33

ADVANCES IN PARASITOLOGY

Edited by

J.R. BAKER R. MULLER

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PARASITOLOGY

VOLUME 33

Editorial Board

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C. Combes Laboratoire de Biologie Animale, Université de Perpignan, Avenue de Villeneuve, 66860 Perpignan Cedex, France

J.P. Kreier Department of Microbiology, College of Biological Sciences, Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210–1292, USA

W.H.R. Lumsden 17A Merchiston Crescent, Edinburgh EH10 5AX, UK

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M. Yokogawa Department of Parasitology, School of Medicine, Chiba University, Chiba, Japan

Advances in PARASITOLOGY

Edited by

J.R. BAKER

Cambridge, England

and

R. MULLER

International Institute of Parasitology
St Albans, England

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CONTRIBUTORS TO VOLUME 33

I. BURGESS, *Medical Entomology Centre, University of Cambridge, Cambridge Road, Fulbourn, Cambridge, CB1 5EL, UK*

A. CHABAUD, *Biologie Parasitaire, Muséum National d'Histoire Naturelle, 61 Rue Buffon, 75231 Paris cedex 05, France*

B. FRIED, *Department of Biology, Lafayette College, Easton, Pennsylvania 18042, USA*

I. LANDAU, *Biologie Parasitaire, Muséum National d'Histoire Naturelle, 61 Rue Buffon, 75231 Paris cedex 05, France*

F. MILORD, *Direction générale de la santé publique, de la Montérégie, 25 Boul. Taschereau, Greenfield Park (Québec) J4V 2G8, Canada*

J. PÉPIN, *Service des Maladies Infectieuses, Centre Hospitalier Universitaire, 3001, 12ème Avenue Nord, Sherbrooke, Québec J1H 5N4, Canada*

K. ROHDE, *Department of Zoology, University of New England, Armidale NSW 2351, Australia*

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PREFACE

In this volume we have tried, as usual, to provide a mixture of parasitological topics with, we hope, something to interest everyone. The volume starts with a thorough and up-to-the-minute review of chemotherapy of human African trypanosomiasis by Drs J. Pépin and F. Milord in Québec, Canada. This is a particularly timely review because the political upheavals in parts of sub-Saharan Africa in the last decade or so have resulted in a resurgence of epidemics of sleeping sickness, and also because some new drugs are at last coming into use — although unfortunately, as the authors point out, the market is not sufficiently lucrative for the lengthy development and testing necessary for the development of new drugs to be commercially worthwhile. The only hope is that compounds being developed to combat some of the parasitic infections associated with AIDS will also prove useful in treating human African trypanosomiasis. However, since the last comprehensive review in 1980, considerable progress has been made in the use of the existing drugs and in the use of drug combinations. All these matters, and more, are fully and succinctly covered in this review, with the benefit of the authors' first-hand knowledge derived from their plentiful experience in the field.

The next chapter is a thorough study of the biology of the fascinating group of malaria parasites that parasitize the rodent *Thamnomys rutilans* in Central and West Africa. The authors, Dr Irène Landau and Professor Alain Chabaud, are the leaders of the group at the *Muséum National d'Histoire Naturelle* in Paris that has been studying this group of *Plasmodium* species in depth and detail for many years. Apart from their intrinsic interest as a group of closely related species which have evolved to fill certain specific and closely defined biogeographical niches (the concept of *vicariance*), these parasites have provided material for studies which have yielded information of theoretical and potentially practical significance in relation to human malaria, of which the notion of chronotherapy, timing the administration of drugs with respect to the parasite's life cycle to obtain the maximum effect, is one example. However, the main emphasis of this fascinating review is on the parasites themselves; it is, as the authors subtitle it, a "zoological study".

In almost all digeneans except the schistosomes the stage infective to the definitive host is ingested as a metacercarial cyst and in the next chapter Professor Bernard Fried reviews recent work on the mechanisms of excystment in various families of the Digenea. As well as comparing the *in vitro* excystation process in 50 species, there is valuable information on the range of studies that can be carried out with excysted metacercariae: studies on morphology, behaviour, immunology and on the growth, development and differentiation of metacercariae excysting *in vivo* and *in vitro* (including in hens' eggs). The structure of the cyst layers of many species has been

compared and the extreme variation in both structure and chemical composition noted. While there has been little recent research on the extrinsic factors involved in excystation, there has been some fascinating new information on intrinsic factors, particularly on the role of thiol proteases and lipophilic factors.

Next, Professor Klaus Rohde gives a detailed and up-to-date account of the "minor" groups of the parasitic platyhelminthes, the Aspidogastrea, Amphilinidea and Gyrocotylidea. In addition, aspects of symbiotic turbellarians are discussed that may cast light on the phylogeny of parasitic platyhelminthes. In recent years, major advances have been made in the study of the life cycles of Aspidogastrea (in particular *Lobatostoma manteri*) and Amphilinidea (*Austramphilinia elongata*), and of the ultrastructure of Aspidogastrea, Amphilinidea, Gyrocotylidea and symbiotic Turbellaria. These ultrastructural studies, especially those on spermatogenesis, protonephridia, tegument/epidermis and sensory receptors, in conjunction with work using cladistic techniques on DNA sequences of some key taxa, have shown that all major groups of parasitic platyhelminthes form one monophylum, the Neodermata. The sister group of Neodermata is not found among the turbellarian Rhabdocoela, as usually assumed. Evidence is not yet complete, but it seems that the Neodermata (possibly plus the Fecampiidae) have split off a turbellarian line very early in evolution, i.e. that a large taxon comprising Proseriata, Dalyelliida, Tricladida, etc. may represent the sister group of the Neodermata (perhaps plus the Fecampiidae).

