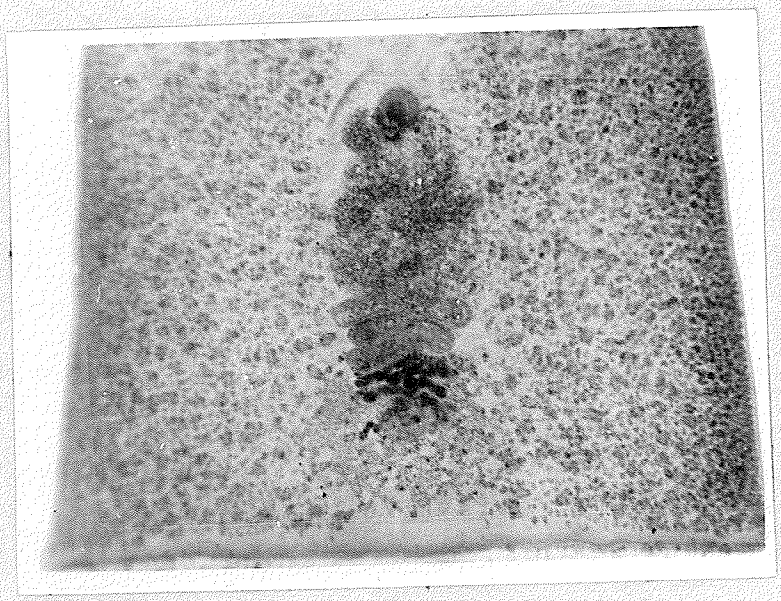

FIGURE 18.

D. latus LAVDOWSKY'S FIXATIVE - HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

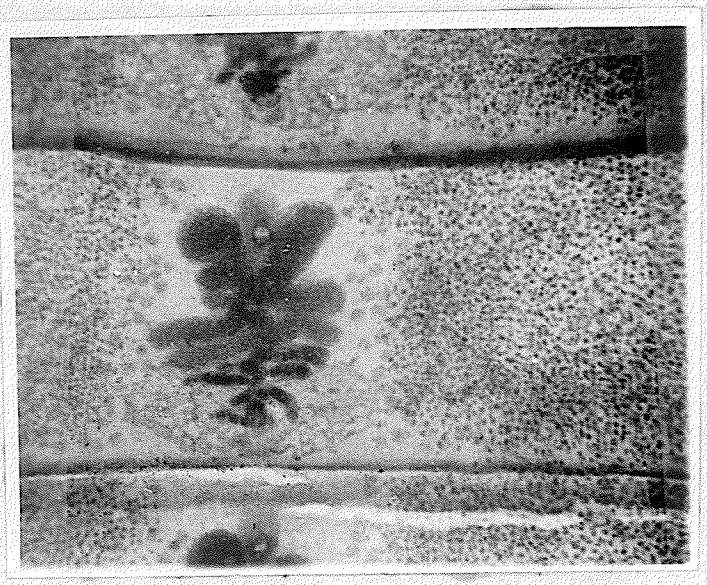

FIGURE 19.

D. latus PETRUNKEVITCH'S FIXATIVE - HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

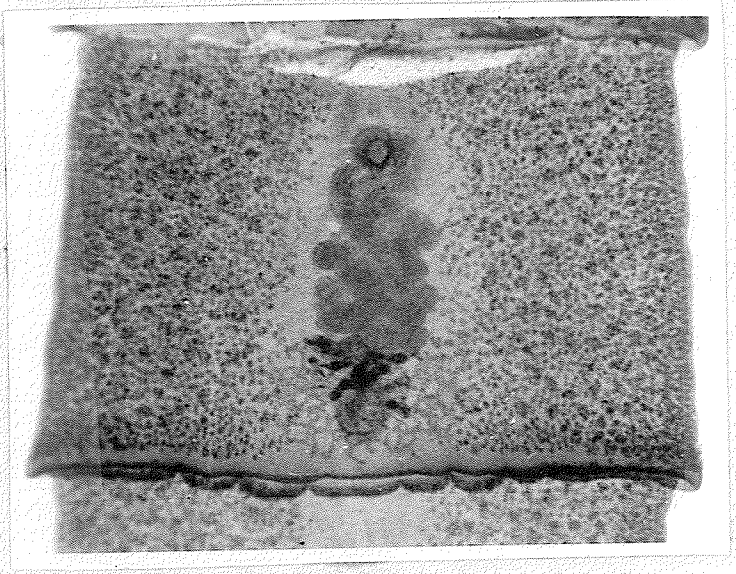

FIGURE 20.

D. latus SCHAUDINN'S FIXATIVE - HARRIS' HAEMATOXYLIN STAIN (ISA VARIANT)

LILLIE'S ACID HAEMALUM

Specimens were immersed in this stain for 45 minutes with destaining by 5 per cent acid alcohol for 2 to 20 minutes.

Allen's B-15- results were noticeably poorer than with previous stains; testes and ovary were lightly stained.

Bouin's- vitellaria stained deeply; testes and ovary were barely visible; see FIGURE 21.

Demke's- all major internal structures were clearly marked; slight contraction of specimen was noted after mounting.

Gilson's- suitable results were obtained; specimen required 2 hours destaining to lighten musculature.

Lavdowsky's- stain gave inferior results compared with previous haematoxylins.

Schaudinn's- testes lacked deep staining; other major structures were well defined; see FIGURE 22.

Susa- staining results were satisfactory; slight contraction was noted after mounting.

Lillie's haematoxylin stain exhibited affinities for the parenchymal musculature of all the specimens. The stain also lacked the penetrating power shown by other haematoxylins.

FIGURE 21.

D. latus BOUIN'S FIXATIVE - LILLIE'S ACID HAEMALUM STAIN

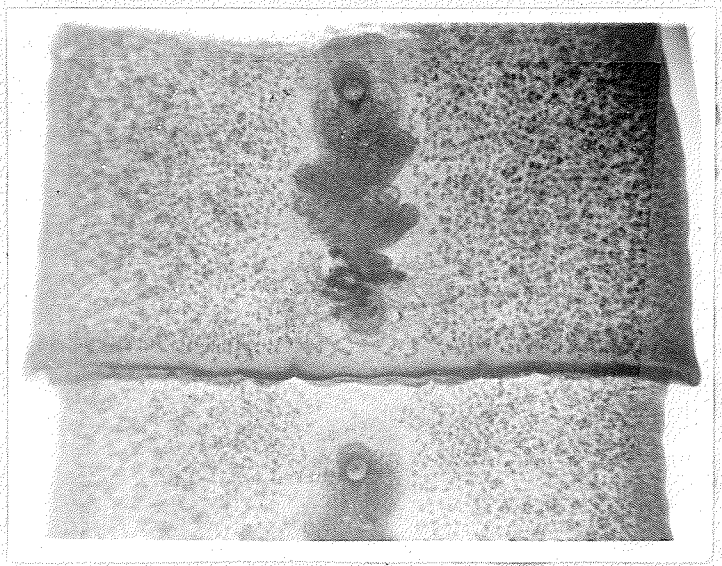

FIGURE 22.

D. latus SCHAUDINN'S FIXATIVE-LILLIE'S ACID HAEMALUM STAIN

MANN'S ACID HAEMATEIN

The stain was allowed to act on the specimens for 45 minutes with destaining by 5 per cent acid alcohol for 10 to 25 minutes.

Alcohol- a slight improvement was noted with this combination; see FIGURE 23 and compare it with FIGURE 9; the two stains were made identically except for the use of haematoxylin in Ehrlich's stain and haematein in Mann's.

Bouin's- stain was used for both 45 minutes and 48 hours; neither specimen showed the genitalia deeply stained; see FIGURES 24 and 25 respectively.

Cleverdon's- internal structures did not take up stain; vitellaria also remained unstained.

Demke's- suitable results were obtained; good destaining action occurred in the musculature.

Gilson's- stain was highly effective; see FIGURE 26; increased staining time of 30 hours did not improve results.

Helly's- typically poor results obtained after this fixative are shown in FIGURE 27.

Lavdowsky's- suitable results were obtained.

Petrunkevitch's- suitable results were obtained.

Schaudinn's- excellent results were obtained.

Susa- Mann's stain proved to be one of the best after this fixative; see FIGURE 28.

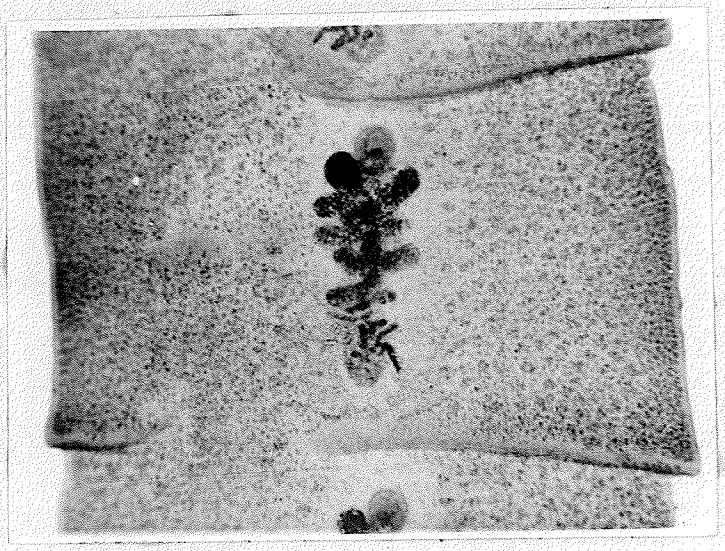

FIGURE 23.

