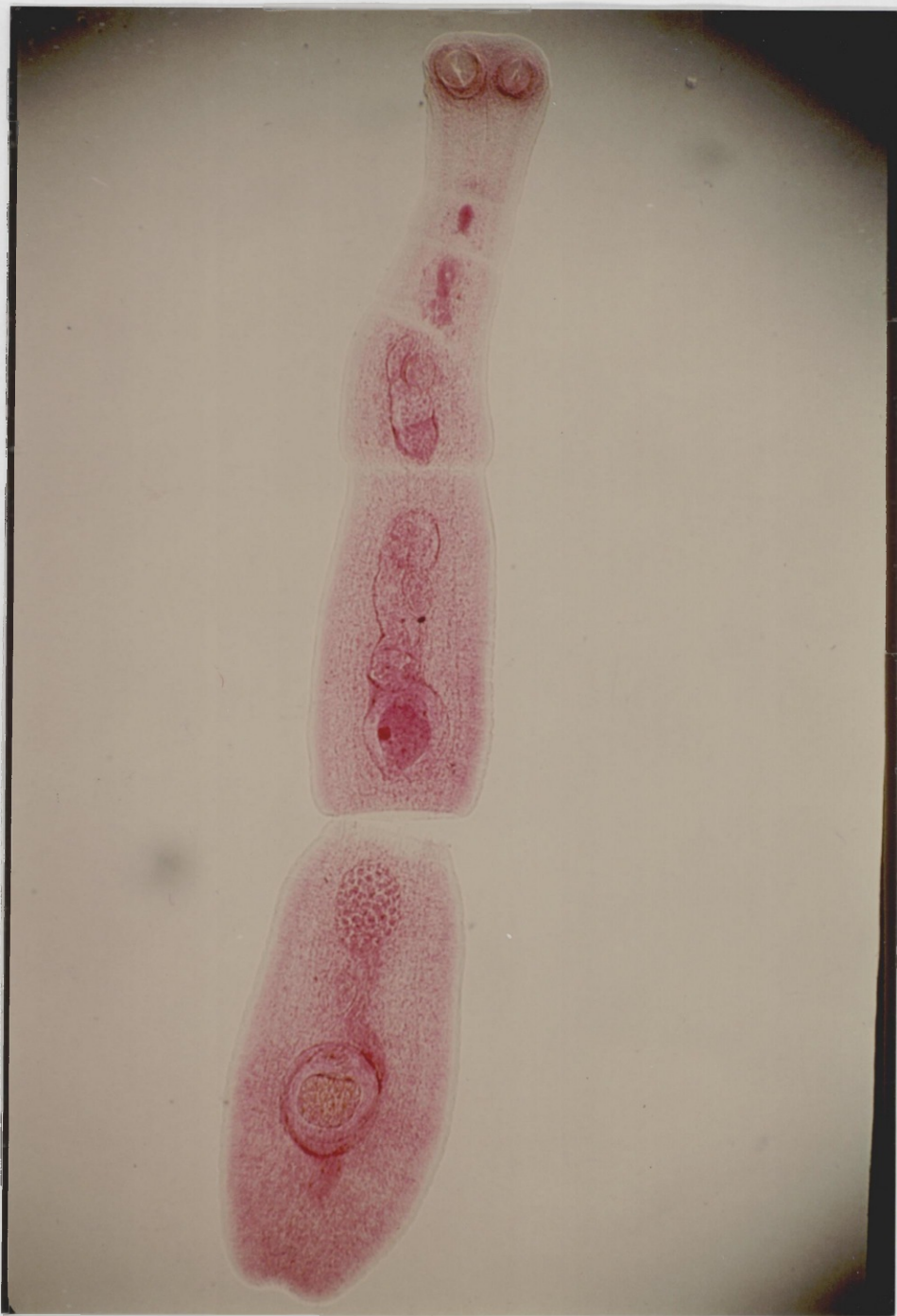




Frontispiece. Adult Mesocestoides corti from dog, 38 days p.i.

Gower's carmine. x 40.

IN VITRO CULTURE OF MESOCESTOIDES CORTI (CESTODA)

By

SHIOU-JENG ONG

A thesis submitted for the degree of
Doctor of Philosophy of the University of London
and the
Diploma of Membership of the Imperial College of
Science and Technology

Department of Pure and Applied Biology,
Imperial College of Science and Technology,
London SW7.

June 1984.

Dedicated to my mother, Hsen-Niu C. Ong,

and

the memory of my father, Quai Ong.

ABSTRACT

The in vitro culture of tetrathyridia of Mesocestoides corti to sexually mature adults has been successfully achieved. Tetrathyridia began segmenting in vitro within one week. This is compatible with the in vivo rate of development in the dog. In the most successful experiments, up to 100% of tetrathyridia developed into strobilate adults after 11 days. Factors favouring segmentation and sexual differentiation were investigated. Although sexually mature adults were grown consistently from undifferentiated tetrathyridia in vitro, eggs containing motile oncospheres were only obtained sporadically. Possible reasons for the low production of embryonated eggs are discussed.

Asexual multiplication of both tetrathyridia and strobilate adults was observed in some cultures. It was found that a diphasic medium was more favourable for asexual multiplication than a monophasic medium; this phenomenon appeared to be associated with penetration of the solid phase by the organisms. The consistency of the physical environment, rather than nutritional requirements, is thus believed to be associated with the asexual multiplication of tetrathyridium and adult M.

corti.

The success of cultivation was also evaluated by comparing development in vitro with that in vivo. The ultrastructure of the tegument of tetrathyridium, pre-segmentation stage and strobilate adult was examined. In particular, the possible functions and significance of various types of microtriches and vesicles observed in the distal cytoplasm of syncytial tegument cells, at various stages, are discussed.

ACKNOWLEDGEMENTS

This study was carried out with the financial support from the National Science Council of the Republic of China (1981 and 1982) and the Committee of Vice-Chancellors and Principals of the Universities of the United Kingdom (1983), to whom I am deeply indebted.

I wish to express my sincere thanks to my supervisor, Professor J.D. Smyth, for all his help, encouragement and constant enthusiasm throughout the course of this study and also for his prompt reading and advisory commenting on the manuscript. I would also like to thank my advisor, Dr. D.P. McManus, for all his help, as well as his assistance in biochemical techniques and for commenting on the manuscript.

My thanks are also due to all my colleagues, in this department, who gave assistance with readiness, and in particular to the following: Dr. N.J. Barrett, Mrs. G. Adams, Mrs. Annie Merry, Mrs. Nina Dowson, Mr. John Harmer (Workshop); Mr. Ian French, Mr. Sinclair Stammers (Photographic Unit), Mr. Dave Hopkins, Mr. Norman Costa (EM Unit), Mrs. Merlyn Singh, Mr. Paul Morris (Animal Unit) and especially Miss Ida Sontora.

I am grateful to Dr. C.N.L. Macpherson for carrying out dog experiments in Kenya, and to Dr. Marcus French for providing him with the facilities to do so, in the laboratory in Lodwar.

The statistical advice given by Dr. Chien-Jen Chen (Department of Public Health, College of Medicine, National Taiwan University, Taiwan) is also acknowledged.

I am indebted to Dr. R.C.A. Thompson (Division of Veterinary Biology, School of Veterinary Studies, Murdoch University, Australia) for providing me with the specimens of M. corti maintained in his laboratory. Thanks are also due to Dr. D.B. Conn (Department of Biological Sciences, University of Cincinnati, Ohio) for sending me the manuscript of his paper on "Fine structure of the gravid paruterine organ and embryonic envelopes of Mesocetoides lineatus (Cestoda)", and to Dr. Geogio Gabella (University College, London) for the interpretation of some ultrastructural micrographs.

I would like to thank Dr. C.N.L. Macpherson, Dr. N.J. Davey and particularly Dr. R. Madhavi (Department of Zoology, Andhra University, Waltair, India) for correcting my English and commenting on various parts of the manuscript.

I duly appreciate all those named and unnamed who generously extended their friendship and companionship towards me during my stay in this country.

Finally, I wish to thank my husband, for his understanding, my brothers and especially my sister, Shiou-ching, for their moral support and continual encouragement from miles away. To my mother, I am more than grateful. This work would not have been completed without her extra financial support.

CONTENTS

ABSTRACT, 4

ACKNOWLEDGEMENTS, 6

CONTENTS, 9

LIST OF FIGURES, 13

LIST OF TABLES, 13

LIST OF PLATES, 13

ABBREVIATIONS, 14

CHAPTER 1 : GENERAL INTRODUCTION, 15

1.1 Basic aims, 15

1.2 Taxonomy, 16

1.3 Life-cycle, 17

1.4 Morphology and asexual multiplication of
tetrathyridia, 19

1.5 Scope of this study, 22

CHAPTER 2 : MATERIALS AND GENERAL METHODS, 32

2.1 Maintenance of Mesocestoides corti tetrathyridia
in mice, 32

2.2 Collection of tetrathyridia, 32

2.3 In vitro culture, 33

2.3.1 General aseptic technique, 33

2.3.2 Preparation of culture media, 34

2.3.3 Culture vessels, 37

2.3.4 Routine replacement of media, 37

2.3.5 Agitation of cultures, 37

2.4 Experimental infection of dogs, 38

- 2.5 Morphology and histology, 38
- 2.6 Electron microscopy, 39
 - 2.6.1 Scanning electron microscopy (SEM), 40
 - 2.6.2 Transmission electron microscopy (TEM), 41

CHAPTER 3 : EFFECTS OF SOME CULTURE FACTORS ON
SEGMENTATION RATES IN VITRO, 44

- 3.1 Review of literature on cultivation of cestodes from metacestode to adult, 44
- 3.2 Some basic problems of in vitro cultivation, 52
- 3.3 Methods, 54
 - 3.3.1 Pretreatment, 55
 - 3.3.2 Number of tetrathyridia per culture, 56
 - 3.3.3 Gas phase, 56
 - 3.3.4 pH, 57
 - 3.3.5 Medium, 58
- 3.4 Results, 58
 - 3.4.1 Pretreatment, 59
 - 3.4.2 Number of tetrathyridia per culture, 59
 - 3.4.3 Gas phase and pH, 60
 - 3.4.4 Medium, 61
- 3.5 Discussion, 63
 - 3.5.1 Pretreatment, 63
 - 3.5.2 Gas phase and pH, 66
 - 3.5.3 Medium, 68
 - 3.5.4 Egg production, 70

CHAPTER 4 : DEVELOPMENT IN VIVO AND IN VITRO, 88

- 4.1 Morphological changes and the behaviour of tetrathyridia prior to segmentation in the monophasic medium S10E, 88
- 4.2 Development in vivo, 89
- 4.3 Development in vitro, 91
- 4.4 Effects of oxygen on the development of M. corti in vitro, 93
- 4.5 Embryogenesis in vitro, 97
 - 4.5.1 Fertilization, 97
 - 4.5.2 Cleavage and cellular differentiation, 99
 - 4.5.3 Mature eggs in the paruterine organ : with special reference to the embryonic envelopes, 100
 - 4.5.3.1 The egg mass in the paruterine organ of M. corti obtained from the dog intestine, 101
 - 4.5.3.2 Mature eggs from in vitro cultured worms, 103

CHAPTER 5 : ULTRASTRUCTURAL CHANGES IN THE TEGUMENT DURING LARVAL-ADULT TRANSFORMATION, 127

- 5.1 Introduction, 127
- 5.2 Scanning electron microscopy of tetrathyridia at various developmental stages, 128
 - 5.2.1 Invaginated tetrathyridia from experimental mice, 129
 - 5.2.2 Tetrathyridia with no sign of segmentation, 129
 - 5.2.3 The pre-segmentation stage, 131
- 5.3 Transmission electron microscopy of the tegument of the tetrathyridia at various developmental stages in vitro, with emphasis on the apical scolex, 132
 - 5.3.1 Tetrathyridia with no sign of segmentation, 133

5.3.2 The pre-segmentation stage, 133

5.4 The tegumental ultrastructure of the adult M. corti in vivo, 134

5.5 Functional considerations, 135

CHAPTER 6 : SUMMARY AND GENERAL DISCUSSION, 153

6.1 In vitro cultivation, 153

6.2 The tegument, 157

REFERENCES, 165

APPENDICES, 193

LIST OF FIGURES

Fig. 1 - 1	p. 23
Figs. 3 - 1 to 3 - 7	pp. 73 - 84
Figs. 4 - 1 to 4 - 3	pp. 106 - 108

LIST OF TABLES

Table 1 - 1	p. 24
Tables 3 - 1 & 3 - 2	pp. 85 - 86
Table 4 - 1	p. 109
Table 5 - 1	p. 138

LIST OF PLATES

Plate 2 - 1	p. 43
Plate 3 - 1	p. 87
Plates 4 - 1 to 4 - 11	pp. 110 - 126
Plates 5 - 1 to 5 - 7	pp. 139 - 152

ABBREVIATIONS

B	basal lamina	od.	oviduct
Ba	base (microthrix)	OM	"oncospherical membrane"
C	capsule	ov.	ovary
CB	cytoplasmic bridge	PC	perinuclear cytoplasm
cir.	cirrus	PG	penetration gland
c.p.	cirrus pouch	PM	plasma membrane
c.v.d.	common vitelline duct	Sh	shaft (microthrix)
DC	distal cytoplasm	u.	uterus
E	embryophore	ut.d.	uterine duct
Em	embryo	v.	vitellaria
Ep	embryonic epithelium	vag.	vagina
G	germinative cells	W	wall
Gl	glycogen	ZA	zonula adherens
g.o.	genital opening		
H	hooks		
HM	hook muscle		
I	inner envelope		
M	mitochondria		
Ms	muscle layers		
N	nucleus		
Nu	nucleolus		
O	outer envelope		
oc.	oocapt		

CHAPTER 1

GENERAL INTRODUCTION

1.1 Basic aims

As is well recognised (Weinstein, 1966; Smyth, 1969a; Silverman and Hansen, 1971), the in vitro culture of helminths, particularly cestodes, enables the factors in the growth, differentiation and reproduction of parasites to be studied under controlled conditions in the absence of the host. An added advantage is that living rather than dead specimens can be obtained for morphological and ultrastructural studies and thus provide more accurate information.

The second larval stage of Mesocestoides corti, the tetrathyridium, serves as a very good model for these biological studies because it has the ability for both sexual differentiation and asexual multiplication (Specht and Voge, 1965). Moreover, the tetrathyridium can be maintained easily in the laboratory by intraperitoneal passage through experimental mice (Specht and Voge, 1965).

Once optimal conditions for growth and development are

established, the immunology, biochemistry, physiology and chemotherapy of the parasite can then be studied effectively by means of in vitro culture techniques (Weinstein, 1966; Silverman, 1965; Smyth, 1969b).

1.2 Taxonomy

Mesocestoides corti is a cyclophyllidean parasite classified as below.

Order Cyclophyllidea Beneden in Braun, 1900

Family Mesocestoididae Perrier, 1897

characterised by medially situated genital atrium.

Genus Mesocestoides Vaillant, 1863

characterised by the presence of paruterine organ.

Species corti Hoeppli, 1925

Voge (1955) regarded M. variabilis Mueller, 1927 and M. manteri Chandler, 1942 as synonyms of M. corti. The revised diagnosis was presented:

"Scolex usually but not always followed by a neck. Scolex diameter 247-616 μm ; sucker diameter 90-200 μm x 99-300 μm . Immature and mature proglottids always trapezoidal; mature proglottids wider than long or longer than wide. Testis number 20-86 per proglottid;

testis diameter 26-88 μm . Cirrus pouch width 54-117 μm ; cirrus diameter 6-10 μm . Vitelline glands usually posterior to ovaries in relaxed specimens, lateral to ovaries in contracted specimens or in relatively wide proglottids. Ovary and vitelline glands variable in shape and size. Egg capsule width 239-447 μm ; egg mass width 215-354 μm . Adults usually found in carnivores."

Although the family Mesocestoididae is assigned to the order Cyclophyllidea by the criterion of the presence of four suckers on the scolex, the classification is still open to question. Mesocestoidid adults possess two compact vitelline glands and a medially situated genital atrium. These are not the characters of cyclophyllideans. The developmental patterns from the hatched oncosphere to the tetrathyridium determined from in vitro culture (Voge, 1967) resembles those of proteocephalideans (Voge, 1969). Wardle, McLeod & Radinovsky (1974) established a new order, Mesocestoididea, but it has not been adopted by most parasitologists. Moreover, the classification and speciation of the genus Mesocestoides is also rather controversial (Witenberg, 1934; Voge, 1955; Chertkova and Kosupko, 1977; Loos-Frank, 1980a).

1.3 Life-cycle

The distribution of mesocestoidid parasites is cosmopolitan (Table 1-1), but the life cycle is still incompletely known. It is suspected (Fig. 1-1) that two intermediate hosts are required (Markowski, 1933; Soldatova, 1944; Voge, 1969). The first intermediate host is thought to be a coprophagous arthropod, but there is only limited evidence to support this view. Soldatova (1944) discovered the larvae of Mesocestoides lineatus, at various developmental stages from oncospheres to the stage of "cysticeroid" (described as "proceroid-like" by Voge, 1967) in oribatid mites which were ascribed an active role in the completion of the life cycle of this parasite. It is assumed that when the proceroid-like stage in the body cavity of the oribatid mite is eaten by the second intermediate host, it becomes a tetrathyridium. The second intermediate host can be an amphibian (James and Ulmer, 1967), reptile (Voge, 1953; Dyer, 1971; Mankau and Widmer, 1977), bird (Markowski, 1933; Kaliner, 1972; Kugi, 1971, 1973) or mammal including the dog, cat, fox, rodent etc. It is assumed that the second intermediate hosts are acting as paratenic hosts rather than as intermediate hosts, but the paratenic host is obligatory since the definitive hosts, which can be the dog, cat, fox, rodent, skunk, opossum etc., are more likely to eat a small vertebrate carrying tetrathyridia rather than a mite (Baer, 1971). However, observations from in vitro

cultivation from oncosphere to fully developed tetrathyridium (Voge and Seidel, 1968) support the hypothesis of the requirement for two intermediate hosts as the tetrathyridial stage does not develop unless blood or haemoglobin is added to the medium. This suggests that a vertebrate intermediate host is required in nature (Voge, 1969). Tetrathyridia occupy the body cavities and invade the viscera of the infested hosts. When eaten by the definitive hosts, tetrathyridia develop to strobilar stages in the small intestine. Of particular significance, the tetrathyridia can also multiply asexually in the intestine of the definitive hosts before transition to the adults takes place (Eckert, von Brand and Voge, 1969). Human infection with tapeworms of the genus Mesocestoides is sporadic. Eighteen cases have so far been reported in the world. Eleven occurred in Japan (Morisita, Nagase and Moriyama, 1975), one in Korea (Choi, Kim and Choi, 1967), one in Africa (Fain and Herin, 1954) and five in the United States (Hutchison and Martin, 1980).

1.4 Morphology and asexual multiplication of tetrathyridia

The tetrathyridium of Mesocestoides corti is a metacestode of special interest. It resembles a plerocercoid but has an invaginated scolex bearing four

suckers, lacking hooks and a rostellum. It is symmetrical, flat and tapering posteriorly. It varies in length from less than one to over 10 mm, depending on the host and duration of the infection (Novak, 1972).

The asexual multiplication of Mesocestoides tetrathyridia in laboratory animals was first described by Specht & Voge (1965) and, thereafter, discussed by some investigators (Voge and Coulombe, 1966; Hart, 1967; Novak, 1972; Hess, 1972, 1980). It is now clear that the tetrathyridium divides by longitudinal splitting from the scolex region (Novak, 1972; Hess, 1972, 1980) while all other multiplying cestode larvae proliferate by budding.

A longitudinal cleft near the midline of the scolex marks the beginning of longitudinal fission (Novak, 1972; Hess, 1972, 1980), but the division stops in the middle of the tetrathyridium body (Hess, 1980). There are two suckers in each half of the scolex. One or both of the scoleces separate from the parental body. A new pair of suckers forms in each daughter scolex, but they may appear before separation occurs (Novak, 1972). The fragment without suckers does not regenerate a scolex and later degenerates.

By using electron microscopy, Hess (1980) believed that a polynucleated cell mass which he called 'apical

massif' played an important role in the mode of multiplication by antero-posterior fission of tetrathyridia. It is located at the top of the solex between two pairs of suckers and he concluded it possessed 'cytomorphogenetic power'. During asexual multiplication, the apical cleft splits the apical massif, which increases during the predivision phase, into two parts. Subsequently, the lateral area of the apical massif differentiates into a tegumental syncytium, subtegumental muscles, glycogen-storing parenchyma cells and a part of the analagen of the suckers. While, the centre of it remains undifferentiated. The apical cleft advances posteriorly, splitting the scolex into two halves during the lateral differentiation of the apical massif.

Polycephalic tetrathyridia, with several scoleces, were observed by Hart (1967) and Novak (1972) in mice with a long-standing infection. Such organisms transplanted into a new host break into the usual tetrathyridia and large branching acephalic fragments (Novak, 1972). On the other hand, a scolex containing supernumerary suckers, in the absence of an apical cleft, was also observed by Novak (1972), and has sometimes been observed in in vitro culture conditions which apparently are not suitable for asexual multiplication of tetrathyridia (present thesis).

1.5 Scope of this study

Tetrathyridia of M. corti, recovered from the peritoneal cavity of experimentally infected mice, were used for in vitro culture. Experiments were set up in an attempt to determine the optimal conditions for segmentation and sexual differentiation in vitro. In order to evaluate the success of in vitro cultivation, dogs were infected orally with tetrathyridia and autopsied at various periods post infection. The development in vitro was compared with that in vivo at the level of light microscopy and transmission electron microscopy. The ultrastructural changes in the tegument during larval-adult transformation were especially examined.

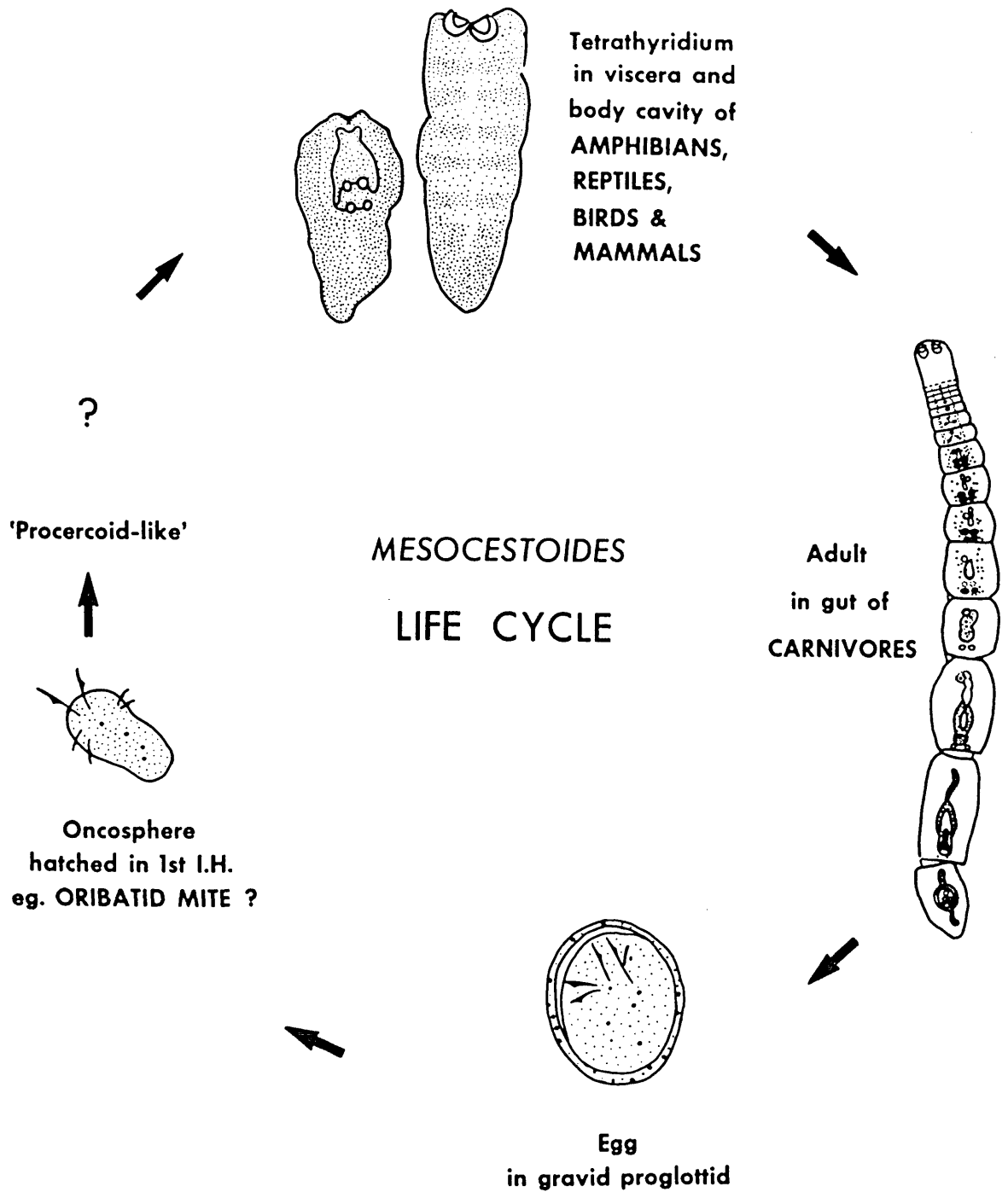


Fig. 1-1 Life cycle of Mesocestoides sp. The stages shown in the mite are based only on the observations of Soldatova (1944) and require confirmation.

Table 1-1 Infested hosts and geographical distribution of Mesocestoides spp.

Parasites/Stage	Hosts	Localities	References
<u>M. corti</u> /Strobilae (S)	1. mouse	USA	Hoepli, 1925
	2. <u>Procyon lotor</u> (raccoon)	USA	Ogren, 1956
	3. dog	USA	Winters, 1981
<u>M. corti</u> /Tetrathyridia (T)	4. <u>Sceloporus occidentalis</u> <u>biseriatus</u> , Hallowell, 1854 (lizard)	USA	Specht & Voge, 1965
<u>M. manteri</u> /S	5. <u>Lynx rufus</u> (bobcat)	USA	Chandler, 1942b
	6. <u>Urocyon littoralis</u> (gray fox)	USA	Voge, 1953
<u>M. variabilis</u> /S	7. 1 st human case	USA	Chandler, 1942a
	8. raccoon, dog	USA	Chandler, 1942b
	9. <u>Spilogale interrupta</u> (spotted skunk)	USA	Self & McKnight, 1950

10. dog, cat	USA	Douglas, 1951
11. <u>Martes P. pennanti</u> (fisher)	USA	Meyer & Chitwood, 1951
12. <u>Vulpes macrotis</u> , <u>Canis latrans</u> (coyote), <u>Lynx rufus</u> , <u>Didelphis virginiana</u> (opossum)	USA	Voge, 1953
13. opossum	USA	Babero, 1957
14. skunk	Mexico	Little, 1966
<u>M. variabilis</u> /T		
15. lizard, snake	USA	Voge, 1953
<u>M. paucitesticulus</u> /S		
16. <u>Nyctereutex procyonoides</u> (badger)	Japan	Sawada & Kugi, 1973
17. <u>Vulpes vulpes japonica</u>	Japan	Kugi, 1977
<u>M. carnivoricolus</u> /S		
18. carnivores	USA	Grundmann, 1956
<u>M. lineatus</u> /S		
19. <u>Nyctereutex procyonoides</u>	USSR	Antipin, 1946
20. dog, fox	Cyprus	Watson, 1949
21. dog	Spain	Tarazona Vilas, 1955

22. coyote	USA	Wood & Haldiman, 1957
23. dog	Tunisia	Juminer & Durand, 1960
24. dog	Poland	Fagasinski & Piusinski, 1961
25. dog	USA	Honess, 1962
26. human case	Japan	Ito, Honda & Ishiguro, 1962
27. human case	Japan	Morisita, Kobayashi, Goto, Eguchi, Moriyama & Ohashi, 1964
28. rodents : <u>Citellus relitus</u> , <u>Apodemus sylvaticus</u> , <u>Lepus tolai</u>	SSR	Tokobaev, 1964
29. human case	Japan	Kamegai, Ichihara & Nonobe, 1967
30. human case	Japan	Tanaka, Miyamoto & Kaneko, 1967
31. dog	Iran	Sadighian, 1969
32. carnivores	Alaska	Shults, 1970
33. carnivores	Afghanistan	Tenora & Kullmann, 1972
34. white fox	USSR	Pereverzeva, 1972
35. human case	USA	Gleason, Kornblum & Walzer, 1973
36. human case	Japan	Kagei, Kihata, Shimizu, Urabe & Ishii, 1974
37. human case	Japan	Morisita, Nagase, Moriyama & Matumoto, 1975
38. dog	USA	Williams, Westheimer & Banman, 1975
39. <u>Vulpes vulpes crucigera</u> (wild red fox)	UK	Thompson, 1976

M. lineatus/T

40. dog	France	Ssolonitzin, 1933
41. hen, porcupine	Turkey	Maskar, 1953
42. <u>Pitymys multiplex</u>	Italy	Pujatti, 1953
43. dog	Poland	Fagasinski & Piusinski, 1961
44. kitten	Bulgaria	Shcherbak & Sharpilo, 1966
45. <u>Erinaceus europaeus</u> (hedgehog)	Italy	Macchioni, 1967
46. midday gerbil	USSR	Babaev, 1970
47. <u>Microtus arvalis</u> (common vole)	SSR	Movsesyan, Chubarin & Kurbert, 1978

M. lineatus/"Cysticeroid"

48. Oribatid mite	USSR	Soldatova, 1944
-------------------	------	-----------------

M. litteratus/S

49. dog	Japan	Kugi, 1969
50. carnivores	Afghanistan	Tenora & Kullmann., 1972
51. <u>Felis silvestris</u> (wild cat)	Scotland	Burt, Pike & Corbett, 1980

M. litteratus/T

52. <u>Corvus frugilegus</u> (rook)		Markowski, 1933
53. green pheasant, copper pheasant	Japan	Kugi, 1973

M. leptothylacus/S

54. Vulpes vulpes Germany Loos-Frank, 1980b

M. leptothylacus/T

55. Microtus arvalis Germany Loos-Frank, 1980b

M. latus/S

56. opossum USA Chandler, 1942b

57. Felis domestica USA Self & McKnight, 1950

Mephitis varians
(striped skunk),

Procyon lotor
(raccoon)

58. Mephitis mephitis Canada Choquette, 1951

59. skunk USA Wood & Haldiman, 1957

M. kirbyi/S

60. Canis latrans USA Chandler, 1944

61. coyote USA Butler & Grundmann, 1954

62. carnivores Alaska Shults, 1970

63. Canis lupus (wolf) Canada Choquette, Gibson, Kuyt & Pearson, 1973

M. dissimilis/S

64. Myonax sanguineus proteus Tanganyika Baer, 1933
(mongoose)

M. jonesi/S

65. gray fox

USA

Giordia, 1955

M. petrowi/S

66. Vulpes vulpes

USSR

Sadykhov, 1971

M. tenuis/S

67. dog

India

Banerjee, Das Gupta, Ray & Maulick, 1974

M. beringi/S

68. Alopex lagopus

USSR

Chertkova & Kosupko, 1975

M. erschowi/S

69. fox, bear

USSR

Chertkova & Kosupko, 1975

M. zacharovae/S

70. cat, jackal,
Felis sylvestris

USSR

Chertkova & Kosupko, 1975

Species unidentified/S

71. Procyon lotor lotor
(raccoon)

USA

Ingram, 1941

72. Didelphis virginiana

USA

Byrd & Ward, 1942

73. human case,
domestic cat

Astrida

Fain & Herin, 1954

74. human case

Korea

Choi, Kim & Choi, 1967

75. human case

USA

Gleason & Healy, 1967

76. dog	Canada	Speckmann & Webster, 1975
77. human case	USA	Gutierrez, Buchino & Schubert, 1978
78. dog	Japan	To, Morimoto & Kugi, 1978
79. <u>Vulpes macrotis</u>	USA	Bjotvedt, Tomkiewicz & Golightly, 1980
80. human case	USA	Hutchison & Martin, 1980

Species unidentified/T

81. rodents :	Cyprus	Watson, 1949
<u>Acomys dimidiatus</u> ,		
<u>Rattus rattus</u>		
82. monkey	Egypt	Ezzat & Gaafar, 1951
83. rodents,		
insectivores,		
birds : poultry, partridge	Astrida	Fain & Herin, 1954
84. genet cat	Kenya	Buck, Pester & Buckley, 1959
85. Amphibians :	USA	James & Ulmer, 1967
<u>Bufo americanus</u> ,		
<u>B. cognatus</u> ,		
<u>Rana pipens</u>		
86. Insectivora	Afghanistan	Tenora & Kullmann, 1972
87. <u>Cnemidophorus sexlineatus</u>	USA	Dyer, 1971
(lizard)		
88. <u>Marmota marmota</u>	Switzerland	Hörning & Tenora, 1971

89. <u>Phasianus colchicus</u> <u>versicolor</u> (pheasant)	Japan	Kugi, 1971
90. domestic fowl	Kenya	Kaliner, 1972
91. chicken	Kenya	Kaliner, 1974
92. dog	USA	Orthoefer, Baker & Kennedy, 1974
93. <u>Acridotheres tristis</u>	USSR	Chertkova, Ilyasov & Yusupov, 1975
94. <u>Papio</u> sp. (baboon)	East Africa	Reid & Reardon, 1976
95. reptiles : lizard, rattlesnake	USA	Mankau & Widmer, 1977
96. dog	USA	Barsanti, Jones, Bailey & Knipling, 1979
97. dog	USA	Greve, Hanson & McGill, 1979
98. <u>Vulpes macrotis</u>	USA	Bjotvedt, <u>et al.</u> , 1980
99. <u>Paguma larvata</u> (masked palm civet)	Taiwan	Jensen, Schmidt & Kuntz, 1983

CHAPTER 2

MATERIALS AND METHODS

2.1 Maintenance of Mesocestoides corti tetrathyridia in mice

Tetrathyridia of M. corti, originally obtained from Dr. M. Voge (UCLA), were maintained in TO mice (LACA and TF1 mice were sometimes used) by intraperitoneal injection. 20 to 25 tetrathyridia in 0.5 ml of Hanks' balanced salt solution (HBSS) (Flow Laboratories Ltd., UK), containing 100 IU penicillin and 100 µm streptomycin per ml and 20 mM HEPES (Flow), were used for each mouse.

2.2 Collection of tetrathyridia

Only the tetrathyridia recovered aseptically from the peritoneal cavity of an infected mouse (three to nine months post infection) were used for in vitro culture (Plate 2-1). The infected mouse was killed with chloroform and the abdomen was painted twice with 1% iodine solution in 90% ethanol, in a lamina flow cabinet. Metal instruments for dissecting mice were sterilised by

boiling in water. A small incision was made in the abdominal wall and the tetrathyridia were removed from the peritoneal cavity, using a 1 ml sterile serological pipette, to a 30 ml sterile Universal container (Sterilin Ltd., UK). A small amount of warm Hanks' saline was used to rinse the peritoneal cavity, if necessary. The larvae were then sterilised by washing with at least three changes of warm Hanks' saline containing antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml) and 20 mM HEPES, for about 10-20 minutes each time. Washing was performed on a rotator apparatus rotating at 20 revolutions per minute in a 38°C incubator.