The volume concludes with a masterly review of all aspects of scabies and its causative organism by Dr Ian Burgess, from Cambridge, UK. This disease, in spite of having been known from the time of Aristotle, is still imperfectly understood. It appears to have a cyclical periodicity, with major outbreaks occurring at intervals of 20 to 30 years, for reasons which are not yet entirely clear. All aspects of the condition and the responsible mite are covered in this chapter, from the anatomy of *Sarcoptes scabiei* itself through the pathology and epidemiology of the disease to its control and the prospects for its future eradication. Ten years ago a writer on the subject (J.O'D. Alexander, in "Arthropods and Human Skin"; Springer, Heidelberg and New York, 1984) stated that "Ideally, a worldwide mass treatment campaign should eradicate scabies" but this ideal is very far from being achieved. As Dr Burgess concludes, any real progress towards global eradication of scabies depends on political as well as clinical effort instead of the "often half-hearted, inadequate and inconsistent" measures all too frequently applied at the present time even in so-called "developed" countries.

J.R. BAKER
R. MULLER

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The Treatment of Human African Trypanosomiasis

J. Pépin

Service des Maladies Infectieuses, Centre Hospitalier Universitaire, 3001, 12 ème Avenue Nord, Sherbrooke, Québec J1H 5N4, Canada

and

F. Milord

Direction générale de la santé publique, de la Montérégie, 25 Boul. Taschereau, Greenfield Park (Québec) J4V 2G8, Canada

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1. THE DISEASE

The last two comprehensive reviews on treatment of human African trypanosomiasis (HAT) were published in 1970 and 1980 (Apted, 1970a, 1980). Since then, there have been at last some innovations in the treatment of sleeping sickness, although most of the currently used drugs have been available for more than 50 years, a very rare situation in medicine. The optimism of the late 1950s concerning eventual eradication of HAT has proved to be premature. A resurgence of trypanosomiasis has occurred in several countries, linked to civil unrest or wars which resulted in the cessation, for only a few years, of the activities of national control programmes in Zaire, Angola, Uganda, Sudan and Tchad. Thus, for the next few decades at least, there will be tens of thousands of patients needing treatment each year for HAT throughout the African continent.

It is beyond the scope of this chapter to review the epidemiology or clinical features of HAT, and several references are available for those interested in such matters (Apted, 1970b, c; Scott, 1970; WHO, 1986). There has been no recent progress regarding the description of clinical signs of HAT, and relevant chapters of textbooks written during the last few decades are still appropriate. We will try only to provide answers to the questions a clinician has to address before selecting the therapeutic regimen for a patient with HAT or deciding if the patient needs further therapy.

1.1. Is the Patient Infected with Trypanosomes?

In most instances, trypanosomes will have been detected by examination of the blood, the lymph node aspirate, the cerebrospinal fluid (CSF), or any combination thereof. The presence of cervical lymphadenopathy, known as Winterbottom's sign, is seen in fewer than half of the early-stage cases and in less than a quarter of late-stage cases; when present, especially as large soft and mobile lymph nodes, the examination of lymphatic fluid will often reveal the presence of trypanosomes. Examination of blood can also allow a diagnosis to be made. Usually an examination of a wet blood film or of a Giemsa-stained thick film will be made first; if these fail to reveal the presence of trypanosomes, the buffy coat should be examined in a microhematocrit tube; if available, the most sensitive assay for detection of bloodstream trypanosomes is the miniature anion-exchanger centrifugation technique (MAECT) (Lumsden *et al.*, 1977). As the disease progresses,

trypanosomes are found less frequently in the blood and lymph nodes, and more frequently in the CSF. The double centrifugation technique is the most sensitive method for finding trypanosomes in the CSF (Cattand *et al.*, 1988). Asymptomatic carriers or patients who were infected with trypanosomes and were spontaneously cured have been reported but are thought to be very rare, so that all individuals in whom trypanosomes have been seen should be treated, even if they have no symptoms.

Centers dealing with a large number of cases have to treat occasional patients in whom trypanosomes have not been seen; these patients are known as "clinical cases". They live in an endemic focus, have symptoms compatible with late-stage HAT, and their CSF shows an abnormal number of lymphocytes. If available, a serological assay will demonstrate the presence of antibodies against *T. b. gambiense*, and their total immunoglobulin M (IgM) levels will be elevated. With experienced laboratory technicians, clinical cases should not represent more than 5% of the total number of cases. Our experience in Zaire is that virtually all of them will respond to melarsoprol or eflornithine, confirming the diagnosis.

More difficult is knowing how to handle patients who have a positive serology (performed during a case-finding survey), in whom trypanosomes cannot be detected, who are asymptomatic and have a normal CSF — the "serological suspects". Most authorities recommend not treating them, but following them closely with sequential parasitological assays. In high-prevalence areas, it may be acceptable to treat them — with pentamidine, suramin, a combination of these two drugs, or diminazene, treatments that result in little toxicity — in order to sterilize more rapidly the human reservoir, but this approach should be considered experimental (WHO, 1986; H. Bruneel, personal communication).

1.2. Is it the Gambiense or Rhodesiense Subspecies?

It is impossible to distinguish morphologically between the two pathogenic subspecies. As the geographic distribution of *T. b. gambiense* and *T. b. rhodesiense* shows little overlap, this is usually not a problem, even in countries endemic for both parasites such as Uganda, Sudan, Angola and Ethiopia. For the rare traveller who has been in areas endemic for both subspecies, there are a few research laboratories that can perform isoenzyme determinations or deoxyribonucleic acid (DNA) probes which will allow this distinction to be made. If these are not available, and there is some doubt about the subspecies involved, we recommend treating patients with rapidly progressive disease with the *T. b. rhodesiense* protocols, whereas those whose symptoms have been developing over a period of months should be treated with *T. b. gambiense* protocols.