D. latus ALCOHOL FIXATIVE - MANN'S ACID HAEMALUM STAIN

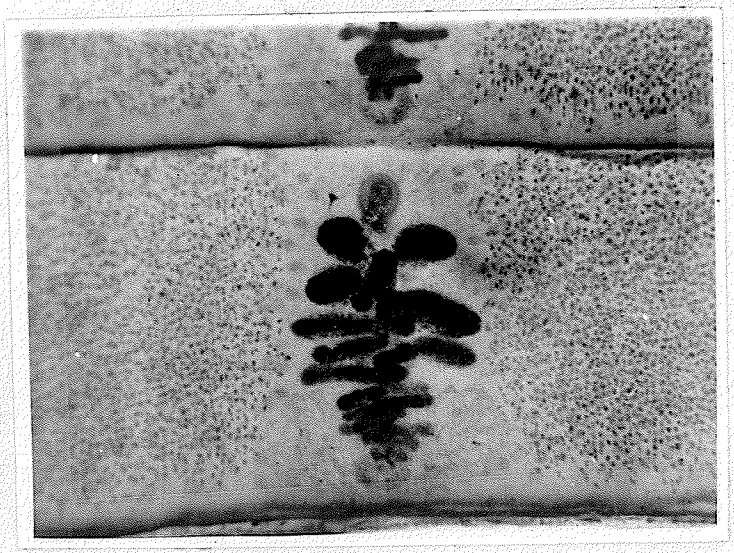

FIGURE 24.

D. latus BOUIN'S FIXATIVE - MANN'S ACID HAEMALUM STAIN

(STAINING TIME: 45 MINUTES)

FIGURE 25.

D. latus BOUIN'S FIXATIVE - MANN'S ACID HAEMALUM STAIN

(STAINING TIME: 48 HOURS)

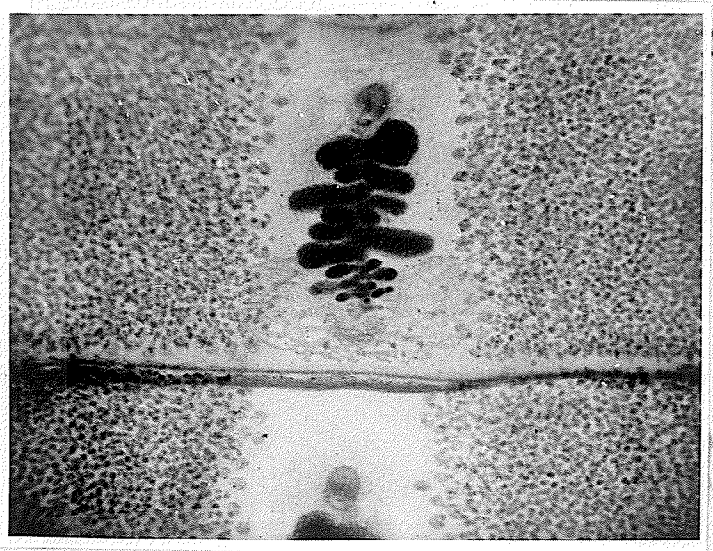

FIGURE 26.

D. latus GILSON'S FIXATIVE - MANN'S ACID HAEMALUM STAIN

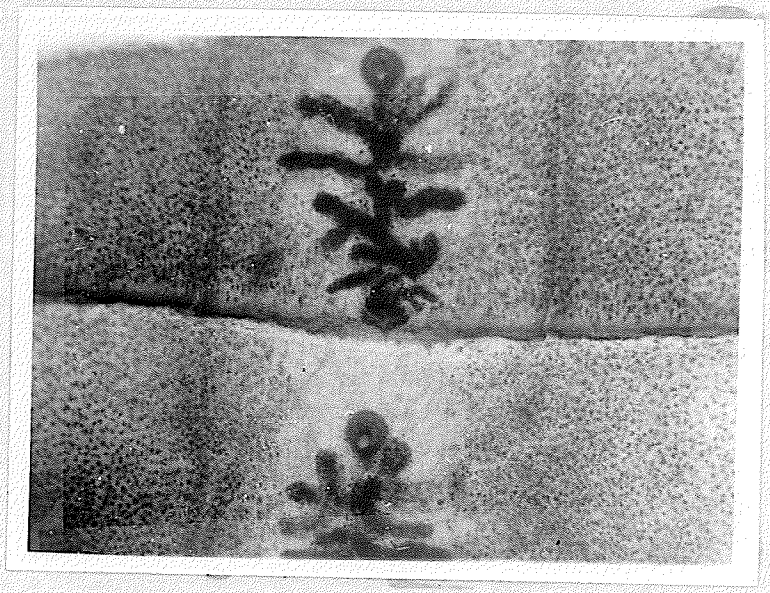

FIGURE 27.

D. latus HELLY'S FIXATIVE - MANN'S ACID HAEMALUM STAIN

FIGURE 28.

D. latus SUSA FIXATIVE - MANN'S ACID HAEMALUM STAIN

GOWER'S ALUM - CARMINE

This stain was devised for use with trematodes. Specimens were stained for 6 days. Destaining with 5 per cent acid alcohol for 5 to 10 minutes was done only where indicated.

Allen's B-15- internal structures showed light staining; see FIGURE 29.

Demke's- specimen was destained; genitalia were lightly stained; excessive contraction resulted after mounting.

Gilson's- specimen was destained; results were unsatisfactory; genitalia were only lightly stained; excessive contraction occurred after mounting.

Kleinenberg's- specimen was destained; internal structures were clearly stained; excessive contraction occurred after mounting.

Petrunkevitch's- suitable results were obtained; see FIGURE 30.

Schaudinn's- combination proved satisfactory; see FIGURE 31.

Tellyesniczky's- specimen was destained; testes lacked stain; other structures showed light staining; see FIGURE 32.

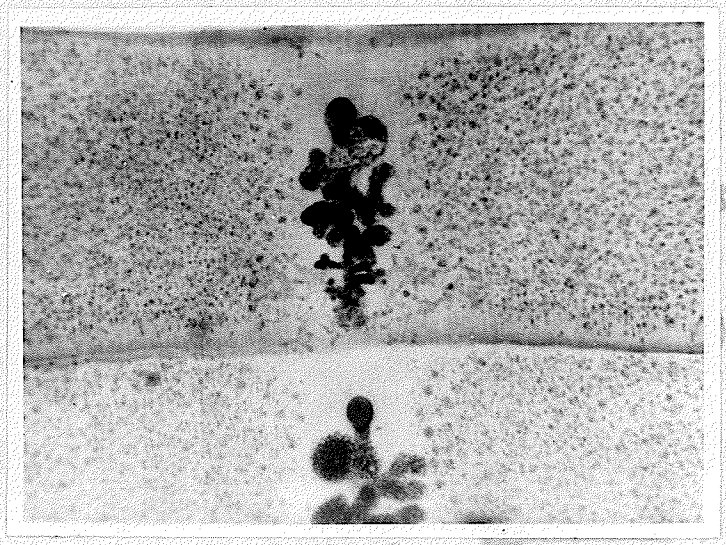

FIGURE 29.

D. latus ALLEN'S B-15 FIXATIVE-GOWER'S ALUM - CARMINE STAIN

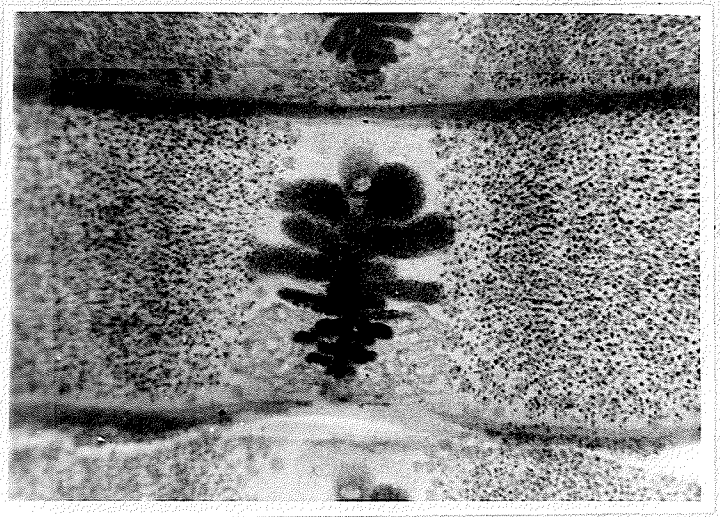

FIGURE 30.

D. latus PETRUNKEVITCH'S FIXATIVE - GOWER'S ALUM - CARMINE STAIN

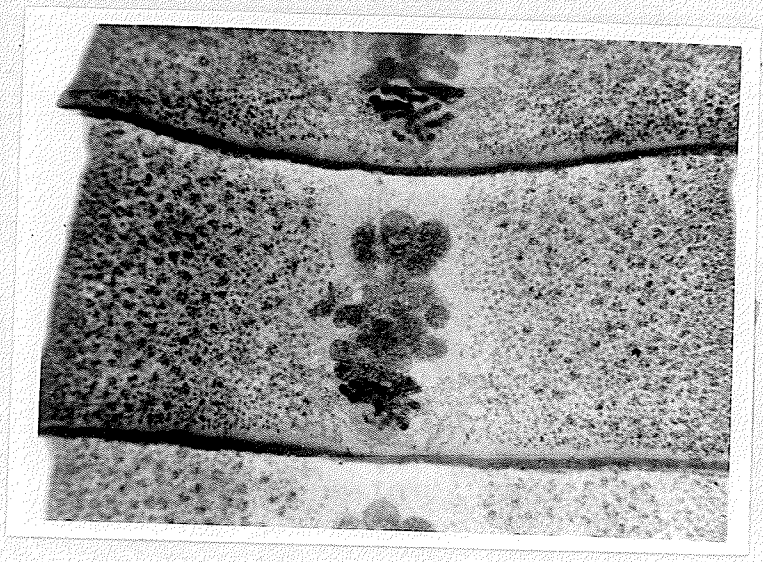

FIGURE 31.