2.3 In vitro culture

2.3.1 General aseptic techniques

Standard aseptic techniques were employed throughout the in vitro culture work. All procedures were carried out in a lamina flow cabinet which was cleaned and swabbed with 70% ethanol before use.

Used pipettes were immediately immersed in an easily removed disinfectant, about 6% solution of sodium hypochlorite (British Drug Houses Chemicals Ltd., UK)

which was afterwards rinsed off with running water. Pipettes were then soaked in the detergents, 'PYRONEG' (Diversey Ltd., UK) and 'LIPSOL' (Lip Equipment and Services Ltd., UK) for one hour each before final washing with water for one hour. Other glassware was washed thoroughly in 'PYRONEG'. 3 N HCl or 'LIPSOL' was employed to remove any residues if necessary.

After being rinsed repeatedly in distilled water and dried in an oven, the pipettes and bottles etc. were put in autoclave bags. The mouthpieces of pipettes or apertures in glass apparatus were plugged with non-absorbent cotton wool and the screw caps of bottles were loosened before autoclaving at 1-2 kg/cm² pressure at 121°C for 20 minutes.

2.3.2 Preparation of culture media

The liquid culture medium, S10E, was made up of commercially available basic medium CMRL 1066 supplemented with serum, yeast extract, glucose and dog bile as below.

CMRL 1066 with glutamine (Flow)	130 ml
Foetal Bovine Serum (Flow)	50 ml
Yeast Extract (GIBCO) in HBSS	18 ml

30% Glucose (Hopkins and Williams)	
in distilled water	2.8 ml
5% Dog Bile in HBSS	0.7 ml

CMRL 1066 was reconstituted according to the instructions provided by the manufacturer. 10.0 g. of powder was dissolved in a litre of triple distilled water (GIBCO) or high purity water produced by 'NANOPURE II' at 18.3 or higher megohms/cm of electrical resistivity. Additionally, 1.32 g. of sodium bicarbonate (Analar R, BDH) and 7.39 g. of HEPES (Flow) were added to give the final concentration in S10E.H [using HEPES (H) as a buffer], 0.85 g/l and 20 mM respectively. The pH was adjusted to approximately 7.3 by adding a few drops of 1 N NaOH before passing the solution through a sterile 0.2 μ m millipore filter (Millipore Ltd.) by vacuum filtration or pressure filtration. After incubation at 38°C for 48 hours, the sterile CMRL 1066 were added with antibiotics (Flow) at the concentration of penicillin 100 IU/ml and streptomycin 100 μ g/ml. Antibiotics were supplied as a mixed solution of penicillin 5000 IU/ml and streptomycin 5000 μ g/ml. Therefore, 2 ml of antibiotics solution was added per 100 ml of CMRL 1066. The reconstituted 1066 was then stored at 4°C.

Both 5% (w/v) yeast extract and 5% (v/v) dog bile were made up in Hanks' saline. Hanks' saline was

reconstituted as described for CMRL 1066, containing sodium bicarbonate 0.35 g/l, HEPES 20 mM and antibiotics. 9.9 g. of commercially available powder, 0.35 g. of sodium bicarbonate and 4.77 g. of HEPES were dissolved in a litre of triple distilled water or high purity water. Other procedures were the same as described above for the preparation of CMRL 1066. The solution of yeast extract or dog bile was filtered beforehand using non-sterile millipore filters of 5, 1.2, 0.8, 0.45 and 0.2 μ m pore size in that order, then finally through a sterile 0.2 μ m millipore filter. After incubation for 48 hours, all sterile solutions were stored at 4°C. However, undiluted dog bile was kept at -20°C.

A coagulated serum base was used as the solid phase of a diphasic medium of which S10E.H was the liquid phase. Two to three mls of newborn calf serum (Flow) was pipetted into a 19 x 105 mm Leighton tissue culture tube (Bellco Glass Inc., USA). This was incubated at 37°C in an inclined position with screw cap loosened until serum became opaque and coagulated. Usually, it took 30-75 minutes to 'set'. The screw cap was tightened as soon as the tube was allowed to cool in the lamina flow cabinet. Tubes with serum base slant were used immediately or stored at 4°C.

2.3.3 Culture vessels

19 x 105 mm Leighton tubes (Bellco) with screw caps were found to be suitable and used in most of the experiments. It is important to check the tops of tubes for cracks. It was only possible to tighten securely tubes with intact mouths. Furthermore, tubes were kept sealed with two layers of parafilm. To maintain the pH and gas phase of cultures as desired, it was found all these precautions must be taken.

2.3.4 Routine replacement of media

Medium was replaced as necessary. Usually, about half volume of the liquid medium was replaced with fresh medium on the third and the sixth days of culture, and three quarters of the old medium was replaced every two days thereafter. For those cultures having high segmentation rates, medium renewal was changed to daily, after two weeks, in order to remove the acidic waste products excreted by the strobilating adults and to replenish nutrients.

2.3.5 Agitation of cultures

Culture tubes were put at an angle of about 10° to the horizontal. They were rotated at a speed about one revolution per five minutes on a roller apparatus in a 38°C incubator.

2.4 Experimental infection of dogs

Due to the lack of facilities for keeping the experimental dogs in the department and the restrictions on the use of dogs as experimental animals in this country, the infection of dogs was kindly done by Dr. Calum N. L. Macpherson in Kenya.

Tetrathyridia obtained from an experimental mouse were washed as previously described in section 2.2. They were then kept in cold Hanks' saline and brought to Kenya. Puppies (approximately four weeks old) were purchased from Turkana and given praziquantel (Droncit R) and canex (R) as directed. The puppies were starved for 18 hours prior to infection. Each puppy received approximately 0.5 ml of sedimented tetrathyridia orally from a plastic pipette. Dogs were starved for 24 hours prior to autopsy at 6, 7, 14, 17, 42 and 55 days post infection.

2.5 Morphology and histology

Cultures were observed regularly using an inverted microscope (Zeiss) and worms were removed as necessary for more detailed study. Live specimens were squashed between the slide and cover-glass on the two ends of which were put two masses of vaseline. The squashed worms were observed microscopically unstained, in 0.1% neutral red or in aceto-orcein (Paul, 1970). Specimens to be prepared as whole mounts were squashed as described above and fixed in mercuric-acetic or 4% formal-saline. Some were relaxed in tap water for 10 minutes then fixed in boiling 4% formal-saline. The fixed specimens were later stained in Gower's carmine or Mayer's acid haemalum (Carleton, 1938). Others were directly fixed in Bouin's fluid, Carnoy's fluid or 4% formal-saline and embedded in wax. 7 μ m sections were cut and stained with haematoxylin and eosin or orange G.

2.6 Electron microscopy

Worms recovered from infected dogs were washed several times in HBSS. They were then fixed in 4% glutaraldehyde (TAAB) buffered by 0.1 M sodium cacodylate (TAAB), pH 7.2, containing 3% sucrose (Analar R, BDH) and 1.5 mM CaCl_2 (BDH) at 4°C. Further preparation could not be done until the specimens were sent back to London. Some specimens

were, therefore, unavoidably held in the fixatives for up to one month.

Worms grown in vitro were fixed in 4% glutaraldehyde in 0.12 M Millonig's phosphate buffer containing 3% sucrose and 0.5 mM calcium chloride or in sodium cacodylate buffer containing sucrose and Ca^{++} , as described above, at 4°C for at least three hours.

2.6.1 Scanning electron microscopy (SEM)

After washing the fixed specimens in three changes of the same buffer containing the same concentrations of sucrose and calcium chloride as used in the fixatives for about 45 minutes at 4°C, the worms were dehydrated in an ascending series of acetone at room temperature for 15 minutes in each stage (25%, 50%, 70% and 90% acetone). They were transferred to two changes of 100% acetone for 30 minutes each followed by critical point drying in a Polaron E 3000 Critical Point Drying Apparatus using liquid carbon dioxide as medium. The worms were then mounted on aluminium stubs with double-sided sticky tabs and coated with gold in a Polaron E 5000 SEM Coating Unit. The stubs were examined under a Philips 500 scanning electron microscope at 25 kv.

2.6.2 Transmission electron microscopy (TEM)

The fixed specimens were washed in three changes of cold buffer wash as previously described in section 2.6.1 for at least 30 minutes each and post-fixed in 1% osmium tetroxide (Agar Aids) in the related buffer containing sucrose and Ca^{++} for one hour at 4°C. After washing in three changes of cold distilled water for 30 minutes, the specimens were dehydrated for 15 minutes at each stage of cold acetone - 25%, 50%, 70% and 90%, then left to reach room temperature. Dehydration was completed in two changes of 100% acetone for 30 minutes each. The specimens were transferred to a 1:1 mixture of 100% acetone and araldite (Agar Aids) and rotated for 90 minutes and then left uncovered overnight on the rotator. This was followed by a further three hours in fresh araldite if necessary. The material was embedded in fresh resin and polymerized at 60°C for 48 hours. Ultrathin sections were cut on a Reichert-Jung OM 4 ultramicrotome with glass knives made on an LKB 7800B knifemaker. Sections were mounted on formvar (Agar Aids) coated copper grids. They were stained with saturated uranyl acetate in 50% ethanol for 10-15 minutes followed by Reynolds' lead citrate (Reynolds, 1963) for about 5 minutes. To avoid the precipitation of lead carbonate on the grids, great care was taken to exclude carbon dioxide when staining

with lead citrate. Grids were examined using a Philips EM 301 electron microscope at 80 kv.

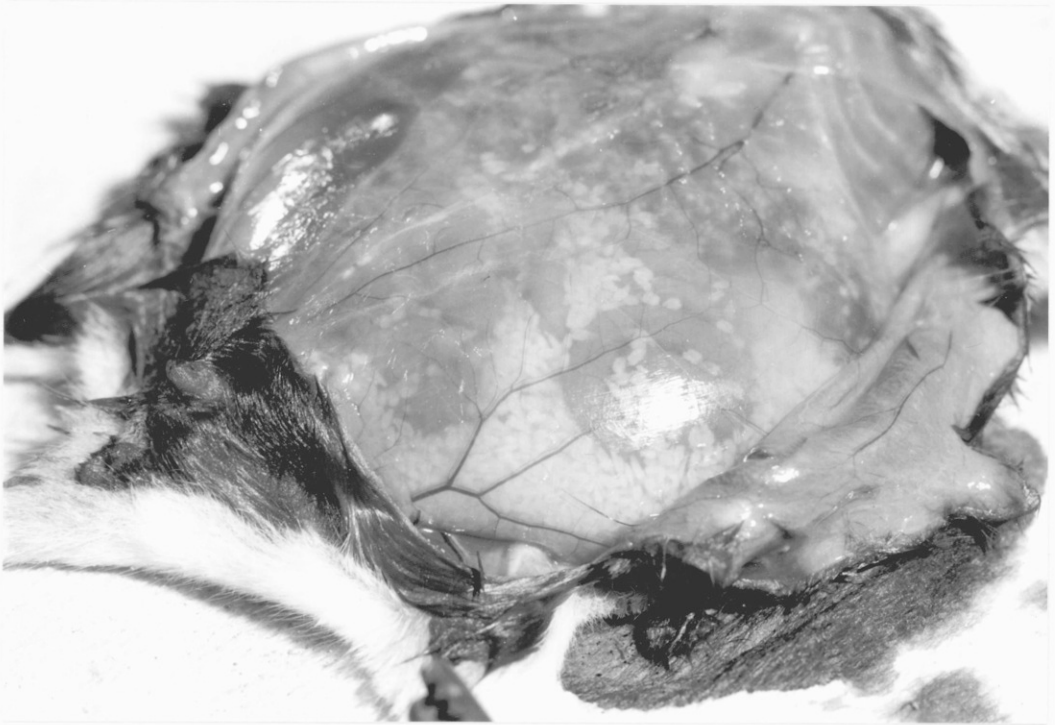


Plate 2 - 1 : Showing the body cavity of a mouse infected
with Mesocestoides corti tetrathyridia.

CHAPTER 3

EFFECTS OF SOME CULTURE FACTORS ON SEGMENTATION RATES IN VITRO

3.1 Review of literature on cultivation of cestodes from metacestode to adult

In vitro culture of cestodes has been reviewed by Clegg and Smyth (1968), Taylor and Baker (1968), Smyth (1969b), Silverman and Hansen (1971), Smyth (1976) and more recently by Voge (1978).

During the last two decades, techniques for in vitro cultivation have undergone remarkable development. Contamination with microorganisms was once a major problem with in vitro culture work but this has now been generally overcome. Smyth (1954) successfully grew the progenetic plerocercoids of Shistocephalus solidus to egg-producing adults by compressing worms within an artificial gut made of semi-permeable cellulose tubing; this procedure was a necessary pre-requisite for insemination. Eggs with up to 77% fertility were eventually obtained. Progenetic plerocercoids of S. solidus have rich carbohydrate and protein reserves and may be cultured to sexual maturity without provision of additional nutrient if the

physico-chemical conditions are carefully controlled. In a series of experiments, Smyth (1946, 1950, 1952) found that a highly buffered medium of pH 7-8 under anaerobic or nearly anaerobic conditions was required for normal development of S. solidus. In addition to this, the culture temperature had to be maintained at 40°C (bird body temperature) and, as mentioned, the worms needed to be compressed to obtain fertile eggs. The in vitro cultivation of such metacestodes provided little information on the nutritional requirements of this species. It did, however, produce data concerning the physico-chemical conditions under which maturation and insemination could take place. On the other hand, the plerocercoids of Spirometra mansonoides, like the majority of pseudophyllidean plerocercoids, are morphologically undifferentiated and have little food reserves. Berntzen and Mueller (1972) successfully cultured plerocercoids to gravid adults which produced infective eggs. A complex medium, medium 115 (Berntzen and Mueller, 1964), and a continuous-flow apparatus (Berntzen, 1966) were employed. It was reported (Berntzen and Mueller, 1964) that an evaginating solution and a gas phase of 5-10% CO₂ in nitrogen were necessary for the growth and differentiation of the plerocercoids of this species. Oxygen was also reported as being toxic to the cultures.

Using a complex medium in a continuous-flow culture

system, Berntzen (1961) cultured cysticercoïds of Hymenolepis diminuta to adults with developing oncospheres. Berntzen (1962) later revised this culture method for H. nana and developed a controlled medium-gas-phase pumping system in which the medium was continually recirculated. Eggs obtained from adults grown in vitro were fed to beetles and normal infective cysticercoïds were recovered. This was the first successful cultivation of a cyclopyllidean tapeworm from metacestodes to patent adults with infective eggs. However, some difficulties were reported (Webster and Cameron, 1963; Clegg and Smyth, 1968) with the preparation of a medium according to the published instructions. Besides this, it was initially shown that when a gas phase of 5% CO₂ in nitrogen was used with medium 101 or 102, a satisfactory culture of H. nana was produced. Later, using medium 126 and with a further improved continuous-flow system, Berntzen (1966) demonstrated that considerably less increase in length took place with this gas phase; 5% O₂ /5% CO₂ /90% N₂ combined with an oxidation-reduction potential of +50 to -50 mv gave optimal growth. Sinha and Hopkins (1967) also failed to repeat the result with H. nana according to Berntzen (1962). They successfully developed a much simpler method for cultivating this parasite to patency using roller tubes and a gas phase of 5% CO₂ in nitrogen. They found that only when liver extract was present in the medium did

worms become gravid. H. diminuta has also been grown in a less complex system (Schiller, 1965); a diphasic medium consisting of a blood-agar base (NNN) in flasks overlaid with Hanks' saline and a gas phase of 3% CO₂ in nitrogen were utilized. It was reported that a higher blood volume in the blood-agar base resulted in larger numbers of gravid proglottids. Embryos produced by the worms grown in the medium with 10%, instead of 30%, blood volume in blood-agar base failed to develop into cysticercoids when fed to beetles. However, worms developed using this system were smaller than those grown in rats and many proglottids were still sterile even though viable oocytes and spermatozoa were abundant. This was probably due to insemination failing to take place. The entire life cycle of H. diminuta was completed in vitro by Graham and Berntzen (1970). Hatched oncospheres of H. diminuta were grown to infective cysticercoids in monolayer cultures of rat fibroblasts. These in vitro-grown cysticercoids were then cultured to gravid adults according to Berntzen (1966). This was the first time that the entire life cycle of a tapeworm was completed in vitro.

Hymenolepis microstoma is a parasite of rodents, occupying the bile duct and, in some hosts, the small intestine. Evans (1970), modifying the technique described by Sinha and Hopkins (1967) for H. nana, successfully grew cysticercoids of H. microstoma to adults

containing eggs which appeared normal on visual examination. In his experiments, ox bile appeared to be essential for egg production. However, Khan and DeRycke (1976) failed to repeat this result. Seidel (1971) used a diphasic culture system and demonstrated that haemin was an essential requirement for strobilization of this species; this was later confirmed by Khan and DeRycke (1976). A preliminary investigation (Seidel, 1971) using ^{14}C -labelled haemin suggested that small quantities of this were incorporated into the organism after three days in culture, the highest counts being recorded just after strobilization. Haemin, or a related tetrapyrrole compound, was thought to be one of the factors required to stimulate strobilization and differentiation of H. microstoma in vitro. It was speculated that the requirement for a tetrapyrrole in vivo, might be satisfied by the presence of conjugated bile pigments as well as coproporphyrin and possibly other tetrapyrroles in the bile duct of the natural host. Using the same culture system, a blood-agar base overlaid with Triple Eagle's medium, 30% horse serum, 0.01 mg% haemin and air as a gas phase, Seidel (1975) induced the in vitro-grown cysticercoids to become gravid, thus completing the entire life cycle of H. microstoma in vitro.

Numerous attempts to cultivate Echinococcus have been made by Smyth and his co-workers. Using a solid phase of

coagulated bovine serum overlaid with a liquid medium of 199 plus 20% hydatid fluid in a gas phase of 8% O₂ / 5% CO₂ / 87% N₂ , Smyth (1967) induced the protoscoleces of Echinococcus granulosus to differentiate into a three-proglottid strobilate stage with the anlagen of the male and female genitalia formed. It was elegantly demonstrated that actual contact with serum base was necessary for strobilization to take place, thus a diphasic medium was a pre-requisite. Later, Smyth, Miller & Howkins (1967) found that medium 858 was superior to medium 199; the segmentation occurred at 22 days, compared with 32 days in the latter. Besides this, sexually mature worms were obtained; fertile eggs were not produced, probably due to lack of insemination. Despite the successful cultivation of E. granulosus of sheep origin, protoscoleces of horse origin surprisingly failed to segment in the same culture system (Smyth and Davies, 1974a; Smyth, 1979). It was thus suggested that the sheep and horse 'strain' of E. granulosus might have different physiological, nutritional or metabolic requirements. This speculation has since been confirmed by further biochemical studies (McManus and Smyth, 1978, 1979). This was the first time that differential growth requirements between strains of parasitic platyhelminths were observed using an in vitro technique. The protoscoleces of Echinococcus multilocularis had also been grown using the

same culture technique (Smyth and Davies, 1975; Smyth and Barrett, 1979). 'Monozoic' forms, with a complete set of mature genitalia, were developed in a monophasic (liquid) medium (in contrast to the E. granulosus sheep strain) as well as in a diphasic system. In some cultures, 'pseudo-segmented' forms with up to three or four sets of genitalia were also obtained. The inter-proglottid membrane of these forms were absent or poorly defined. Again, only unfertilized ova appeared in the uterus. As a result of these experiments, the possibility of using this in vitro culture technique as a taxonomic tool to differentiate between hydatid cysts of the sheep and horse strains of E. granulosus and those of the related species, E. multilocularis was discussed (Smyth and Davies, 1979).

Attempts to culture Taenia spp. have not been very successful, although limited growth was achieved in some cases. Cysticerci of Taenia crassiceps were grown in vitro to the strobilate stage but sexual maturation was not complete (Esch and Smyth, 1976). The culture methods employed were similar to those used by Smyth and Davies (1974b), which were developed for E. granulosus. It was found that pretreatment with enzyme and/or bile salts was not essential for in vitro growth and development of T. crassiceps. However, the development of untreated cysticerci was delayed by up to 13 days. It may be added that the scoleces of most mature T. crassiceps larvae were

already evaginated when removed from the intermediate host. These cysticerci did therefore not require enzyme-bile salt pretreatment for scolex evagination. Previously, evagination was thought to be a necessary pre-requisite for strobilization and organogenesis (Smyth, 1969b; Silverman and Hansen, 1971). The studies with T. crassiceps indicated, however, that evagination and subsequent morphogenesis were distinct events. The enzyme-bile salt treatment did stimulate rostellar evagination, but strobilization and organogenesis could take place without the need for this. On the other hand, a diphasic medium with a coagulated serum base was essential for the support of growth and development of T. crassiceps. Although proglottid formation occurred in the monophasic medium, development always culminated in vesiculation and death within 13 days. Recently, Osuna-Carrillo and Mascaro-Lazcano (1982) used a similar culture medium gassed with 10% O₂/5% CO₂/85% N₂ and successfully cultured the cysticerci of Taenia pisiformis to sexually mature, but non-fertile, adults. An additional procedure in their technique, which was not used by Esch and Smyth (1976) for T. crassiceps, should be noted. After the usual enzyme-bile pretreatment, the cysticerci of T. pisiformis were cultured in a diphasic medium for 24 hours. The liquid phase of this was composed of evaginating solution and culture liquid in a 1:1 proportion, the gas phase consisted of 10% O₂ / 5%

CO₂ / 85% N₂. The active scoleces were then inoculated into a normal diphasic medium which was free of evaginating solution. The effect of this treatment on the development of T. pisiformis in vitro is, however, not clear.

3.2 Some basic problems of in vitro cultivation

The knowledge of the physiological conditions in the parasite habitats, such as the vertebrate intestine, is imperfectly known. To measure precisely the O₂ tension, pCO₂ and pH etc. in the specific intestinal sites is a well-recognized problem (Clegg and Smyth, 1968; Smyth, 1969b).

The role of oxygen in the metabolism of cestodes is still unclear. Although the gas phase of 10% O₂ / 5% CO₂ / 85% N₂ has been used for the cultivation of several dog parasites, for example, Echinococcus spp. (Smyth et al., 1967; Smyth and Davies, 1974b, 1975), Taenia crassiceps (Esch and Smyth, 1976), Taenia pisiformis (Osuna-Carrillo and Mascaro-Lazcano, 1982) and Mesocostoides corti (Thompson, Jue Sue and Buckley, 1982), this percentage might not represent the optimal level for these parasites (Smyth et al., 1967).

The pH of the dog alimentary canal varies along its length (Robinson, Luckey and Mills, 1943; Wilson, 1962). Data given by different authors show some divergence and there is a need for re-examination. In addition, the pH of the crypts of Lieberkühn in which some cestodes are deeply embedded is not clearly known. It is speculated that the pH of this site is more likely to approach that of blood (7.4) than that of the intestinal lumen (Smyth, 1969b). How the intestinal pH affects the development of parasites is unclear. It has been found that Hymenolepis nana has a wide tolerance of pH range (7.0 to 9.0); on the other hand, Hymenolepis diminuta has a small range of pH tolerance (7.0 to 7.8) for maximal growth in vitro (Berntzen, 1962).

The optimal pH for the in vitro culture of some dog parasites has not been very well investigated. A pH of 6.8 was used in the culture system of T. pisiformis (Osuna-Carrillo and Mascaro-Lazcano, 1982). HEPES (pKa 7.3 at 37°C) was the buffer added in the medium for the in vitro cultivation of M. corti by Thompson et al. (1982b), but no further details were given in the published paper. The same is true in the papers regarding the cultivation of Echinococcus sp. However, a pH of 7.4 was reported in the cultivation of E. granulosus (sheep strain) (Smyth et al., 1967).

The physical environment of the intestine is difficult to reproduce in vitro. Using cellulose tubing in the culture of Schistocephalus solidus to simulate the intestinal spatial relationships proved to be essential for the insemination of this parasite (Smyth, 1954). The strobilization of E. granulosus only occurred in the diphasic system, utilizing a protein substrate of heat-coagulated serum base (Smyth, 1967). Actual contact with serum base was experimentally demonstrated to be necessary for strobilization. However, a monophasic medium was as good as a diphasic system for the cultivation of E. multilocularis (Smyth and Davies, 1975; Smyth, 1979).

In order to determine the optimal conditions for the strobilization and sexual differentiation of M. corti tetrathyridia in vitro, experiments were set up as described in the following sections. The success of in vitro cultivation was evaluated by the percentage of transformation from undifferentiated tetrathyridia to strobilating adults, in other words, the segmentation rate. The morphological development in vivo and in vitro will be compared in Chapter four.

3.3 Methods

3.3.1 Pretreatment

The maintenance of M. corti tetrathyridia in laboratory mice and their collection from the body cavity of the infected mouse have been described in Chapter two. After washing in Hanks' saline several times, the tetrathyridia were either (a) used for culture straightaway or (b) incubated in Hanks' saline in a Universal container at 38°C overnight before inoculation. In a further experiment (Ex. No. 30) to test the effect of pretreatment, four groups (c, d, e and f) were additionally set up to identify the effects of some acid or enzyme solutions on the sexual differentiation of tetrathyridia. Washed specimens were treated with the following solutions, then washed thoroughly in Hanks' saline before cultivation.

- (c): Hanks' saline adjusted to pH 2.0 with hydrochloric acid, shaken for 10 minutes in a water bath at 38°C.
- (d): 0.2% pepsin in Hanks' saline (pH 2.0), treated as (c)
- (e): 0.5% trypsin in Hanks' saline (pH 7.4), treated as (c) for 20 minutes
- (f): (d) plus (e)

In this experiment (No. 30), 25 tetrathyridia were inoculated into 10 ml of medium S10E which was buffered with 20 mM HEPPSO (pKa 7.73 at 37°C) instead of HEPES. A gas phase of 5% CO₂ in nitrogen was used. The methods of introducing gas into the culture medium are described in section 3.3.3.

3.3.2 Number of tetrathyridia per culture

The tetrathyridia recovered from the laboratory mouse were at different developmental stages. Care was taken not to include division stages or individuals with only two suckers or those even without scoleces in the inoculum. With the aid of a dissecting microscope, only the tetrathyridia with four suckers were used for in vitro culture.

25 or 100 tetrathyridia were inoculated into 10 ml of monophasic medium S10E and, where possible, four replicates were set up for each group in an experiment.

3.3.3 Gas phase

Gas was sterilized by passage through a millipore filter in the ACRODISC (Gelman Sciences, INC., Michigan)

followed by a sterile Pasteur pipette which was plugged with non-absorbent cotton wool at the proximal end. The tip of the pipette was immersed in the liquid culture medium. Whenever the medium was replaced, the gas was introduced by bubbling in this way for about two minutes. Before starting gassing, it was found important to get rid of the air present in the connecting tubes. This was done by gassing the atmosphere for about two minutes. The following gas phases were tested.

- (a) air
- (b) 5% CO₂ /95% N₂
- (c) 5% CO₂ /10% O₂ /85% N₂
- (d) 20% CO₂ /80% N₂

3.3.4 pH

The pH of the fresh and the utilized media was determined at the time media were replaced. These media were transferred to Universal containers, the screw caps of which were tightened immediately until readings were performed on a Kent EIL 7020 pH meter. To investigate the effect of pH on the segmentation and sexual differentiation of M. corti tetrathyridia in vitro, the following buffers were used at a concentration of 20 mM in the medium.

- (a) 3-(N-Morpholino) propane sulfonic acid (MOPS, pKa 7.01 at 37°C, Research Organics, INC., Ohio)
- (b) N-2-Hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES, pKa 7.3 at 37°C, Flow, UK)
- (c) N-Hydroxyethyl piperazine-N'-2-hydroxy propane sulfonic acid (HEPPSO, pKa 7.73 at 37°C, Research Organics, INC., Ohio)

3.3.5 Medium

Unless otherwise stated, a monophasic medium, S10E, was used in most of the experiments. In the experiment No. 27, the diphasic culture media consisted of a liquid phase of S10E.H and a solid phase of coagulated serum base; development was compared with that in monophasic liquid media. The serum base was prepared as stated in section 2.3.2. The base was punched or not with a sterile Pasteur pipette to make holes into which worms might burrow. 100 tetrathyridia per culture and a gas phase of 5% CO₂/95% N₂ were the other parameters in these cultures.

3.4 Results

Among numerous experiments which were done in the past three years, only a few or representative (see Appendices) of the systems used are presented in detail below.

3.4.1 Pretreatment

It was found, during preliminary experiments, that segmentation only occurred when stock specimens were incubated in Hanks' saline in a Universal container at 38°C overnight before being inoculated into the cultures. Pretreatment with any acid or enzyme solutions, or cultivation immediately after washing with Hanks' saline, resulted in very poor and delayed or no segmentation, even in the presence of the appropriate gas phase and other factors. This result was confirmed in experiment No. 30 which contained six groups of cultures (see section 3.3.1). Only the positive control group (b) showed 20% of segmentation rate after 13 days in vitro, although the rate appeared to be rather low in this case. No sign of segmentation could be found in the other five groups.

3.4.2 Number of tetrathyridia per culture

25 tetrathyridia per culture gave similar, if not better, results to 100 tetrathyridia per culture (Fig.

3-1) in the presence of a suitable pH and a gas phase of 5% CO₂ /95% N₂. The pH was determined from the utilized media.

3.4.3 Gas phase and pH

After numerous experiments, it has been concluded that a gas phase of 5% CO₂ /95% N₂ combined with a pH higher than 7.4 provides the most suitable conditions for segmentation and sexual differentiation. Tetrathyridia began segmenting in vitro within one week. This is compatible with the in vivo rate of development in the dog.

Figure 3-2 showed that 5% CO₂ /95% N₂ gave significantly higher segmentation rates than air. The pH of the utilized media was, again, measured whenever the media were changed. Similarly, 5% CO₂ /95% N₂ was demonstrated to be superior to 5% CO₂ /10% O₂ /85% N₂ (Fig. 3-3) as far as segmentation was concerned.

It was found difficult to maintain the desired pH if a gas phase of 20% CO₂ /80% N₂ was employed. Gassing with a high concentration of CO₂ lowered the pH of the medium and, therefore, the segmentation of tetrathyridia was delayed (Fig. 3-4). This result could partly explain the

delay of segmentation in our early experiments (Barrett, Smyth and Ong, 1982).

The influence of pH, while holding other factors constant is illustrated in Figure 3-5. A pH higher than 7.4 appeared optimum for segmentation to take place. The upper limit was, however, not critically determined.

3.4.4 Medium

Segmentation occurred in the monophasic medium S10E as well as in the diphasic medium using coagulated serum base as a solid phase. However, the segmentation rates were very low in some diphasic cultures which contained very actively multiplying tetrathyridia.

The consistency of the coagulated serum bases was not uniform. Some were less solid than others and tetrathyridia could only migrate in the former. Sometimes, a hard surface coat was formed on the serum base. This prevented the tetrathyridia from penetration too. Only when some tetrathyridia became established in the serum base, did asexual multiplication take place in the diphasic cultures. As mentioned in Chapter one, the asexual multiplication by splitting of the scolex is a unique character of tetrathyridia and in the adult

Mesocestoides (Eckert, von Brand and Voge, 1969; Schmidt and Todd, 1978).

Because some organisms were embedded in the serum base, they had to be removed carefully from it with dissecting needles for worm counts to be made and, therefore, the culture had to be terminated. In experiment No. 27, the comparison between the diphasic media and the monophasic media was made after 11 days in culture (Table 3-1). The pH of the two groups during the first 10 days culture is illustrated in Figure 3-6. The serum base of the diphasic media was not punched in this case and only three (culture No. I, J and L) of the four replicates (I-L) were terminated for worm count. (Culture No. K was continued for further observations on the development and, particularly, the egg production.) The total number of organisms in this group increased from 300 to 445 after 11 days. On the other hand, no multiplication could be found at all in the four tubes (culture No. A-D) of the group using monophasic media. As far as segmentation was concerned, however, there was no significant difference between the diphasic and the monophasic media (87.6% and 93% respectively). Nevertheless, in those diphasic cultures (E-H) containing a punched serum base, the segmentation rates were very low (0 - 8.3%) while the tetrathyridia actively multiplied in

vitro (Table 3-2). It was found that the greater the number (X) of organisms situated in the serum base, the higher was the number (Y) of worms produced in the culture. The correlation coefficient is $r = 0.975$ (d.f. = 5, $p < 0.01$). The linear relationship between the two variables can be expressed in the regression equation, $Y = 11.97 + 1.30X$ (Fig. 3-7).

Although most segmenting adults were free in the liquid phase, some were found in the base. The organisms in the serum base were moving rather rapidly and leaving tracks behind (Plate 3-1). They were sometimes observed emerging from the base.

The multiplication of tetrathyridium and adult M. corti by splitting from the scolex basically conformed to the pattern of those described and illustrated by Novak (1972) and Schmidt & Todd (1978).

3.5 Discussion

3.5.1 Pretreatment

It has been speculated that evagination acts as a trigger, without which differentiation or segmentation

will not take place. Therefore, pretreatment with acid or enzyme solutions before cultivation is usually used to evaginate the scoleces of larval cestodes.

Mesocestoides tetrathyridia, however, usually evaginated spontaneously after washing in Hanks' saline or later in culture. No pretreatment is therefore needed for evagination and the effects of pretreatment before cultivation were not intensively examined in this study. However, evagination of scoleces does not appear to be a sufficient stimulus by itself to initiate the transformation of tetrathyridia to strobilating adults. It has been found that some 'conditioning' of tetrathyridia before cultivation appears to be necessary. This conditioning can be provided, at least to some extent, by incubating the stock specimens in Hanks' saline in a Universal container at 38°C overnight before cultivation.

After incubation overnight, the solution near the specimen surface was acidic, presumably due to the excretion of metabolic waste products. This area was also turbid. Some unidentified material was obviously being released. SEM studies showed that some microtriches of the pre-adult stage obtained in vitro were shed. Micrographs and details of EM studies on the tegument at various developmental stages are given later in Chapter

five. Unless further experiments are performed, one cannot unequivocally relate the particulate material near the specimen surface to the microtriches.

In his studies of Mesocestoides lineatus, James (1968) pointed out that the scolex and neck became separated from the remainder of the tetrathyridial body one hour after feeding to the definitive host (mice). Only the neck and the scolex of tetrathyridium were involved in the formation of the adult worm. Studies on the preserved young adults of M. corti from experimental dogs, which were carried out in Kenya, six days after infection showed that worms at this stage were also devoid of a "tail" portion.