1.3. Is it Early-stage or Late-stage Trypanosomiasis?

History and physical examination are not sensitive enough to identify patients with modest CSF anomalies (white blood cell [WBC] count of 6–50 mm^{-3}), and a lumbar puncture should be performed in all patients with suspected or proven trypanosomiasis. The CSF WBC count is the most widely used indicator to distinguish between early- and late-stage infection. Although the cut-off is somewhat arbitrary, patients with $\geqslant$ 6 WBC mm^{-3} are considered to have late-stage disease and should thus receive drugs appropriate for that stage. Patients with a normal CSF WBC count but with trypanosomes seen in the CSF, a rare occurrence, should also be treated as late-stage disease, even though it is possible that these trypanosomes represent contamination of the CSF with a minute quantity of blood during the lumbar puncture. Less than 1% of patients will have symptoms suggestive of central nervous system (CNS) involvement despite a normal CSF, without trypanosomes. They should also be treated with melarsoprol or eflornithine.

Neujean (1950) showed 40 years ago that the cephalic CSF, obtained by pneumoencephalogram, became abnormal and contained trypanosomes earlier in the infection than lumbar CSF (both during the initial episode or during an eventual relapse), but this technique is invasive and not available even in teaching hospitals. Determination of CSF proteins has no advantage over the more widely available WBC count. CSF IgM assays have never been used outside a few research laboratories (Whittle *et al.*, 1977).

1.4. Has the Treatment been Effective?

Patients treated for Gambian HAT need to be followed for at least 2 years after treatment, and a lumbar puncture should routinely be performed every 6 months for WBC count and trypanosomes detection. For Rhodesian HAT, most authors recommend more frequent lumbar punctures (i.e. every 3 months), at least during the first year. A relapse is easily diagnosed when trypanosomes are found in the CSF (or in other body fluids, a much less common event). If the CSF WBC count is clearly higher than the previous determination and above 50 mm^{-3}, the patient should be considered to have relapsed even if he or she is asymptomatic. If the CSF WBC count is higher than the previous one but still in the intermediate range of 20–49 mm^{-3}, we would treat the patient for a relapse only if there is a recurrence of symptoms. If the patient is asymptomatic and the CSF WBC count is high (>50 mm^{-3}), but lower than the previous one, it is better not to treat as in occasional patients it takes months for the CSF WBC count to return to normal. Indeed the CSF of some patients gets worse immediately after treatment with melarsoprol, with a significant increase in WBC count and protein level; this is known in the French literature as the "orage liquidien"

— the "CSF storm" — and is not predictive of a relapse. In any situation where there is doubt about the presence of a relapse, it is preferable to ask the patient to come back after 2 or 3 months for a repeat lumbar puncture, or sooner if symptoms recur.

2. THE DRUGS

2.1. Pentamidine

2.1.1. *Chemistry and Mode of Action*

Pentamidine is an aromatic diamidine, soluble in water, which has been used in the treatment of trypanosomiasis for more than 50 years. Two forms of the drugs have been used: pentamidine isethionate (relative molecular mass (M_r) = 593) and the closely related pentamidine methanesulfonate (often designated by its trade name Lomidine®, M_r = 533). The production of the latter salt has been discontinued. Recommended doses are usually expressed as pentamidine base (1 mg of pentamidine base is the equivalent of 1.74 mg of pentamidine isethionate and of 1.56 mg of pentamidine methanesulfonate). Pentamidine has been shown to be a reversible inhibitor of trypanosomal S-adenosyl-L-methionine decarboxylase, thereby reducing the synthesis of polyamines (Bitonti *et al.*, 1986a). Other possible modes of action, reviewed elsewhere (Williamson, 1970; Sands *et al.*, 1985), are kinetoplast fragmentation or inhibition of glycolysis, or of DNA, ribonucleic acid (RNA), protein and phospholipid synthesis, or of amino acid transport or of cation efflux. Trypanosomes contain a pentamidine transport system which raises the intracellular concentration of the drug to many times the plasma concentration, increasing its selectivity (Damper and Patton, 1976).

2.1.2. *Pharmacokinetics*

Early trials with pentamidine suggested that it had a prolonged action and a slow rate of excretion. Van Hoof *et al.* (1944) reported that two human volunteers given a single injection of pentamidine (2–3 mg kg^{-1}) and on whom infected tsetse flies were fed every alternate day for 1 year became parasitemic (blood films were examined daily during 1 year) only 295 and 365 days after the injection. This suggested that significant levels of pentamidine persisted for months and was the basis for the "chemoprophylaxis" campaigns.

Most recent studies of pentamidine pharmacokinetics have been performed on acquired immune deficiency syndrome (AIDS) patients with *Pneumocystis carinii* pneumonia (PCP). Using high performance liquid chromatography (HPLC), the elimination half-life after the first dose was 9.4 h after

intramuscular administration, and 6.4 h after intravenous administration; with this latter route, the peak plasma concentration was three times higher than after intramuscular injection (Conte *et al.*, 1986). Renal clearance was 5% of the plasma clearance for intramuscularly treated patients and 2.5% for those treated intravenously, and it was postulated that a major part of the dose may be excreted unchanged in the bile, as there is no identifiable metabolite. In patients who received multiple doses of pentamidine for PCP, drug accumulation occurred and trough concentrations increased progressively without achieving steady state throughout treatment (Conte, 1991). In this latter study, the elimination half-life after the first dose was estimated at 29 h. After the last of an average of 13 daily injections, the mean elimination half-life was 12.0 days and detectable concentrations of pentamidine were documented in all patients except one 6 weeks after the last dose. Pentamidine is thought to be deposited in tissues, mostly the kidneys, for 6–8 weeks (Waalkes *et al.*, 1970).

The only pharmacokinetic study in sleeping sickness patients was performed recently on 11 patients from Côte d'Ivoire treated with the methanesulfonate salt (Bronner *et al.*, 1991). Maximal plasma concentrations varied widely, from 420 to 13420 nmol l^{-1}; after the last of 10 injections (of 3.5–4.5 mg kg^{-1} base) on alternate days, the median plasma concentration was five times higher than after the first dose. Small amounts of pentamidine were found in the CSF of all patients after the last dose, corresponding to 0.5–0.8% of the plasma concentrations. Median half-life was 22.4 h after the first dose and 47.1 h after the last dose. Whether or not the CSF penetration of pentamidine increases in parallel with CSF inflammation is unknown.