D. latus SCHAUDINN'S FIXATIVE-GOWER'S ALUM - CARMINE STAIN

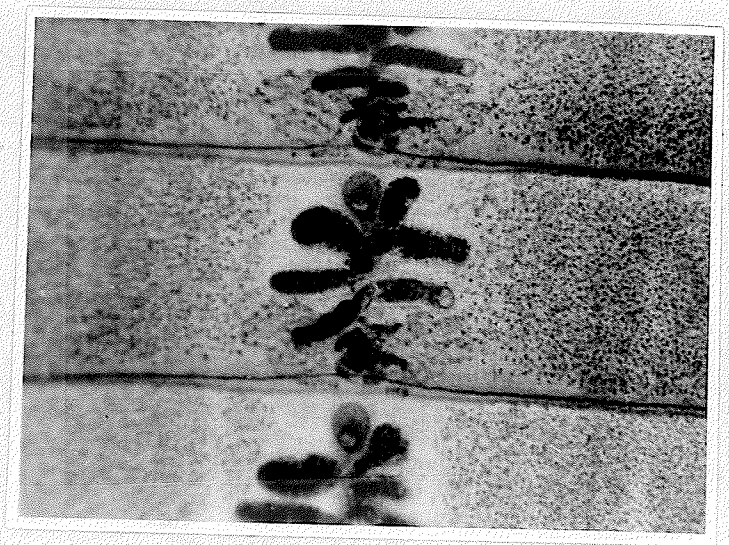

FIGURE 32.

D. latus TELLYESNICZKY'S FIXATIVE - GOWER'S ALUM-CARMINE STAIN

GRENACHER'S BORAX CARMINE

Specimens were immersed in the stain for 3 days. Destaining was done with 5 per cent acid alcohol for 10 to 35 minutes.

Demke's- internal structures lacked stain; specimen contracted after mounting.

Formalin- stain washed out in alcohol series without use of acid alcohol.

Lavdowsky's- results were inferior to those obtained with the haematoxylin stains; see FIGURE 33.

Schaudinn's- genitalia were well defined; see FIGURE 34.

Susa- stain proved to be the most effective after this fixative; vitellaria showed only light staining; see FIGURE 35.

Zenker's- testes showed complete lack of stain; other structures were well defined; see FIGURE 36.

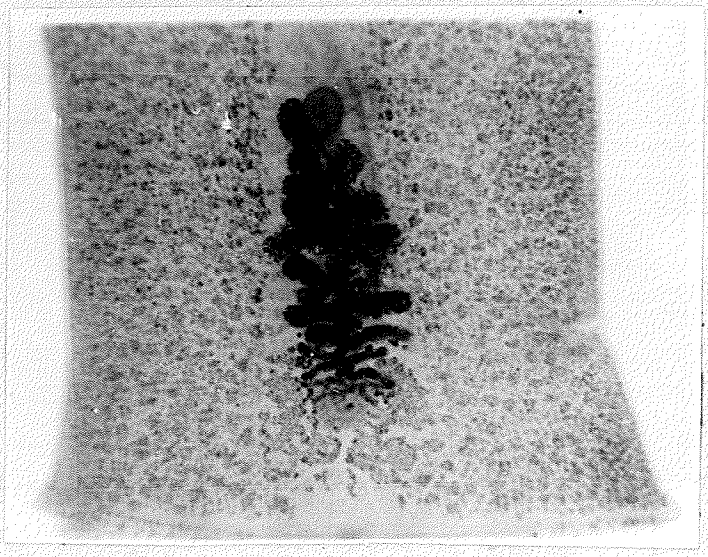

FIGURE 33.

D. latus- LAVDOWSKY'S FIXATIVE - GRENACHER'S BORAX CARMINE STAIN

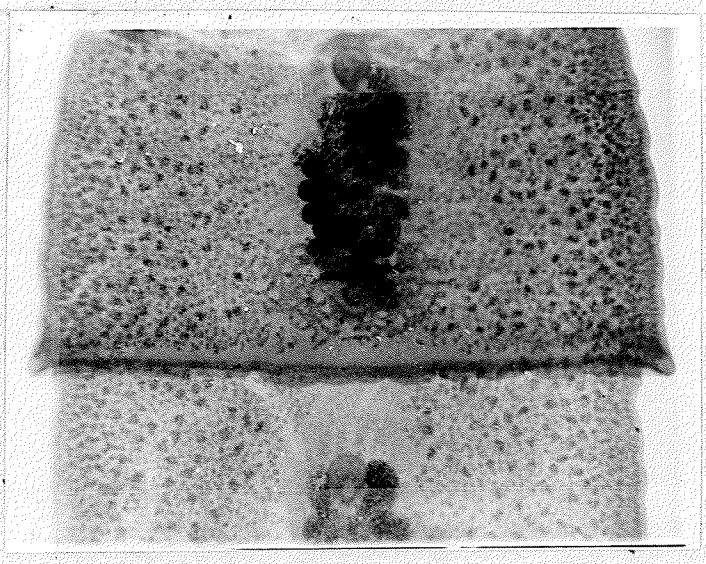

FIGURE 34.

D.latus SCHAUDINN'S FIXATIVE - GRENACHER'S BORAX CARMINE STAIN

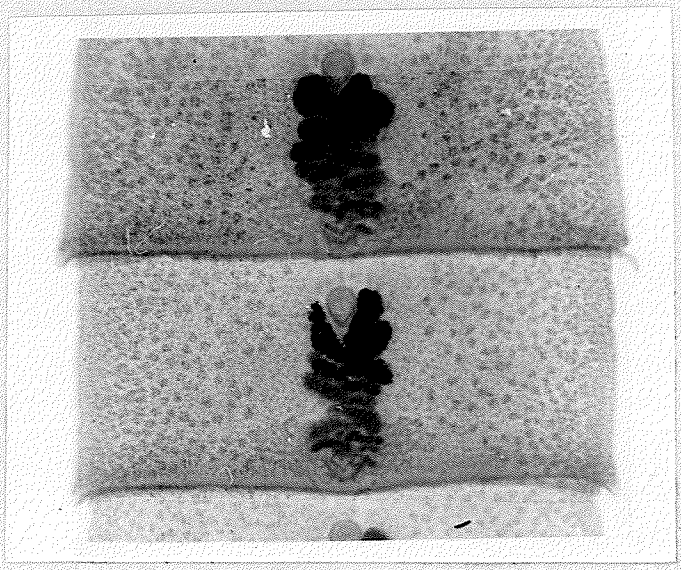

FIGURE 35.

D. latus SUSA FIXATIVE - GRENACHER'S BORAX CARMINE STAIN

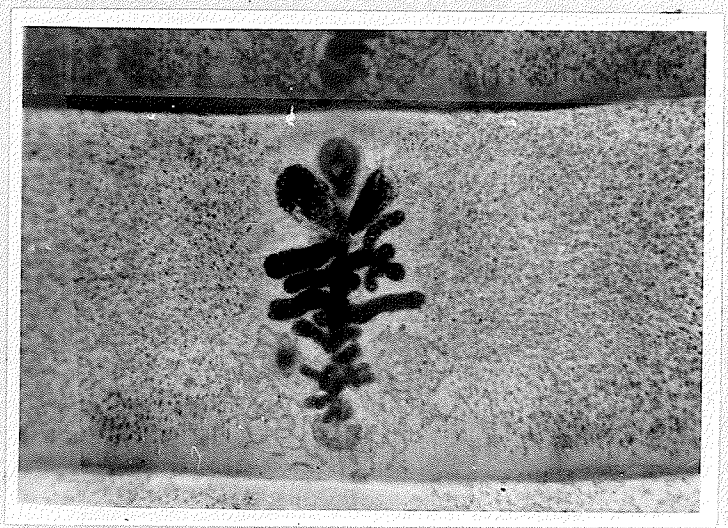

FIGURE 36.

D. latus ZENKER'S FIXATIVE - GRENACHER'S BORAX CARMINE STAIN

GUYER'S ALUM-COCHINEAL

Specimens were stained for 32 hours with destaining in 10 per cent acid alcohol for 10 to 45 minutes.

Demke's- genitalia took up stain; testes were mostly obscured by heavily stained vitellaria; specimen contracted after mounting; see FIGURE 37.

Gilson's- stain proved very effective after this fixative; destaining gave light musculature; see FIGURE 38.

Kleinenberg's- stain gave the best results after this fixative; see FIGURE 39.

Lavdowsky's- results were satisfactory.

Petrunkevitch's- genitalia were well defined.

Schaudinn's- genitalia were well defined.

Susa- results with this stain were inferior to those with Grenacher's borax carmine.

Tellyesniczky's- testes did not take up stain; other structures were suitably stained; see FIGURE 40.