The phenomenon that larval tissue separates from the neck region in culture or in the definitive host and takes no part in the formation of the adult worm has been reported in Spirometra mansonoides (Berntzen and Mueller, 1964, 1972), Taenia serialis (Smyth, 1969a) and Taenia hydatigena (Featherston, 1971). Berntzen & Mueller (1964) also reported that without use of an evaginating solution consisting of medium diluted with saline containing bile salts, trypsin and glutathione, the differentiation and growth of Spirometra mansonoides did not occur, even under the appropriate culture conditions. Esch and Smyth (1976) found that pretreatment was not essential for in vitro

growth and development of Taenia crassiceps but the onset in development of untreated cysticerci was delayed up to 13 days. These results indicated that proper pretreatment before cultivation is required for development, without delay, of M. corti and other cestodes in vitro. It must be emphasised that evagination of scoleces is not a satisfactory indicator that strobilar differentiation will occur.

The pretreatment tests in experiment No. 30 were not, however, very convincing because of the low segmentation rate in the positive control group. The serum used in this experiment was an emergency supply and less growth-promoting factors or some inhibitors were suspected of being present in this serum. It is clearly necessary to carry out more experiments in this area and attempt to develop precisely a suitable pretreatment method.

3.5.2 Gas phase and pH

Experimental results have shown that rate of segmentation was low or delayed in the cultures with a favourable pH but unfavourable gas phase or vice versa. An anaerobic or nearly anaerobic condition, combined with a pH higher than 7.4, has proved to be most suitable so

far. However, segmentation sometimes occurred in aerobic conditions and occasionally even at a rather high segmentation rate. The wide range of host specificity for this parasite is presumably related to its adaptability for various physico-chemical environments.

The gassing method used in this study is simple and easy to apply but it was found difficult to standardize the gassing time, owing to the bubbles produced by gassing. Usually, gassing was performed until bubbles (i.e. froth) came up to the mouth of the culture tube. If, for some reason, the bubbles were not produced, the cultures were gassed for about the same time, generally two minutes.

The marked change in the pH of stock media was noted. Due to the release of CO₂ to the atmosphere, the pH of stock media increased after storage, particularly if only a little volume of medium was left in a bottle. The pH was, if necessary, corrected by further gassing before media were added to cultures. Otherwise, freshly prepared media were used. The pH dropped remarkably when the number of segmenting adults increased in a culture; these worms grew rapidly after about 10 days in vitro. Sub-culturing of the segmenting worms at this stage was necessary to obtain maximum development. A continuous-flow system would be appropriate, but how to control its

physico-chemical conditions properly presents further problems.

3.5.3 Medium

The liquid medium S10E and solid serum base were originally developed for the cultivation of Echinococcus granulosus (Smyth and Davies, 1974b; Smyth, 1979). This system has proved to be suitable for M. corti as well (Barrett et al., 1982; Thompson et al., 1982b). However, as far as segmentation is concerned, the monophasic medium was found to be superior to a diphasic system for this parasite.

Asexual multiplication occurred only in some diphasic cultures containing a suitable serum base into which tetrathyridia could migrate. This result is in compliance with the observation of Voge & Coulombe (1966). They found that increases in numbers in blood agar tubes only occurred if at least some organisms became embedded in the agar. The importance of the consistency of the physical environment for the multiplication of this parasite is becoming evident. It is speculated that nervous rather than nutritional stimulation may be involved in this phenomenon.

It should be mentioned that other factors such as pH also influence the multiplication of this worm (Voge and Coulombe, 1966). This is a possible interpretation of the results obtained in culture No. E of experiment No. 27 (see Table 3-2). Compared to other tubes in the same group, very few organisms were produced asexually in this diphasic culture. It was noted that the pH range (7.2 - 7.4) of this particular tube was, for some reason, somewhat lower than others (> 7.4). Voge & Coulombe (1966) illustrated that a pH range of 7.4 - 7.6 was optimal for asexual multiplication.

The asexual multiplication seen in the monophasic liquid cultures in our previous report (Barrett et al., 1982) was probably related to the fact that dividing tetrathyridia, which originated from the infected mouse, were used in the cultures. The splitting of inoculated dividing tetrathyridia slowed down and, in some cases, ceased in monophasic cultures. This phenomenon resulted in the occurrence of polycephalic tetrathyridia and also strobilating adults containing a split scolex or supernumerary suckers in the liquid medium.

The sensitivity of in vitro cultivation systems is well recognized. Any slight difference may affect the growth and development of cultured worms. The present

techniques have been sufficiently successful to reduce greatly the variation in development among the replicates using identical culture conditions. The growth and development within an individual culture were also reasonably consistent. However, the segmentation rates varied in different experiments. Several sources of variation are unavoidably present. Different batches of the natural components of the culture media such as serum and dog bile gave different results. The different growth-promoting activity of different batches of serum is well recognized in the field of tissue culture and the basis for this is not understood. Therefore, it is strongly recommended to test several serum samples before starting long term experiments. This was what was done, so far as possible, in this study. It is also possible that the state of tetrathyridia obtained from an infected mouse differs from the state of those from another one due to individual incompatibility between worm and host. Finally, a more precise pretreatment may be able to eliminate this variation to a certain degree.

3.5.4 Egg production

Egg production in helminths in culture has been, to date, an intriguing problem. So far, very few species of parasites cultured in vitro have produced normal, fertile

eggs.

In this study, the shed proglottids in most of the cultures were pipetted, from time to time, into wells of depression slides and dissected under a dissecting microscope. Eggs containing motile oncospheres were recovered sporadically. The size of the eggs and the activity of the oncospheres appeared normal. (Details are given in Chapter four.) However, it was difficult to demonstrate the viability of these eggs by further cultivation to the next larval stage due to the lack of a plentiful supply of eggs from the in vitro culture experiments. It was also impossible to conduct feeding tests since the first intermediate host of this parasite is, as indicated earlier, still not known.

The increasing nutritional requirements for rapidly growing strobilar adults presents a difficult problem which must be overcome for the successful in vitro cultivation of a cestode. Attempts to increase egg production by adding various additives, such as blood macerate (20 ml of 50% dog blood in HBSS and 50 ml of newborn calf serum coagulated at 80°C for 75 minutes followed by maceration in 50 ml HBSS), 0.1% dog bile in HBSS or serotonin (5-hydroxytryptamine, 0.1, 0.5 and 1 mM) etc. to the culture medium have so far proved to be unsuccessful.

Some helminths require specific physical conditions before insemination can take place. Efforts to improve insemination by compressing within cellulose tubing, which has been successful with other cestodes (Smyth, 1954) or providing support such as filter paper, agarose (2%, 4%, 6% and 8%), have also failed.

In the natural hosts, gravid proglottids are shed and pass out in the faeces. The physico-chemical conditions, such as gas phase, pH or temperature for gravid proglottids in later stages of development could be quite different from that appropriate for immature or mature proglottids. The correct culture conditions necessary for the production of viable oncospheres in vitro therefore need further study.

Fig. 3-1 The segmentation rates (A) and pH range (B) of the cultures inoculated with 25 or 100 tetrathyridia in 10 ml monophasic liquid medium S10E (Ex. No. 27).

Gas phase : 5% CO₂ / 95% N₂.

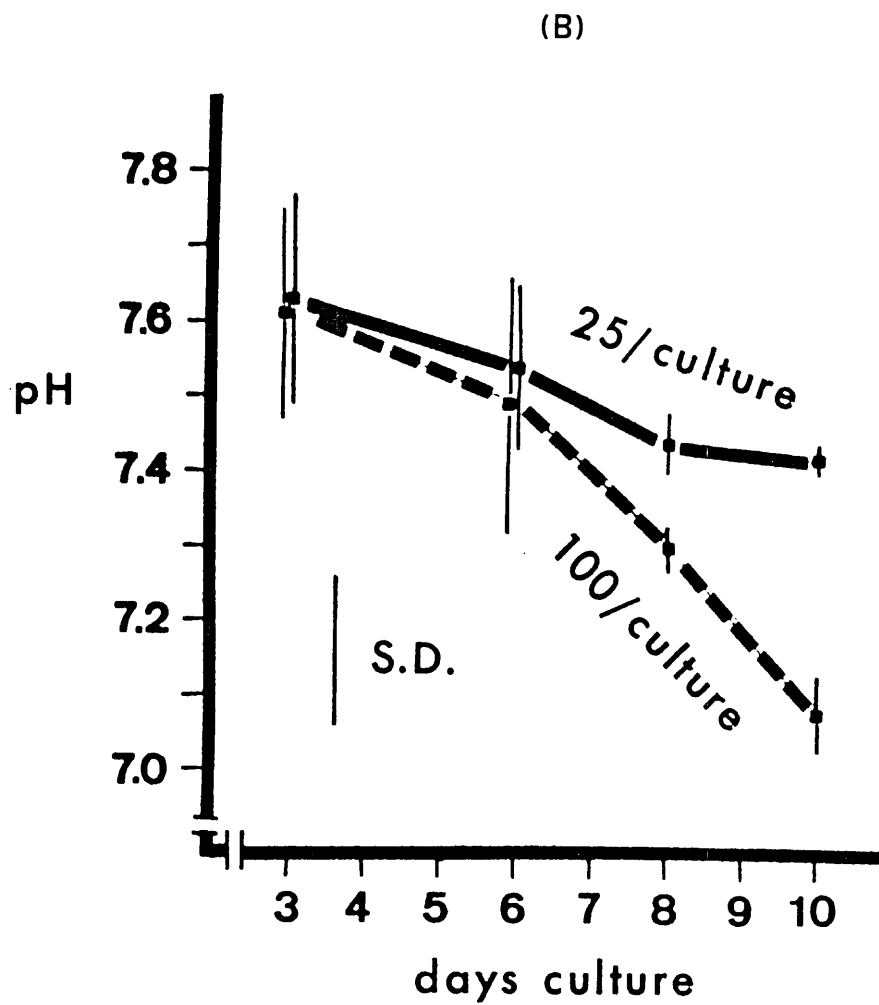
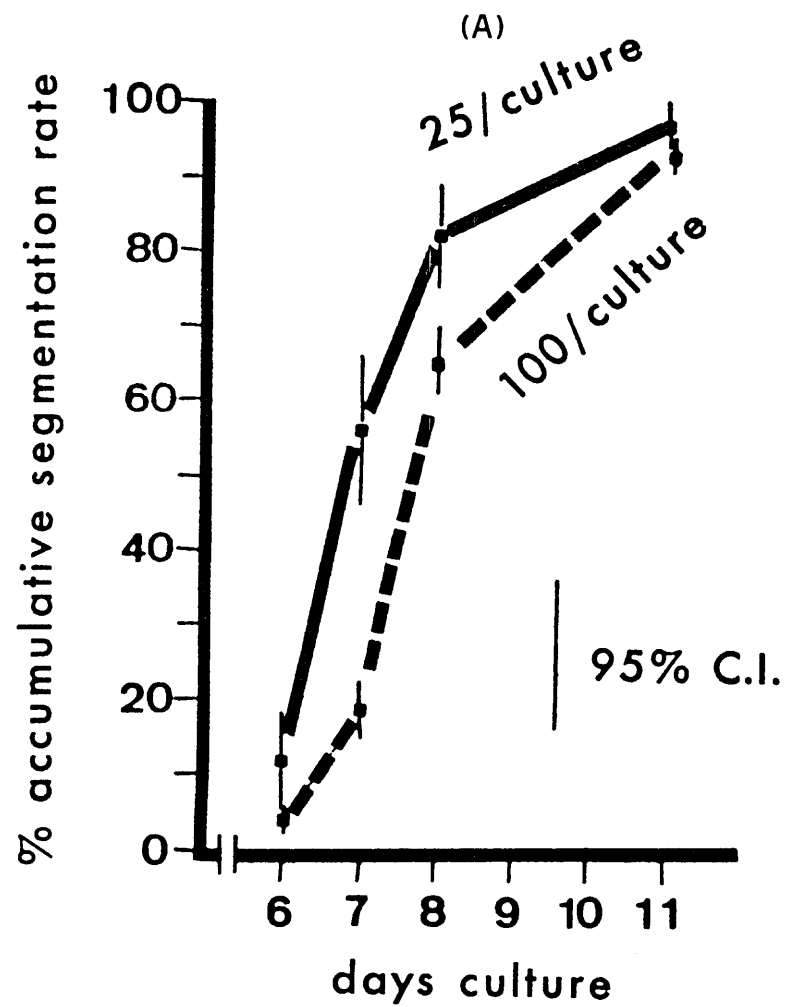


Fig. 3-2 Comparison of segmentation rates under air and 5% CO₂ / 95% N₂. The gas phase of 5% CO₂ / 95% N₂ gave significantly higher segmentation rates (B) than air in the presence of a pH above 7.4 (A) during the first 10 days (Ex. No. 27). (25 tetrathyridia per culture containing 10 ml S10E)

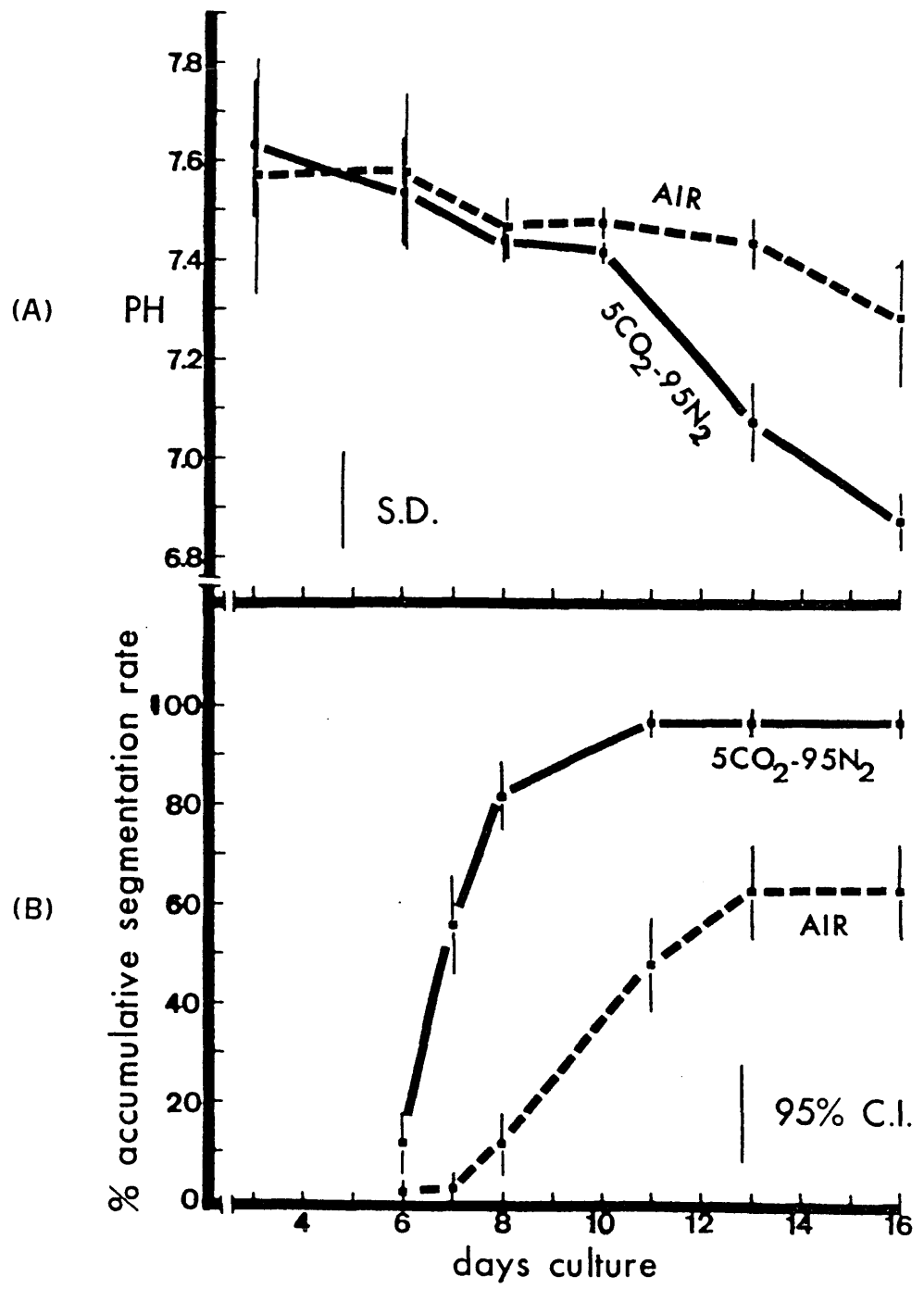


Fig. 3-3 Comparison of segmentation rates under 5% CO₂ / 95% N₂ and 5% CO₂ / 10% O₂ / 85% N₂. The gas phase of 5% CO₂ / 95% N₂ gave significantly higher segmentation rates than 5% CO₂ / 10% O₂ / 85% N₂. The pH was between 7.5 - 7.7 (Ex. No. 29). (25 tetrathyridia per culture containing 10 ml S10E)

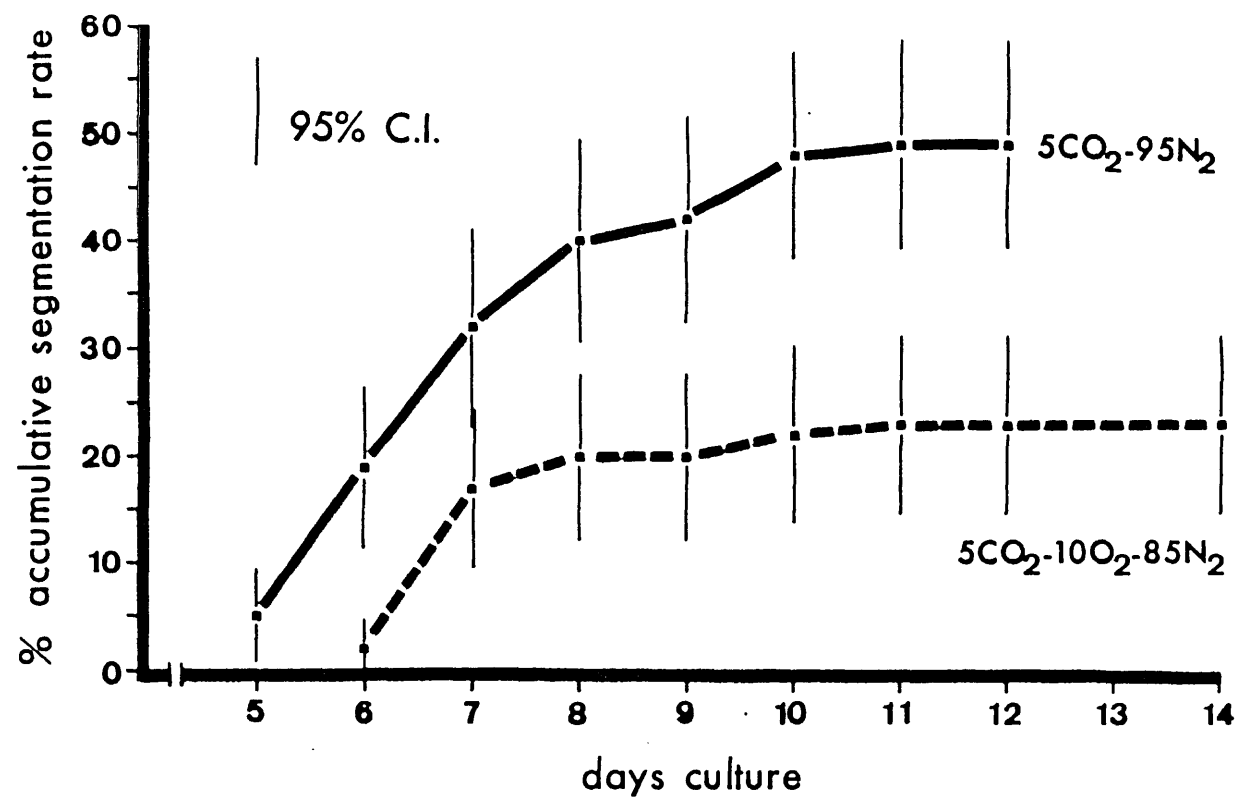


Fig. 3-4 Comparison of segmentation rates under 5% CO₂ / 95% N₂ and 20% CO₂ / 80% N₂. Compared to the gas phase of 5% CO₂ / 95% N₂, segmentation (B) was delayed in 20% CO₂ / 80% N₂, presumably due to the unfavourable pH (< 7.4) (A) caused by gassing with high concentration of CO₂ (Ex. No. 27). (100 tetrathyridia per culture containing 10 ml S10E)

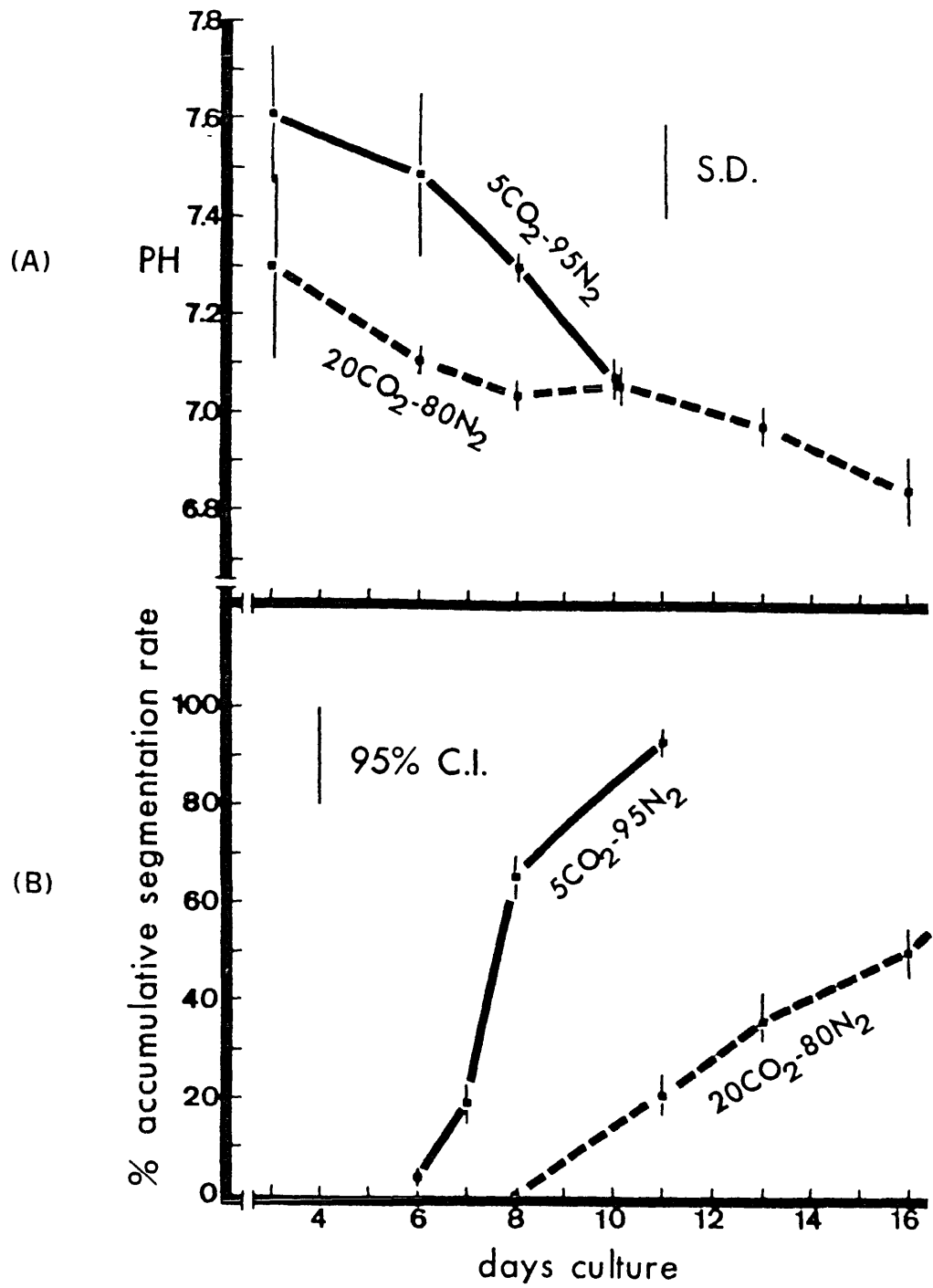
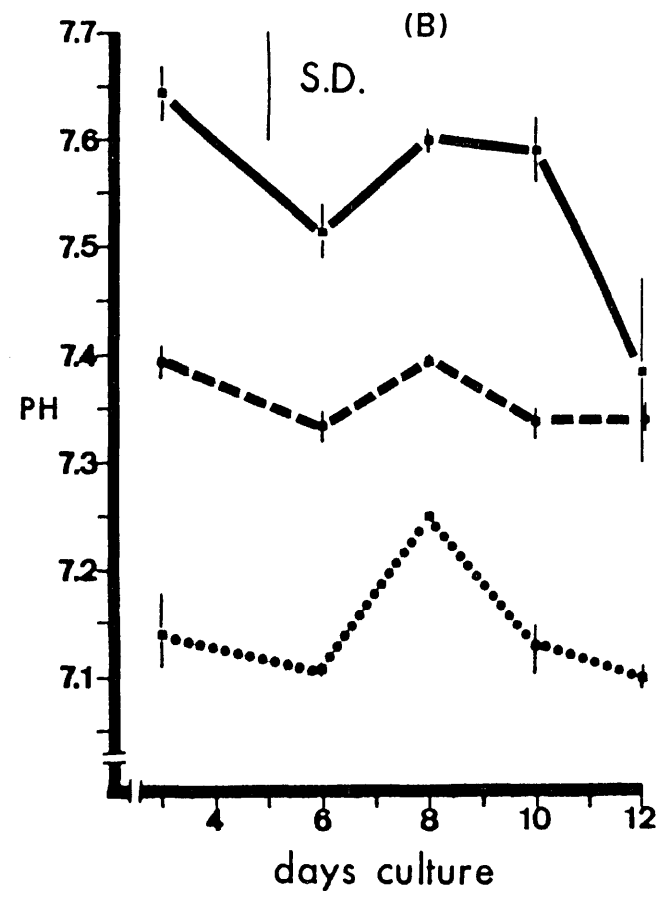
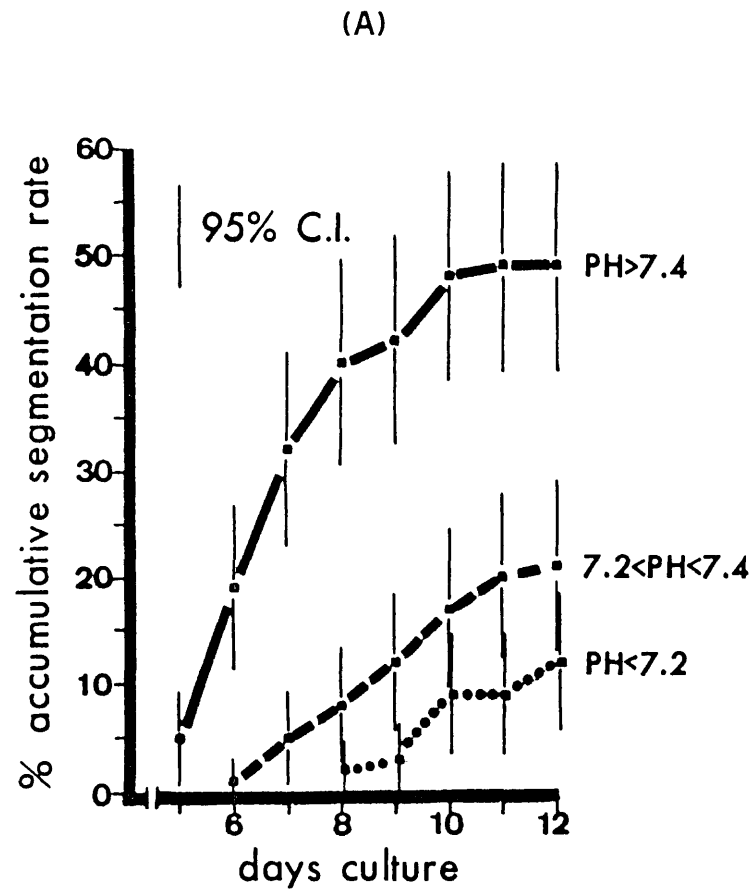


Fig. 3-5 The effect of pH (B) on the segmentation rate (A) (Ex. No. 29). Gas phase : 5% CO₂ / 95% N₂. (25 tetrathyridia per culture containing 10 ml S10E)



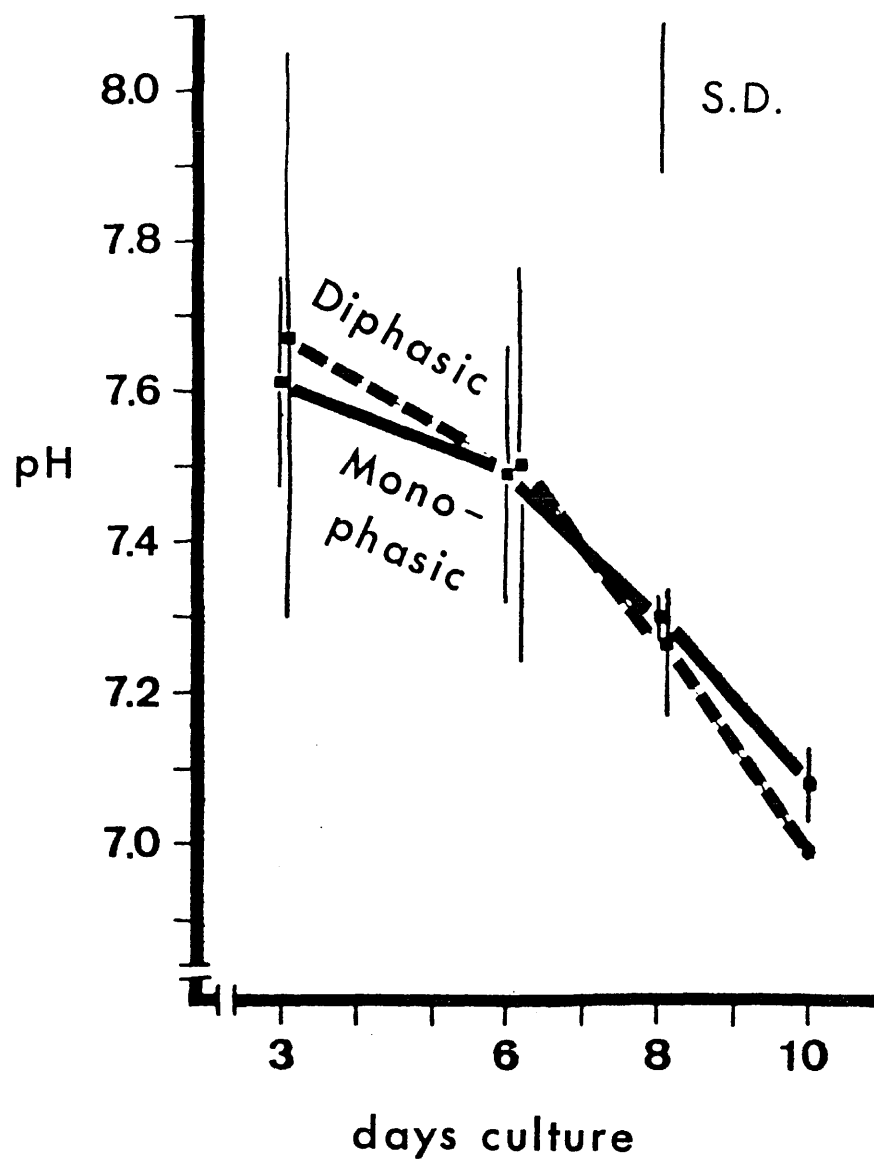


Fig. 3-6 The pH variation of the diphasic cultures No. I, J & L and the monophasic cultures No. A-D of the experiment No. 27.

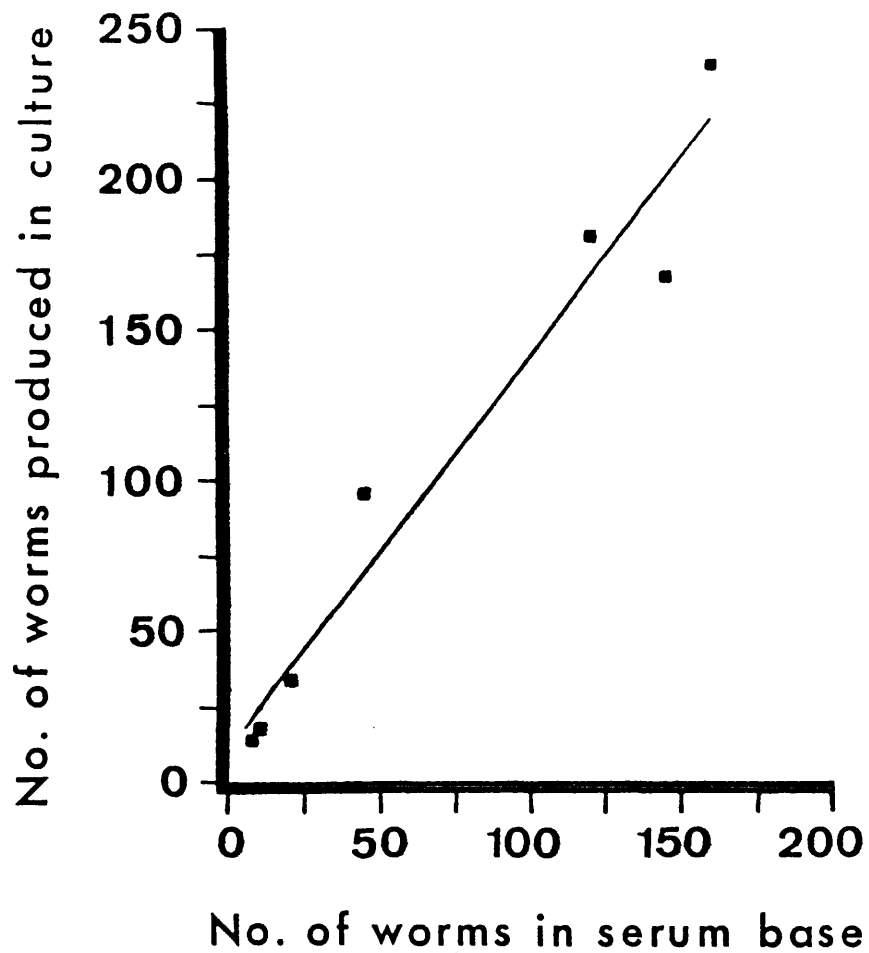


Fig. 3-7 The relationship between number of worms produced in diphasic culture and number of worms situated in its serum base, with regression line $Y = 11.97 + 1.30 X$.

Table 3-1 Comparison of development in monophasic and diphasic media.. No significant difference was found between the segmentation rate in diphasic medium and that in monophasic medium after 11 days in vitro (Ex. No. 27). Gas phase : 5% CO₂ / 95% N₂. (pH refer to Fig. 3-6)

Medium	Culture No. (Number inoculated)	Segmenting adults		Tetrathyridia		Worms recovered	95% confidence interval of segmentation rate
		Number	%	Number	%		
Diphasic medium (Serum base <u>not</u> punched)	I, J & L (300)	390	87.6	55	12.4	445	0.91-0.85
Monophasic medium	A-D (400)	373	93	27	7	400	0.96-0.91

Table 3-2 The distribution and development of the organisms in the diphasic cultures of
experiment No. 27 (11 days culture)*. Gas phase : 5% CO₂/95% N₂.