2.1.3. *Efficacy*

Pentamidine acts relatively slowly as trypanosomes can be detected in the blood and lymph nodes for up to 48 h after the first injection (Van Hoof *et al.*, 1944). The first field trials of pentamidine made during World War II showed that the drug was remarkably effective in patients with early-stage Gambian HAT (Lourie, 1942; Saunders *et al.*, 1944). Although it could cure 96–100% of patients with 0–4 and 5–20 WBC mm^{-3} in the CSF, it was less efficient in patients with 21–100 and >100 WBC mm^{-3}, with cure rates of 86% and 44% respectively (Lourie, 1942). Comparable cure rates of 75% for patients with "intermediate" CSF anomalies (WBC count between 3 and 40 mm^{-3} in one series, between 20 and 100 mm^{-3} in the other) were reported in other series from Sierra Leone and Nigeria (Harding, 1945; Duggan and Hutchinson, 1951).

In an extremely large series from several territories of Afrique Occidentale Française (AOF), Jonchère (1951b) reported that only 718 (7%) of 10 281 patients with early-stage gambiense sleeping sickness subsequently relapsed after a course of five injections. The vast majority (679) of these relapses

were characterized by an increase in CSF WBC count, without trypanosomes being seen in the CSF, the blood or the lymph nodes. The 7% failure rate was virtually identical in Upper Volta, Guinea, Sénégal and Côte d'Ivoire. Le Rouzic (1949a), in another large study in AOF, showed that the failure rates were identical for both salts of pentamidine: 7.6% of 3640 patients treated with pentamidine isethionate relapsed, compared to 7.3% of 2423 patients given the methanesulfonate salt. An identical failure rate of 7% was reported in Upper Volta 15 years later (Dutertre and Labusquière, 1966). Jonchère (1951b) and Neujean and Evens (1958) reported that patients relapsing after pentamidine responded as well to arsenicals as patients who had not received pentamidine before. This was also true among the patients whom we treated in Zaire, and multiresistant strains, described in laboratory work on *T. b. brucei* and *T. b. rhodesiense* (Frommel and Balber, 1987; Bacchi *et al.*, 1990), have not yet been a problem for clinicians.

Neujean and Evens (1958) reported a 16% failure rate among 102 patients with normal CSF treated with pentamidine alone in Kinshasa in the late 1950s, as compared to 3% (1 of 29) of those who received a combination of pentamidine and suramin. It is thus interesting to note that, 15 years later, *T. b. gambiense* strains with reduced sensitivity to pentamidine were documented in Lower-Zaire (Kayembe and Wéry, 1972). It is unclear if this was related to prior massive use of pentamidine chemoprophylaxis in the same area, but increasing degrees of pentamidine resistance were induced in two strains tested in an animal model by the administration of repeated subcurative doses of pentamidine. On the other hand, patients who had developed early-stage HAT after having received one or several injections of pentamidine chemoprophylaxis responded well to curative doses of pentamidine (Jonchère, 1951b). Of 407 patients whom we treated in the 1980s with the pentamidine–suramin combination, 39 (9.6%) subsequently relapsed and had to be treated with melarsoprol (Pépin *et al.*, 1989d). Thus the frequency of resistance to pentamidine seems to be higher in Zaire than in West Africa, but has been rather stable over the last three decades.

2.1.4. *Toxicity*

Pentamidine injection sites are exquisitely tender, making occasional patients reluctant to be treated for an asymptomatic disease. Sterile gluteal abcesses are not uncommon (5%). Hypotension, due to the liberation of histamine, is common but syncope is rarely seen with the intramuscular route (0.02% of patients) and will respond readily to intravenous saline and subcutaneous adrenalin if needed. Hypotension is more frequent if pentamidine is rapidly administered intravenously, but this route is rarely if ever used in the treatment of HAT. When pentamidine was used on a large scale in the chemoprophylaxis of HAT, the estimated rate of death was 1 per 300 000 injections (Jonchère, 1951a; Gall, 1954), but it is likely that serious adverse

effects not related to the drug itself but to faulty injection techniques (gas gangrene, sciatic nerve injury with paralysis of the leg) were underreported. Pruritus, rash, tachycardia, nausea and vomiting are also seen. Hypoglycemia can occur shortly after an injection, as a result of insulin release by the pancreas, and damage to this gland may rarely result in pancreatitis or diabetes mellitus, which was apparently more common with the methanesulfonate than with the isethionate salt. Hypocalcemia and renal failure are well described but more difficult to recognize in rural hospitals. Neutropenia is seen in trypanosomiasis patients, but less often than in AIDS or cancer patients; anaemia, thrombocytopenia, hypomagnesemia, hyperkalemia, arrhythmias, QT interval prolongation and abnormal liver function tests have been reported in the AIDS literature (Sands *et al.*, 1985; Goa and Campoli-Richards, 1987). Pentamidine and related diamidines have shown no evidence of mutagenic or genotoxic effects in laboratory assays (Stauffert *et al.*, 1990), and pentamidine is thought to have no adverse effect on pregnancy (Gall, 1954; Schneider, 1963).

2.1.5. *Availability and Cost*

Despite having been discovered more than half a century ago, the price of pentamidine increased considerably in the last few years (from US$1 to around US$30 per 300 mg vial) as the AIDS epidemic created a new and very lucrative market for the treatment and prophylaxis of PCP. Fortunately, trypanosomiasis patients have been excluded from this law of supply and demand, as the manufacturer (May and Baker, a subsidiary of Rhône Poulenc: Dagenham, RM10 7XS, UK) agreed to provide pentamidine isethionate (Pentacarinate®) free of charge for trypanosomiasis patients, through the World Health Organization.