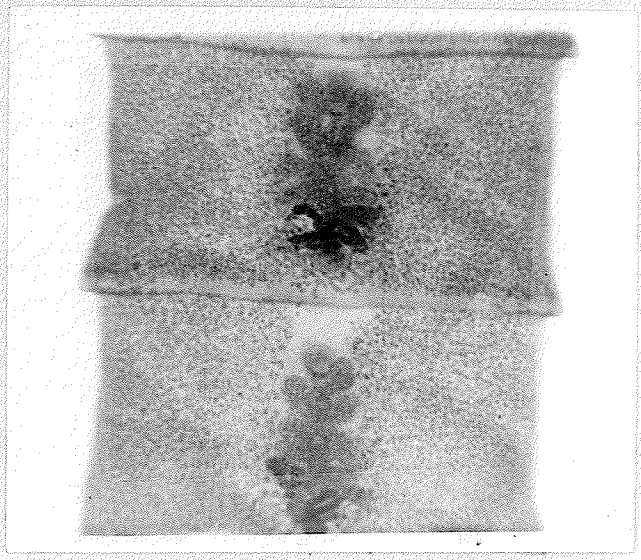

FIGURE 37.

D. latus DEMKE'S FIXATIVE - GUYER'S ALUM - COCHINEAL STAIN

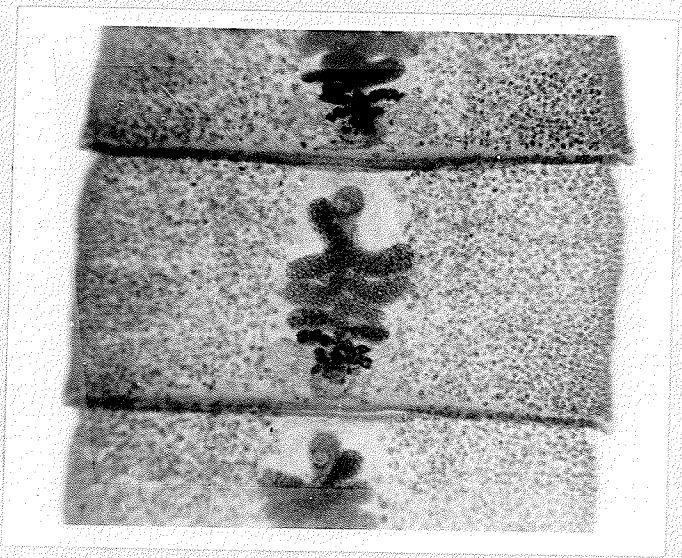

FIGURE 38.

D. latus GILSON'S FIXATIVE - GUYER'S ALUM - COCHINEAL STAIN

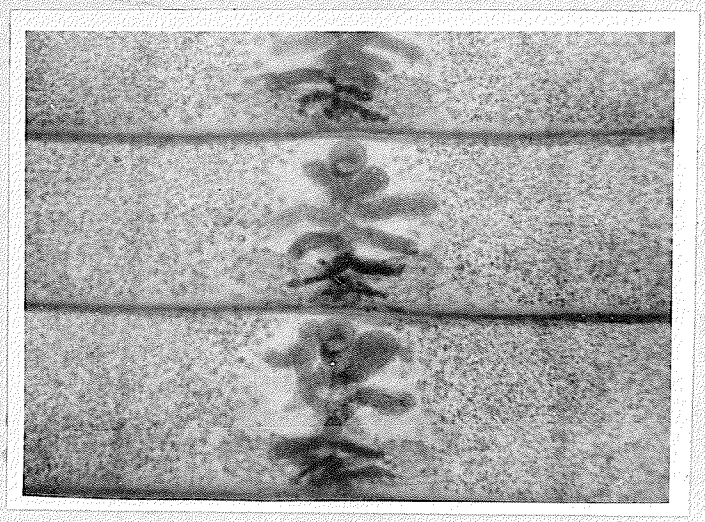

FIGURE 39.

D. latus KLEINENBERG'S FIXATIVE - GUYER'S ALUM - COCHINEAL STAIN

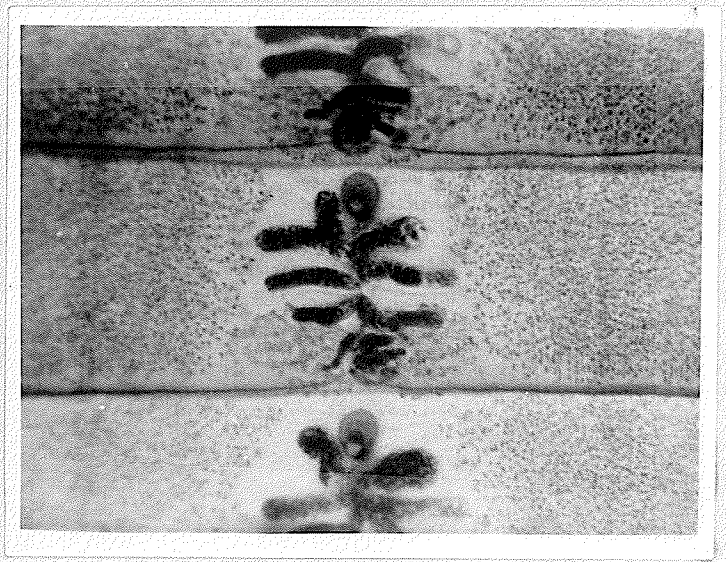

FIGURE 40.

D. latus TELLYESNICZKY'S FIXATIVE-GUYER'S ALUM - COCHINEAL STAIN

MAYER'S PARACARMINE

The stain was allowed to act for 4 days. Destaining of the specimens was done for 3 to 20 minutes using 5 per cent acid alcohol.

Allen's B-15- internal structures showed evidence of light staining; results were mainly poor.

Schaudinn's- genitalia were lightly stained but clearly evident.

GUYER'S PICRO - CARMINE

Specimens were stained for 7 days and destained with 10 per cent acid alcohol for 1 to 10 minutes. Tapeworm segments fixed in Gilson's fixative and Petrunkevitch's fixative completely disintegrated on removal from the stain.

Demke's- most internal structures were well stained; slight contraction occurred after mounting.

Schaudinn's- stain gave good results; see FIGURE 41.

Susa- staining of all the major structures except the ovary occurred; destaining of the parenchymal musculature was complete.

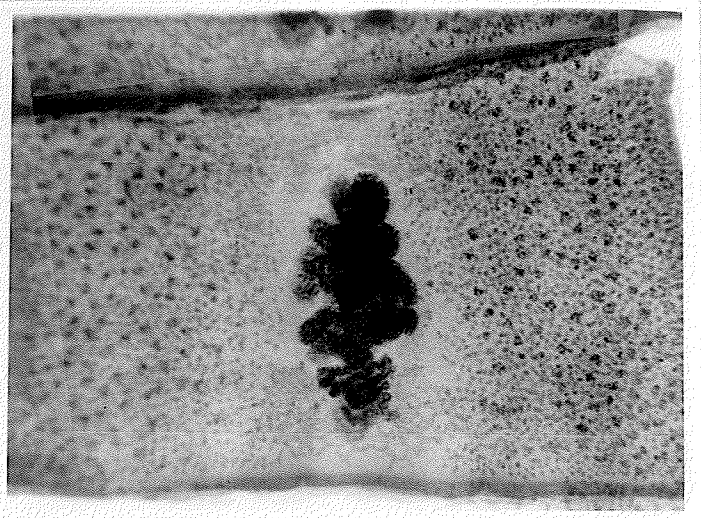

FIGURE 41.

D. latus SCHAUDINN'S FIXATIVE-GUYER'S PICRO - CARMINE STAIN

SCHNEIDER'S ACETO - CARMINE

Specimens were stained for 68 hours. All excess stain was removed in the alcohol series without the use of acid alcohol. The specimen fixed with Gilson's solution disintegrated on removal from the stain.

Alcohol- testes and ovary did not take up the stain; see FIGURE 42.

Demke's- all major internal structures stained deeply; excessive shrinkage occurred after mounting.

Schaudinn's- satisfactory staining resulted; see FIGURE 43.

Petrunkevitch's- genitalia were clearly stained; slight contraction of specimen occurred after mounting.

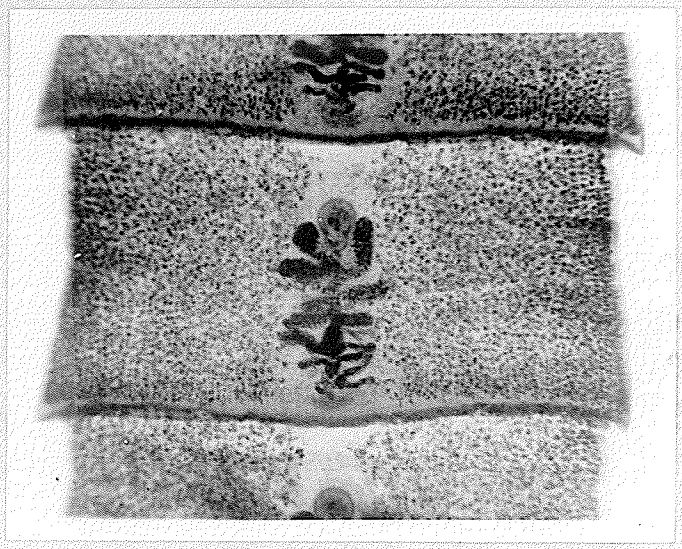

FIGURE 42.

D. latus ALCOHOL FIXATIVE - SCHNEIDER'S ACETO - CARMINE STAIN

FIGURE 43.

D. latus SCHAUDINN'S FIXATIVE-SCHNEIDER'S ACETO - CARMINE STAIN

MALLORY'S ACID FUCHSIN

Specimens were stained for 18 hours. Destaining with 10 per cent acid alcohol required 6 hours. The specimen fixed with Bouin's solution was included to further illustrate the poor staining obtained after this fixative.

Bouin's- testes and ovary were faintly visible; vitellaria and uterus wall were deeply stained; see FIGURE 44.

Demke's- genitalia were stained darkly; musculature showed poor destaining qualities.