Culture No.	No. free in liquid phase			No. situated in serum base			Total No. of adults (a+c)	Total No. of tetrathyridia (b+d)	No. of all organisms (a+b+c+d)	Increase Y
	Adults (a)	Tetrathy- ridia(b)	Total (a+b)	Adults (c)	Tetrathy- ridia(d)	Total (c+d=X)				
Serum base punched										
E	4	103	107	0	10	10	4 (3.4%)	113	117	17
F	21	135	156	2	118	120	23 (8.3%)	253	276	176
G	0	123	123	0	145	145	0	268	268	168
H	3	176	179	0	159	159	3 (0.89%)	335	338	238
Serum base <u>not</u> punched										
I	149	3	152	42	3	45	191 (97%)	6	197	97
J	106	7	113	11	9	20	117 (88%)	16	133	33
L	79	29	108	3	4	7	82 (71%)	33	115	15

* Initial inoculation = 100 tetrathyridia per culture

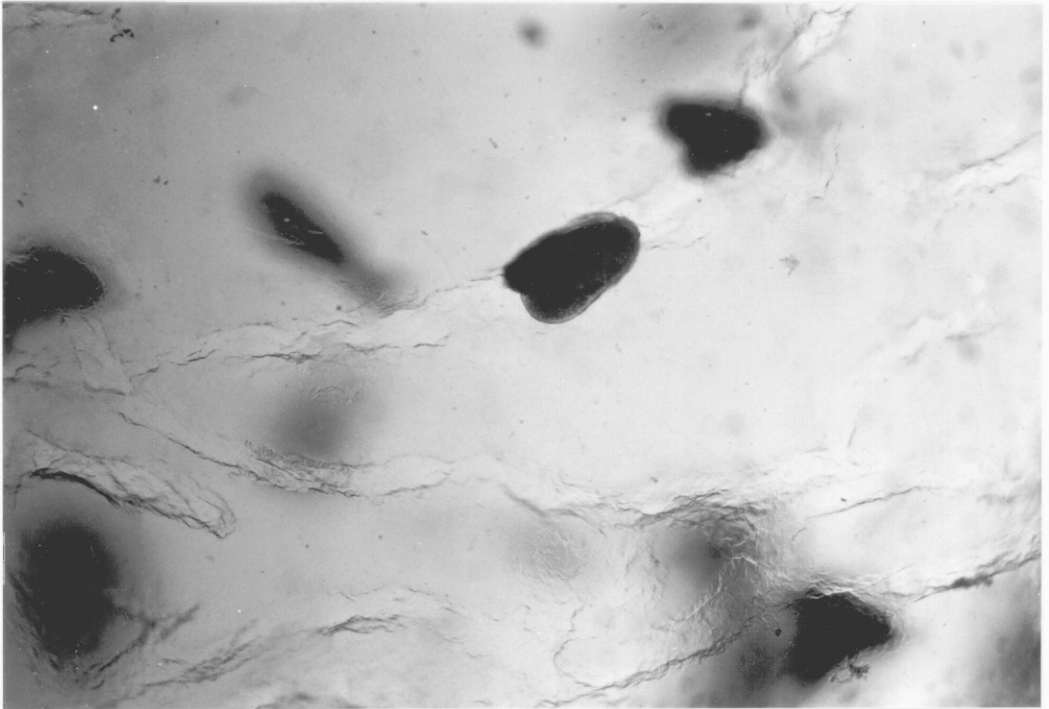


Plate 3 - 1 : The coagulated serum base of a diphasic culture (Ex. No. 27-K, 12 days). The cultured organisms were migrating in the serum base and leaving tracks behind.

CHAPTER 4

DEVELOPMENT IN VIVO AND IN VITRO

4.1 Morphological changes and the behaviour of tetrathyridia prior to segmentation in the monophasic medium S10E.

On introduction of tetrathyridia into a favourable culture, the neck of the tetrathyridium elongated and exhibited rhythmical contraction and extension. Shortly after this, the suckers moved actively and behaved as if "searching" as described for Taenia crassiceps by Esch & Smyth (1976). These worms became translucent, partly because the density of calcareous corpuscles was reduced as a result of increase in body area, owing to the generation of new tissue. On the other hand, evidence from EM studies showed a change in the structure of the tegument at this stage (see Section 5.3.2). This structure might contribute, in part, to the translucent appearance of these worms which later segmented, from day 5 onwards. Several segments appeared simultaneously, on commencing strobilisation. These are the "primary strobilae" according to the definition of Freeman (1973); the germinative cells which produce the gonads of these segments are supposedly distributed along the length of

the body, whereas in the adult the germinative cells are abundant in the region of the neck only.

4.2 Development in vivo

The growth and development of M. corti in the experimentally infected dogs generally followed the pattern described by Thompson et al. (1982b). Worms recovered from the dog six days post infection (p.i.) were mainly small and comprised a few immature proglottids only. At seven days p.i. the length of the adults and the number of the proglottids increased. Some of the proglottids were mature in the sense that the male and female genitalia had developed. Developing oncospheres were first observed in one proglottid of a worm 14 days p.i., but the paruterine organ was in the early stages of development. At 17 days p.i., most adult worms were gravid and the paruterine organ was filled with fertile eggs. The adults continued to elongate and their proglottids increased in number until 14 days p.i., then started decreasing. The adult worms were very short and comprised only a few proglottids after 42 and 55 days in the dog. Data, presented by Dr. Macpherson, on the distribution of the worms in the dogs' small intestine indicated a possible 'crowding effect' after 17 days p.i. (Table 4-1). The increasing population is presumably the

result of asexual proliferation of both tetrathyridia and adult M. corti in the dog intestine.

These observations indicated that tetrathyridia which had been passaged in the laboratory mice for several years were still able to transform to the adults in the dog and produce eggs containing oncospheres. Evidence from biochemical studies suggested that M. corti of two origins represent the same strain. Extracts of M. corti tetrathyridia kindly sent from Australia by Dr. R. C. A. Thompson and our own specimens were compared on their isoenzyme patterns for four enzymes and also on their protein patterns following isoelectric focusing in polyacrylamide gels. Methods and procedures were as described by McManus & Smyth (1979). The enzymes examined were: lactate dehydrogenase, malate dehydrogenase, phosphoglucomutase and phosphoglucose isomerase. The patterns of the four enzymes and the protein were all the same between specimens of the two origins. Cultivation of the two specimens under the same conditions gave similar results too.

Because of the lack of living adults from infected dogs and, perhaps, improper relaxation of worms before fixation, study of the gross morphology of in vivo adults was very difficult. Therefore, observations made on M. corti in the dog by Thompson et al. (1982b) were

alternatively used to assess the in vitro development of this parasite in my study. Meanwhile, a 38-day-old adult sent by Dr. Thompson, which was relaxed in tap water and fixed in boiling 5% formalin, provided a very good sample illustrating the growth and maturation in vivo, after being stained with Gower's carmine (Frontispiece). Its strobila was as short as five proglottids, comprising one immature, one mature and three gravid ones. In the latter, the uterus was filled with developing oncospheres or the paruterine organ was fully developed and contained fertile eggs.

4.3 Development in vitro

The growth, segmentation and organogeny of M. corti in my culture system was at a rate comparable to that in the dog (see Section 4.2) during the first two weeks or so. Thompson et al. (1982b) found the same rate of development in their in vitro system. No visible difference in the development, up to this stage, was noticed among the in vitro adults which were cultured in anaerobic or aerobic conditions.

The sexual differentiation process in vitro has been described in detail in a previous paper (Barrett et al., 1982) and generally conformed to those illustrated by

Thompson et al. (1982b). Figure 4-1 cited from the preceding paper (Barrett et al., 1982) summarised diagrammatically the maturation sequence of M. corti cultured in vitro. The male genitalia matured slightly earlier than the female reproductive system, as shown in the in vivo worms (Thompson et al., 1982b). Sexually mature proglottids (stage 5 and 6) were achieved within 5-6 days of segmentation. The paruterine organ started developing (stage 7-9) from 8 days after segmentation commenced. A mass of cells arose from the posterior end of the uterus in the mature proglottids (Plate 4-1A) and developed a thick fibrous wall of the paruterine organ. Its lumen was derived directly from the lumen of the uterus, in accordance with the observations of Byrd & Ward (1943). It was not uncommon to see developing oncospheres in the uterus when it was transforming to a paruterine organ (Plate 4-1B).

Beyond this stage, however, the development in vivo and in vitro showed some deviation. The same was true between aerobic and anaerobic cultures. The effects of oxygen on the development of M. corti cultured in vitro are discussed in the following section (4.4). In anaerobic cultures, very few paruterine organs were, as those of in vivo worms, full of embryonated eggs and assumed ovoid or spherical shape (Plates 4-1C, 4-2A). More often were found a few ova with glistening envelopes in a somewhat empty paruterine organ

(Plate 4-2B). Sometimes hooks were scattered in the organ, which was packed with cells, whereas the profile of the oncospheres or the eggs could not be clearly recognised (Plate 4-2C). On dissecting this organ, no normal eggs were recovered. It appeared that the developing oncospheres, which were found in the transforming uterus, failed to ripen in most cases and eventually degenerated in the paruterine organ. "Gravid" proglottids containing a degenerated paruterine organ became dark and lost in the medium. Meanwhile, new proglottids were produced rapidly. This resulted in a very long adult consisting of many immature and mature proglottids (Plates 4-3, 4-4, 4-5) in contrast to the short, gravid adults in vivo (Frontispiece). If additives such as blood macerate or dog bile were added to the medium, even larger worms with more immature and mature proglottids developed in the culture. (In other words, these additives failed to improve the egg production of the cultured worms.) The growth of the strobilating adults was not observed to cease in an anaerobic culture, unless there was a 'crowding effect'.

4.4 Effects of oxygen on the development of M. corti in vitro.

Using a comparable culture system under aerobic conditions (10% O₂ /5% CO₂ /85% N₂), sexually mature

strobilated adults of M. corti were grown from undifferentiated tetrathyridia in vitro by Thompson et al. (1982b). They reported, however, that all reproductive structures were gradually resorbed after 18 days. In addition, the growth and strobilisation ceased after 30-35 days in their culture. This phenomenon was also observed in my aerobic cultures.

In experiment No. 25, three groups of cultures were established, one anaerobic (20% CO₂ /80% N₂) and the other two aerobic (air and 5% O₂ /10% CO₂ /85% N₂ respectively). All other conditions were held constant. Adult worms were removed on days 6, 8, 13 or 14, 17, 20, 21 and/or 22, 27 or 28 and studied alive. Some were fixed in boiling formal-saline after relaxation in tap water for 10 minutes and then stained in Gower's carmine. It was found that the resorption of genitalia became apparent in the group cultured under air for 20 days and in those using 5% O₂ /10% CO₂ /85% N₂, as a gas phase, it was noticed after 28 days. However, no such phenomenon was seen in the anaerobic group during this experimental period.

In one experiment (NO. 13-C-V), 36 immature young adults were obtained from the anaerobic culture No. 13-C and distributed into four 50 ml tissue culture flasks (Costar, USA), i.e. 9 adults per t. c. flask containing 25 ml of S10E.H. 20 ml of medium was replaced and gassed twice a

week. Four gas phases were used respectively (a) 20% CO₂ /80% N₂, (b) 10% CO₂ /90% N₂, (c) 5% O₂ /15% CO₂ /80% N₂ and (d) 10% O₂ /5% CO₂ /85% N₂. The growth and development was monitored over a 40 day period. Although worms appeared more active in the aerobic cultures (c and d), the development in these four flasks did not show much difference during the first week. It was noted, however, that the mature segments became opaque, then dark within 10 days in the aerobic cultures (c and d). The total length of the worms did not increase apparently after this stage. These worms were also slim and poorly-developed. This phenomenon was worse in flask (d) than (C). Contrary to this, the adults cultured in anaerobic conditions (a and b) continued to produce new segments throughout the whole period and developed into very long and broad worms composed of many immature and mature proglottids, especially in flask (b). There were some unsatisfactory features of this particular experiment. Firstly, the experimental specimens all originated from an anaerobic culture which was gassed with 20% CO₂ /80% N₂. The influence of varying the gas phases halfway is not known. Secondly, observations were made on a single culture, because of the difficulty of obtaining enough young adults, exactly under the same culture conditions and also at similar developmental stages. Thirdly, the pCO₂ was not controlled. Despite these disadvantages, the report of Thompson et al. (1982b) and the findings in the present study suggested that anaerobic

conditions were more satisfactory than aerobic conditions for the development of M. corti in vitro.

In the early stages, the worms appeared more active in the aerobic cultures, probably because more energy was generated through aerobiosis. This is a well-known effect of oxygen on the tissues of higher animals, and it has been demonstrated to be true for M. corti tetrathyridia by Köhler & Hanselmann (1974). An active metabolism under aerobic conditions may result in the accumulation of lipid to a higher extent for fatty acids are known to be end-products of carbohydrate metabolism (Smyth, 1969b; von Brand, 1973). The accumulation of lipid in the parasite tissue could possibly explain the darkening of mature adult in an aerobic culture. Smyth (1967) reported that visible accumulation of fine lipid droplets darkened Echinococcus granulosus cultured in vitro.

It is also possible that the change in the refraction (i.e. darkening) implies a structural change in the tegument. The uptake of nutrients through the tegument could be consequently impaired, and the resorption of reproductive structures may have occurred as a result of abnormal nutrition. Thompson et al. (1982b) also suggested that the failure to cater for the nutritional requirements of mature parasites cultured in vitro, and those from dogs transferred to in vitro cultures, induced the resorption of

genital organs. Uptake problems rather than nutrition deficiency of the culture medium is believed to be involved in this matter since resorption did not occur in anaerobic cultures using the same medium. It is not known if lipid accumulation is associated with the failure of tegumental uptake.

4.5 Embryogenesis in vitro

The terminology of various layers of embryonic envelopes has been very confusing and nonuniform. The classification proposed by Rybicka (1972) will be followed below.

4.5.1 Fertilization

In a mature proglottid, motile sperm was abundant in the convoluted duct within the cirrus pouch (Plate 4-6A). The cirrus is eversible and was seen frequently to protrude through the common genital opening (Plate 4-6B), which is located in the midline of the dorsal surface of the segment (Byrd & Ward, 1943). Both the male and female ducts open to the genital atrium, internal to the genital opening (Plate 4-7). Self-copulation was sometimes observed in the live specimens examined microscopically on the slide. The

everted portion of the cirrus was seen to go directly into the female genital opening while the eversion was in progress (Plate 4-6C). By the time the cirrus had completely everted, it was deeply inserted into the thickened terminal portion of vagina (Plate 4-6D). Although the transfer of sperm was not observed, motile sperm was occasionally found in the vagina close to the oviduct (Plate 4-8A). According to Rybicka (1964), the first contact between the oocyte and the sperm takes place in the oviduct. A muscular oocapt was usually conspicuous (Plate 4-8B) and opened into the oviduct which continued posteriorly and then looped anteriorly before it branched into two tubes (Fig. 4-2). One of these tubes connected with the vagina. The other one was joined by the common vitelline duct and then became a narrow duct, the uterine duct, leading to the sigmoid uterus (Fig. 4-3). In his study of the development of the oncosphere of M. corti, Ogren (1956) reported that oocytes in the uterine duct contained sperm. In the present study, an oocyte containing sperm was observed in the uterus (Plate 4-8C). The oocyte and a single vitelline cell were enclosed by a membrane-like capsule (Plate 4-8D). According to Rybicka (1966), materials which were eliminated from the vitelline cells formed the capsule before the oocyte entered the uterus; immediately after entering the uterus, the oocyte commenced maturation division. Ogren (1956) found that during oocyte maturation, the sperm nucleus enlarged until it was of a size equal to the female pronucleus; the

fusion of male and female pronuclei completed fertilization. In the present culture system, usually, only a few fertilized oocytes, enclosed by a capsule, were found in the uterus.

4.5.2 Cleavage and cellular differentiation

The cleavage of cestode embryos has been reviewed in detail by Rybicka (1966). According to this author, two cleavage patterns (called here I & II) are distinguished in cyclophyllideans according to the time at which the equal division of macromeres occurs. In M. corti, the first cleavage pattern (I) was observed by Ogren (1956). Thus, the first division of the zygote is equal, resulting in the formation of two identical macromeres. Further unequal divisions produce three types of blastomeres, macromeres, mesomeres and micromeres (Rybicka, 1966). The 2-cell, 3-cell and 4-cell stages, which were derived from the first three cleavages of the zygote, of an embryo in a cultured worm appeared similar to those reported by Ogren (1956). Divisions continued and a mixture of cells of several sizes then made detailed observation difficult. By the end of cleavage (pattern I), one of the macromeres divides again equally and the embryo enters the preoncospherical phase (Rybicka, 1966). In the preoncospherical phase, the micromeres degenerate and the macromeres develop into a

syncytial cytoplasmic layer, the outer envelope, surrounding the embryo (Ogren, 1956; Rybicka, 1966). At the same time, the mesomeres multiply and differentiate into the oncosphere and, speculatively, its inner envelope; the latter is also a syncytial cytoplasmic layer beneath the outer envelope (Ogren, 1956; Rybicka, 1966). Plate 4-9A showed the envelopes of a developing oncosphere in the uterus of a 20 day-old worm cultured in vitro. However, the presence of an intact outer envelope, in this particular sample, is questionable. The reasons for this comment are discussed later in Section 4.5.3.

4.5.3 Mature eggs in the paruterine organ: with special reference to the embryonic envelopes.

Increasing evidence has indicated that the embryonic envelopes play a vital role in the oncosphere development and its survival both within and outside the host (Rybicka, 1966; Coil, 1975; Lethbridge, 1980; Wharton, 1983). The capsule in cyclophyllideans probably provides some transitory protection for the cleaving embryo before the true embryonic envelopes are formed (Rybicka, 1966). The precursors of the outer envelope are macromeres which inherit the whole yolk material from the ovum (Rybicka, 1966; 1973). The function of the outer envelope is, therefore, regarded as a store of nutritive material for the

embryo. In some species of cyclophyllideans, the outer envelope disintegrates after the shell formation is complete (Rybicka, 1972; Fairweather and Threadgold, 1981). Although the exact origin of the cyclophyllidean egg shell is not fully understood, it is generally believed that the shell materials are secreted by the outer envelope (Rybicka, 1973; Fairweather and Threadgold, 1981). The inner envelope gives rise to the embryophore and the "oncospherical membrane" (Rybicka, 1966, 1972; Fairweather and Threadgold, 1981). The embryophore is a rigid layer of keratin-like protein secreted by the degenerating nuclei of the cells forming the inner envelope (Rybicka, 1966; Lethbridge, 1980). In the taeniids, the embryophore, together with the "oncospherical membrane", forms the only barrier between the oncosphere and the external environment (Lethbridge, 1980), as the outer capsule is very thin and is lost in faecal eggs (Smyth, 1969 b).

4.5.3.1 The egg mass in the paruterine organ of M. corti obtained from the dog intestine.

The oncosphere of a mature egg of M. corti is enclosed by a thin "oncospherical membrane" and a syncytial cytoplasmic layer of the inner envelope (Plate 4-9B). The embryophore occupies the outer limits of the inner envelope and is bounded by the envelope's plasma membrane (Plate

4-10A). The outer embryophore appears somewhat regularly indented and the outer membrane of the inner envelope is convoluted accordingly. The boundary of the outer envelope cannot be easily recognized since no shell material is deposited and all mature eggs are so closely packed in the paruterine organ (Plates 4-9B, 4-10B). Cellular organelles such as polysomes, endoplasmic reticulum, mitochondria, annulate lamellae and nuclei etc. are distributed, outside the embryophore, among the eggs (Plate 4-9B). These organelles are normally found in the outer envelope, which, in other cyclophyllideans, degenerates once shell formation is complete (Rybicka, 1973; Fairweather and Threadgold, 1981). Fragments of disintegrated capsule (Plate 4-10D) and intercellular junctions (Plate 4-10C) are sometimes observed in this intermediate area. Conn, Etges & Sidner (personal communication) also found small desmosomes and maculae occludens scattered among the egg mass in the paruterine organ of Mesocestoides lineatus. On the whole, all eggs look as if they were embedded in a cellular matrix (Plate 4-10D). Through this peculiar method of protection and the confinement of all eggs in the paruterine organ, the need for individual protective shells may consequently be reduced.

It should be mentioned that the specimens examined were recovered directly from the dog intestine rather than collected in the faeces where the shed gravid proglottids

were naturally found. These observations could, therefore, only represent a transitory picture of embryonic envelopes. Even so, Conn et al. (personal communication) discovered very similar pictures of embryonic envelopes in the gravid proglottids of M. lineatus taken from faeces for study.

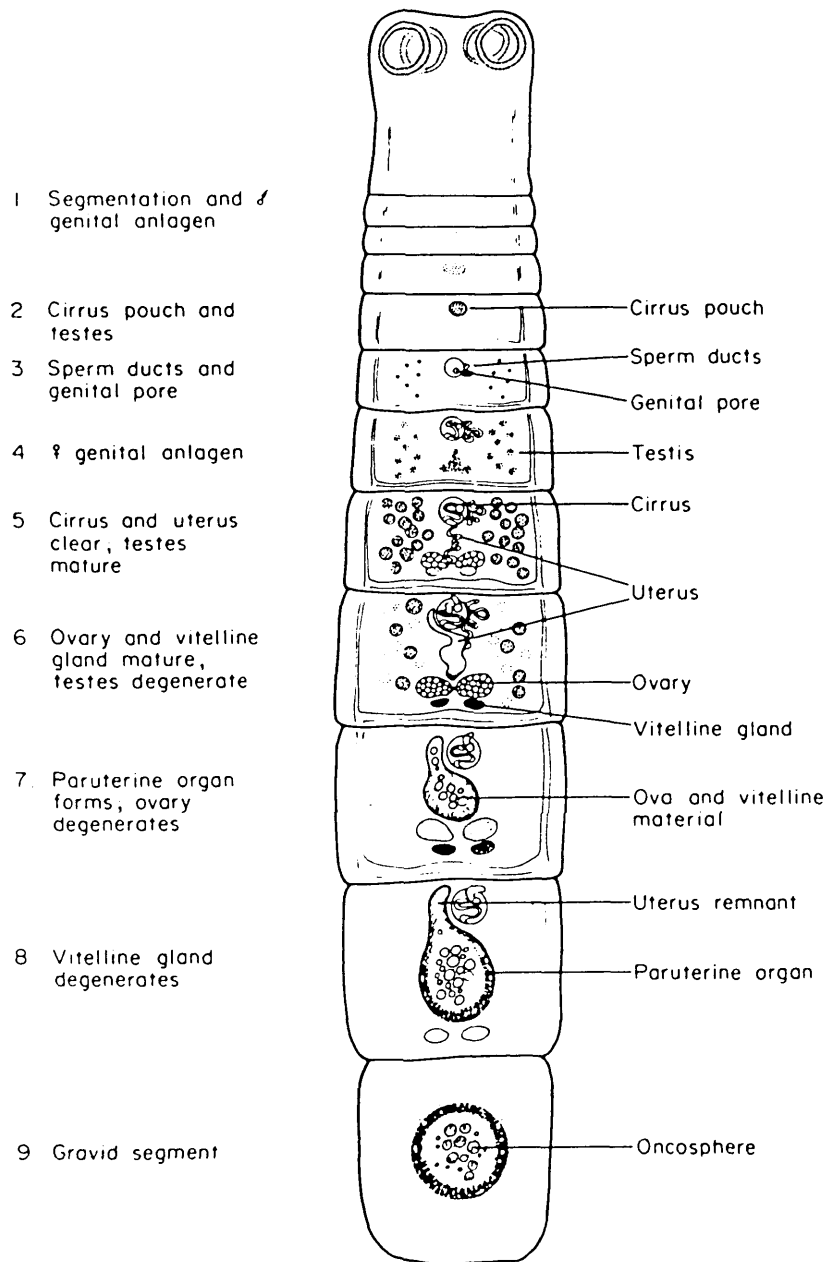
4.5.3.2 Mature eggs from in vitro cultured worms.

The mature eggs in a gravid proglottid obtained in vitro were studied. The "oncospherical membrane" and the embryophore are present around the oncosphere (Plate 4-11, A & B) as observed in the eggs of in vivo worms (Section 4.5.3.1). In the latter, however, much more beta-glycogen was found in the region between the "oncospherical membrane" and the embryophore (Plate 4-10D). A thin layer of outer envelope, which could not be easily identified in the egg mass of in vivo worms, with enlargements containing reticular bodies (Plate 4-11A) was seen adjacent to the embryophore. Shell appeared to be absent. The oncospheres were not studied in detail but their morphology appeared similar to that of other cestodes. The embryonic epithelium, consisting of a vesicular cytoplasm, is separated from other structures of the oncosphere by a basal lamina (Plate 4-11B). The hooks, musculature, penetration glands (Plate 4-11B) and germinative cells (Plate 4-11C)

located opposite to the hook region were also observed.

As shown in Plate 4-1B, a few developing eggs are somewhat individually present in the uterus in vitro rather than close together as they are in vivo. The outer envelope can hence be recognized from the more or less isolated eggs in the paruterine organ, providing these eggs are able to reach maturity incidentally, without the protection of a cellular matrix (see Section 4.5.3.1). If this cellular matrix indeed represents the outer envelope, it would also play a role in supplying nutrients for the developing embryo (see Section 4.5.3). Since a shell is absent, it seems difficult to sustain these cellular organelles around the embryo without forming a coherent egg mass. Using an inverted microscope, ova were observed showing considerable movement, up and down, along a comparatively narrow uterus while the parasite was in the culture tube containing liquid medium. Failure to build up an egg mass might be the reason why the developing oncospheres sometimes could not normally develop and later degenerated in the paruterine organ of an in vitro worm (see Section 4.3 and Plate 4-2C). Possibly for the same reason, free hooks were occasionally found in the uterus (Plate 4-11D). According to this interpretation, a sufficient number of fertilized eggs become important for creating a crowded uterine 'environment' so that all eggs can adhere together by intercellular junctions. Through this peculiar

method of protection, the developing oncospheres could perhaps mature and withstand the disruptive effects of the contractile movement of the uterus.



Diagrammatic representation of the stages of sexual differentiation encountered in *M. corti* cultured *in vitro* from the tetrathyridium.

Fig. 4-1 (from Barrett, Smyth and Ong, 1982)

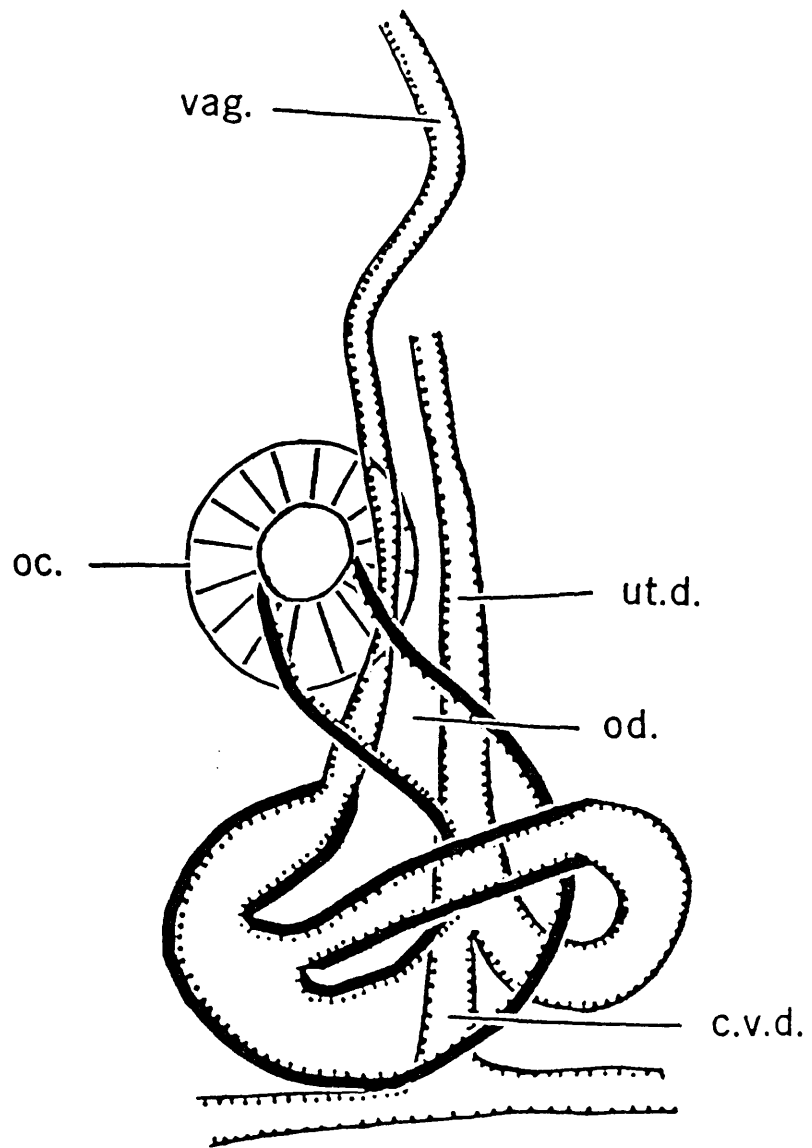


Fig. 4-2 Part of female reproductive system (free-hand from living specimen) : common vitelline duct (c.v.d.); oocapt (oc.); oviduct (od.); uterine duct (ut.d.); vagina (vag.).

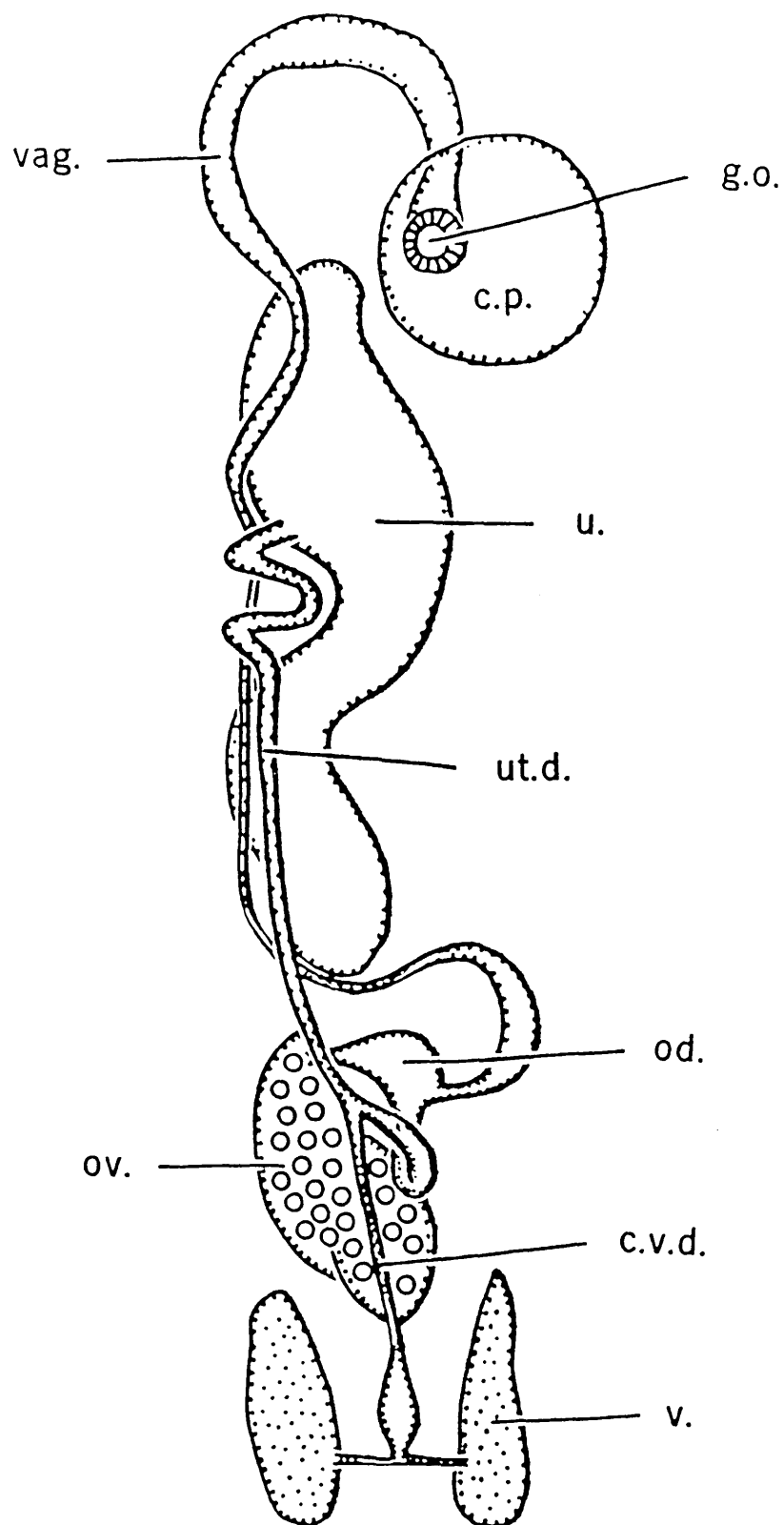


Fig. 4-3 Female reproductive system (free-hand from a 'stretched' specimen) : common vitelline duct (c.v.d.); genital opening (g.o.); oviduct (od.); ovary (ov.); uterus (u.); uterine duct (ut.d.); vitellaria (v.); vagina (vag.).

Table 4-1 Distribution of the worms in the small intestine of domestic dogs which were experimentally infected with Mesocestoides corti tetrathyridia in Kenya.
(data by courtesy of Dr. C. N. L. Macpherson)

Dog No.	Days infected	Location of worms along intestine (cm from pylorus)						Total No. of worms
		25	50	75	100	125	150	
1	6	130	68	24	1	-	-	223
2	7	-	4	74	4	-	-	82
3	14	50	350	400	70	-	-	870
4	17	> 500	> 1000	> 1000	400	-	-	^b 3000
5	42	> 300	> 1000	> 1000	400	-	-	^b 3000
^a 6	55	> 500	> 500	> 300	> 500	> 500	> 500	^b 4000

a Heavy infection to 320 cm from pylorus

b Almost 60% conversion of larvae to adults

NB All larger values are estimates.

Each dog received approximately 0.5 ml of sedimented tetrathyridia orally.

Plate 4-1 Mesocestoides corti in vitro. (living specimens)

4-1A : The uterus (u) of a mature segment. A mass of cells arose from the posterior end of the uterus and later developed a thick fibrous wall of paruterine organ. x 360.

4-1B : Developing oncospheres in the uterus. x 230.

4-1C : Embryonated eggs in the gravid proglottid from a 62 days culture (Ex. No. 13-F). x 96.