2.2. Diminazene

2.2.1. *Chemistry and Mode of Action*

Diminazene aceturate is, like pentamidine, an aromatic diamidine, soluble in water, with $M_r = 587$. Its mode of action is at least partially through irreversible inhibition of trypanosomal S-adenosyl-L-methionine decarboxylase, an enzyme involved in the synthesis of polyamines (Bitonti *et al.*, 1986a). It also selectively blocks kinetoplast DNA synthesis (Newton and LePage, 1967). It is said to kill trypanosomes more slowly than suramin (de Raadt *et al.*, 1966).

2.2.2 *Pharmacokinetics*

The pharmacokinetics of diminazene have not been studied in humans. In animals, the half-life is between 11 and 14 h for sheep, dogs and goats, and

63 h for cattle (Klatt and Hadju, 1976; Aliu and Odegaard, 1985; Anika and Onyeyili, 1989). In the sheep, plasma protein binding was estimated at 65–85% (Aliu and Odegaard, 1985). Diminazene is to some extent absorbed when given orally, but its usefulness by that route of administration has not been further studied since the first report by Bailey (1968).

2.2.3. *Efficacy*

The manufacturer of diminazene has never been interested in marketing it for human trypanosomiasis, as the cost of more sophisticated toxicological studies and licensing was judged too high in view of the very limited market. Nevertheless, clinicians and national control programs in several endemic countries have used it extensively, as the drug is effective, usually well tolerated, and cheap. However, the uncomfortable legal and ethical situation has resulted in very few of these data being published.

For rhodesiense early-stage trypanosomiasis, a regimen of three doses (5 mg kg^{-1} per dose, every alternate day) was used in two series in Uganda, and only 2 of 78 patients relapsed (Onyango *et al.*, 1970; Temu, 1975). More recently, only 7 of 200 similar patients treated with the same dosage subsequently relapsed (Abaru and Matovu, 1984). In gambiense early-stage HAT, diminazene (2 mg kg^{-1} per injection for 7 daily injections) cured 16 of 17 Nigerian patients (Hutchinson and Watson, 1962). In Zaire, Ruppol and Burke (1977) reported having treated several hundreds early-stage patients, 15% of whom eventually required melarsoprol as their CSF examinations suggested a relapse. They recommended a regimen of three injections separated by 48 h, with 7 mg kg^{-1} being given each time. Diminazene has also been used as pre-treatment before melarsoprol in late-stage Rhodesian HAT, in series too small to allow evaluation of its efficacy in that setting (De Raadt *et al.*, 1966), but there was a later report from the same center about two patients with late-stage disease in whom diminazene itself seem to have precipitated a fatal reactive encephalopathy (De Raadt, 1967).

2.2.4. *Toxicity*

Brain damage after diminazene administration was noted in dogs (Losos and Crockett, 1969), but this toxicity is apparently species-dependent; it has been seen in camels and horses but not in bovines, rats or mice (East African Trypanosomiasis Research Organization, 1975). Despite its having been used in thousands of patients suffering from HAT, little toxicity has been documented. Considering that the drug is not approved for human use, it is possible that clinicians who witnessed significant adverse effects preferred to be discreet. Our personal experience is that the drug is generally very well tolerated. Its injection is much less painful than that of pentamidine, and we have not seen sterile gluteal abcesses.

In the few published studies, no toxicity was noted in 50 patients in Uganda (Temu, 1975) or in 17 patients in Nigeria, apart from mild albuminuria (Hutchinson and Watson, 1962). In a more recent series from Uganda, neurological toxicity was seen in seven patients (five with numbness of the legs, one with paralysis, one with coma) among 99, but no long-term toxicity was seen 1–9 years later, even with these seven patients (Abaru *et al.*, 1984). An American patient given diminazene for the treatment of babesiosis developed a Guillain–Barré syndrome (Ruebush *et al.*, 1979). Fever, nausea and vomiting have also been reported (Abaru and Matovu, 1984). Ruppol and Burke (1977), without giving precise numbers, stated that among a group of several hundred patients no death or adverse effect was seen apart from fever in a few of them. H. Bruneel (personal communication) gave a single dose (7 mg kg^{-1}) to 282 "serological suspects" in central Zaire without any adverse effect.

2.2.5. *Availability and Cost*

Diminazene is produced by Hoechst and sold under the trade name Berenil®, with "restricted for veterinary usage" clearly written on the package. A 1.05 g sachet costs approximately US$1, so that a course of three injections (7 mg kg^{-1} per injection) would cost only US$1.50.

2.3. Suramin

2.3.1. *Chemistry and Mode of Action*

Suramin is a sulfated naphthylamine, developed more than 70 years ago. It has M_r = 1429, and is soluble in water. Its mode of action is poorly understood, as it inhibits numerous enzymes, including L-α-glycerophosphate oxidase (Fairlamb and Bowman, 1977; Gutteridge, 1985), RNA polymerase (Hawking, 1978) and many others that probably have no relation to its trypanocidal effect: hyaluronidase, urease, hexokinase, fumarase, trypsin. It is relatively slowly cidal, trypanosomes disappearing from blood and lymph nodes 12–36 h after injection (Hawking, 1978). Suramin deteriorates in air and should be injected immediately after it is prepared.

2.3.2. *Pharmacokinetics*

Suramin is bound to plasma proteins of all kinds (albumin, globulins, fibrinogen) rapidly after injection, is thus slowly excreted and can be found in the blood for up to 3 months after injection; this was the basis for its use in chemoprophylaxis (Hawking, 1978). Using a colorimetric method, Hawking (1940) showed that there was much variability in suramin pharmacokinetics between individuals. After a single 1-g dose, suramin was detected in the

plasma for 5–8 days and after a course of five to six doses it could be detected for 150–250 days. He also demonstrated that the CSF penetration of suramin was negligible, and confirmed this by a biological method. The same variability had been observed in nine human volunteers given a single 1-g injection and exposed to trypanosomes (by direct inoculation or through the bites of infected tsetse flies), the interval to detectable parasitemia varying between 73 and 327 days (Duke, 1936). With a 2-g dose, the duration of protection ranged between 103 and 180 days.