Lavdowsky's- suitable staining resulted.

Gilson's- suitable staining resulted; destain action of musculature went to completion.

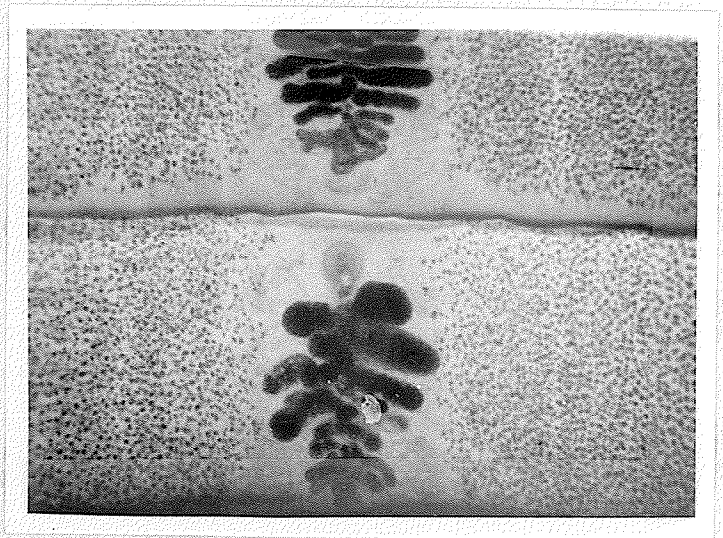

FIGURE 44.

D. latus BOUIN'S FIXATIVE - MALLORY'S ACID FUCHSIN STAIN

RISER'S COELESTIN BLUE B

Specimens were stained in an undiluted solution for 3 minutes. The use of acid alcohol was not required with this stain.

Allen's B-15- testes and ovary were lightly stained; vaginal canal was dark; see FIGURE 45.

Bouin's- musculature showed good differentiation; testes and ovary lacked stain; see FIGURE 46.

Demke's- suitable results were obtained; microscopic examination showed deeply stained testes; see FIGURE 47.

Formalin- stain proved to be one of the best for this fixative; both ovary and testes showed light staining; see FIGURE 48.

Gilson's- suitable staining resulted; see FIGURE 49.

Lavdowsky's- suitable staining resulted; see Figure 50.

Petrunkevitch's- suitable staining resulted.

Schaudinn's- suitable staining resulted.

Susa- suitable staining resulted.

Zenker's- specimens fixed in this solution were not reported with other stains because of the inability to stain the testes; 2 hours staining with Coelestin blue B showed darkly stained testes but destaining the parenchymal musculature became impossible.

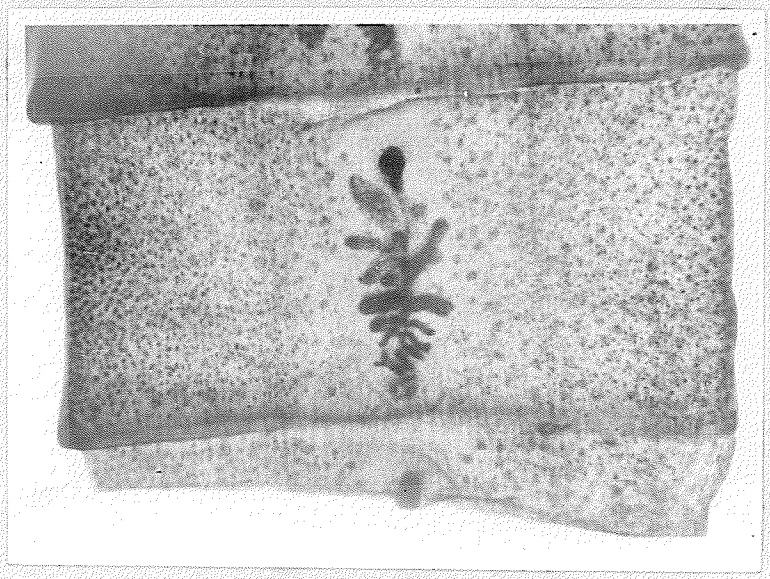

FIGURE 45.

D. latus ALLEN'S B-15 FIXATIVE - RISER'S COELESTIN BLUE B STAIN

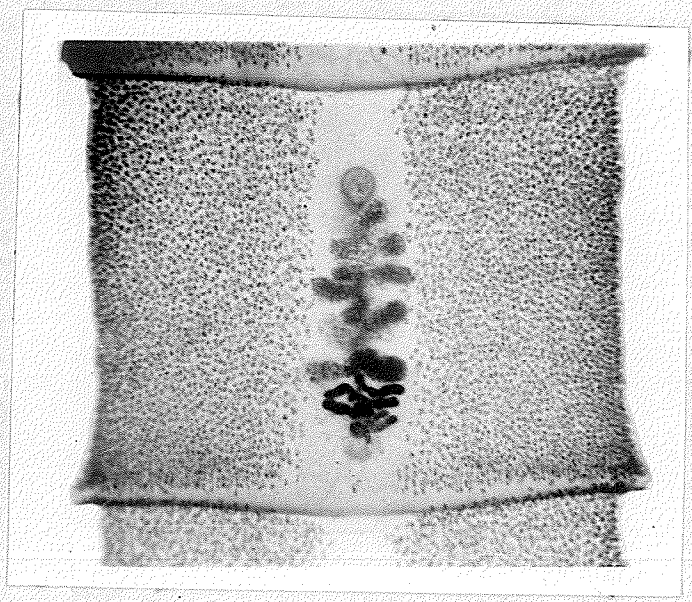

FIGURE 46.

D. latus BOUIN'S FIXATIVE - RISER'S COELESTIN BLUE B STAIN

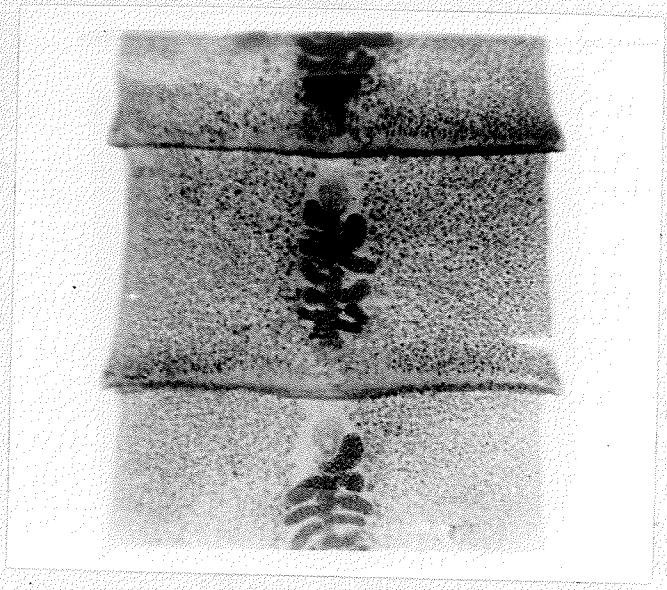

FIGURE 47.

D. latus DEMKE'S FIXATIVE - RISER'S COELESTIN BLUE B STAIN

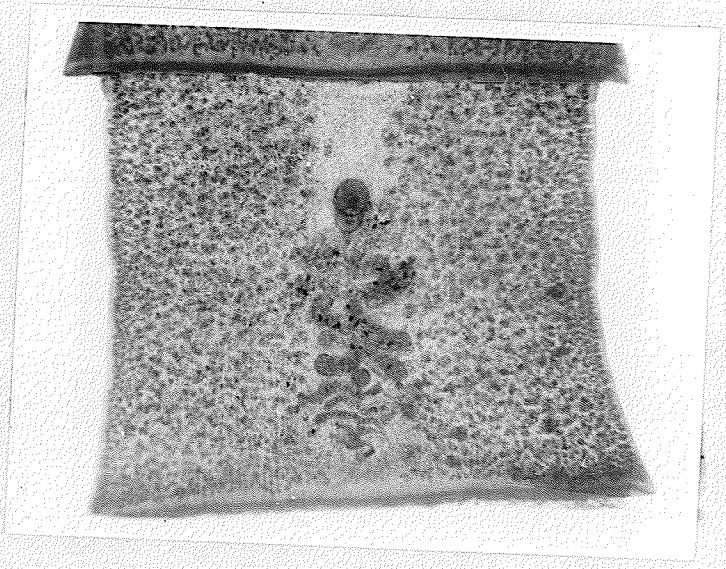

FIGURE 48.

D. latus FORMALIN FIXATIVE - RISER'S COELESTIN BLUE B STAIN

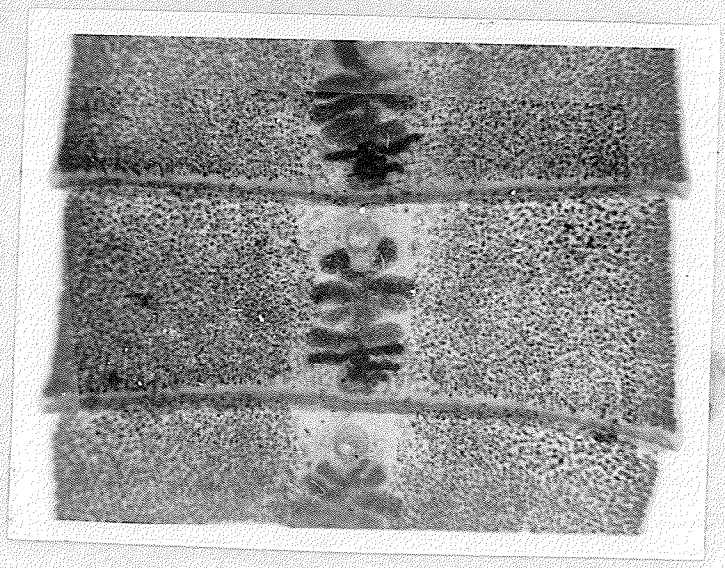

FIGURE 49.