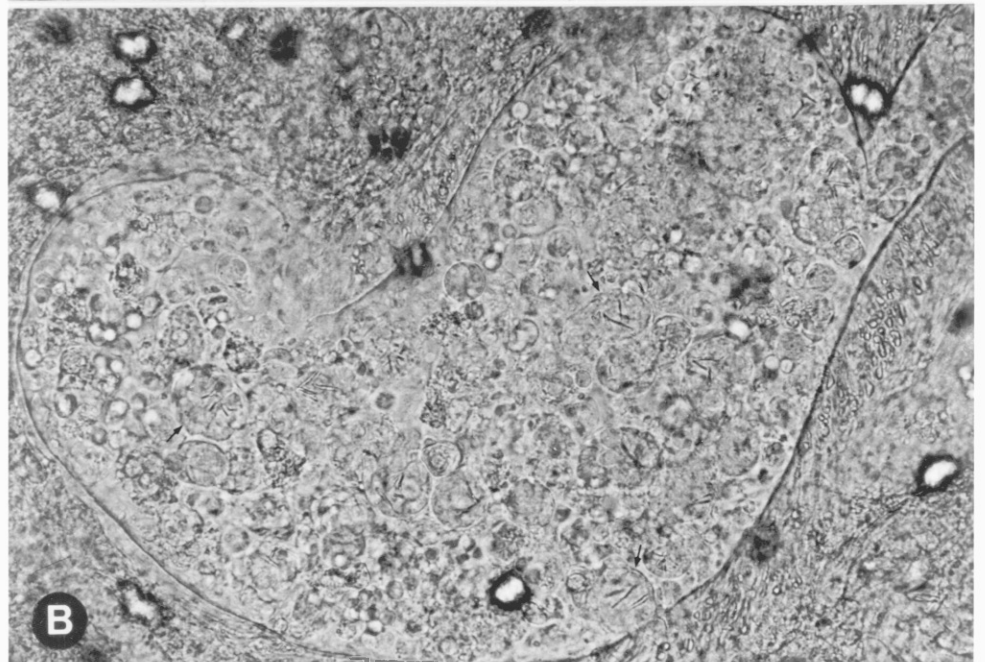
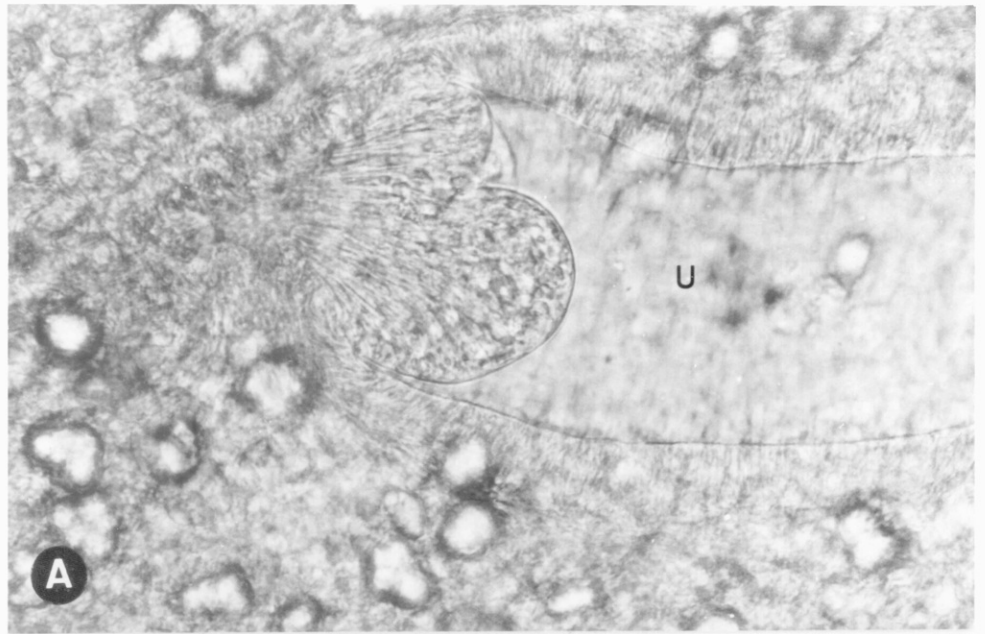
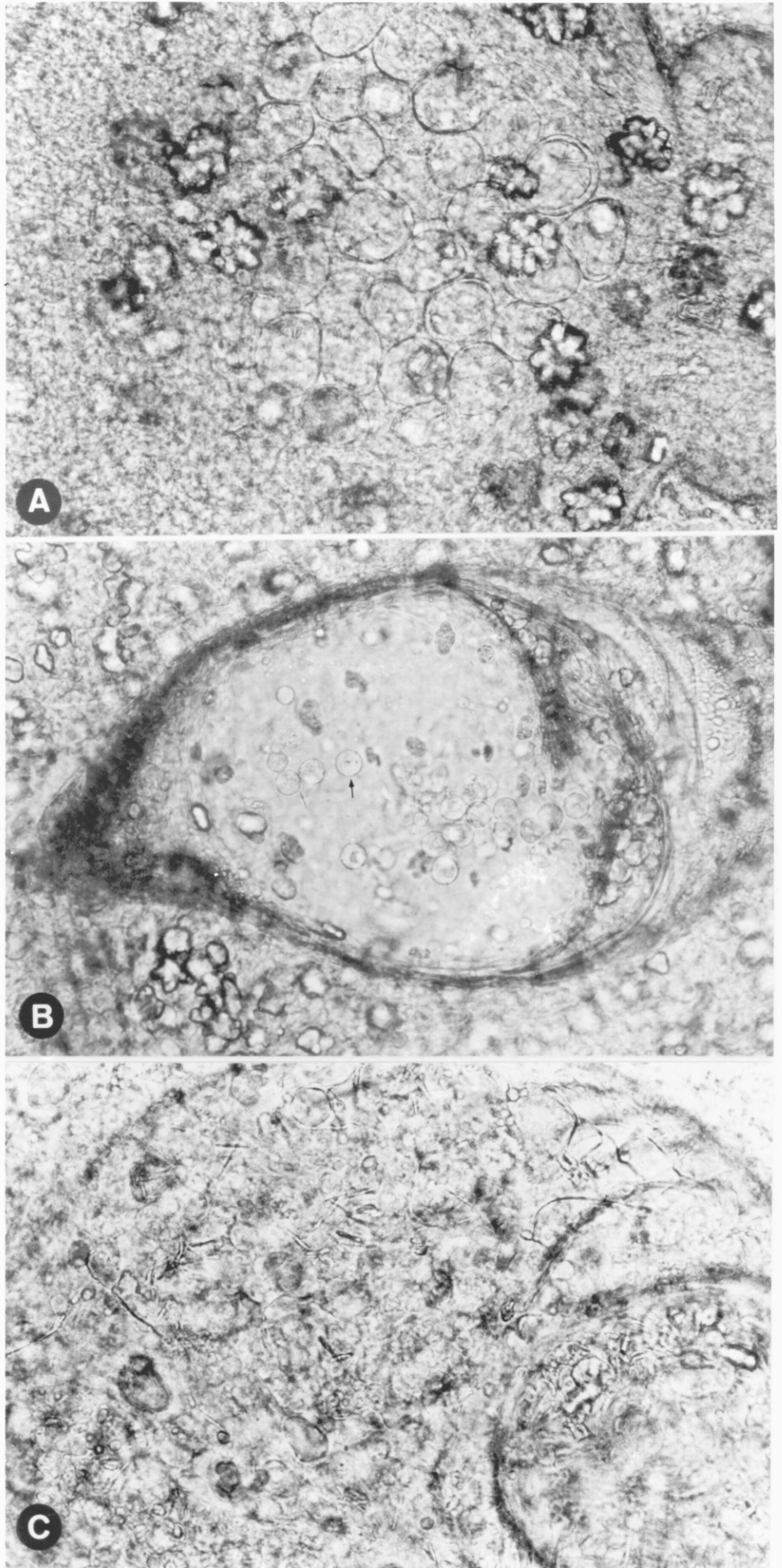


Plate 4-2 The paruterine organ of Mesocestoides corti in vitro.
(living specimens)

4-2A : Enlargement from Plate 4-1C. Embryonated eggs
were obtained sporadically. x 230.

4-2B : Ova with glistening envelopes in a paruterine
organ. x 230.

4-2C : Hooks scattered in a paruterine organ. The
profile of the eggs could not be recognised.
x 360.



Plates 4-3 to 4-5 A well-developed Mesocestoides corti in vitro,
22 days. Gower's carmine. x 39.

4-3 : Anterior,

4-4 : Middle,

4-5 : Posterior.

Plate 4-3



Plate 4-4



Plate 4-5

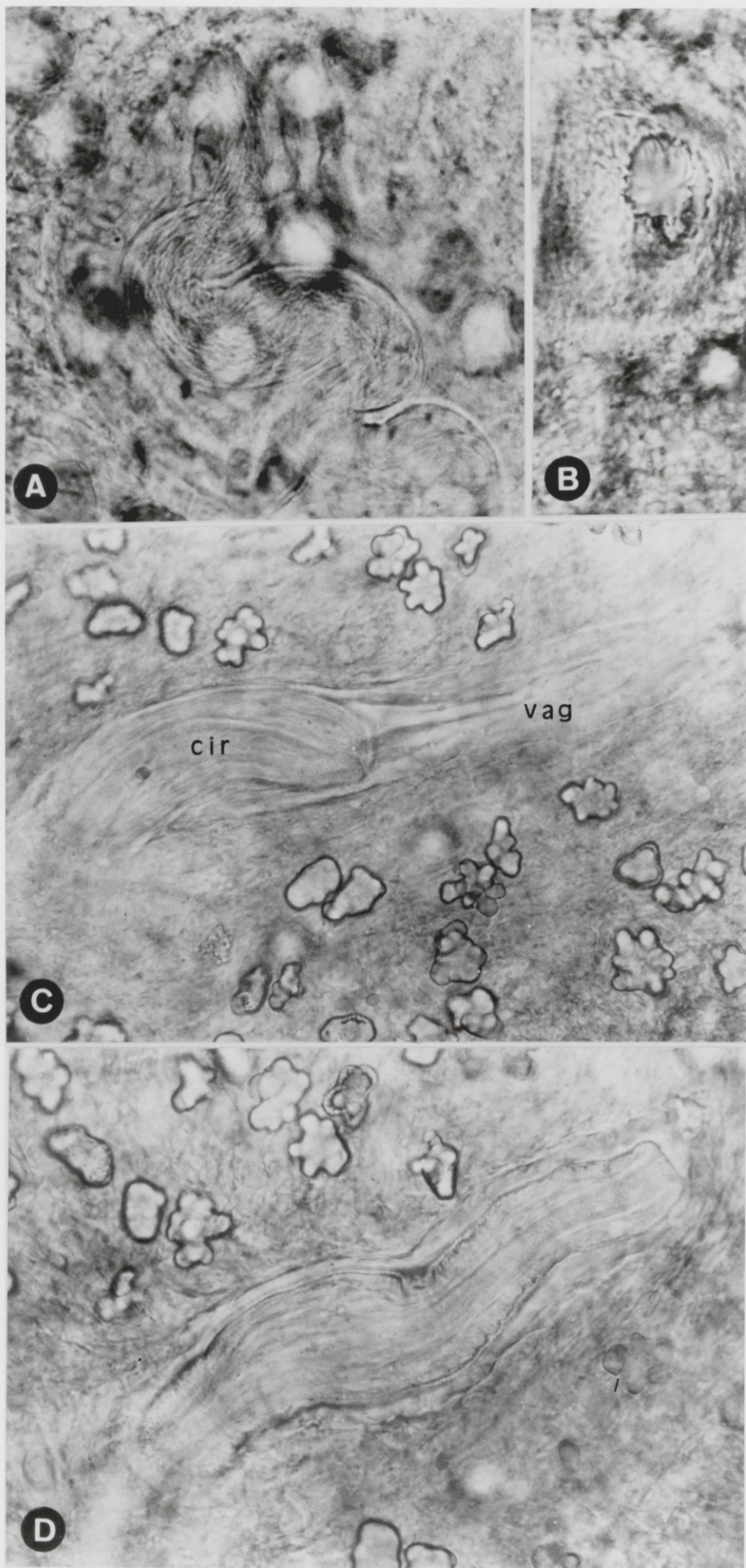


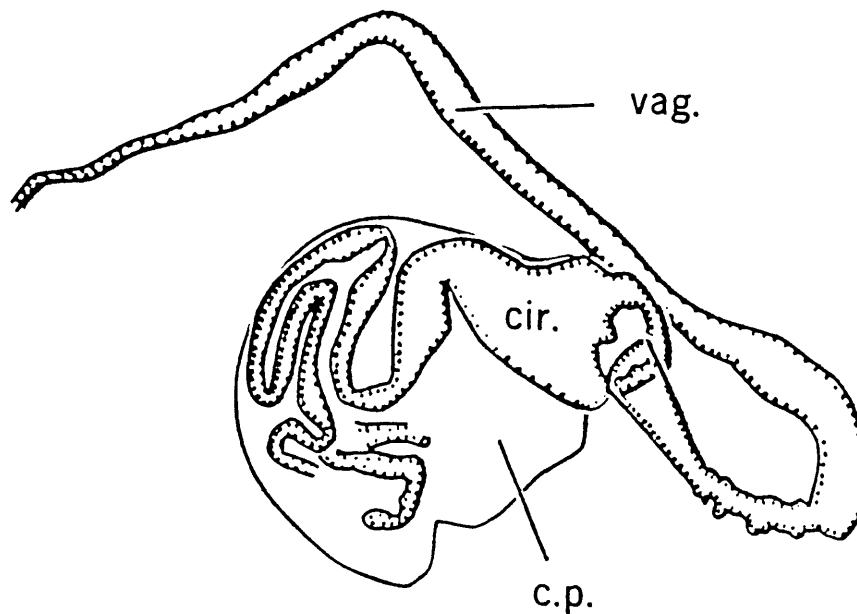
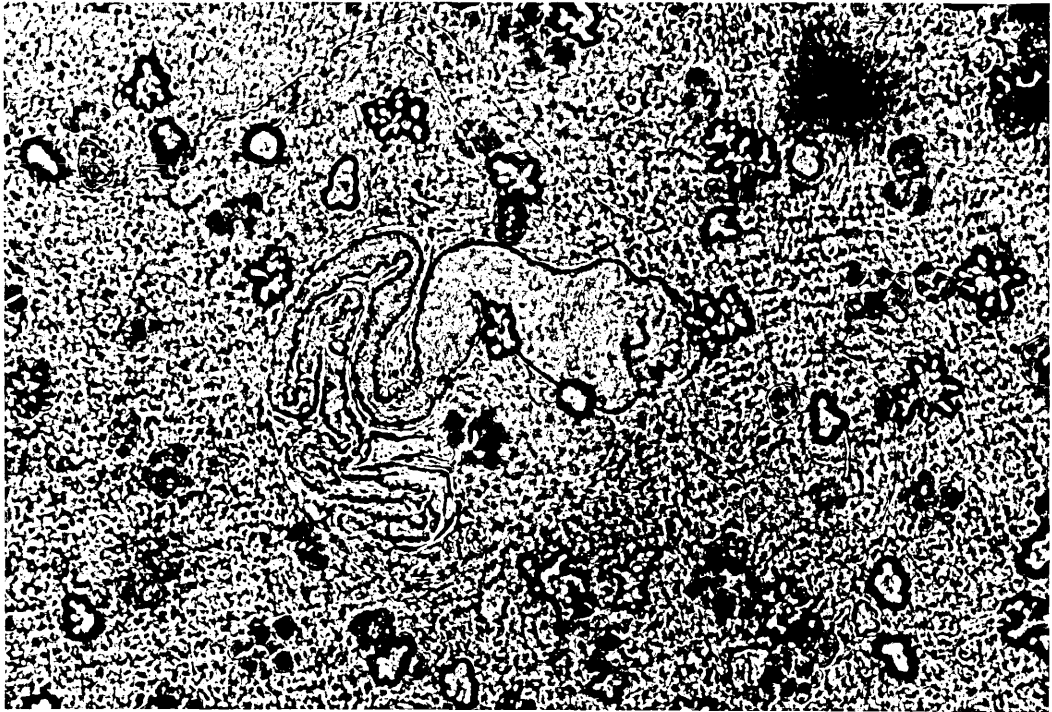
Plate 4-6 Mesocestoides corti in vitro. (living specimens)

4-6A : Motile sperm in the convoluted duct within a
cirrus pouch (Ex. No. 25-B, 14 days). x 540.

4-6B : Sphincter of genital opening located in the
midline of the dorsal surface of proglottid.
x 570.

4-6C & 4-6D : Self-copulation in vitro. (C) x 340,
(D) The cirrus (cir) deeply inserted
into the vagina (vag). x 440.





(tracing from photograph)

Plate 4-7 : Vagina (vag.) and cirrus pouch (c.p.) of a 'squashed' living specimen. Both the male and female ducts open to the genital atrium, internal to the genital opening. x 230.

Plate 4-8 Mesocestoides corti in vitro. (living specimens)

4-8A : Motile sperm in the vagina close to oviduct
(Ex. No. 25-Q, 14 days). x 1140.

4-8B : Oocapt (oc) and oviduct (od) (Ex. No. 25-N,
17 days). x 1150.

4-8C : An oocyte containing sperm (arrow) in uterus
(Ex. No. 25-Q, 14 days). x 1150.

4-8D : Early stage of embryogenesis. The oocyte and
a single vitelline cell (arrow) were enclosed
by a membrane-like capsule (Ex. No. 25-Q, 14
days). x 1980.

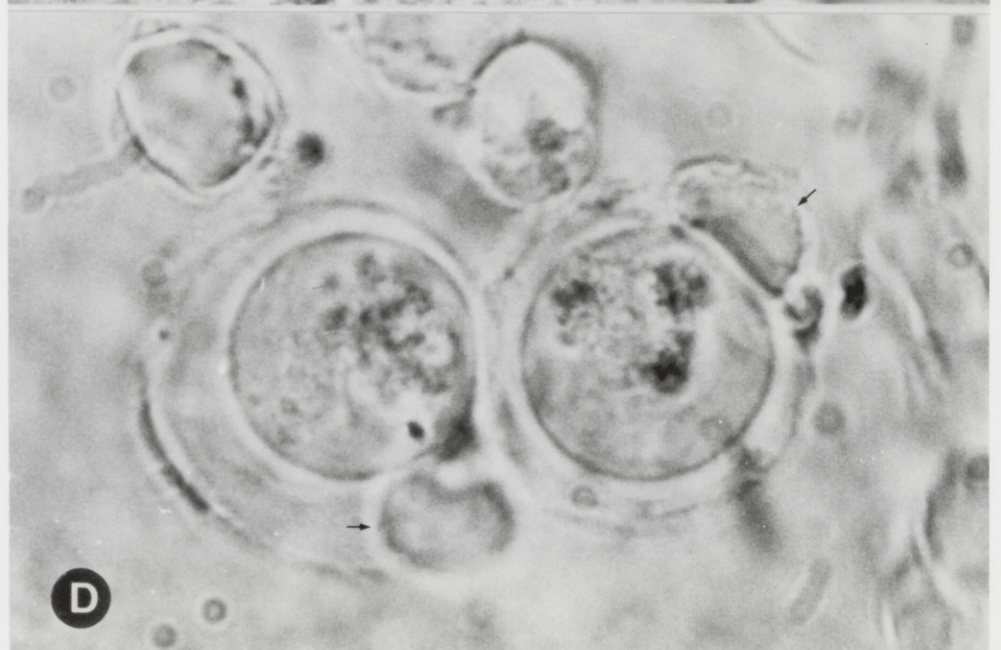
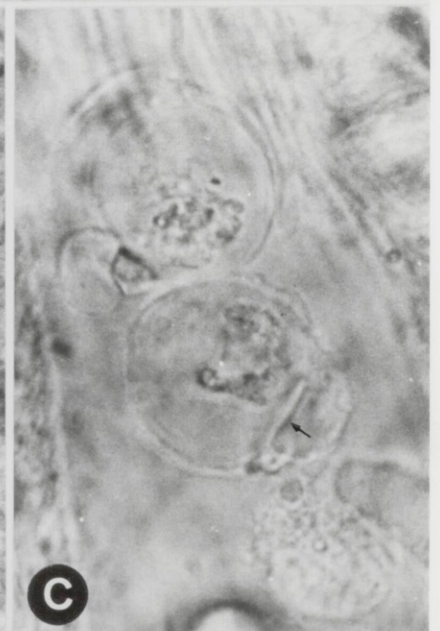
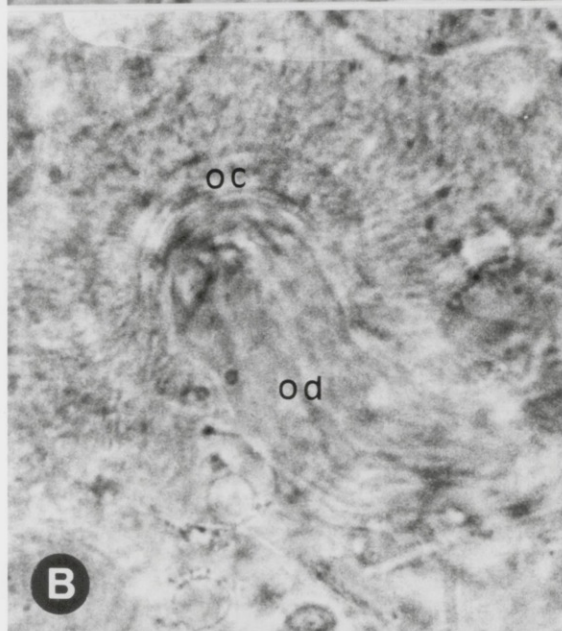
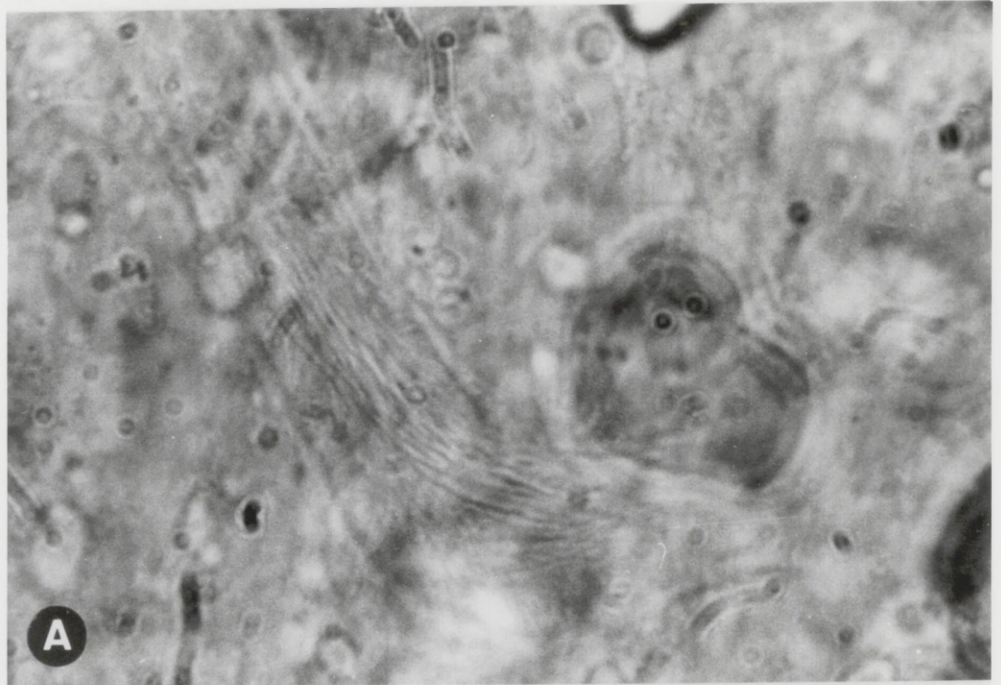


Plate 4-9A : A developing oncosphere in the uterus of a 20 day-old worm cultured in vitro. uranyl acetate & lead citrate. Bar = 2 μ m.

Plate 4-9B : The egg mass in the paruterine organ of Mesocestoides corti in vivo, 17 days p.i. uranyl acetate & lead citrate. Bar = 1 μ m.

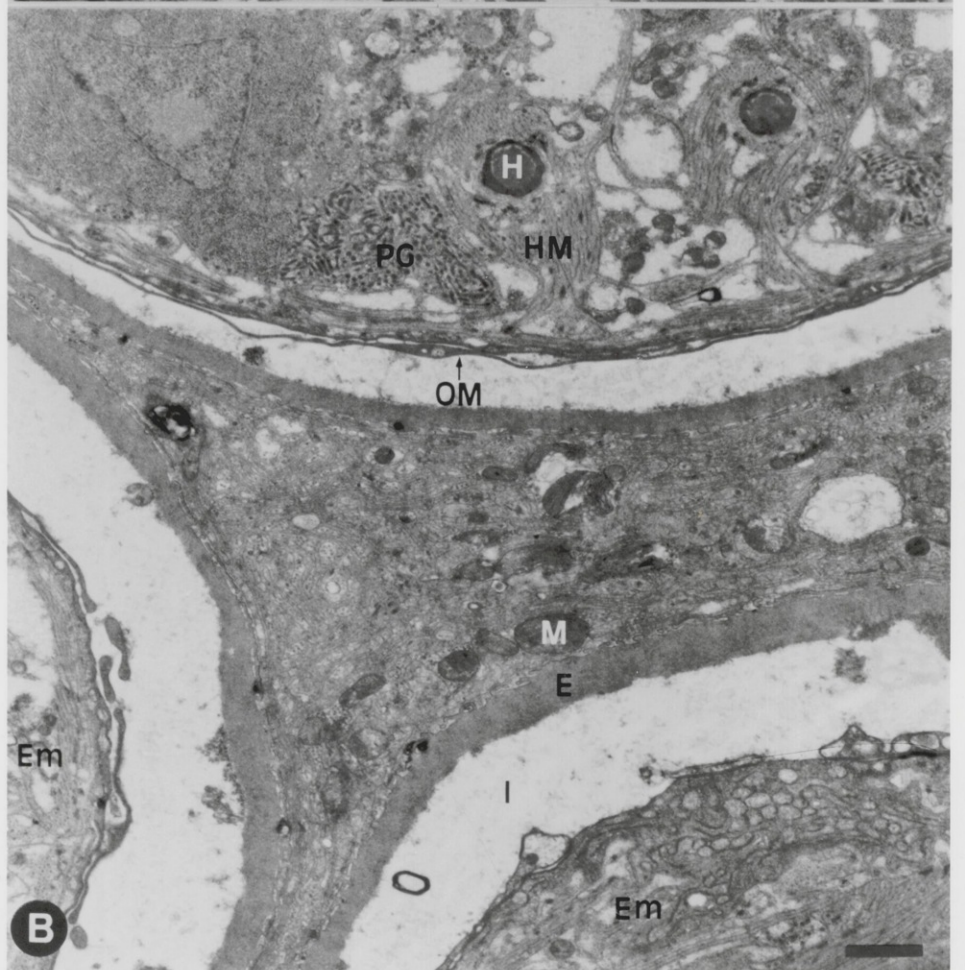


Plate 4-10A : The embryophore (E). The outer embryophore appears somewhat regularly indented and the outer membrane (PM) of the inner envelope is convoluted accordingly. uranyl acetate & lead citrate. Bar = 0.2 μ m.

Plate 4-10B : Longitudinal section through a gravid proglottid of Mesocestoides corti in vivo showing closely packed eggs in the paruterine organ. haematoxylin & eosin. x 150.

Plate 4-10C : Zonula adherens (ZA). Intercellular junctions are observed in the egg mass in the paruterine organ of in vivo worms. uranyl acetate & lead citrate. Bar = 1 μ m.

Plate 4-10D : The egg mass in the paruterine organ of Mesocestoides corti in vivo, 17 days p.i. uranyl acetate & lead citrate. Bar = 5 μ m.

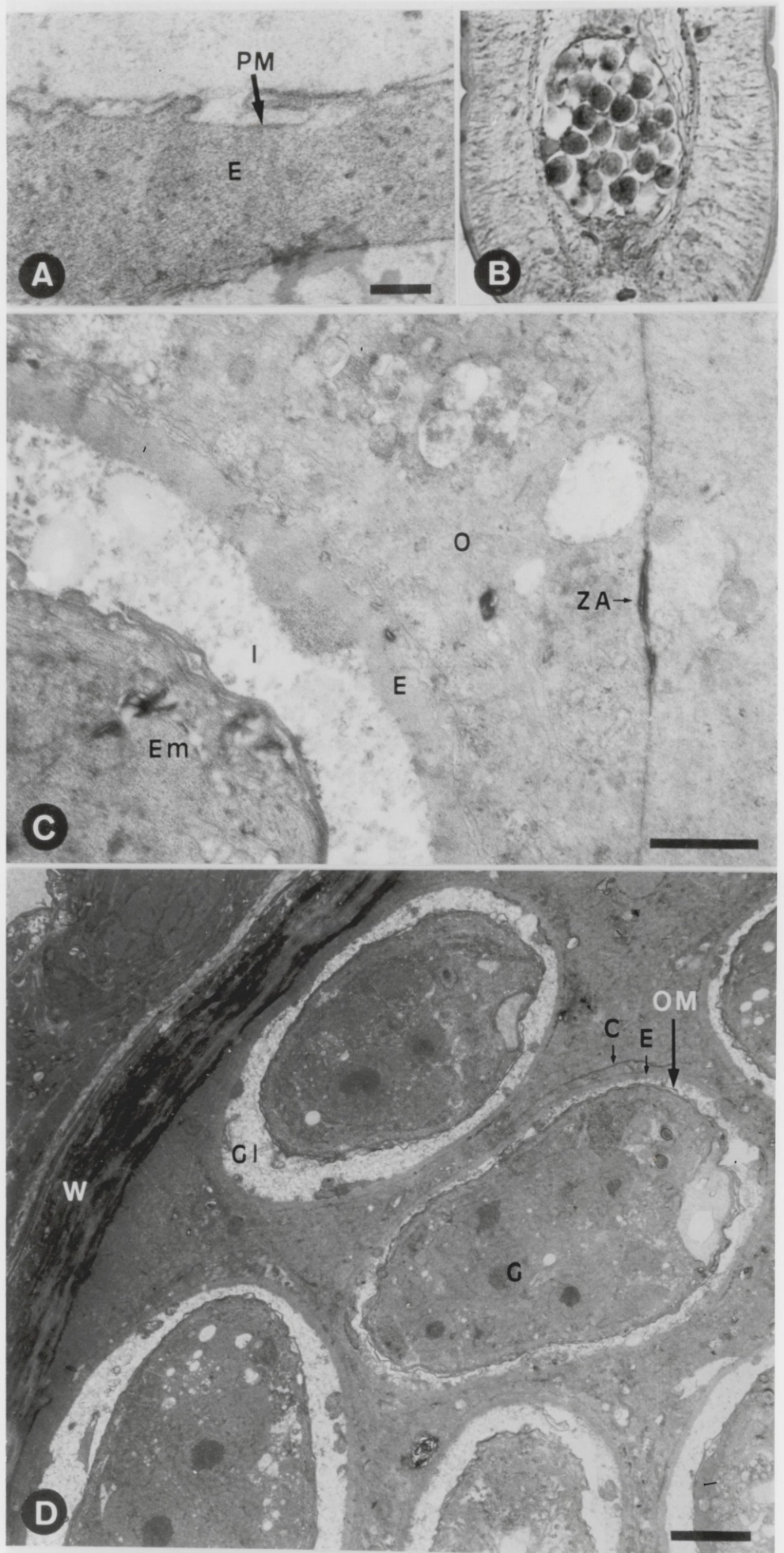
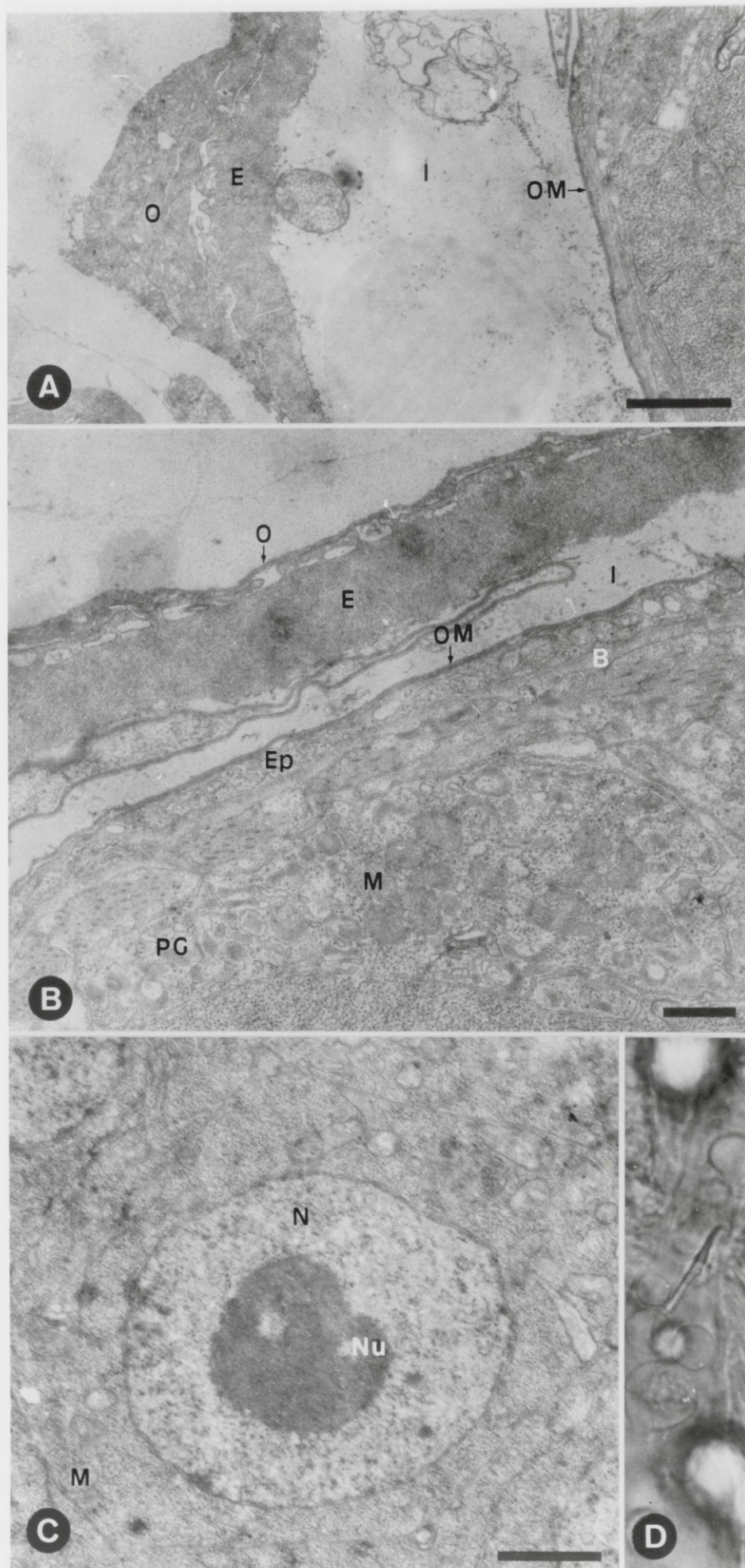


Plate 4-11A & 4-11B : Mature eggs in the paruterine organ of
Mesocestoides corti in vitro, 50 days.
uranyl acetate & lead citrate. (A) Bar
= 1 μm , (B) Bar = 0.5 μm .