As subcutaneous and intramuscular injections cause local inflammation and necrosis, suramin is normally given intravenously. If given concomitantly with pentamidine, both drugs will form a complex that is thought to be less toxic and more trypanocidal than either drug alone (Giumaraes and Lourie, 1951; Williamson, 1970). Preparations containing both drugs have never become commercially available because they induced severe reactions at the site of injection, but this principle underlies the widely used combination of intravenous suramin and intramuscular pentamidine (Neujean and Evens, 1958).

Very little research was done on suramin for decades, until it was found to have some activity against reverse transcriptase of retroviruses (Mitsuya *et al.*, 1984), and more recently as an anticancer drug (Pinedo and Van Rijswijk, 1992). It was later shown to be useless in human immunodeficiency virus (HIV) infection (Cheson *et al.*, 1987) and quite toxic in that context, but at least modern technology was used to confirm earlier pharmacokinetic data. Four patients with HIV infection were given a total of 6.2 g of suramin; 99.7% of the drug was found to be bound to plasma proteins. After the last dose, the half-life of suramin was 44–54 days, one of the longest half-lives ever documented for drugs given to humans. Total plasma suramin levels were higher than 100 $\mu g\ ml^{-1}$ for several weeks, and urinary excretion eventually accounted for most of the elimination of the drug (Collins *et al.*, 1986).

2.3.3. *Efficacy*

Cure rates of 96% were reported 50 years ago with suramin monotherapy for early-stage Gambian HAT (Harding, 1945), and Apted (1953, 1980) even considered suramin to be almost 100% effective in patients with early-stage Gambian or Rhodesian trypanosomiasis. However, Neujean documented a 28% failure rate among early-stage gambiense patients in the Belgian Congo and quoted other workers having seen a similar 25–35% frequency of treatment failure (Neujean, 1950; Neujean and Evens, 1958). For many reasons (treatment failures, duration of treatment, need for intravenous administration, cost) suramin monotherapy has rarely been used for Gambian early-stage disease during the last 20 years and there are no contemporary data concerning its efficacy.

In early-stage Rhodesian HAT, suramin is thought to be more effective

than pentamidine (Nash, 1960; Schneider, 1963; Apted, 1980; WHO, 1986). In a retrospective study in Kenya, up to 49% of patients who had been given suramin without having had a lumbar puncture, because they had no overt sign of CNS involvement, subsequently relapsed, demonstrating again the absolute necessity of examining the CSF of all patients before deciding on treatment (Wellde *et al.*, 1989). Among 29 patients who were given suramin after a lumbar puncture showing a WBC count less than 6 mm^{-3}, only 2 (6.9%) relapsed. In Tanzania, 31% of 49 early-stage patients treated with suramin eventually relapsed, as well as 7 of 11 (64%) patients given suramin despite borderline CSF anomalies (WBC count 7–10 mm^{-3}) (Veeken *et al.*, 1989). We are not aware of other recent series so that it is difficult to assess if there has been an increase in the failure rate in the last decades. Patients who relapse following suramin should receive melarsoprol, and the failure rate with this drug will be comparable to that seen when it is used as the first-line treatment.

Suramin, because of its poor CNS penetration, is not an efficient treatment for late-stage trypanosomiasis, although it will clear the hemolymphatic system of trypanosomes (with a temporary improvement of symptoms) and is used for that purpose in patients with CSF anomalies, in the hope that this pre-treatment will reduce the toxicity of melarsoprol, especially the Jarish–Herxheimer reactions (Apted, 1953; Buyst, 1975). Indeed, some patients with mildly abnormal CSF will be cured by suramin alone (Keevill, 1934; Neujean, 1950; Wellde *et al.*, 1989), but the failure rate is too high despite a transient improvement in the cell count of the cephalic and lumbar CSF (Chesterman, 1924; Neujean, 1950), and suramin alone should never be used for these patients. Suramin, like pentamidine, is also used by mobile teams to "sterilize" patients in their villages until they reach the hospital where melarsoprol will be administered.

Suramin has been found, in various animal models, to have synergistic effects with other trypanocidal drugs, including eflornithine (Clarkson *et al.*, 1984; Bacchi *et al.*, 1987), nifurtimox (Raseroka and Ormerod, 1986), metronidazole (Raseroka and Ormerod, 1985), Ro 15–0216 (Zweygarth and Rottcher, 1987) and other 5-nitroimidazoles (Jennings *et al.*, 1983), but such synergism has never been demonstrated in human trypanosomiasis.

2.3.4. *Toxicity*

A test dose of 200 mg is recommended by some authors, but severe idiosyncratic reactions (anaphylactic shock) are very rare (less than 1 per 2000 patients) (Apted, 1980). Febrile reactions are sometimes seen after the first injection, more often in the rhodesiense disease (10% in Kenya; Wellde *et al.*, 1989), and are due to trypanosome lysis or to pyrogens in the drug. In our series of 620 patients given melarsoprol, four died shortly after admission, before receiving melarsoprol but after receiving the pre-treatment dose of

suramin (Pépin *et al.*, 1989a). These patients were moribund upon admission, and it is impossible to know if suramin precipitated the fatal outcome. For these rare patients, we would now empirically give at least one dose of steroids before suramin. With the single injection (as pre-treatment) or with the two injections (for early-stage patients, in combination with pentamidine) that we used in Zaire, adverse effects are rare apart from the occasional fever or urticarial reaction. When suramin is used in monotherapy so that several injections have to be given, renal toxicity is the most common problem, but it is usually only mild proteinuria. Polyneuropathy and stomatitis have been reported in the trypanosomiasis literature. Keratopathy and various types of self-limited skin reactions have also been described in cancer patients receiving high-dose suramin (Holland *et al.*, 1988; LaRocca *et al.*, 1990), as were liver dysfunction, adrenal insufficiency, thrombocytopenia and neutropenia in AIDS patients (Cheson *et al.*, 1987). Concomitant onchocerciasis will increase the risk of hypersensitivity reactions with suramin and, if possible, should be treated first with ivermectin.