D. latus GILSON'S FIXATIVE - RISER'S COELESTIN BLUE B STAIN

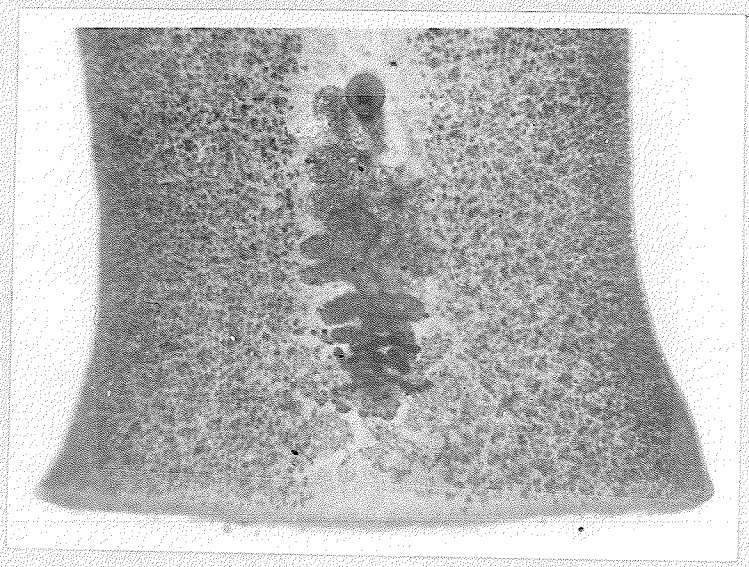

FIGURE 50.

D. latus LAVDOWSKY'S FIXATIVE - RISER'S COELESTIN BLUE B STAIN

CONGO RED

Specimens were placed in a 1 per cent aqueous solution of this stain for 48 hours. No destaining of the specimens was necessary. With the exception of one specimen all showed insufficient staining.

Lavdowsky's- internal structures were stained a deep reddish - orange color; poor contrast was noted between the genitalia and parenchymal musculature.

GUYER'S SAFRANIN STAIN

Specimens were immersed in this stain for 94 hours. Destaining of tissues was not required. The stain did not produce suitable contrast between the internal structures of the specimens.

PAPPENHEIM'S METHYL GREEN - PYRONIN STAIN

This aniline stain was allowed to act for 24 hours. Only one specimen retained the stain in tissues other than the ova to a suitable extent.

Gilson's- vitellaria, testes, and ovary were light blue; parenchymal tissues were light red; ova were red, green, and brown, the latter being tanned eggs far down the uterus.

CHAPTER VII

CLEARING AGENTS

Gray (1954) published detailed information on the properties and actions of numerous clearing agents for sectioned tissues. The writer has not found comparable information for wholemount specimens especially tapeworms. Various clearing agents were examined with a view to establishing their usefulness, if any, for tapeworm material of the size of D. latus.

Four groups of clearing agents were examined, namely, essential oils, synthetic clearing agents, higher alcohols, and mixtures. Tapeworm material fixed in Zenker's solution was used for the clearing tests. Successive proglottides from the same tapeworm body were stained with Coelestin blue B. Preliminary handling of specimens was identical in all respects. All specimens were dehydrated to the absolute alcohol stage to eliminate any effects of water. Clearing agents were allowed to act for a minimum of 24 hours. It is pointed out that although a clearing agent proved ineffective with D. latus its use with smaller, less muscular specimens is often recommended. An example was terpineol which proved useless with D. latus but ideal with small fragile cestodes and trematodes. Following are the observed results of 23 clearers with their respective refractive index where known.

ESSENTIAL OILS

Oil of Cajeput- R.I. 1.47; agent produced slight optical clearing; specimen remained pliable and unshrunken.

Oil of Cedarwood- R.I. 1.50; both Bush and B.D.H. brands were used;no suitable optical clearing occurred; slight hardening and excessive shrinkage was noted.

Oil of Cloves- R.I. 1.53; suitable clearing resulted; parenchymal tissues took up a yellow color; specimen remained pliable and unshrunken; see FIGURE 51.

Oil of Lilac- R.I. 1.48; slight optical clearing resulted; specimen remained pliable and unshrunken.

Oil of Origanum- poor optical clearing resulted; specimen remained pliable and unshrunken.

Oil of Thyme- R.I. 1.50; poor optical clearing resulted; specimen hardened but remained unshrunken.

SYNTHETIC CLEARING AGENTS

Aniline- R.I. 1.59; excellent optical clearing resulted for a limited time; specimen then showed clouding in the uterus region; specimen remained pliable and unshrunken.

Benzene- R.I. 1.50; good optical clearing occurred followed by cloudiness; hardening and shrinkage resulted.

Carbon Tetrachloride- R.I. 1.47; no optical clearing occurred; specimen hardened.

Creosote, Beechwood- very good optical clearing resulted; specimen remained pliable and unshrunken; clearing agent removed excess stains only from specimens; see FIGURE 52.

Ethyl benzoate- R.I. 1.51; poor optical clearing resulted; specimen remained pliable but shrinkage occurred.

Ethylene glycol monoethyl ether- no optical clearing occurred; specimen showed slight hardening and no shrinkage.

Methyl benzoate- R.I. 1.52; poor optical clearing occurred; specimen remained soft and unshrunken; see FIGURE 53.

Methyl salicylate- R.I. 1.54; clearing agent produced the best results; hardening resulted and only slight shrinkage; no cloudiness appeared at the end of 9 months in specimens cleared from 95 per cent alcohol; see FIGURE 54.

Terpineol- R.I. 1.48; no clearing occurred with D. latus; specimen remained pliable and unshrunken; satisfactory results were had with small trematodes; see FIGURE 55.

Toluol- R.I. 1.50; no optical clearing occurred; shrinkage and hardening of specimen resulted.

Xylol- R.I. 1.50; poor optical clearing resulted; shrinkage and hardening of specimen occurred.

HIGHER ALCOHOLS

Amyl alcohol- no optical clearing occurred.

Cyclohexanol- no optical clearing occurred.

Tertiary butyl alcohol- no optical clearing occurred.

MIXTURES

Cole's clearer- (composed of xylol, toluol, beechwood creosote, aniline); poor optical clearing resulted; parenchymal musculature showed cloudiness; specimen remained pliable and unshrunken; see FIGURE 56.

Supercedrol- (composition unknown); solution recommended for sections; no clearing resulted; specimen remained pliable and unshrunken.

Weigert's clearer- (composed of xylol, phenol); poor optical clearing resulted; specimen destained within 24 hours; specimen remained soft and unshrunken.

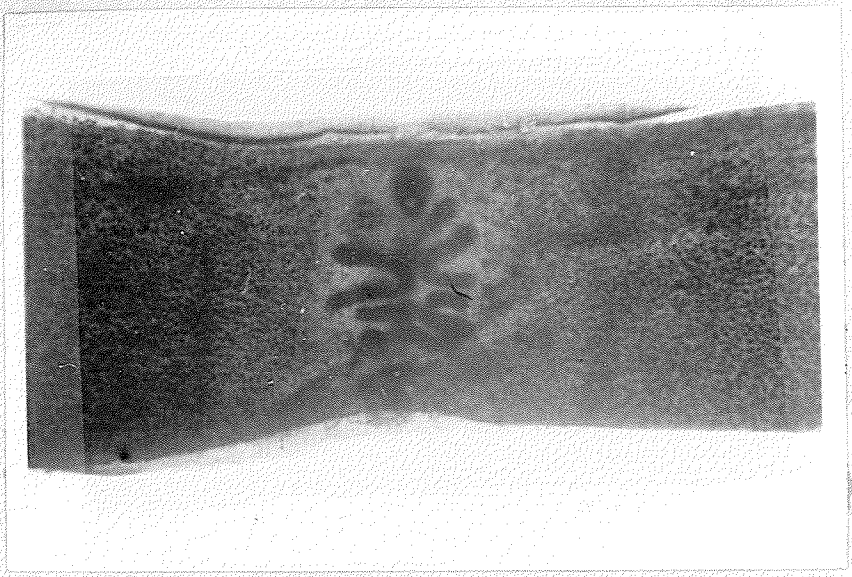

FIGURE 51.

D. latus EFFECTS OF CLOVE OIL AS A CLEARING AGENT

FIGURE 52.

D. latus EFFECTS OF BEECHWOOD CREOSOTE AS A CLEARING AGENT

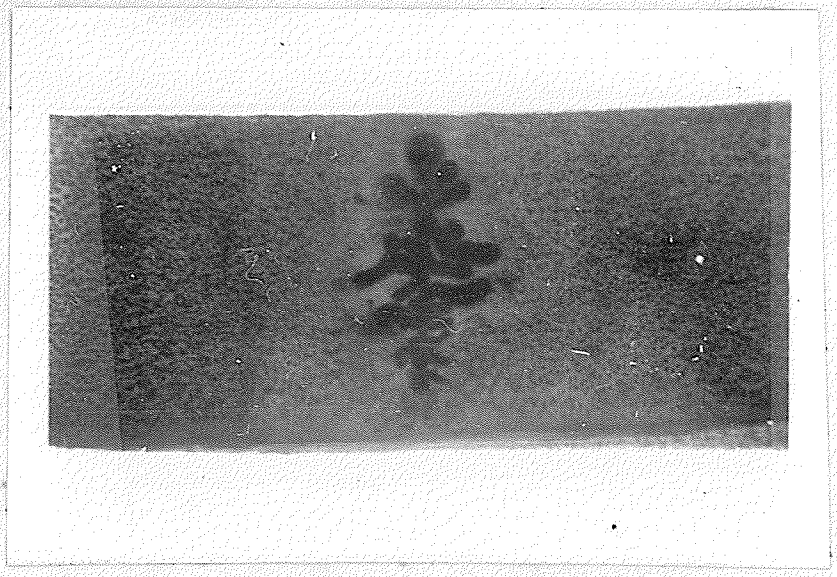

FIGURE 53.