Plate 4-11C : The germinative cells of oncosphere in the
paruterine organ of Mesocestoides corti in vitro,
50 days. uranyl acetate & lead citrate. Bar = 1 μm .

Plate 4-11D : A free hook in the uterus of Mesocestoides corti
in vitro, 28 days. (living specimen) x 840.



CHAPTER 5

ULTRASTRUCTURAL CHANGES IN THE TEGUMENT DURING LARVAL-ADULT TRANSFORMATION

5.1 Introduction

It has long been recognised that the body covering of cestodes is not only protective, but is also a metabolically active surface (Lee, 1966; Smyth, 1969b; Lumsden, 1975). Since cestodes lack a digestive tract, all nutrients must enter and waste materials be eliminated through the body covering which is now most commonly referred to as the tegument (Rothman, 1959). The structure and terminology of the cestode tegument have been reviewed by Smyth (1969b, 1972). The tegumental projections on the free surface of cestodes are termed 'microvilli' (Lee, 1966; 1972) or 'microtriches' (singular 'microthrix'; Rothman, 1959). The latter is more frequently used. Each microthrix consists essentially of a distal electron-dense 'shaft' and a proximal medullary 'base' (Jha and Smyth, 1969). It has become evident that more than one type of microtriches are present in the different body regions of the same stage and at the different stages of the life cycle (Table 5-1). The

polymorphism of microtriches suggests multiple functions of the cestode tegument. Although many roles have been ascribed (Rothman, 1963; Lee, 1966, 1972; Bråten 1968a,b; Smyth, 1969b; Jha and Smyth, 1969; Lumsden, 1975; Hess and Guggenheim, 1977; Thompson et al. 1980, 1982a), the precise function of microtriches is still not clear. Similarly, numerous vacuoles, vesicles and membrane-bound bodies of various sizes have been reported in the tegument of many cestodes (Rothman, 1963; Oaks and Lumsden, 1971; Featherston, 1972; Kwa, 1972; Blitz and Smyth, 1973; Hayunga and Mackiewicz, 1975; Hopkins and Law, 1978; Specian and Lumsden, 1980; Threadgold and Hopkins, 1981; Richards and Arme, 1982; Threadgold and Dunn, 1983), but their functions are still not perfectly known. Smyth (1972) emphasized that the examination of the changes which occur during the transformation of the parasite, from one stage to the next stage of the life cycle, could provide some clues as to the functions of the structures of the cestode tegument. The satisfactory in vitro culture system in the present study enabled the tegument of M. corti at various developmental stages to be investigated. Possible functions of some structures of the tegument are discussed.

5.2 Scanning electron microscopy of tetrathyridia at various developmental stages

5.2.1 Invaginated tetrathyridia from experimental mice

The invaginated tetrathyridia which were obtained from the abdominal cavity of the experimentally infected mice were examined topographically. The whole surface of the body, into which the scolex invaginated, appeared sculptural at low magnification (Plate 5-1, A & B). Transverse openings of various sizes were scattered irregularly throughout the body surface. Under high magnification, short, filamentous microtriches (Plate 5-1C) were the only type (called here filamentous type I) observed on the body surface of the invaginated tetrathyridia. These microtriches often occurred in groups with the tips of several microtriches gathered together, forming a group. The significance of this phenomenon is not known.

5.2.2 Tetrathyridia with no sign of segmentation

The ultrastructure of the tegument of the evaginated M. corti tetrathyridia, which are maintained in mice, has been studied in detail by Hess & Guggenheim (1977), Voge et al. (1979) and Hess (1980). According to these authors, two types of microtriches occur on the tegument

of the tetrathyridium. One is filamentous and the other blade-like. Hess & Guggenheim (1977) reported, however, that the base of the filamentous microtriches of the scolex region was shorter than that of the posterior part of the tetrathyridia, and the shaft of the former (7 μm) was strikingly longer than the latter (1.1 μm). My observations agree with theirs but I have divided these filamentous microtriches into two types, type I (the posterior part of tetrathyridia) (Plate 5-1, D & E) and type II (scolex region).

After cultivation under unfavourable conditions in a monophasic medium, tetrathyridia grew slightly in size rather than transformed to the adult stage; their tegumental structure generally conformed to that described for the evaginated tetrathyridia, obtained from experimental mice, by the above authors. The long, filamentous microtriches (type II) predominated on the apical scolex (Plate 5-2A) as well as the lining of the suckers (Plate 5-2B). The blade-like microtriches were seen on the sucker rims and the surface immediately adjacent to the suckers, as illustrated by Voge et al. (1979). Posterior to this region, filamentous microtriches (type I ?) mingled with blade-like microtriches. The proportion of the two types of microtriches in this body area appeared individually different (Plate 5-2, C & D). In contrast to this,

regional variations occur in tetrathyridia which are maintained in mice (Hess and Guggenheim, 1977). Voge et al. (1979) stated that the 'long slender' microtriches predominate in this area. The sculptural body, containing only filamentous type I microtriches, of the invaginated tetrathyridium, which was described in section 5.2.1, becomes the posterior part of this tetrathyridium (Plate 5-2E).

5.2.3 The pre-segmentation stage

Prior to segmentation in the monophasic medium S10E, the tetrathyridia became translucent under light microscopy (for details see Section 4.1). At this pre-segmentation stage, the tegument of the body area was thrown into folds and gave a wrinkled appearance at low magnification under the scanning electron microscope (Plate 5-3A). At high magnification, the microtriches on the folded tegument appeared rough and disordered (Plate 5-3B). Shed microtriches were seen occasionally (Plate 5-3C). The surface of the scolex, however, did not change ultrastructurally. Two types of microtriches (filamentous type II and blade-like) were observed (Plate 5-3, D & E). Their distribution on the scolex was shown in detail in Plate 5-3A. A sculptural 'tail' was still present at this stage.

5.3 Transmission electron microscopy of the tegument of the tetrathyridia at various developmental stages in vitro, with emphasis on the apical scolex.

The ultrastructure regarded as the basic pattern of cestodes has been described in the tegument of M. corti tetrathyridia by Hess (1980). The external level of the tegumental syncytium is an anucleate cytoplasmic region which is connected to the inner perinuclear cell bodies by cytoplasmic connections (tubules or bridges etc.). Based on the terminology applied to trematodes by Burton (1964), the 'distal cytoplasm' is used for the surface syncytium, and the 'perinuclear cytoplasm' for the cytoplasm in the region of the cell bodies sunken in the parenchyma, in this study. The cytoplasmic bridges extend from the tegumental cell bodies through the superficial muscle layers and penetrate the basal lamina beneath the distal cytoplasm. The free surface of the distal cytoplasm densely bears microtriches, which are invested with plasmalemma. According to Hess (1980), the distal cytoplasm is of uniform structure on the whole body of the tetrathyridium. The following organelles and inclusions were observed: mitochondria, vesicles containing flocculent substances of low electron density, and disc-shaped bodies. The latter lie perpendicular to the tegumental surface except over the suckers where they are

parallel.

In the present study, emphasis was placed on the tegumental ultrastructure of the apical scolex, where only filamentous type II microtriches were scanned during the larval-adult transformation in vitro.

5.3.1 Tetrathyridia with no sign of segmentation

In the apical scolex of a tetrathyridium which showed no sign of segmentation, the distal cytoplasm of the tegument contained mitochondria, dense disc-shaped bodies, and various sizes of membrane bound vesicles with flocculent contents (Plate 5-4A). In addition, electron dense, ovoid bodies were centrally or eccentrically situated in some vesicles. It is not known if they are actually derived from the disc-shaped bodies or represent another type of organelle in this region.

5.3.2 The pre-segmentation stage

In the pre-segmentation stage, large vesicles (approximately 300 - 500 nm), containing electron-translucent material, densely fill the perinuclear and distal cytoplasm of the tegument in the

apical scolex (Plate 5-4B). These vesicles were also present in the rest of the body surface (except the sculptural 'tail') but were reduced in number and size (approximately 200-400 nm) (Plate 5-4C). The translucent appearance at this stage under light microscope might be associated, in part, with the occurrence of these vesicles. Large vesicles persisted in the tegument of the apical scolex of the adult worms both in vitro and in vivo. Details of the latter are given in the following section.

5.4 The tegumental ultrastructure of adult M. corti in vivo

The tegumental ultrastructure of adult M. corti cultured in vitro was generally identical to that in vivo although the suckers of the latter presented the appearance of muscularity which was absent from the former. Therefore, only the tegument of the in vivo adult is illustrated ultrastructurally below.

At low magnification, the whole body surface of the adult worm appeared wrinkled (Plate 5-5A) under the scanning electron microscope. At high magnification, the scolex was covered with microtriches which resembled those on the scolex of tetrathyridia, in spite of being more

densely arranged. The long, filamentous (type II) microtriches were present on the apical scolex and the lining of the suckers (Plate 5-5, B & C). Other regions of the scolex and the area posterior to the scolex were covered by blade-like microtriches (Plate 5-5, D & E). On the folded tegument of strobilae (Plate 5-5F), small blade-like microtriches occurred uniformly (Plate 5-5G). The elongated, cylindrical base (approximately 0.92 μm in length and 0.10-0.12 μm in diameter) of this microtrich and the deep folds of the strobilar tegument were revealed by transmission electron microscopy (Plate 5-6, A & B). Small membrane-limited, oval vesicles (approximately 100 x 60 nm) distributed along the free border of the distal cytoplasm (Plate 5-6, A & C), were especially prominent.

The large, flocculent vesicles, which first appeared in the pre-segmentation stage, and the electron-dense disc-shaped bodies occurred in the syncytial tegument of the apical scolex of the adult stage (Plate 5-7A). Both the distal and the perinuclear cytoplasm in this region were extensively vesiculated (Plate 5-7B). The ultrastructure, suggestive of fusion between the vesicle membrane and the tegument plasmalemma, was sometimes observed (Plate 5-7, C & D).

5.5 Functional considerations

SEM studies have shown that the blade-like microtriches occurred on the sucker rims and the adjacent area in both larval and adult stages. The location and structure of these blade-like microtriches suggest that they may play an important role in maintaining position. As they traverse the infected tissues in vivo, tetrathyridia were observed migrating rapidly in the serum base of a suitable diphasic culture. The blade-like microtriches covering the anterior part of the larvae possibly prevent backward movement and, therefore, assist in locomotive movements. As to the adult stage, these blade-like microtriches supposedly assist in retaining the unarmed scolex in the intestine of the host.

The elongate, proximal base occurred in the filamentous type I microtriches, which were observed on the body surface of the invaginated tetrathyridia, as well as the small blade-like microtriches on the strobilar tegument. This portion of the two types of microtriches is considered to be the main absorptive zone of the larval and the adult tegument respectively. The deep folds which appear frequently on the strobilar tegument further increase the surface area for absorption. Plate 5-6A showed that the two absorptive sides inside a deep fold were kept apart by the small blade-like shafts. This would perhaps allow a free flow of nutrients in the fold.

The long, filamentous (type II) microtriches were present only on the apical scolex. Their functional significance cannot be deduced from the limited information in this study, but they are likely to represent an important structural adaptation.

The apical scolex of the pre-segmentation stage and the adult stage was extensively vesiculated. The appearance of the large membrane bound vesicles in the apical scolex coincides with the onset of the rapid somatic growth after the pre-segmentation stage. It is possible that these vesicles contribute to the amplification of the surface membrane by incorporating the vesicle membrane into the tegumental plasmalemma. Since these vesicles are also present in the perinuclear cytoplasm and the cytoplasmic bridges, they are likely to be derived from the Golgi complex in the perinuclear cytoplasm of the tegumental cells.

Table 5-1 : The distribution of the various types of microtriches in cestodes

Reference	Species	Filamentous		Blade-like (BL)	Small BL with long base
		(long base)	(long shaft)		
Rothman (1963)	<u>Hymenolepis diminuta</u>		scolex	scolex (cone shaped)	proglottids
Baron (1968)	<u>Taenia crassiceps</u> cysticercus	bladder wall		scolex	
Bråten (1968b)	<u>Diphyllbothrium latum</u>			plerocercoid	adult
Yamane (1968)	<u>Diphyllbothrium erinacei</u>			plerocercoid, adult scolex	proglottids
Yamane (1969)	<u>Diplogonoporus grandis</u>				gravid proglottids
Jha & Smyth (1971)	<u>Echinococcus granulosus</u>		rostellum		
Featherston (1972)	<u>Taenia hydatigena</u>		top rostellum	sucker, rostellum	gravid proglottids
Lumsden, Oaks & Mueller (1974)	<u>Spirometra mansonoides</u>	plerocercoid (?) (digiform)		proceroid, plerocercoid (conoid type)	
Andersen (1975)	<u>Diphyllbothrium</u> spp.		inner surface of bothrium	scolex	mature proglottids
Hess & Guggenheim (1977)	<u>Mesocetoides corti</u> tetrathyridium	posterior extremity	scolex	scolex	
Voge, Sogandares-Bernal & Martin (1979)	<u>M. corti</u> tetrathyridium		sucker linings	sucker rims	
Thompson, Hayton & Jue-Sue (1980)	<u>Proteocephalus tidswelli</u>		apical organ	rostellum, neck (giant BL) proglottid	
Thompson, Houghton & Zaman (1982)	<u>E. granulosus</u>		scolex (cylindrical, slender)	strobila (flattened BL)	
Novak & Dowsett (1983)	<u>T. crassiceps</u> cysticercus	(rostellum, bladder; thin filament-SEM)		scolex, sucker rims (thick cylindrical)	
Threadgold & Dunn (1983)	ditto	bladder wall (?)	pre-bulb region	pre-bulb region	

Plate 5-1 The invaginated tetrathyridia from mice and the
filamentous type I microtriches.

5-1A & 5-1B : Showing the sculptural body surface of
the invaginated tetrathyridia from mice.
Bar = 100 μm .

5-1C : Short, filamentous (type I) microtriches on the
body surface of the invaginated tetrathyridia.
Bar = 1 μm .

5-1D : The filamentous type I microtriches on the posterior
part of a tetrathyridium, 6 days in culture.
Bar = 1 μm .

5-1E : Showing the long (0.65 μm) base (Ba) of the
filamentous type I microtriches. Bar = 0.1 μm .

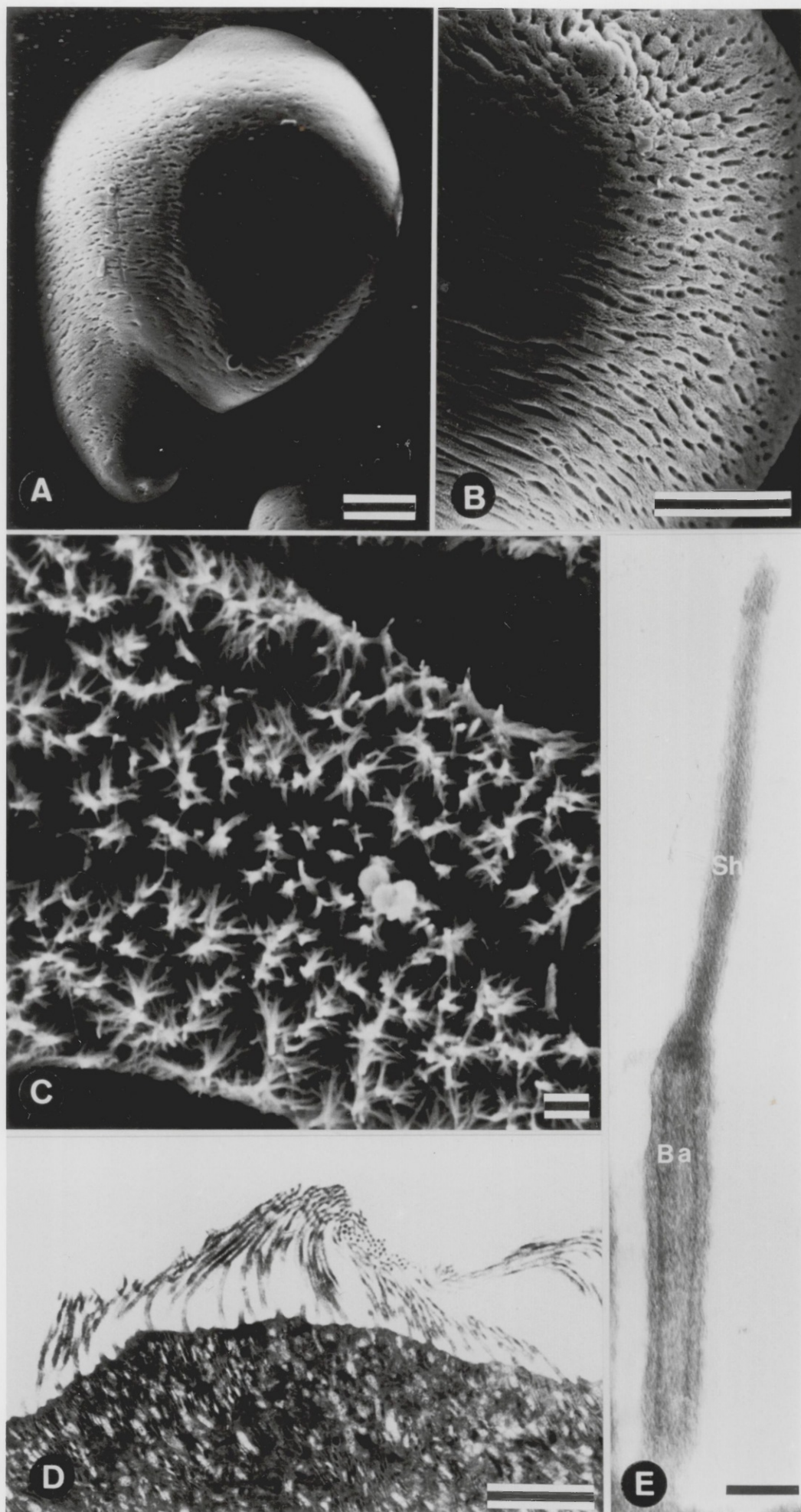


Plate 5-2 Scanning electron microscopy of the tetrathyridia
with no sign of segmentation.

5-2A & 5-2B : The long, filamentous (type II) microtriches
present in : (A) the apical scolex of a
tetrathyridium, 50 days in culture, Bar =
1 μm ; (B) the lining of the suckers of a
tetrathyridium, 51 days in culture, Bar =
10 μm .

5-2C & 5-2D : The intermingled area of tetrathyridia, 51
days in culture. Note the proportion of
the filamentous and the blade-like microtriches
is individually different. Bar = 1 μm .

5-2E : Showing the sculptural 'tail' of a tetrathyridium,
3 days in culture. Bar = 100 μm .

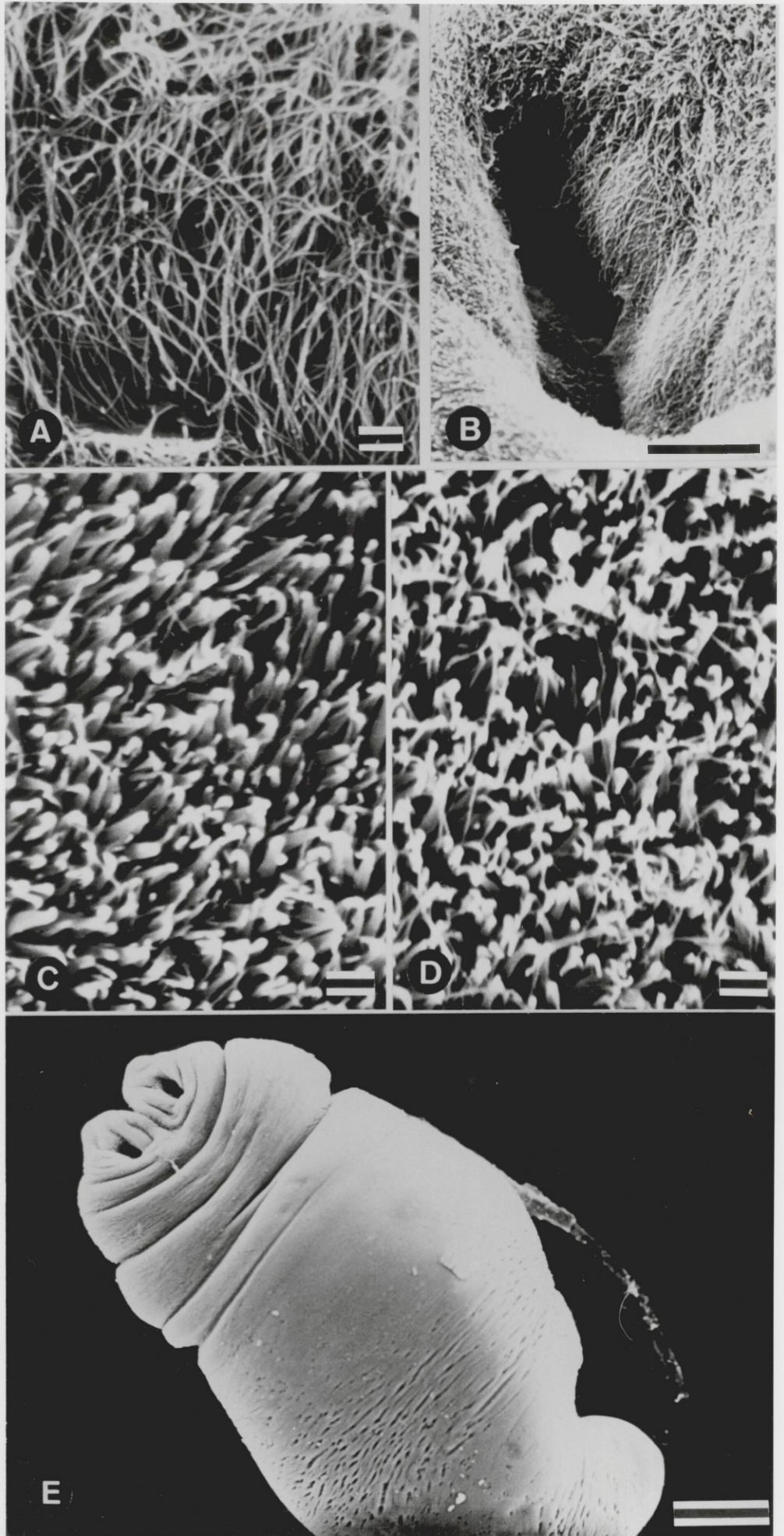


Plate 5-3 Scanning electron microscopy of the pre-segmentation stage.

5-3A : Note the wrinkled tegument and the distribution of the two types of microtriches, the filamentous type II (-) and the blade-like (+), on the scolex.
Bar = 100 μ m.

5-3B : The microtriches on the folded tegument appeared rough and disordered. 6 days in culture.
Bar = 1 μ m.

5-3C : Shed microtriches (arrow) were seen occasionally.
6 days in culture. Bar = 1 μ m.

5-3D : The filamentous type II microtriches on the apical scolex. 6 days in culture. Bar = 1 μ m.

5-3E : The blade-like microtriches in the region immediately posterior to scolex. 6 days in culture. Bar = 1 μ m.

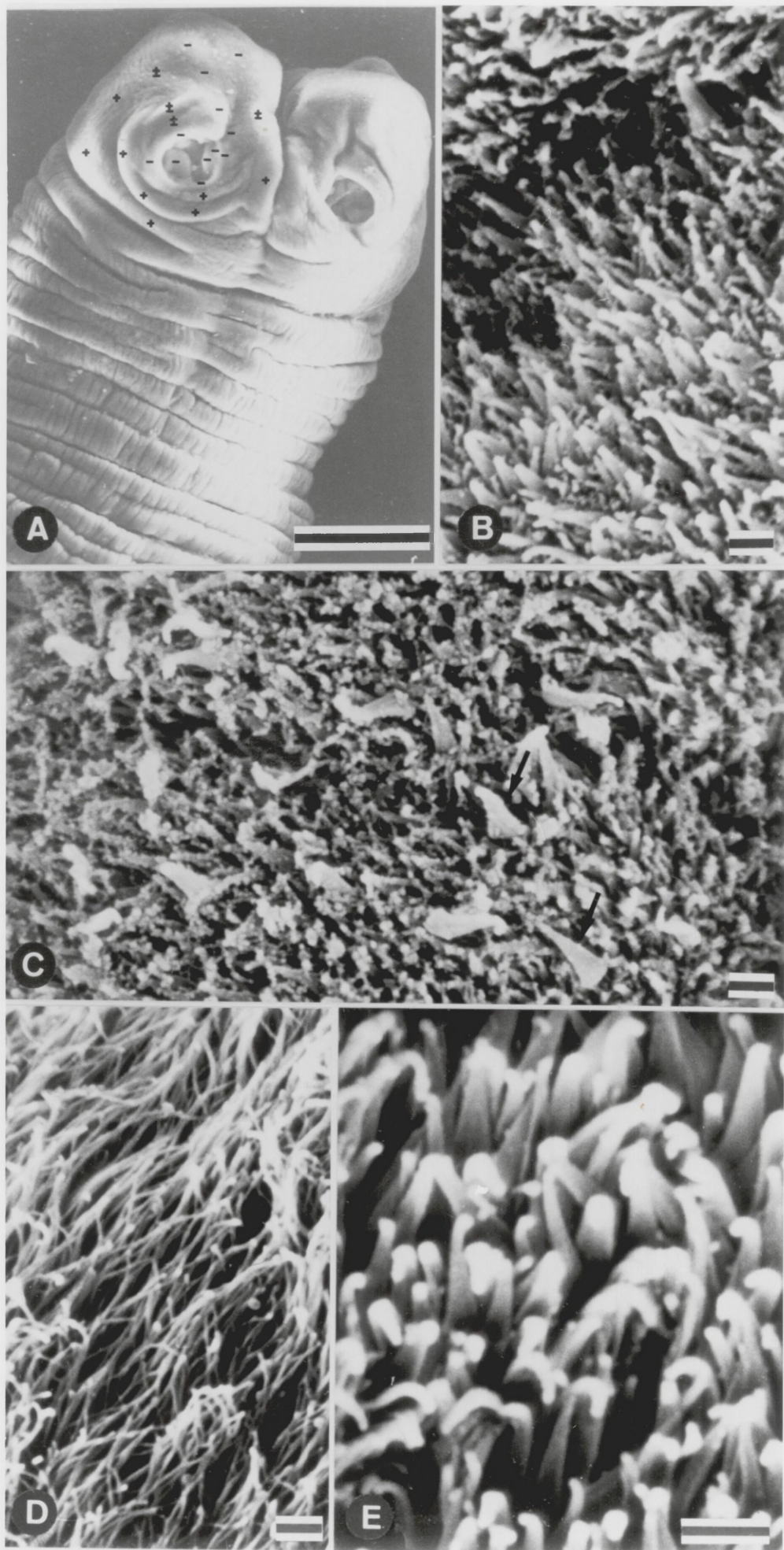


Plate 5-4 Transmission electron microscopy of the apical scolex
of the tetrathyridia at different developmental stages.

5-4A : The tegument of the scolex of a tetrathyridium
with no sign of segmentation. 6 days in culture.
Bar = 0.5 μm .

5-4B & 5-4C : The pre-segmentation stage.

(B) The vesiculated apical scolex.

Bar = 1 μm .

(C) The large, electron-translucent
vesicles also present in the tegument
of the 'neck' region. Bar = 1 μm .

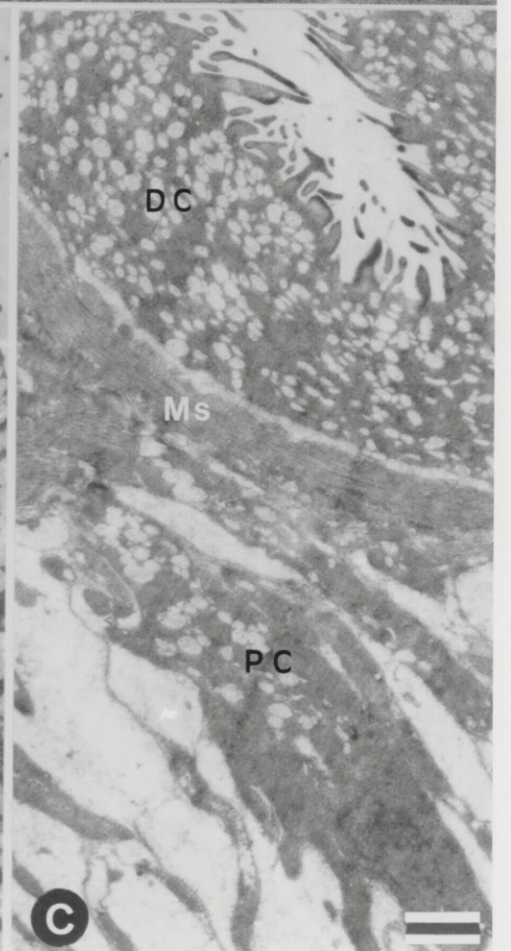
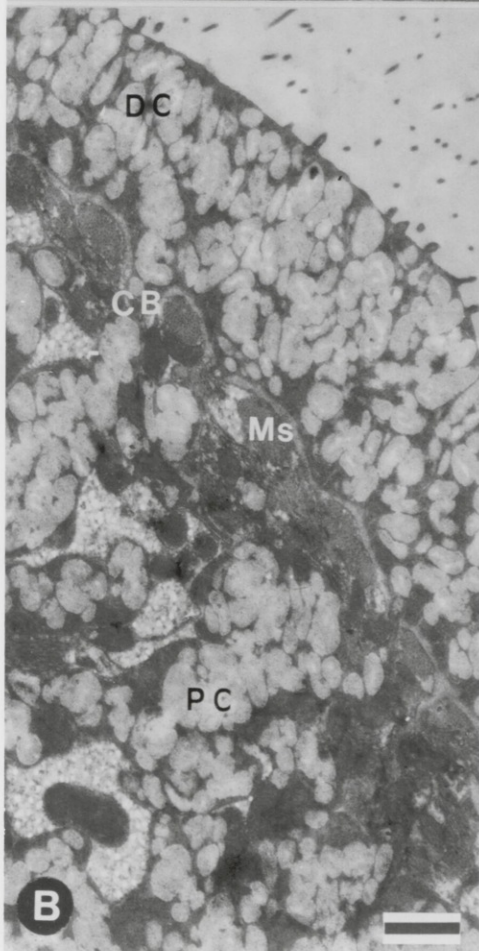
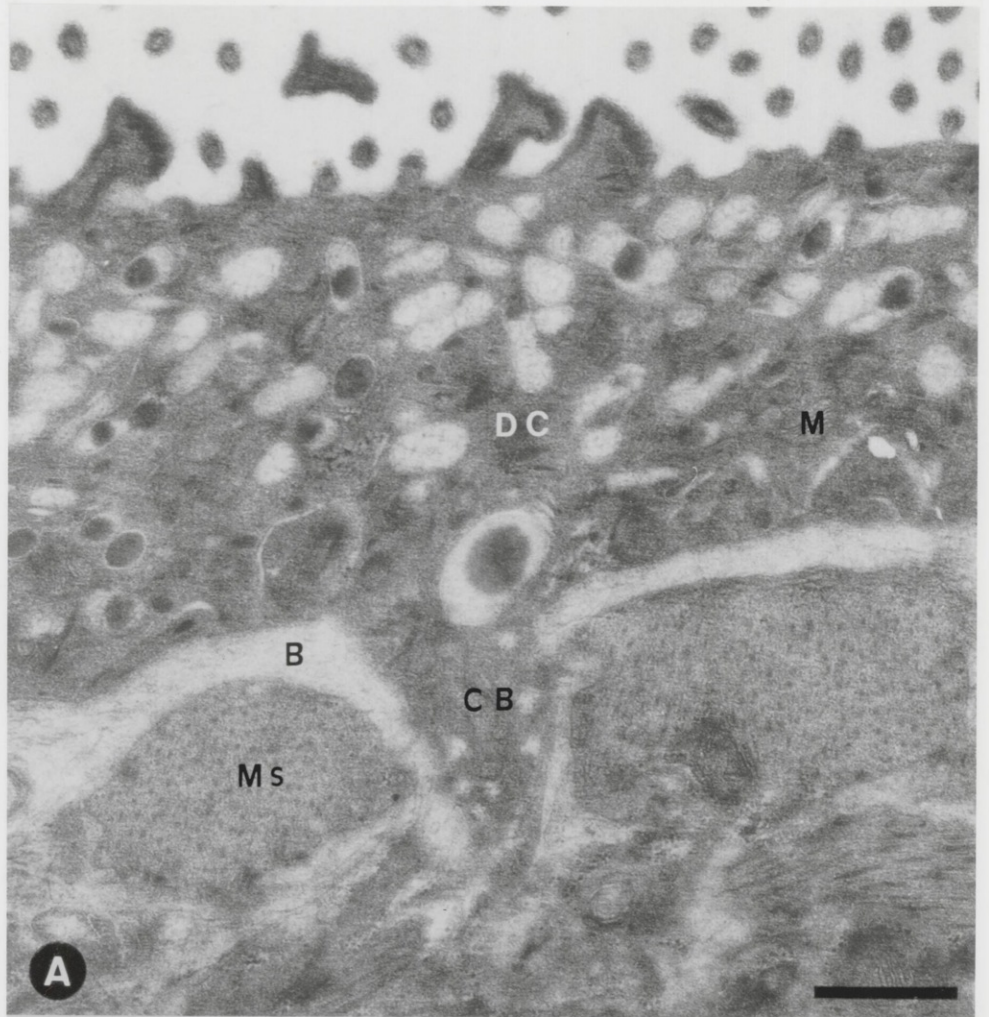


Plate 5-5 Scanning electron microscopy of the adult M. corti
from dogs.

5-5A : The whole body surface appeared wrinkled.

42 days p.i. Bar = 100 μm .

5-5B & 5-5C : The long, filamentous (type II)
microtriches present in : (B) the
apical scolex, 6 days p.i. Bar = 1 μm ;
(C) the lining of suckers, 42 days p.i.
Bar = 1 μm .

5-5D & 5-5E : The blade-like microtriches present in :
(D) the scolex, 17 days p.i. Bar = 1 μm ;
(E) the area posterior to the scolex, 17
days p.i. Bar = 1 μm .

5-5F : Showing the folded tegument of strobilae. 7 days
p.i. Bar = 10 μm .

5-5G : The small blade-like microtriches occurred uniformly
on the strobilar tegument. 42 days p.i. Bar = 1 μm .