2.3.5. *Availability and Cost*

Suramin is produced by Bayer and sold as 1-g vials under the trade name Bayer 205® (previous trade names, sometimes used in the older literature, were Germanin®, Antrypol® and Moranyl®, among others). It costs approximately US$7 per 1-g vial.

2.4. Melarsoprol

2.4.1. *Chemistry and Mode of Action*

Melarsoprol, also known as Mel B, was synthesized half a century ago by the late Dr Friedheim (1949, 1951), by the addition of BAL (British anti-lewisite, dimercaprol), a heavy metal chelator, to the arsenic of melarsen oxyde, also developed by him. It has M_r = 398, and is insoluble in water, alcohol or ether. Thus it is dissolved in propylene glycol, and sold as a 5-ml ampoule (instead of 6 ml previously) of the 3.6% solution. It has to be administered intravenously and, if injected subcutaneously, the propylene glycol will induce a severe chemical cellulitis.

The cellular target for melarsoprol has recently been shown to be trypanothione; its irreversible binding to trypanothione results in a compound called Mel T (Fairlamb *et al.*, 1989). Trypanothione synthesis is also reduced by ornithine decarboxylase inhibitors, so that theoretically eflornithine and melarsoprol could have synergistic effects. Melarsoprol is also a potent inhibitor of pyruvate kinase, a key glycolytic enzyme (Flynn and Bowman, 1969).

2.4.2. *Pharmacokinetics*

Remarkably little is known about the pharmacokinetics of melarsoprol. The currently used treatment schemes are those developed originally by Friedheim and Neujean (with very minor modifications). The 1-week drug-free intervals between the first, second and third series are unique among the hundreds of antimicrobial regimens developed for other infectious diseases, and one wonders why this could be better than giving lower doses of melarsoprol, every day for 2–3 weeks, with the same cumulative dosage.

Studies using total arsenic levels have shown that 30–60% of the melarsoprol is excreted, mostly (70–80%) in the feces, with only 10–20% being detected in the urine in the first 48 h and none thereafter (Monnet and Baylet, 1951b; Cristau *et al.*, 1975). Another study using total arsenic determinations suggested that the CSF penetration of melarsoprol is poor (Monnet and Baylet, 1951a). Melarsoprol CSF concentrations were also estimated by a bioassay using trypanosomes *in vivo* (Hawking, 1962), which showed that the CSF levels were only 0.5–5% (mean 0.8%) of simultaneous plasma levels. Similar findings have been described with an experimental enzyme-linked immunosorbent assay (ELISA) which, with a single patient, showed that there was virtually no melarsoprol in the CSF after the first injection, and approximately 3.5% of serum levels after the second injection (Maes *et al.*, 1988). The remarkable efficacy of melarsoprol in patients with late-stage disease is thus more a consequence of its extraordinary activity against the parasite than of good CSF penetration.

Assays to measure melarsoprol levels in blood or CSF have never been exploited on a large scale, so that basic quantities, such as its elimination half-life, are unknown. Preliminary results with the experimental ELISA suggested that free melarsoprol levels drop rapidly after the first injection, but that there is substantial accumulation starting with the second dose (Maes *et al.*, 1988). This is in line with Hawking's (1962) findings with the bioassay.

2.4.3. *Efficacy*

Melarsoprol remains the most active trypanocidal drug available. Despite the poor CSF penetration, trypanosomes in the CSF are slower moving and fewer in number only 5–8 h after the first injection, and disappear from the bloodstream and lymph nodes even more rapidly, within 30–120 min (Ceccaldi, 1952; Whittle and Pope, 1972). Clinically, even patients with advanced sleeping sickness will feel better within 1 week of initiation of melarsoprol therapy. However, the important determinant of efficacy in trypanosomiasis treatment is the proportion of patients who relapse during follow-up.

The minimal effective dose of melarsoprol is unknown. Studies with the sheep model have suggested that 0.9–1.8 mg kg^{-1} day^{-1} (25–50% of currently recommended regimens for humans) may be sufficient (Bouteille *et al.*, 1988), but direct extrapolation from animal models to human disease is

always risky. In Guinea-Bissau, 16 patients with normal CSF were treated with a single injection (4 mg kg^{-1}) of melarsoprol and all were apparently cured (Pinto, 1954); this was later confirmed in the Belgian Congo (Neujean and Evens, 1958) and by a much larger series in Upper Volta (Burkina Faso), where only 26 (4.8%) of 535 patients with early-stage Gambian disease relapsed after a single injection of melarsoprol (Dutertre and Labusquière, 1966). Good results with a single dose of another arsenical drug, Mel W, were also reported for patients with early-stage Gambian HAT (Schneider, 1963). More recently, in Zaire, there was no increase in the relapse rate when we decreased from 3 series of 4 injections (for a cumulative dose of approximately 67 ml) to 3 series of 3 injections (approximately 50 ml) the treatment of gambiense patients with a CSF showing 100 or more WBC mm^{-3} (unpublished data). In Kenya, where various regimens were used for patients with Rhodesian disease, 2 of 140 (1.4%) of those given a total of more than 30 ml of melarsoprol relapsed, compared to 11 of 115 (9.6%) of those who received less than 30 ml (Wellde *et al.*, 1989). This was in line with Apted's (1957) findings three decades earlier; he experimented with various dosages in late-stage Rhodesian disease (total dose between 7.5 and 27.5 ml) and recommended that at least 25–30 ml be given to keep the relapse rate to a minimum. Thus it is certainly possible that a large number of patients have been overtreated, and the regimens recommended in the third section of this chapter represent maximal cumulative doses of 34.5–37.5 ml, in an attempt to take these facts into consideration.