D. latus EFFECTS OF METHYL BENZOATE AS A CLEARING AGENT

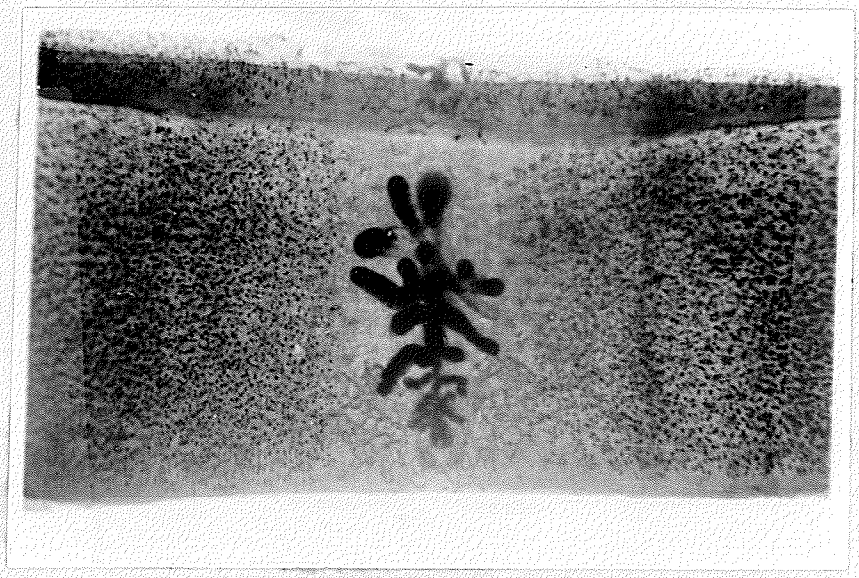

FIGURE 54.

D. latus EFFECTS OF METHYL SALICYLATE AS A CLEARING AGENT

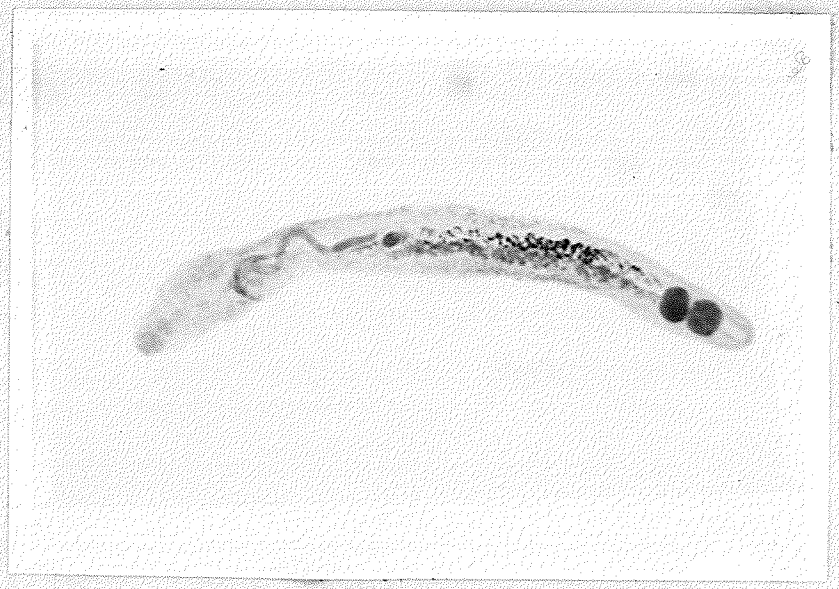

FIGURE 55.

EFFECTS OF TERPINEOL AS A CLEARING AGENT ON A SMALL TREMATODE

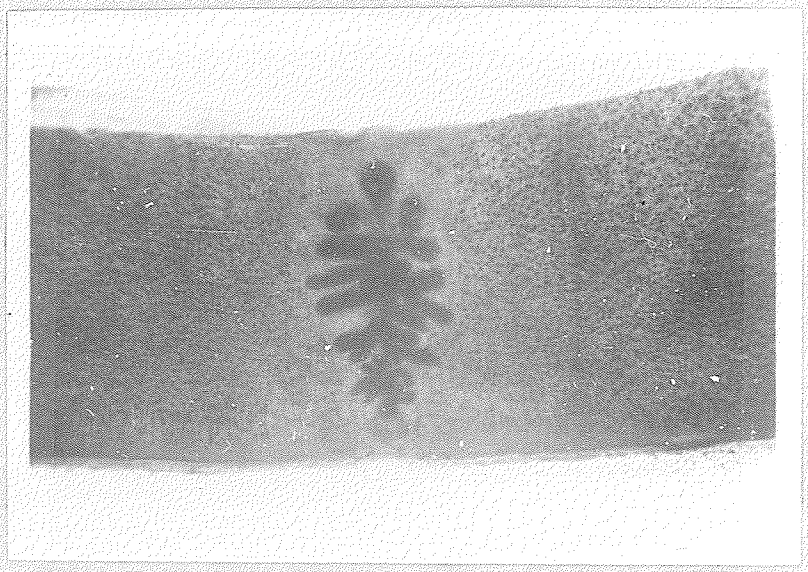

FIGURE 56.

D. latus EFFECTS OF COLE'S MIXTURE AS A CLEARING AGENT

CHAPTER VIII

SPECIAL TECHNIQUES FOR TAPEWORM WHOLEMOUNTS

Catechol treatment- Smyth (1954) used an aqueous catechol solution to tan polyphenol oxidase, an enzyme of the shell - producing regions of the female genitalia of trematodes and certain cestode groups. For contrast he suggested carmine "to stain the remainder of the genitalia not containing the enzyme." D. latus was tested and found to exhibit a similar tanning reaction. Smyth immersed small alcohol- fixed specimens in a 0.2 per cent aqueous catechol solution at 40°C for 30 to 90 minutes followed by dehydration, clearing, and mounting. The catechol technique when applied to D. latus required a 0.5 per cent solution at 40°C for 19 hours to produce results. Sixteen differently fixed specimens, when tested, exhibited varying degrees of tanning. The Lewitsky - fixed specimen showed the darkest tanning of shell - producing regions. See FIGURE 57. No extensive tanning of the vitelline ducts of any specimen occurred in the tests. The Kleinenberg - fixed specimen gave almost as good results with catechol whereas specimens fixed in alcohol as suggested by Smyth showed comparatively light tanning. In view of the better staining results obtained after Kleinenberg's fixative than after Lewitsky's fixative, the former would prove more useful for contrast staining with the usual nuclear stains.

Double staining of tapeworms- Only limited success was had with the use of two stains for preparing tapeworm wholemounts. The use of borax carmine and methyl green proved effective. The carmine stain was allowed to act on D. latus for 22 hours followed by immediate use of 2 per cent aqueous methyl green for 6 hours. Destaining, dehydration, and clearing showed internal structures having a light purple color while the ova stained various shades of green and brown. The parenchymal musculature and vitellaria took up little stain.

Neither Janus green and coelestin blue B nor light green and Delafield's haematoxylin produced any effective contrasts of internal structures.

An attempt was made, without success, to produce a polychrome stain effect on D. latus by the use of borax carmine and coelestin blue B. The specimen took on a purple color which was very striking but no color contrast of the genitalia was evident.

Destaining techniques- Tapeworms stained by the regressive method usually require the use of a destaining agent to remove stain from internal muscle layers. Some species of tapeworms present the additional problem of having a diffused, follicular type of vitellarium, which when stained can successfully obscure observation of internal structures. In carrying out the destaining

techniques the writer had more success with the parenchymal musculature than with the vitellaria of tapeworms. The use of acid alcohol proved the most controllable though not the most successful destaining agent. On D. latus specimens acid alcohol proved inadequate for destaining the vitellaria but very useful for lightening muscle layers. Free chlorine, made by the interaction of $KClO_3$ and concentrated HCl in a test tube partly stoppered with cotton wadding, was applied to deeply stained tapeworm proglottides. The proglottides had been previously blotted dry of adhering alcohol. The chlorine effectively bleached muscle layers and greatly lightened the vitellaria. The only difficulty encountered was the rapidity with which chlorine would bleach the entire specimen. The use of a 2 per cent aqueous solution of sodium hypochlorite (5 - 6 per cent available chlorine) was effective if not allowed to overact. Too long immersion in this solution caused excessive loss of stain at the clearing stage.

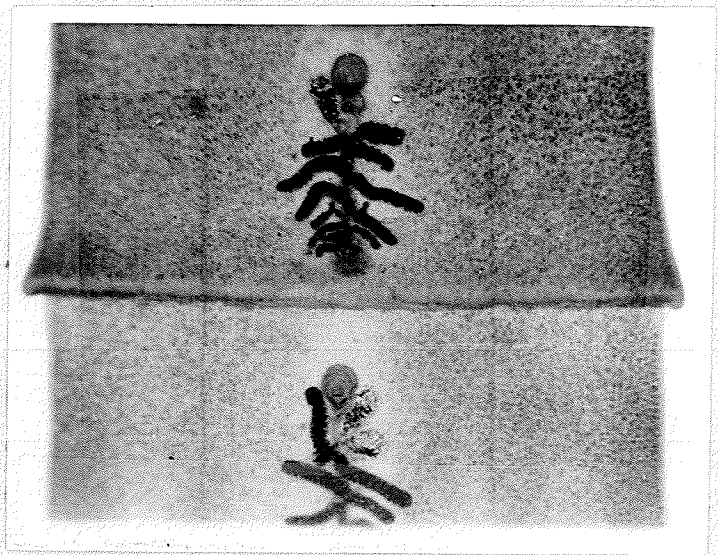

FIGURE 57.