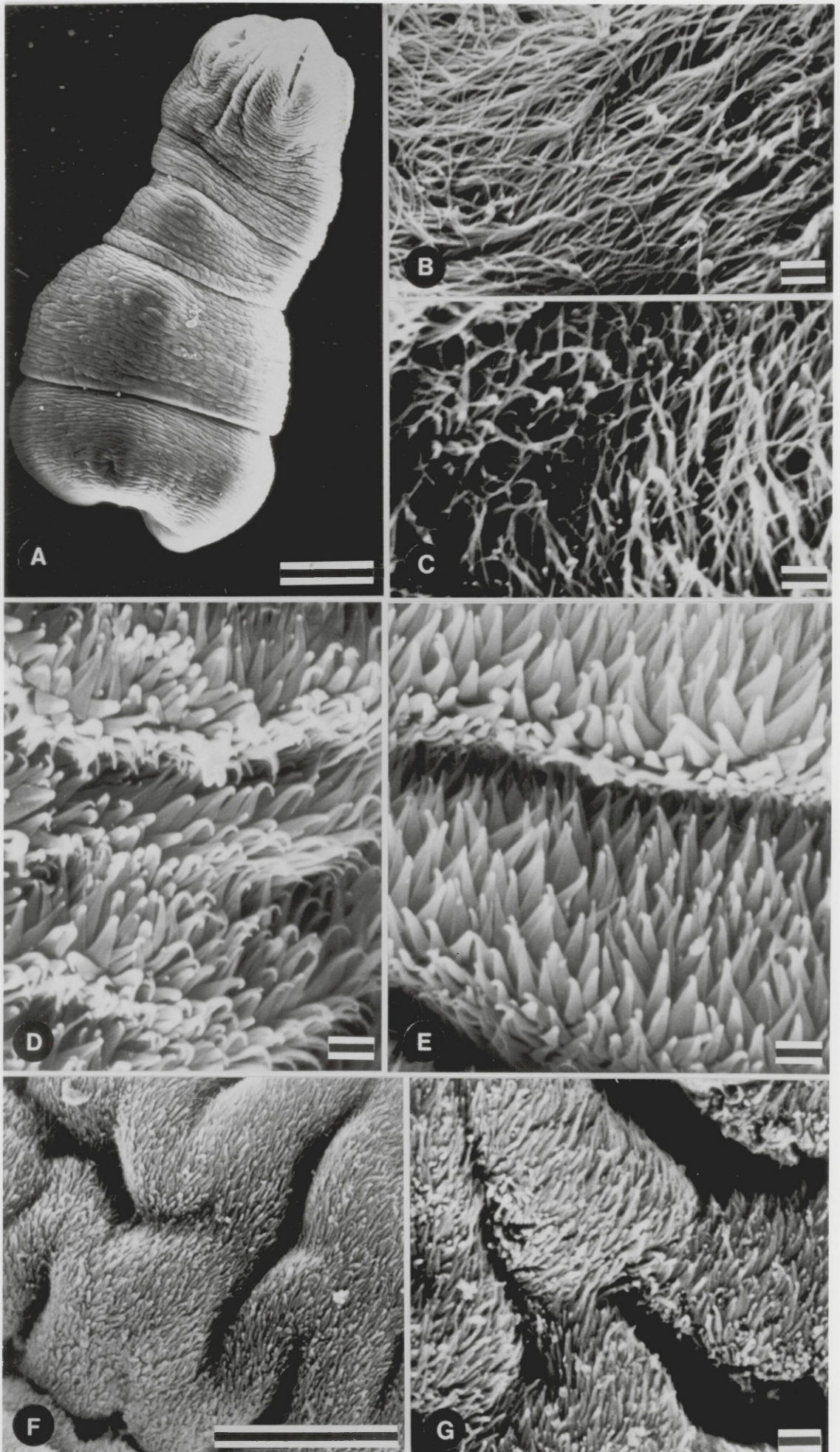


Plate 5-6 Transmission electron microscopy of the strobilar
tegument of M. corti from dogs, 17 days post infection.

5-6A : Showing a deep fold on the tegument and the small
vesicles along the free border of the distal cytoplasm
(DC). Bar = 2 μ m.

5-6B : The elongate, cylindrical base (Ba) of the small
blade-like microtriches. Bar = 0.5 μ m.

5-6C : The small membrane-limited oval vesicles (arrow)
along the surface border. Bar = 0.5 μ m.

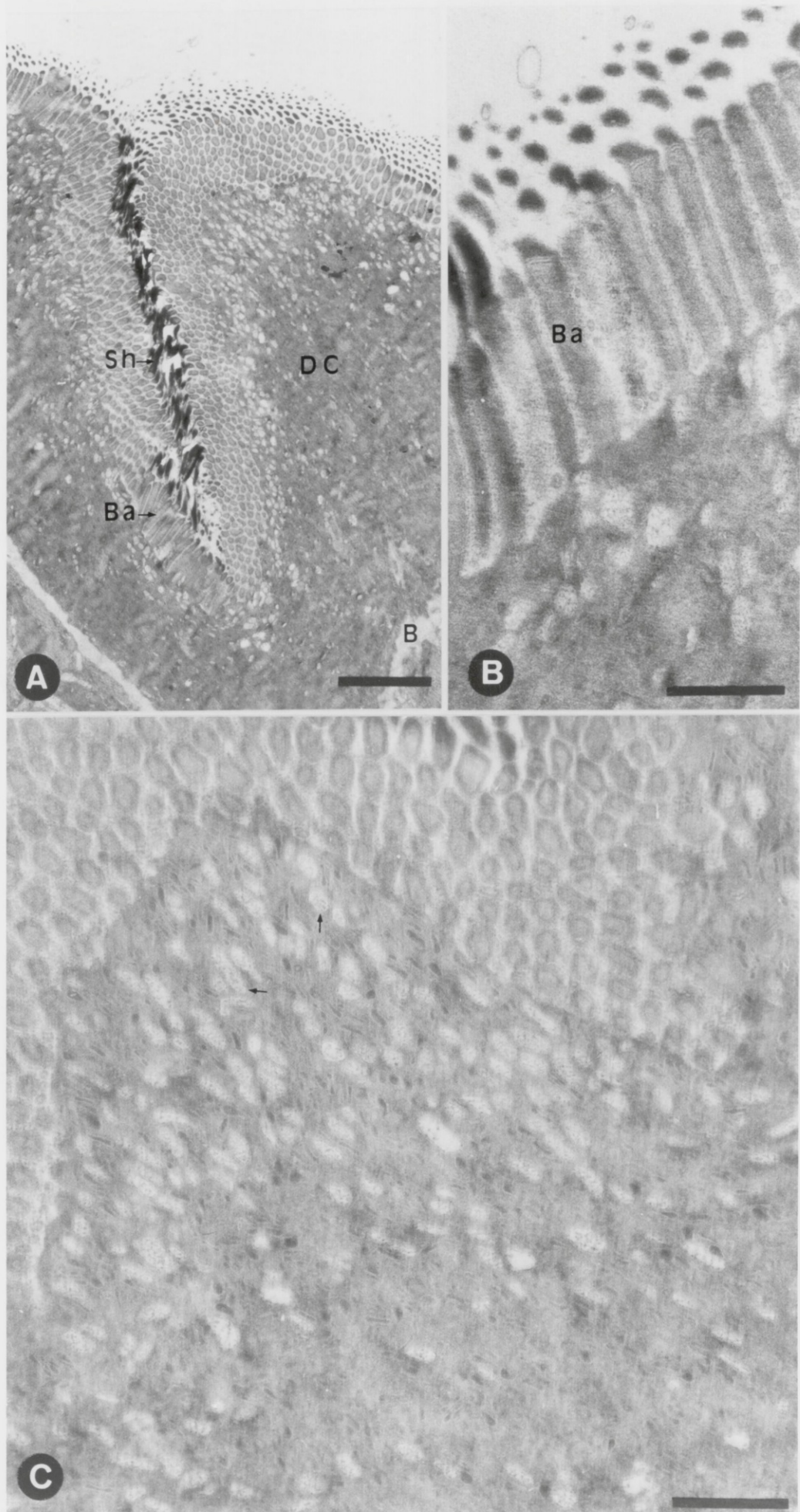
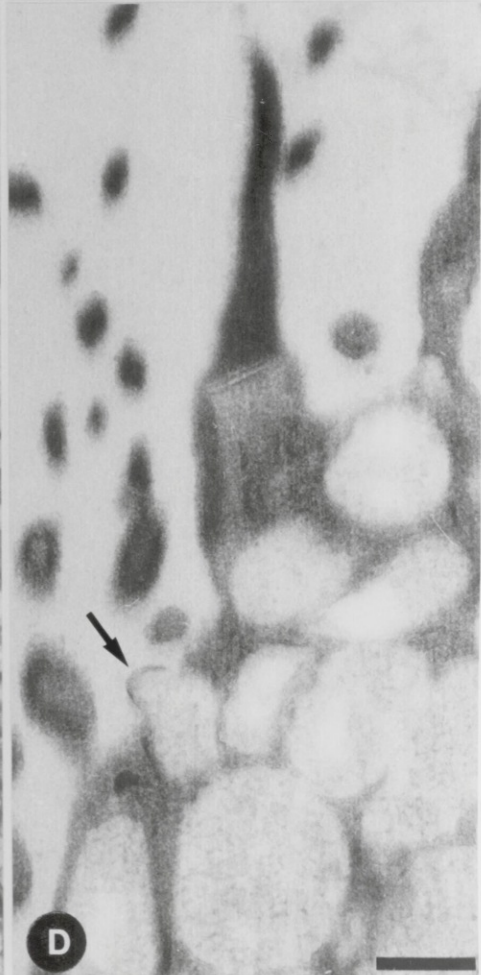
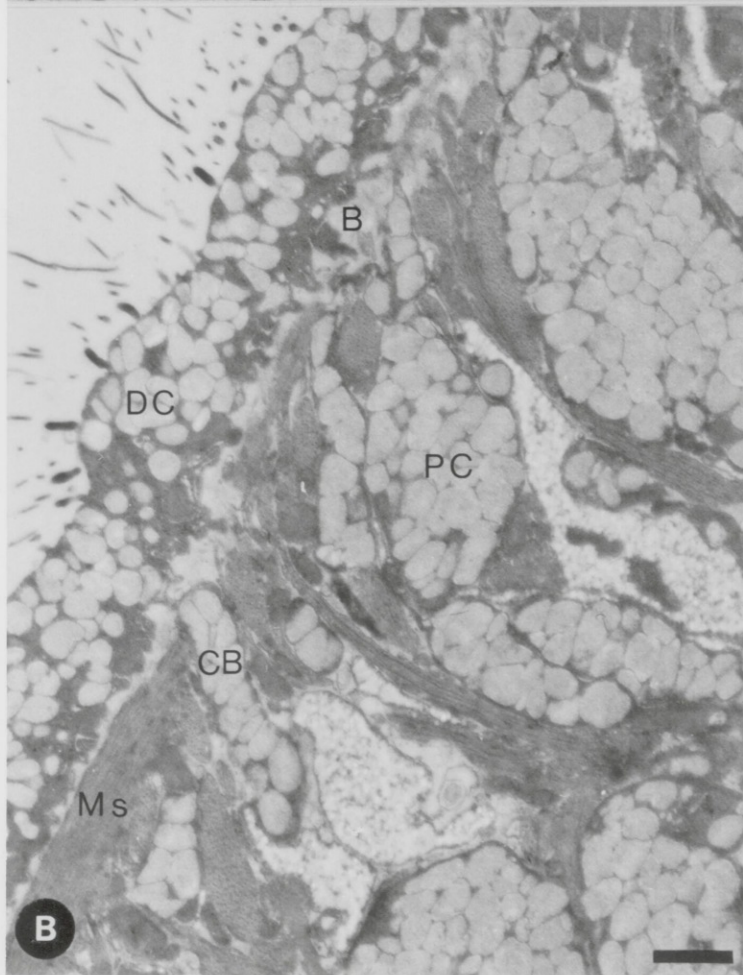
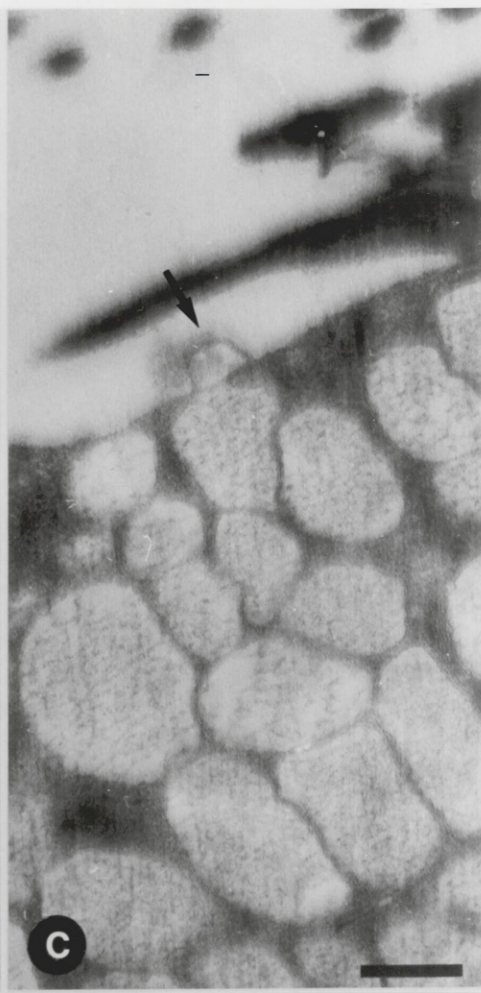
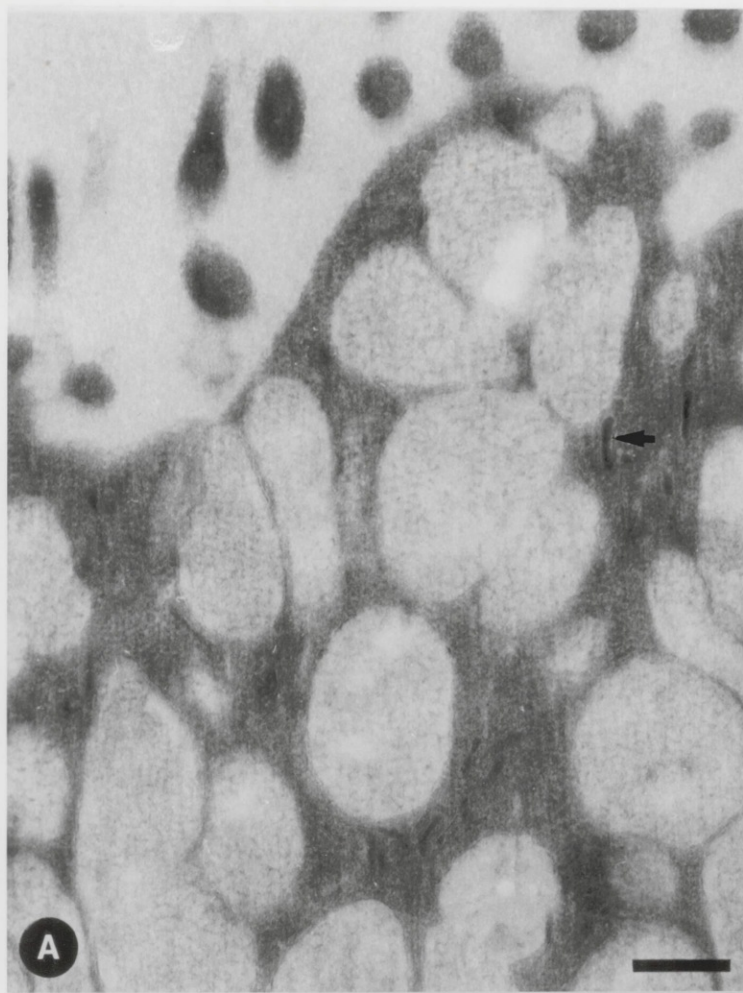


Plate 5-7 Transmission electron microscopy of the apical scolex
of the adult M. corti from dogs, 17 days post infection.

5-7A : The large, flocculent, membrane-bound vesicles
and the electron-dense disc-shaped bodies (arrow)
were observed in the distal cytoplasm of tegument.
Bar = 0.2 μ m.

5-7B : Both the distal (DC) and the perinuclear cytoplasm
(PC) of the syncytial tegument were vesiculated.
Bar = 1 μ m.

5-7C & 5-7D : Note the apparent fusion between the vesicle
membrane and the tegument plasmalemma.
Bar = 0.2 μ m.



CHAPTER 6

SUMMARY AND GENERAL DISCUSSION

6.1 In vitro cultivation

Tetrathyridia of Mesocestoides corti, maintained in the laboratory mice by intraperitoneal infection, were used for in vitro culture. It has been found that some 'conditioning' of tetrathyridia before cultivation appears to be necessary for strobilar differentiation. This conditioning can be provided, at least to some extent, by incubating the stock specimens in Hanks' saline in a Universal container at 38°C overnight before inoculation. Further studies to develop precisely a suitable pretreatment method is clearly necessary. It is emphasised that evagination of scoleces is not a satisfactory indicator that segmentation and sexual differentiation will occur.

25 or 100 tetrathyridia were introduced into a Leighton tube (19 x 105 mm) containing 10 ml of monophasic medium S10E. A diphasic medium with coagulated serum base was as effective as a monophasic medium for segmentation, providing the organisms did not actively multiply in the

former. The consistency of the coagulated serum bases was not uniform. Asexual multiplication occurred only in some diphasic cultures containing a suitable serum base into which tetrathyridia could migrate. It was demonstrated that the greater the number of organisms present in the serum base, the higher was the number of worms produced in culture. In those diphasic cultures which contained very actively multiplying tetrathyridia, the segmentation rates were very low.

A gas phase of 5% CO₂ /95% N₂ combined with a pH higher than 7.4 was found to provide the most suitable conditions for segmentation and sexual differentiation. Under the most favourable conditions, tetrathyridia began segmenting in vitro within one week and sexually mature proglottids appeared within 5-6 days of segmentation. These are compatible with the in vivo rate of development in the dog.

Regarding the most appropriate gas phase for strobilar differentiation in vitro, it has been shown that anaerobic conditions were more satisfactory than aerobic conditions for the development of M. corti in vitro. The question of the availability of oxygen in the vertebrate intestine has been a matter of controversy. It has also been demonstrated that the intestinal oxygen tension (and the pH & gastrointestinal function) can be significantly

affected by the presence of H. diminuta (Podesta and Mettrick, 1974a). In the rat normal intestine, the aqueous phase of the luminal contents has a pO_2 of 40-50 mmHg; in the solid particulate phase (distal ileum and colon), anoxic conditions are likely to prevail in the central lumen. However, in the parasitized intestine fluid absorption is reduced; the luminal contents are well mixed by intestinal motility and anoxic conditions do not occur. Notwithstanding, the parasitized intestine does appear to provide sufficient oxygen for aerobic metabolism. It is, however, well recognized that some helminths (e.g. Schistosoma) have a predominantly anaerobic metabolism even when living in an aerobic environment (Smyth and Halton, 1983). Little is known regarding the metabolic pathways followed by M. corti and this is clearly an area requiring further investigation.

A pH higher than 7.4 appeared more suitable for the segmentation of M. corti tetrathyridia in vitro. In contrast, Podesta & Mettrick (1974b) found that lowering the pH of luminal fluids increases both fluid and glucose transport by H. diminuta. According to these authors, the rate of glucose absorption by H. diminuta under acidic conditions is double that under alkaline pH. In the early stage (first two weeks) of infection of M. corti, the luminal pH might not be lowered by the small number of tetrathyridia, which became successfully established in

the dog intestine and which start segmenting during this period. However, when the population of tetrathyridia and adult M. corti increases remarkably (after 17 days in the dog) by asexual multiplication, the infected gut is likely to become acidified by the parasites. The maturation of proglottids appear to benefit from the acidic environment, but further segmentation is retarded. This hypothesis reconciles the developmental sequence of adult worms in the dog. The proglottids increased in number until 14 days p.i. then started decreasing. At 17 days p.i., most adult worms were gravid. After 42 and 55 days in the dog, the adult M. corti were very short and comprised only a few proglottids.

Although sexually mature adults were grown consistently from undifferentiated tetrathyridia in vitro, fully developed oncospheres were only obtained sporadically. It is apparent that insemination in vitro cannot be compared with that in vivo, in spite of the fact that self-copulation was sometimes observed. However, a sufficient amount of fertilized eggs seems to be necessary for building up a protective egg mass, because a protective shell is absent from M. corti eggs. Failure to form an egg mass might be the reason why the developing oncospheres could not normally develop in vitro and later degenerated in the paruterine organ. In accordance, it is interesting to note that all the egg-producing cestodes

cultured in vitro belong to the category of which the eggs possess a protective shell or capsule, for example Schistocephalus solidus (Smyth, 1954), Spirometra mansonoides (Berntzen and Mueller, 1972), Hymenolepis nana (Berntzen, 1962; Sinha and Hopkins, 1967), H. diminuta (Schiller, 1965; Berntzen, 1966; Graham and Berntzen, 1970) and H. microstoma (Evans, 1970; Seidel, 1975). Through the protection of the egg shell, the amount of fertilized eggs should have little effect on the maturation of individual eggs in these species and, therefore, fully mature eggs are easily obtained in vitro. On the other hand, it is difficult to rule out the possibility that specific nutritional requirements or other physiological factors may be responsible for the low rate of egg production of M. corti cultured in vitro.

6.2 The tegument

The body surface of the invaginated tetrathyridia, which were obtained from the abdominal cavity of experimental mice, were covered with short filamentous type I microtriches. The elongated proximal base of this microthrix is considered to be the main absorptive zone of the tetrathyridial tegument. Similar microtriches on the bladder wall of the cysticercus of Taenia crassiceps were reported by Baron (1968). It should be mentioned,

however, that the tetrathyridial body into which the scolex invaginates is not cystic, but is parenchymatous.

The scolex of the tetrathyridia at various developmental stages in vitro and adult M. corti in vivo were described ultrastructurally. No major variation was observed, under scanning electron microscope, during the larval-adult transformation. The long, filamentous type II microtriches predominated on the apical scolex and the lining of suckers. The blade-like microtriches, which possibly play an important role in maintaining position, were present on the sucker rims and the surface adjacent to the suckers. These observations do not appear to be restricted to M. corti. Table 5-1 indicates that these microtriches are probably of universal occurrence on the scolex of cyclophyllidean and pseudophyllidean cestodes.

Using transmission electron microscopy, the large, membrane-bound, electron-translucent vesicles were first observed in the tegument ('scolex-neck' region) of the pre-segmentation stage and persisted in that of the apical scolex of the adult worms both in vitro and in vivo. The occurrence of these vesicles coincides with the onset of the rapid somatic growth after the pre-segmentation stage. Moreover, apparent fusion between the vesicle membrane and the tegument plasmalemma was sometimes observed. It is

proposed that these vesicles contribute to the amplification of the surface by incorporating the vesicle membrane into the tegumental plasmalemma. According to this explanation, it would be logical to assume that the flocculent contents of these vesicles are involved in the formation of surface glycocalyx. Preliminary ultrastructural cytochemical studies, using the periodic acid-thiocarbohydrazide-osmium (PATCO) technique (Threadgold, 1976), also indicated that these vesicles were carbohydrate-rich. In spite of the fact that the ultrastructural studies do not clarify the origin of these large, membrane-bound vesicles, it is speculated that they are derived from the Golgi apparatus in the perinuclear cytoplasm of the tegumental cells. Oaks & Lumsden (1971) presented cytochemical evidence for the localization of carbohydrate-containing material, which they related to the formation of the glycocalyx, within the Golgi cisternae and vesicles in the perikarya and within vesicles concentrated beneath the brush border in the distal tegument cytoplasm of Lacistorhynchus tenuis and Hymenolepis diminuta. With autoradiography studies, Bennett, Leblond & Haddad (1974) have shown that in rat intestinal epithelia the Golgi apparatus is the site of completion of synthesis of glycoproteins which later migrate to the cell surface by fusion of Golgi-formed vesicles. In other words, the vesicle membrane provides the components of plasma membranes and the glycoprotein

contents contribute to the cell coats. Although the trigger for strobilisation is still unclear, it is very likely that whatever initiates strobilisation coincidentally stimulates the synthetic activity of the tegumental cells in the apical scolex of M. corti.

The vesiculated nature of the scolex tegument has been reported from some cestodes. The large (300-400 nm), electron-dense, membrane-bound, ovoid granules are unique to the rostellar tegument of H. diminuta (Specian and Lumsden, 1980). According to these authors, they are apparently produced by cells located principally in the rostellum, but are not limited to the part of the surface syncytium overlying the rostellum. These ovoid granules occupy the distal cytoplasm of the scolex tegument to a point about half way to the suckers. Concomitant with an increased fuchsinophilia, the ovoid granules become more numerous, appearing first at 3-4 days post-infection. The granules persist despite the loss of fuchsinophilia by 35 days p.i. Histochemical and cytochemical studies (Specian and Lumsden, 1981) reveal that these granules contain protein and neutral carbohydrate-rich material, and they are apparently elaborated from the cyton Golgi regions. However, their function has not been ascertained. Earlier, Rothman (1963) also found that the scolex tegument of H. diminuta was composed of closely packed dense granules which do not resemble any structure seen in

the strobilar tegument; the possibility that the tegument of the worm may arise from the molecular constituents of the scolex tegumental granules has been considered. In the ultrastructural studies of the tegument of Taenia hydatigena, Featherston (1972) pointed out that the distal cytoplasm on the top of the rostellum was extensively vacuolated; the same was true in the neck region of worms up to 7 days old. In worms older than 7 days, the neck region tends to be compact and resembles the typical cytoplasm. The vacuoles found in the 'scolex-neck' region are described, by this investigator, as relatively large, lacking a definite limiting membrane, and in a majority of cases containing no electron dense particles. Due to the low magnification of the pictures shown in the published paper, the absence of limiting membrane requires further confirmation. The rostellar tegument of developing Raillietina cesticillus is also densely vesiculated, according to Blitz & Smyth (1973). These vesicles have a flocculent content and measure 300-500 nm. Although these authors speculated that they might be pinocytotic vesicles, there is no direct evidence for this. It is notable, however, that the contents of these large vesicles are variably electron-dense, translucent, lucid or flocculent in different species. Whether this variability is a result of technical or physiological differences remains obscure.

The high synthetic activity of the tegumental cells in the apical scolex of M. corti is reminiscent of a group of modified tegumental cells, previously referred to as the rostellar gland (Smyth, 1964), in the apical rostellum of Echinococcus granulosus (Thompson, Dunsmore and Hayton, 1979). According to these authors, their secretory product was paraldehyde-fuchsin (PAF) negative and did not contain carbohydrate; it was thought to be a lipoprotein. The cells only became active as the worm reached maturity (30-35 days post infection). Possible functional activities of the secretion were postulated to be associated with hook formation, nutrition, regulation, adhesion or protection. Because the time at which these cells become active is different from that of the tegumental cells in the apical scolex of M. corti, the latter may have a different role.

Small blade-like microtriches uniformly occurred on the strobilar tegument of M. corti. The absorptive function of the adult tegument is possibly related to the elongated, cylindrical base of this microthrix. The deep folds which appeared frequently on the strobilar tegument might further increase the surface area for absorption. The small blade-like shafts kept the two absorptive sides of a deep fold apart, and a free flow of nutrients was supposedly allowed in the fold. This type of microthrix

has been observed on the strobilar tegument of H. diminuta (Rothman, 1963), D. latum (Bråten, 1968b), Diphyllbothrium erinacei (Yamane, 1968), Diplogonoporus grandis (Yamane, 1969), T. hydatigena (Featherston, 1972) and Diphyllbothrium spp. (Andersen, 1975). The small blade-like microtriches, with elongated proximal base, may be regarded as the general pattern of the microtriches on the strobilar tegument of cyclophyllidean and psedophyllidean cestodes.

In general, the present observations do not detract from the hypothesis of tegumental functions put forward by previous workers. The occurrence of microtriches, as the microvilli of the surface of intestinal epithelia, increases the surface area or amount of membrane for absorption (Lee, 1966; Bråten, 1968b; Taylor and Thomas, 1968; Jha and Smyth, 1969; Smyth, 1972; Lee, 1972; Lumsden, 1972, 1975; Read, 1973; Threadgold and Dunn, 1983). The electron-dense shafts at the distal end of microtriches possibly increase friction between parasite and the host, and, therefore, play an important role in maintaining position or the migration of parasites in the host (Rothman, 1963; Lee, 1966; Berger and Mettrick, 1971; Hess and Guggenheim, 1977; Thompson et al. 1980). Lee (1966) and Thompson et al. (1980, 1982a) also suggested that the electron-dense shafts might keep the absorptive surfaces of parasite and host slightly apart so that

nutrients could flow between them.

The cultivation of M. corti from oncosphere to tetrathyridium has been completed in vitro by Voge & Seidel (1968). Although all the optimum conditions for the various phases have not been precisely defined, the techniques in this study have been sufficiently successful to produce sexually mature strobilate adults consistently from undifferentiated tetrathyridia in vitro. Once fully developed oncospheres can be obtained and hatched in quantity from the in vitro cultured adults, it should be possible to maintain the whole life cycle of M. corti in the laboratory.

REFERENCES

- Andersen, K. (1975). Comparison of surface topography of three species of Diphyllbothrium (Cestoda, Pseudophyllidea) by scanning electron microscopy. Int. J. Parasit., 5 : 293-300.
- Antipin, D.N. (1946). [Treatment of Mesocestoides infestation in the raccoon-dog.] Collected papers on helminthology dedicated by his pupils to K.I. Skryabin in his 40th year of scientific, educational and administrative achievement. pp.34-36.
- Babaev, YA. (1970). [Helminth fauna of certain species of Gerbillinae in Turkmenia.] In : Academician K.I. Skryabin and the development of helminthological science in Turkmenia. (Tashliev, A.O. and Shagalina, L.M., eds.) pp. 92-104. Ashkhabad:Izdat."ILIM"
- Babero, B.B. (1957). Some helminths from Illinois opossums. J. Parasit., 43 : 232.
- Baer, J.G. (1933). "Contribution à l'étude de la Faune helminthologique africaine." Revue Suisse de Zoologie, XL(1) : 31-84.
- Baer, J.G. (1971). Animal parasites. George Weidenfeld and Nicolson Ltd., London.
- Banerjee, K.N., Das Gupta, C.K., Ray, S. and Maulick, K.K. (1974). Incidence of Mesocestoides tenuis Meggitt,

- 1931 in a pet dog. Indian J. of Animal Health, 13 : 179.
- Baron, P.J. (1968). On the histology and ultrastructure of Cysticercus longicollis, the cysticercus of Taenia crassiceps Zeder, 1800, (Cestoda; Cyclophyllidea). Parasitology, 58 : 497-513.
- Barrett, N.J., Smyth, J.D. and Ong, S.J. (1982). Spontaneous sexual differentiation of Mesocestoides corti tetrathyridia in vitro. Int. J. Parasit., 12 : 315-322.
- Barsanti, J.A., Jones, B.D., Bailey, W.S. and Knipling, G.D. (1979). Diagnosis and treatment of peritonitis caused by a larval cestode Mesocestoides spp. in a dog. Cornell Veterinarian, 69 : 45-53.
- Bennett, G., Leblond, C.P. and Haddad, A. (1974). Migration of glycoprotein from the Golgi apparatus to the surface of various cell types as shown by radioautography after labeled fucose injection into rats. J. Cell Biol., 60 : 258-284.
- Berger, J. and Mettrick, D.F. (1971). Microtrichial polymorphism among hymenolepid tapeworms as seen by scanning electron microscopy. Trans. Am. Microsc. Soc., 90 : 393-403.
- Berntzen, A.K. (1961). The in vitro cultivation of tapeworms. I. Growth of Hymenolepis diminuta (Cestoda : Cyclophyllidea). J. Parasit., 47 : 351-355.
- Berntzen, A.K. (1962). In vitro cultivation of tapeworms.

- II. Growth and maintenance of Hymenolepis nana (Cestoda : Cyclophyllidea). J. Parasit., 48 : 785-797.
- Berntzen, A.K. (1966). A controlled culture environment for axenic growth of parasites. Ann. N. Y. Acad. Sci., 139 : 176-189.
- Berntzen, A.K. and Mueller, J.F. (1964). In vitro cultivation of Spirometra mansonoides (Cestoda) from the proceroid to the early adult. J. Parasit., 50 : 705-711.
- Berntzen, A.K. and Mueller, J.F. (1972). In vitro cultivation of Spirometra spp. (Cestoda) from the plerocercoid to the gravid adult. J. Parasit., 58 : 750-752.
- Bjotvedt, G., Tomkiewicz, S.M.,JR. and Golightly, R.T.,JR. (1980). A description of helminths in a kit fox (Vulpes macrotis). Veterinary Medicine and small Animal Clinician, 75 : 881-884.
- Blitz, N.M. and Smyth, J.D. (1973). Tegumental ultrastructure of Raillietina cesticillus during the larval-adult transformation, with emphasis on the rostellum. Int. J. Parasit., 3 : 561-570.
- von Brand, T. (1973). Biochemistry of parasites. 2nd ed. Academic Press, New York & London.
- Bråten, T. (1968a). An electron microscope study of the tegument and associated structures of the proceroid of Diphyllbothrium latum (L.). Z. Parasitenk., 30 :

95-103.

Bråten, T. (1968b). The fine structure of the tegument of Diphyllbothrium latum (L.) : A comparison of the plerocercoid and adult stages. Z. Parasitenk., 30 : 104-112.

Buck, S.C., Pester, F.R.N. and Buckley, J.J.C. (1959). 'Tetrathyridium' from genet cat, Kenya. [Demonstration] Trans. R. Soc. Trop. Med. Hyg., 53 : 304.

Burt, M.D.B., Pike, A.W. and Corbett, L.K. (1980). Helminth parasites of wild cats in north-east Scotland. J. Helminth., 54 : 303-308.

Burton, P.R. (1964). The ultrastructure of the integument of the frog lung-fluke, Haematoloechus medioplexus (Trematoda : Plagiorchiidae). J. Morphology, 115 : 305-318.

Butler, J.M. and Grundmann, A.W. (1954). The intestinal helminths of the coyote, Canis latrans, in Utah. J. Parasit., 40 : 440-443.

Byrd, E.E. and Ward, J.A. (1942). Segmental anatomy of an opossum cestode, Mesococcestoides [Mesocestoides] sp. J. of the Tennessee Academy of Science, 17 : 341-342.

Byrd, E.E. and Ward, J.W. (1943). Observations on the segmental anatomy of the tapeworm, Mesocestoides variabilis Mueller, 1928, from the opossum. J. Parasit., 29 : 217-226.

Carleton, H.M. (1938). Histological technique. Oxford

University Press.

- Chandler, A.C. (1942a). First record of a case of human infection with tapeworms of the genus Mesocestoides. Am. J. Trop. Med., 22 : 493-496.
- Chandler, A.C. (1942b). Mesocestoides manteri n.sp. from a lynx, with notes on other North American species of Mesocestoides. J. Parasit., 28 : 227-231.
- Chandler, A.C. (1944). A new species of Mesocestoides, M. kirbyi, from Canis latrans. J. Parasit., 30 : 273.
- Chertkova, A.N., Il'yasov, I.N. and Yusupov, M.Yu. (1975). [Materials for the study of Mesocestoididae cestodes.] Materialy Nauchnykh Konferentsii Vsesoyuznogo Obshchestva Gel'mentologov, 27 : 176-180.
- Chertkova, A.N. and Kosupko, G.A. (1975). [Cestodes of the genus Mesocestoides found in domestic and wild animals in USSR and some principles of their systematics.] Trudy Vsesoyuznogo Instituta Gel'mintologii im. K.I. Skryabina (Teoreticheskie Problemy Veterinarnoi Gel'mintologii), 22 : 193-211.
- Chertkova, A.N. and Kosupko, G.A. (1977). [Revision of the suborder Mesocestoidata Skrjabin, 1940] Trudy Vseso. Inst. Gel'mint. im. K.I. Skry. (Prob. Veter. Gel'mint.), 23 : 141-153.
- Choi, W.Y., Kim, B.C. and Choi, H.S. (1967). The first case of human infection with tapeworms of the genus Mesocestoides in Korea. Korean J. Parasit., 5 : 60-64.

- Choquette, L.P.E. (1951). "Helminthes parasites trouvés chez la mouffette Mephitis mephitis dans la province de Quebec." Can. J. Comp. Med., 15 : 62-64.
- Choquette, L.P.E., Gibson, G.G., Kuyt, E. and Pearson, A.M. (1973). Helminths of wolves, Canis lupus L., in the Yukon and Northwest territories. Can. J. Zool., 51 : 1081-1091.
- Ciordia, H. (1955). Mesocetoides jonesi, n.sp., from the gray fox, with descriptions of the chromosome complement and a dicephalic specimen. J. Tennessee Acad. Sci., 30 : 57-63.
- Clegg, J.A. and Smyth, J.D. (1968). Growth, development, and culture methods : parasitic platyhelminths. In : Chemical zoolgy vol. II (Florkin, M. and Scheer, B.T. eds.) pp. 395-462. Academic Press, New York and London.
- Coil, W.H. (1975). The histochemistry and fine structure of the embryophore of Shipleya inermis (Cestoda). Z. Parasitenk., 48 : 9-14.
- Conn, D.B., Etges, F.J. and Sidner, R.A. (1983). Personal communication.
- Douglas, J.R. (1951). New parasite records from California dogs. Cornell Veterinarian, 41 : 342-346.
- Dyer, W.G. (1971). Some helminths of the six-lined lizard, Cnemidophorus sexlineatus, in South Dakota. Proc. Helminth. Soc. Wash., 38 : 256.

- Eckert, J., von Brand, T. and Voge, M. (1969). Asexual multiplication of Mesocestoides corti (Cestoda) in the intestine of dogs and skunks. J. Parasit., 55 : 241-249.
- Esch, G.W. and Smyth, J.D. (1976). Studies on the in vitro culture of Taenia crassiceps. Int. J. Parasit., 6 : 143-149.
- Evans, W.S. (1970). The in vitro cultivation of Hymenolepis microstoma from cysticeroid to egg-producing adult. Can. J. Zool., 48 : 1135-1137.
- Ezzat, M.A.E. and Gaafar, S.M. (1951). Tetrathyridium sp. in a Syke's monkey (Cercopithecus albigularis) from Giza Zoological Gardens, Egypt. J. Parasit., 37 : 392-394.
- Fagasinski, A. and Piusinski, W. (1961). "Inwazja Tetrathyridium elongatum (Blumberg, 1882) u psa." Wiadomosci Parazytologiczne, 6 : 533-536.
- Fain, A. and Herin, V. (1954). "Notes à propos d'un cas d'infestation humaine par un Mesocestoïde à Astrida (Ruanda-Urundi). Présence de ce cestode chez le chat et découverte de larves du type Tetrathyridium chez divers vertébrés." Annales de la Société Belge de Médecine Tropicale, 34 : 893-900.
- Fairweather, I. and Threadgold, L. T. (1981). Hymenolepis nana : The fine structure of the embryonic envelopes. Parasitology, 82 : 429-443.
- Featherston, D.W. (1971). Taenia hydatigena. II.

- Evagination of cysticerci and establishment in dogs.
Exptl Parasit., 29 : 242-249.
- Featherston, D.W. (1972). Taenia hydatigena. IV.
Ultrastructure study of the tegument. Z. Parasitenk.,
38 : 214-232.
- Freeman, R.S. (1973). Ontogeny of cestodes and its
bearing on their phylogeny and systematics. Adv.
Parasit., 11 : 481-557.
- Gleason, N.N. and Healy, G.R. (1967). Report of a case of
Mesocestoides (Cestoda) in a child in Missouri. J.
Parasit., 53 : 83-84.
- Gleason, N.N., Kornblum, R. and Walzer, P. (1973).
Mesocestoides (Cestoda) in a child in New Jersey
treated with niclosamide (Yomesan). Am. J. Trop. Med.
Hyg., 22 : 757-760.
- Graham, J.J. and Berntzen, A.K. (1970). The monoxenic
cultivation of Hymenolepis diminuta cysticercoids with
rat fibroblasts. J. Parasit., 56 : 1184-1188.
- Greve, J.H., Hanson, R.L. and McGill, L.D. (1979).
Treatment of parasitic ascites in a dog. J. Am. Vet.
Med. Asso., 174 : 828-829.
- Grundmann, A.W. (1956). A new tapeworm, Mesocestoides
carnivoricolus, from carnivores of the Great Salt Lake
Desert region of Utah. Proc. Helminth. Soc. Wash., 23
: 26-28.
- Gutierrez, Y., Buchino, J.J. and Schubert, W.K. (1978).
Mesocestoides (Cestoda) infection in children in the

- United States. J. Pediatrics, 93 : 245-247.
- Hart, J.L. (1967). Studies on the nervous system of tetrathyridia (Cestoda : Mesocestoides). J. Parasit., 53 : 1032-1039.
- Hayunga, E.G. and Mackiewicz, J.S. (1975). An electron microscope study of the tegument of Hunterella nodulosa Mackiewicz and McCrae, 1962 (Cestoidea : Caryophyllidea). Int. J. Parasit., 5 : 309-319.
- Hess, E. (1972). "Contribution a la biologie larvaire de Mesocestoides corti Hoeppli, 1925 (Cestoda, Cyclophyllidea). Note preliminaire. Revue Suisse de Zoologie, 79 : 1031-1037.
- Hess, E. (1980). Ultrastructural study of the tetrathyridium of Mesocestoides corti Hoeppli, 1925 : tegument and parenchyma. Z. Parasitenk., 61 : 135-159.
- Hess, E. and Guggenheim, R. (1977). A study of the microtriches and sensory processes of the tetrathyridium of Mesocestoides corti Hoeppli, 1925, by transmission and scanning electron microscopy. Z. Parasitenk., 53 : 189-199.
- Hoeppli, R.J.C. (1925). Mesocestoides corti, a new species of cestode from the mouse. J. Parasit., 12 : 91-96.
- Honess, R.F. (1962). A note on Mesocestoides lineatus (Goeze, 1782) from dogs in Colorado. Proc. Helminth. Soc. Wash., 29 : 147.

- Hopkins, C.A. and Law, L.M. (1978). Schistocephalus solidus : pinocytosis by the plerocercoid tegument. Exptl Parasit., 44 : 161-172.
- Hörning, B. and Tenora, F. (1971). [The present state of knowledge of cestodes of marmots (genus Marmota).] Věst. Čsl. Spol. Zool., 35 : 103-106.
- Hutchison, W.F. and Martin, J.B. (1980). Mesocestoides (Cestoda) in a child in Mississippi treated with paromomycin sulfate (Humatin). Am. J. Trop. Med. Hyg., 29 : 478-479.
- Ingram, W.M. (1941). The helminth fauna of a raccoon. J. Parasit., 27 : 539-540.
- Ito, J., Honda, J. and Ishiguro, M. (1962). [The second record of a case of human infection with Mesocestoides lineatus in Japan (Cestoda).] Jap. J. Parasit., 11 : 71-75.
- James, H.A. (1968). Studies on the genus Mesocestoides (Cestoda : Cyclophyllidea). PhD Dissertation. Iowa State Univ., Ames, Iowa.
- James, H.A. and Ulmer, M.J. (1967),. New amphibian host records for Mesocestoides sp. (Cestoda : Cyclophyllidea). J. Parasit., 53 : 59.
- Jensen, L.A., Schmidt, G.D. and Kuntz, R.E. (1983). A survey of cestodes from Borneo, Palawan, and Taiwan, with special reference to three new species. Proc. Helminth. Soc. Wash., 50 : 117-134.
- Jha, R.K. and Smyth, J.D. (1969). Echinococcus granulosus

- : ultrastructure of microtriches. Exptl Parasit., 25 : 232-244.
- Jha, R.K. and Smyth, J.D. (1971). Ultrastructure of the rostellar tegument of Echinococcus granulosus with special reference to biogenesis of mitochondria. Int. J. Parasit., 1 : 169-177.
- Juminer, B. and Durand, M. (1960). "Polyparasitisme grave chez un chien tunisien." Archives de l'Institut Pasteur de Tunis, 37 : 391-394.
- Kagei, N., Kihata, M., Shimizu, S., Urabe, M. and Ishii, A. (1974). [The 10th case of human infection with Mesocestoides lineatus (Cestoda : Cyclophyllidea) in Japan.] Jap. J. Parasit., 23 : 376-382.
- Kaliner, G. (1972). [Occurrence and differential diagnosis of tetrathyridiasis in the domestic fowl.] Zentralblatt für Veterinärmedizin B, 19 : 136-143.
- Kaliner, G. (1974). Bronchitis caused by tetrathyridia (Mesocestoididae, Cestoda) in chickens. Avian Diseases, 18 : 255-257.
- Kamegai, S., Ichihara, A., Nonobe, H. and Machida, M. (1967). The 6th and 7th records of human infection with Mesocestoides lineatus (Cestoda) in Japan. Reo. Bull. Meguro. Parasit. Mus., 1 : 1-7.
- Khan, Z.I. and DeRycke, P.H. (1976). Studies on Hymenolepis microstoma in vitro. II Effect of yeast extract on development and maturation. Z.

- Parasitenk., 50 : 73-79.
- Köhler, P. and Hanselmann, K. (1974). Anaerobic and aerobic energy metabolism in the larvae (tetrathyridia) of Mesocestoides corti. Exptl Parasit., 36 : 178-188.
- Kugi, G. (1969). [A canine case of Mesocestoides sp.] Jap. J. Parasit., 18 : 455-458.
- Kugi, G. (1971). [The tetrathyridium larva of Mesocestoides sp. from the Japanese pheasant, Phasianus (Colchius) versicolor.] Jap. J. Parasit., 20 : 139-141.
- Kugi, G. (1973). [Studies on life-history of Mesocestoides spp. II. Tetrathyridium of Mesocestoides litteratus from the green pheasant and the copper pheasant.] J. Vet. Med., Tokyo, 586 : 265-267.
- Kugi, G. (1977). [Mesocestoides paucitesticulus from the wild fox, Vulpes vulpes japonica in Japan.] Jap. J. Parasit., 26 : 25-27.
- Kwa, B.H. (1972). Studies on the sparganum of Spirometra erinacei. III. The fine structure of the tegument in the scolex. Int. J. Parasit., 2 : 35-43.
- Lee, D.L. (1966). The structure and composition of the helminth cuticle. Adv. Parasit., 4 : 187-254.
- Lee, D.L. (1972). The structure of the helminth cuticle. Adv. Parasit., 10 : 347-379.
- Lethbridge, R.C. (1980). The biology of the oncosphere of cyclophyllidean cestodes. Helminth. Abstracts - Ser.

A, 49 : 59-72.

Little, J.W. (1966). The occurrence of Oschmarenia (Chandleria) wallacei (Cestoda : Anoplocephalidae) in central Mexico. Am. Midl. Nat., 76 : 533.

Loos-Frank, B. (1980a). "Mesocestoides leptothylacus n. sp. und das nomenklatorische Problem in der Gattung Mesocestoides vaillant, 1863 (Cestoda, Mesocestoididae)." Tropenmed. Parasit., 31 : 2-14.

Loos-Frank, B. (1980b). The common vole, Microtus arvalis Pall. as intermediate host of Mesocestoides (Cestoda) in Germany. Z. Parasitenk., 63 : 129-136.

Lumsden, R. D. (1972). Cytological studies on the absorptive surfaces of cestodes. VI. Cytochemical evaluation of electrostatic charge. J. Paraist., 58 : 229-234.

Lumsden, R.D. (1975). Surface ultrastructure and cytochemistry of parasitic helminths. Exptl Parasit., 37 : 267-339.

Lumsden, R.D., Oaks, J.A. and Mueller, J.F. (1974). Brush border development in the tegument of the tapeworm, Spirometra mansonoides. J. Parasit., 60 : 209-226.

Macchioni, G. (1967). [Infection in the hedgehog (Erinaceus europaeus L., 1758) by larvae of Mesocestoides lineatus (Goeze, 1782) Railliet, 1893. Identification of the larval forms by experimental infection of the dog; anatomo-histopathological study of the parasitic disease.] Annali della Facolta di

- Medicina Veterinaria, Pisa, 19 : 325-339.
- Mankau, S.K. and Widmer, E.A. (1977). Prevalence of Mesocestoides (Eucestoda : Mesocestoididea) tetrathyridia in southern California reptiles with notes on the pathology in the Crotalidae. Jap. J. Parasit., 26 : 256-259.
- Markowski, S. (1933). "Contribution à la connaissance du developpement de la larvæ Tetrathyridium variabile (Diesing, 1850)." Comptes Rendus Mensuels des Séances de l'Académie Polonaise des Sciences et des Lettres. (Classe de Sciences Mathématiques et Naturelles.), 5 : 5-6.
- Maskar, U. (1953). [Concerning the experimentally produced development of the dog tapeworm from the Dithyridium of hens and porcupine.] Schweiz. Arch. Tierheilk., 95 : 188-193.
- McManus, D.P. and Smyth, J.D. (1978). Differences in the chemical composition and carbohydrate metabolism of Echinococcus granulosus (horse and sheep strains) and E. multilocularis. Parasitology, 77 : 103-109.
- McManus, D.P. and Smyth, J.D. (1979). Isoelectric focusing of some enzymes from Echinococcus granulosus (horse and sheep strains) and E. multilocularis. Trans. R. Soc. Trop. Med. Hyg., 73 : 259-265.
- Meyer, M.C. and Chitwood, B.G. (1951). Helminths from fisher (Martes p. pennanti) in Maine. J. Parasit., 37 : 320-321.

- Morisita, T., Kobayashi, M., Goto, M., Eguchi, T., Moriyama, K. and Ohashi, M. (1964). [The third record of a case of human infection with Mesocestoides lineatus (Cestoda) in Japan.] Jap. J. Parasit., 13 : 101-104.
- Morisita, T., Nagase, K., Moriyama, K. and Matumoto, Y. (1975). [The 11th case of human infection with Mesocestoides lineatus in Japan.] Jap. J. Parasit., 24 : 353-356.
- Movsesyan, S.O., Chubarin, F.A. and Kurbert, A.V. (1978). [The biology of Mesocestoides lineatus (Goeze, 1782).] Vsesoyuznoe Obshchestvo Gel'mintologov [Scientific and applied problems in helminthology. 100th birthday anniversary of academician K.I. Skryabin (1878-1978).] pp. 68-71. Moscow : "Nauka".
- Novak, M. (1972). Quantitative studies on the growth and multiplication of tetrathyridia of Mesocestoides corti Hoeppli, 1925 (Cestoda : Cyclophyllidea) in rodents. Can. J. Zool., 50 : 1189-1196.
- Novak, M. and Dowsett, J.A. (1983). Scanning electron microscopy of the metacestode of Taenia crassiceps. Int. J. Parasit., 13 : 383-388.
- Oaks, J.A. and Lumsden, R.D. (1971). Cytological studies on the absorptive surface of cestodes. V. Incorporation of carbohydrate-containing macromolecules into tegument membranes. J. Parasit., 57 : 1256-1268.

- Ogren, R.E. (1956). Development and morphology of the oncosphere of Mesocestoides corti. A tapeworm of mammals. J. Parasit., 42 : 414-428.
- Orthoefer, J.G., Baker, N.F. and Kennedy, P.C. (1974). Peritonitis due to an intermediate stage of cestode in a dog with lymphosarcoma. J. Am. Vet. Med. Assn., 165 : 537-538.
- Osuna-Carrillo, A. and Mascaro-Lazcano, M.C. (1982). The in vitro cultivation of Taenia pisiformis to sexually mature adults. Z. Parasitenk., 67 : 67-71.
- Paul, J. (1970). Cell and tissue culture. 4th ed. E. & S. Livingstone, Edinburgh & London.
- Pereverzeva, E.V. (1972). [Helminth fauna of white foxes on Wrangel island.] In : Probelmy parazitologii. Trudy VII Nauchnoi Konferentsii Parazitologov USSR Part II. pp. 121-123. Kiev, USSR : Izdatel'stvo "Naukova Dumka".
- Podesta, R.B. and Mettrick, D.F. (1974a). Pathophysiology of cestode infections : Effect of Hymenolepis diminuta on oxygen tensions, pH and gastrointestinal function. Int. J. Parasit., 4 : 277-292.
- Podesta, R.B. and Mettrick, D.F. (1974b). Components of glucose transport in the host-parasite system, Hymenolepis diminuta (Cestoda) and the rat intestine. Can. J. Physiol. Pharmacol., 52 : 183-197.
- Pujatti, D. (1953). "Forme larvali di Mesocestoides

- lineatus Goeze in Pitymys multiplex Fatio." Doriana. Genoa. 1 (37) : 7 [Supplement to Annali del Museo Civico di Storia Naturale "G. Doria".]
- Read, C. (1973). Contact digestion in tapeworms. J. Parasit., 59 : 672-677.
- Reid, W.A. and Reardon, M.J. (1976). Mesocetoides in the baboon and its development in laboratory animals. J. Med. Primatology, 5 : 345-352.
- Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol., 17 : 208-212.
- Richards, K.S. and Arme, C. (1982). The microarchitecture of the structured bodies in the tegument of Caryophyllaeus laticeps (Caryophyllidea : Cestoda). J. Parasit., 68 : 425-432.
- Robinson, C.S., Luckey, H. and Mills, H. (1943). Factors affecting the hydrogen ion concentration of the contents of the small intestine. J. Biol. Chemistry, 147 : 175-181.
- Rothman, A.H. (1959). The physiology of tapeworms, correlated to structures seen with the electron microscope. J. Parasit., 46 : (Suppl.) 10.
- Rothman, A.H. (1963). Electron microscopic studies of tapeworms : The surface structures of Hymenolepis diminuta (Rud., 1819; Blanchard, 1891). Trans. Am. Microsc. Soc., 82 : 22-30.
- Rybicka, K. (1964). Gametogenesis and embryonic

- development in Dipylidium caninum. Exptl. Parasit., 15 : 293-313.
- Rybicka, K. (1966). Embryogenesis in cestodes. Adv. Parasit., 4 : 107-186.
- Rybicka, K. (1972). Ultrastructure of embryonic envelopes and their differentiation in Hymenolepis diminuta (Cestoda). J. Parasit., 58 : 849-863.
- Rybicka, K. (1973). Ultrastructure of macromeres in the cleavage of Hymenolepis diminuta (Cestoda). Trans. Am. Microsc. Soc., 92 : 241-255.
- Sadighian, A. (1969). Helminth parasites of stray dogs and jackals in Shahsavari area, Caspian region, Iran. J. Parasit., 52 : 372-374.
- Sadykhov, I.A. (1971). [New species of cestode, Mesocetoides petrowi n.sp., from the intestine of Vulpes vulpes.] Sbornik Rabot Po Gel'mintologii posvyashchen 90-letiyu so dnya rozhdeniya Akademika K.I. Skryabina. pp. 351-353. Moscow : Izdatel'stvo "KOLOS".
- Sawada, I. and Kugi, G. (1973). A new cestode, Mesocetoides paucitestisculus, from a badger Nyctereutex procyonoides, in Japan. Jap. J. Parasit., 22 : 45-47.
- Schiller, E.L. (1965). A simplified method for the in vitro cultivation of the rat tapeworm Hymenolepis diminuta. J. Parasit., 51 : 516-518.
- Schmidt, J.M. and Todd, K.S. (1978). Life cycle of

- Mesocestoides corti in the dog (Canis familiaris).
Am. J. Vet. Research, 39 : 1490-1493.
- Seidel, J.S. (1971). Hemin as a requirement in the development in vitro of Hymenolepis microstoma (Cestoda : Cyclophyllidea). J. Parasit., 57 : 566-570
- Seidel, J.S. (1975). The life cycle in vitro of Hymenolepis microstoma (Cestoda). J. Parasit., 61 : 677-681.
- Self, J.T. and McKnight, T.J. (1950). Platyhelminths from fur bearers in the Wichita Mountains Wildlife Refuge, with especial reference to Oochoristica spp." Am. Midl. Nat., 43 : 58-61.
- Shcherbak, M.M. and Sharpilo, V.P. (1966). [Data on the systematics, ecology, and parasitofauna of the Black Sea coast of Bulgaria.] In : [Ecology and histroy of the vertebrate fauna of the Ukraine.] pp. 160-168. Nauk. dumka : Kiev.
- Shults, L.M. (1970). Mesocestoides kirbyi and M. lineatus : Occurrence in Alaskan carnivores. Tran. Am. Microsc. Soc., 89 : 478-486.
- Silverman, P.H. (1965). In vitro cultivation procedures for parasitic helminths. Adv. Parasit., 3 : 159-222.
- Silverman, P.H. and Hansen, E.L. (1971). In vitro cultivation procedures for parasitic helminths : recent advances. Adv. Parasit., 9 : 227-258.
- Sinha, D.P. and Hopkins, C.A. (1967). In vitro cultivation of the tapeworm Hymenolepis nana from

- larva to adult. Nature, London, 215 : 1275-1276.
- Smyth, J.D. (1946). Studies on tapeworm physiology. I.
The cultivation of Schistocephalus solidus in vitro.
J. Exptl. Biol., 23 : 47-70.
- Smyth, J.D. (1950). Studies on tapeworm physiology. IV.
Further observations on the maturation of
Schistocephalus solidus under sterile conditions in vitro. J. Parasit., 36 : 371-381.
- Smyth, J.D. (1952). Studies on tapeworm physiology. VI.
Effect of temperature on the maturation in vitro of
Schistocephalus solidus. J. Exptl. Biol., 29 :
304-309.
- Smyth, J.D. (1954). Studies on tapeworm physiology. VII.
Fertilization of Schistocephalus solidus in vitro.
Exptl. Parasit., 3 : 64-71.
- Smyth, J.D. (1964). Observations on the scolex of
Echinococcus granulosus, with special reference to the
occurrence of secretory cells in the rostellum.
Parasitology, 54 : 515-526.
- Smyth, J.D. (1967). Studies on tapeworm physiology. XI.
In vitro cultivation of Echinococcus granulosus from
the protoscolex to the strobilate stage.
Parasitology, 57 : 111-133.
- Smyth, J.D. (1969a). Parasites as biological models.
Parasitology, 59 : 73-91.
- Smyth, J.D. (1969b). The physiology of cestodes. W.H.

- Freeman & Co., San Francisco; Oliver & Boyd, Edinburgh & London.
- Smyth, J.D. (1972). Changes in the digestive-absorptive surface of cestodes during larval/adult differentiation. In : Functional aspects of parasite surfaces. Symposia of the British Society for Parasitology, 10 : 4-70. (Taylor, A.E.R. and Muller, R. eds) Blackwell Scientific Publications, Oxford, London, Edinburgh & Melbourne.
- Smyth, J.D. (1976). Introduction to animal parasitology. Hodder & Stoughton, London, Sydney, Auckland & Toronto.
- Smyth, J.D. (1979). Echinococcus granulosus and Echinococcus multilocularis : In vitro culture of the strobilar stages from protoscoleces. Angewandte Parasitologie, 20 : 137-147.
- Smyth, J.D. and Barrett, N.J. (1979). Echinococcus multilocularis : Further observations on strobilar differentiation in vitro. Revista Iberica de Parasitologia, 39 : 39-53.
- Smyth, J.D. and Davies, C.J. (1979). In vitro differentiation of Echinococcus as a taxonomic tool, with special reference to its use in "strain" identification. Helminthologia (Bratisl), 16 : 5-12.
- Smyth, J.D. and Davies, Z. (1974a). Occurrence of physiological strains of Echinococcus granulosus

- demonstrated by in vitro culture of protoscoleces from sheep and horse hydatid cysts. Int. J. Parasit., 4 : 443-445.
- Smyth, J.D. and Davies, Z. (1974b). In vitro culture of the strobilar stage of Echinococcus granulosus (sheep strain) : A review of basic problems and results. Int. J. Parasit., 4 : 631-644.
- Smyth, J.D. and Davies, Z. (1975). In vitro suppression of segmentation in Echinococcus multilocularis with morphological transformation of protoscoleces into monozoic adults. Parasitology, 71 : 125-135.
- Smyth, J.D., Miller, H.J. and Howkins, A.B. (1967). Further analysis of the factors controlling strobilization, differentiation and maturation of Echinococcus granulosus in vitro. Exptl. Parasit., 21 : 31-41.
- Smyth, J.D. and Halton, D.W. (1983). The physiology of trematodes. 2nd ed. Cambridge University Press, Cambridge, London, New York, New Rochelle, Melbourne & Sydney.
- Soldatova, A.P. (1944). A contribution to the study of the development cycle in the cestode Mesocestoides lineatus (Goeze, 1782), parasitic of carnivorous mammals. Comptes Rendus (Doklady) de l'Académie des Sciences de l'URSS, 45 : 310-312.
- Specht, D. and Voge, M. (1965). Asexual multiplication of Mesocestoides tetrahyridia in laboratory animals. J.

- Parasit., 51 : 268-272.
- Specian, R.D. and Lumsden, R.D. (1980). The microanatomy and fine structure of the rostellum of Hymenolepis diminuta. Z. Parasitenk., 63 : 71-88.
- Specian, R.D. and Lumsden, R.D. (1981). Histochemical, cytochemical and autoradiographic studies on the rostellum of Hymenolepis diminuta. Z. Parasitenk., 64 : 335-345.
- Speckmann, G. and Webster, W.A. (1975). Natural infection and treatment of a dog with Mesocestoides tapeworms. Can. Vet. J., 16 : 26-27.
- Ssolonitzin, J.A. (1933). "Mehrfacher Tetrathyridios der Serosen Höhlen des Hundes." Zeitschrift für Infektionskrankheiten, Parasitare Krankheiten und Hygiene der Haustiere, XLV : 144-156.
- Tanaka, H., Miyamoto, K. and Kaneko, K. (1967). [A human case of Mesocestoides lineatus.] Jap. J. Parasit., 16 : 369-374.
- Tarazona Vilas, J.M. (1955). "Cestodes parasito de vertebrados en la provincia de Huesca." Revista Iberica de Parasitologia, Tomo Extraordinario, pp. 109-122.
- Taylor, A.E.R. and Baker, J.R. (1968). The cultivation of parasites in vitro. Blackwell Scientific Publications, Oxford.
- Taylor, E.W. and Thomas, J.N. (1968). Membrane (contact) digestion in the three species of tapeworm Hymenolepis

- diminuta, Hymenolepis microstoma and Moniezia expansa.
Parasitology, 58 : 535-546.
- Tenora, F. and Kullmann, E. (1972). [The cestodes of
Insectivora and Carnivora in Afghanistan,]
Helminthologia (Bratisl), 11 : 127-139.
- Thompson, R.C.A. (1976). The occurrence of Mesocestoides
sp. in British wild red foxes (Vulpes vulpes
crucigera). J. Helminth., 50 : 91-94.
- Thompson, R.C.A., Dunsmore, J.D. and Hayton, A.R. (1979).
Echinococcus granulosus : Secretory activity of the
rostellum of the adult cestode in situ in the dog.
Exptl. Parasit., 48 : 144-163.
- Thompson, R.C.A., Hayton, A.R. and Jue Sue L.P. (1980).
An ultrastructural study of the microtriches of adult
Proteocephalus tidswelli (Cestoda : Proteocephalidea).
Z. Parasitenk., 64 : 95-111.
- Thompson., R.C.A., Houghton, A. and Zaman, V. (1982a). A
study of the microtriches of adult Echinococcus
granulosus by scanning electron microscopy. Int. J.
Parasit., 12 : 579-583.
- Thompson, R.C.A., Jue Sue, L.P. and Buckley, S.J. (1982b).
In vitro development of the strobilar stage of
Mesocestoides corti. Int. J. Parasit., 12 : 303-314.
- Threadgold, L.T. (1976). Fasciola hepatica :
Ultrastructure and histochemistry of the glycocalyx
of the tegument. Exptl. Parasit., 39 : 119-134.
- Threadgold, L.T. and Dunn, J. (1983). Taenia crassiceps :

- Regional variations in ultrastructure and evidence of endocytosis in the cycticercus' tegument. Exptl. Parasit., 55 : 121-131.
- Threadgold, L.T. and Hopkins, C.A. (1981). Schistocephalus solidus and Ligula intestinalis : Pinocytosis by the tegument. Exptl. Parasit., 51 : 444-456.
- To, Y., Morimoto, T. and Kugi, G. (1978). [Studies on zoonoses. 7. Second report of a dog infected with a cestode of the genus Mesocestoides.] 37-38 Japanese Society of Parasitology (Proceedings of the 31st south Japan regional meeting in Kurume city, 11-12 November 1978, Abstracts) Jap. J. Parasit., (1979) 28 (1, suppl.) : 22-29.
- Tokobaev, M.M. (1964). [Rodents as the final hosts of cestodes of the suborder Mesocestoidata Skryabin, 1940.] Trudy Inst. Zool., Alma-Ata, 22 : 210-211.
- Voge, M. (1953). New host records for Mesocestoides (Cestoda : Cyclophyllidea) in California. Am. Midl. Nat., 49 : 249-251.
- Voge, M. (1955). North american cestodes of the genus Mesocestoides. University of California Publications in Zoolgy, 59 : 125-156.
- Voge, M. (1967). Development in vitro of Mesocestoides (Cestoda) from oncosphere to young tetrathyridium. J. Parasit., 53 : 78-82.

- Voge, M. (1969). Systematics of cestodes - present and future. In : Problems in systematics of parasites. (Schmidt, G.D. ed.), pp. 49-72. University Park Press, Baltimore.
- Voge, M. (1978). Cestoda. In : Methods of cultivating parasites in vitro. 2nd ed. (Taylor, A.E.R. and Baker, J.R. eds.) pp. 193-225. Academic Press, London, New York & San Francisco.
- Voge, M. and Coulombe, L.S. (1966). Growth and asexual multiplication in vitro of Mesocestoides tetrathyridia. Am. J. Trop. Med. Hyg., 15 : 902-907.
- Voge, M. and Seidel, J.S. (1968). Continuous growth in vitro of Mesocestoides (Cestoda) from oncosphere to fully developed tetrathyridium. J. Parasit., 54 : 269-271.
- Voge, M., Sogandares-Bernal, F. and Martin, J.H. (1979). Fine structure of the tegument of Mesocestoides tetrathyridia by scanning and transmission electron microscopy. J. Parasit., 65 : 562-567.
- Wardle, R.A., McLeod, J.A. and Radinovsky, S. (1974). Advances in the zoology of tapeworms, 1950-1970. University of Minnesota Press, Minneapolis.
- Watson, J.S. (1949). Tetrathyridium larvae of Mesocestoides in rodents in Cyprus. J. Parasit., 35 : 383-387.
- Webster, G.A. and Cameron, T.W.M. (1963). Some preliminary observations on the development of

- Echinococcus in vitro. Can. J. Zool., 41 : 185-195.
- Weinstein, P.P. (1966). The in vitro cultivation of helminths with reference to morphogenesis. In : Biology of parasites. (Souslby, E.J.L. ed.) Academic Press, New York.
- Wharton, D.A. (1983). The production and functional morphology of helminth egg-shells. Parasitology, 86 : 85-97.
- Williams, J.F., Westheimer, J. and Banman, W.R. (1975). Mesocestoides infection in the dog. J. Am. Vet. Med. Asso., 166 : 996-998.
- Wilson, T.H. (1962). Intestinal absorption. W.B. Saunders, London.
- Winters, J.B. (1981). Mesocestoides corti in a dog : a case report. Veterinary Medicine & Small Animal Clinician, 76 : 62.
- Witenberg, G. (1934). Studies on the cestode genus Mesocestoides. Archivio Zoologico Italiano, 20 : 467-509.
- Wood, I.B. and Haldiman, J.T. (1957). New locality and host records for the cestode genus Mesocestoides. J. Parasit., 43 : 598.
- Yamane, Y. (1968). On the fine structure of Diphyllbothrium erinacei with special reference to the tegument. Yonago Acta Medica, 12 : 169-181.
- Yamane, Y. (1969). An electron-microscope study of the tegument of Diplogonoporus grandis. Yonago Acta

Medica, 13 : 25-29.

APPENDIX I The experimental protocol and culture system of Ex. No. 27.

Culture No.	Medium	Gas phase	Initial inoculum per culture	pH range during the 1st wk
A - D	Monophasic	5% CO ₂ /95% N ₂	100	> 7.4
E - H	Diphasic (serum base punched)	5% CO ₂ /95% N ₂	100	> 7.4
I - L	Diphasic (serum base <u>not</u> punched)	5% CO ₂ /95% N ₂	100	> 7.4
M - P	Monophasic	5% CO ₂ /95% N ₂	25	> 7.4
Q - T	Monophasic	Air	25	> 7.4
U - X	Monophasic	20% CO ₂ /80% N ₂	100	< 7.4

APPENDIX II The experimental protocol and culture
system of Ex. No. 29.

Culture No.	Gas phase	pH range during the 1st wk
A - D	5% CO ₂ /10% O ₂ / 85% N ₂	< 7.2 (MOPS)*
E - H	5% CO ₂ /95% N ₂	< 7.2 (MOPS)
I - L	5% CO ₂ /10% O ₂ /85% N ₂	7.2 - 7.4 (HEPES)
M - P	5% CO ₂ /95% N ₂	7.2 - 7.4 (HEPES)
Q - T	5% CO ₂ /10% O ₂ /85% N ₂	> 7.4 (HEPPSO)
U - X	5% CO ₂ /95% N ₂	> 7.4 (HEPPSO)

* The buffers shown in the brackets were used in the
culture media.

Initial inoculation : 25 tetrathyridia per culture containing
10 ml S10E.

APPENDIX III The experimental protocol and culture
system of Ex. No. 30.

Culture No.	Pretreatment
A - D	- (Tetrathyridia were used for culture immediately after washing with HBSS.)
E - H	Hanks' saline (pH 2.0), 38°C, 10 min.
I - L	0.2% pepsin (pH 2.0), 38°C, 10 min.
M - P	0.5% trypsin (pH 7.4), 38°C, 20 min.
Q - T	0.2% pepsin (pH 2.0), 38°C, 10 min. then 0.5% trypsin (pH 7.4), 38°C, 20 min.
U - X	Hanks' saline (initial pH 7.3), 38°C, overnight

Constant factors :

1. 25 tetrathyridia per culture containing 10 ml S10E buffered with HEPPSO.
2. Gas phase : 5% CO₂/95% N₂