D. latus EFFECTS OF CATECHOL TREATMENT ON SHELL - PRODUCING REGIONS OF A LEWITSKY - FIXED SPECIMEN

CHAPTER IX

HISTOLOGICAL STAINING OF TAPEWORMS

Efforts were directed towards developing a new stain combination suitable for use with tapeworm sections. Triaenophorus nodulosus was selected for the experimental staining. The worms had been previously relaxed with menthol, fixed in Gilson's solution, and stored in 70 per cent alcohol. The following staining sequence, which proved successful, was repeated seventeen times for verification purposes. Stains utilized were Riser's coelestin blue B, eosin B (water and alcohol soluble), and light green.

PROCEEDURE

1. Worms were upgraded to absolute ethyl alcohol with 1 hour allowed in each solution.
2. Transferred to abs. alcohol and aniline oil, 50:50 for 1 hour.
3. Transferred to pure aniline oil for 1 hour.
4. Transferred to aniline oil and chloroform, 50:50 1 hour.
5. Transferred to chloroform, 2 changes for 30 min. each.
6. Transferred to chlorowax, 50:50 at 52°C for 1 hour.
7. Transferred to parawax at 52°C, 2 changes for 1 hour each.
8. Worms were embedded and sectioned at 7 microns.
9. Sections were deparaffinized in 2 changes of xylol for 15 min. each.

10. Sections were placed in abs. ethyl alcohol for 5 min. then placed directly into distilled water for 5 min.
11. Sections were stained in undiluted coelestin blue B for 3 minutes.
12. Excess stain was removed in distilled water.
13. Sections were stained in 2 per cent aqueous Eosin B for 6 to 7 minutes.
14. Excess stain was removed in distilled water.
15. Sections were placed in 1 per cent aqueous phosphotungstic acid for 20 to 30 seconds. The time was dependent on the desired intensity of coelestin blue B.
16. Sections were rinsed rapidly in distilled water.
17. Sections were stained in a solution of 40 drops of 1 per cent aqueous light green diluted in 50 cc. of distilled water for a maximum of 30 seconds or until slight darkening was noted under a binocular microscope.
18. Sections were rinsed in distilled water and dehydrated rapidly in 2 changes of abs. ethyl alcohol.
19. Sections were plunged into xylol at the moment of desired differentiation.
20. Sections were mounted in xylol - based Permount.

Results- See FIGURE 58. The cuticle was dark green; subcuticular layers--blue - green; vitellaria--blue with red nuclei; parenchymal musculature--red; parenchyma--light green; testes and ovary--light blue; uterus wall--

dark green; cirrus pouch wall and sperm ducts--red. The stains showed no indication of fading.

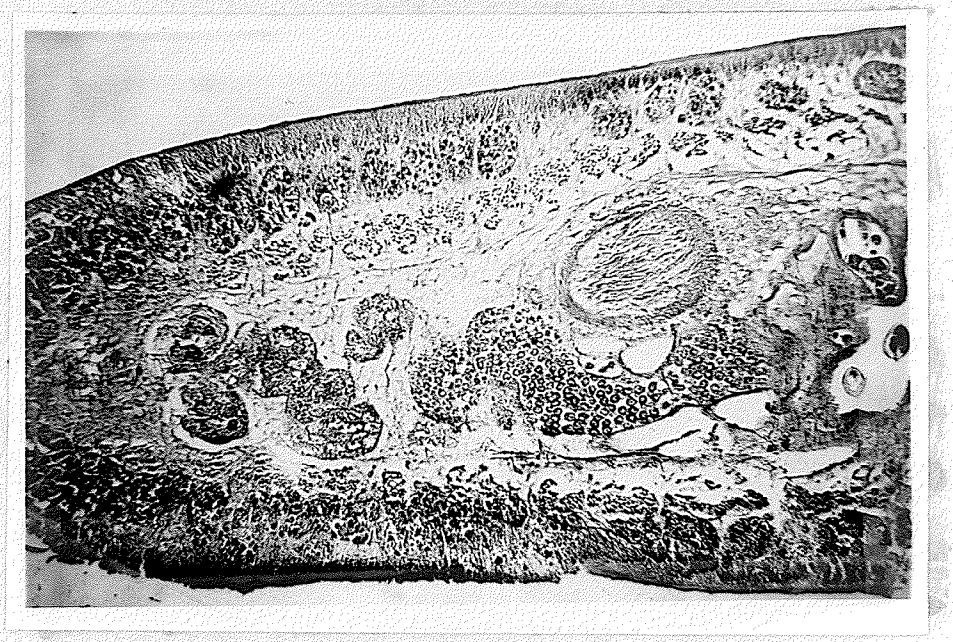

FIGURE 58.

T. nodulosus STAINED WITH COELESTIN BLUE B, EOSIN B, AND LIGHT GREEN

CHAPTER X

DISCUSSION

This section is an evaluation of the results of the fixative - stain tests.

The writer does not know to what extent the successful fixative - stain techniques can be applied to species of tapeworms other than D. latus. There are many differences in size and structure and possibly sufficient chemical differences amongst tapeworms which could limit the application of a particular fixative - stain technique. On the other hand, these differences may be sufficiently unimportant, thus a successful fixative - stain combination with one species could be applied with equal success to an entirely different species of tapeworm. The writer felt he could make the following statements authoritatively only in reference to the species on which the techniques were carried out.

It was found, as has been stated by other workers, that obtaining successful results with a stain depended largely on the fixative used. An example of this was the results of Delafield's haematoxylin after Bouin's fixative and after Gilson's fixative. It was observed that some fixatives, notably Allen's B-15 caused the same internal structures to appear--testes in this case--regardless of

the stain used. Obviously, the presence of urea and/or chromic acid in this fixative had increased the density or improved the staining of this structure. Ethyl alcohol and formalin as fixatives lacked the qualities shown by most of the other fixatives. Haematoxylin stains exhibited little combining power after these solutions, especially with such an easily stained structure as the ovary. Lavdowsky's solution however, a mixture of alcohol and formalin with acetic acid added, showed much greater compatibility with haematoxylin stains. Bouin's solution proved most unsatisfactory as a wholemount fixative whether with aqueous or alcoholic stains. Mann's and Delafield's haematoxylin stains were the only ones which showed some indication of giving results after this fixative. Cleverdon's fixative, as well as Tellyesniczky's and Helly's, lacked the qualities necessary for a tapeworm fixative. They showed very little compatibility with the various stains. Further, the latter two solutions served only to increase the opacity of stained specimens. Demke's solution, in the opinion of the writer, gave commendable results. Stained structures invariably showed good definition and differentiation. The only limiting factor of Demke's solution appeared to be the tendency of specimens to shrink in xylol - based Permount. Gilson's fixative was one of the best used in the tests. In combi-

nation with haematoxylin it permitted rapid and precise staining of all genitalia. Its only drawbacks were the extensive preliminary handling to remove mercuric chloride precipitates and the eventual fragility caused to specimens. Kleinenberg's fixative showed an improvement over Bouin's fixative but prevented most stains from combining with the testes. Petrunkevitch's fixative produced variable results with both haematoxylin and carmine stains. The tendency existed for the testes and not the ovary to stain after this fixative. Like Gilson's solution it caused extreme softening of the specimens. Schaudinn's fixative gave consistently good results; it proved superior to Gilson's fixative in two respects by (1) being compatibile with more stains, especially a number of the carmines, and (2) by fixing the worms in a fairly firm state. Susa fixative showed favorable results with seven of the seventeen stains used. Good staining resulted with both haematoxylin and carmine stains. Trichloroacetic acid was likely the causative agent in blackening and shrivelling the ova contents in specimens fixed with this solution. Zenker's fixative was not entirely satisfactory. Its combination with coelestin blue B was unexcelled but the testes of Zenker - fixed specimens showed a definite tendency to remain unstained. The writer does not believe the presence of potassium dichromate, as in Zenker's fixative, materially

improved wholemount preparations. Specimens fixed with this chemical exhibited the poorest destaining action of the parenchymal musculature.

The results obtained with haematoxylin stains were far superior to those obtained with carmine stains. A lack of high contrast between the genitalia and parenchymal musculature appeared to be the biggest defect of carmine. Of the aniline stains tested, only coelestin blue B matched the effectiveness of the haematoxylin stains. Lillie's acid haemalum gave the poorest results of the haematoxylin stains while Mann's and Harris' haematoxylin (Isa variant) gave excellent results, especially after the mercuric chloride fixatives. Of the carmine stains, Guyer's alum - cochineal and Grenacher's borax carmine proved the most successful. Coelestin blue B was preferred by the writer over all stains. It caused exceptionally high contrast, showed compatibility with the most fixatives, and stained rapidly. Gray (1954) in reference to oxazine stains of which coelestin blue B is one of four in the group, remarked that, "it is a matter of some astonishment to those who have employed them that they have not received wider acceptance as a substitute for hematoxylin." The stain had the additional advantage of staining from both an aqueous and alcoholic solution.

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