Before melarsoprol became available, up to 80% of patients with late-state gambiense trypanosomiasis were resistant to tryparsamide and slowly deteriorated to a fatal outcome. For patients with late-stage rhodesiense disease, no drug had ever been found to be effective. Thus melarsoprol represented a major life-saving breakthrough (Apted, 1970a). In the first large series with gambiense HAT treated with melarsoprol, in Cameroun, only 3.3% of 394 tryparsamide-resistant patients were not cured (Friedheim, 1951). The failure rate has been remarkably uniform between countries and within long periods of time, given that the series summarized in Table 1 differed with regard to treatment regimens, severity of disease before treatment, and completeness of follow-up. In Zaire, we documented a 6.2% rate of relapse in a large series of 1083 patients treated in the 1980s (unpublished data), which compared favorably with the 9% rate reported from Kinshasa three decades earlier (Neujean and Evens, 1958). There may be a small focus stretching over Lower-Zaire and northern Angola with a very high (30–40%) frequency of resistance to melarsoprol, but it has not been described in scientific publications, and is apparently less of a problem now due to regression of the incidence of HAT in that area (Ruppol and Burke, 1977). We believe that this remarkable stability of the rate of resistance to melarsoprol over four decades of widespread use can be explained simply by the fact that patients with melarsoprol-resistant HAT rarely, if ever, have parasites in the

bloodstream, and are thus not infective to tsetse; thus any possibly resistant trypanosomes are not passed on to other hosts.

A similar picture emerges for rhodesiense HAT (Table 2). Relapse rates of 10% were reported from Tanzania in 1957 and 5% from Uganda in 1963, the latter rate being identical to that seen in Zambia in the early 1970s and in Kenya a few years ago. These numbers contradict the usual belief that rhodesiense patients experience fewer relapses than their gambiense counterparts (Schneider, 1963; Buyst, 1975).

The degree of resistance to melarsoprol is, however, different between the two subspecies. Patients with rhodesiense trypanosomiasis who relapse after melarsoprol will often respond favorably to a second course of the same drug (Apted, 1957; Ogada, 1974; Wellde *et al.*, 1989); should they relapse again, additional courses are ineffective (Robertson, 1963b; Ogada, 1974). Our experience in Zaire, which was shared by colleagues from other countries four decades ago (Ceccaldi, 1953; Neujean and Evens, 1958), is that patients with gambiense HAT who relapse after a first course of melarsoprol given at the standard dose will rarely be cured by additional courses of melarsoprol. Before the availability of eflornithine, these patients would slowly deteriorate despite up to eight courses of melarsoprol, and would ultimately die from a terminal infectious complication. Absolute resistance to melarsoprol (i.e. persistence of trypanosomes during the initial melarsoprol treatment) is rare and has not been described in recent reports, but most hospitals will not routinely perform repeat CSF and blood examinations for trypanosomes during treatment. Laboratory studies with mice infected with *T. b. rhodesiense* and with flow cytofluorimetry have suggested that resistance to melarsoprol is probably caused by a reduction in the uptake of the drug by the parasite (Frommel and Balber, 1987; Yarlett *et al.*, 1991).

We have recently examined potential risk factors for relapses after melarsoprol treatment among a cohort of more than one thousand patients with Gambian sleeping sickness in Zaire: the most important was the presence of trypanosomes in the CSF (unpublished data). This same subgroup of patients is also more likely to develop reactive encephalopathy during melarsoprol treatment so that there is certainly a trade-off between treatment failures and encephalopathies, depending on the aggressiveness of the therapeutic regimen. Males were twice as likely to relapse as females, maybe because females have a more active humoral immune response and higher IgM levels than males (Brabin and Brabin, 1992), or because men tend to seek medical care later than women. Relapses were no more frequent among patients who had received prednisolone coverage during treatment than among those who had not.

2.4.4. *Toxicity*

Drug-induced encephalopathy is the most important complication of melarsoprol treatment. Two types of encephalopathy, reactive and hemorrhagic,

Table 1 Efficacy and toxicity of melarsoprol in late-stage *T.b. gambiense* infection.

Country	Regimens used	Number treated	Failure rate (%)	Death rate (%)	Encephalopathy rate (%)	Reference
AOF[a]	—	174	7	4	—	Le Rouzic, 1949b
Cameroun	Full doses	394	3	—	—	Friedheim, 1951
AOF[a]	Various regimens	—	11	2.5	—	Jonchère *et al.*, 1953
Zaire	Full doses	294	9	3	—	Neujean and Evens, 1958
Bénin	Various regimens	391	9	4	—	Richet *et al.*, 1959
Burkina Faso	Full doses	1669	6	2	—	Dutertre and Labusquière, 1966
Côte d'Ivoire	Full doses	112	—	10	5	Bertrand *et al.*, 1973
Congo	Full doses	422	4	2	—	Frézil and Coulm, 1977
Congo	Full doses	1809	3	—	—	Ginoux *et al.*, 1984a
Congo	Full doses	363	—	4	4	Ginoux *et al.*, 1984b
Zaire	Full doses	598	5.5	6	8	Pépin *et al.*, 1989a

[a] AOF, Afrique Occidentale Française (French West Africa).

have been described by pathologists and clinicians (Robertson, 1963a; Adams *et al.*, 1986; Haller *et al.*, 1986), but we find this distinction useless, as these terms probably merely describe various degrees of severity of the same condition, and we will use "reactive encephalopathy" to describe both. Clinically, reactive encephalopathy is diagnosed when there is a rapid deterioration of the patient's level of consciousness and/or seizures; this is often followed within hours by pulmonary edema and a fatal outcome in more than half of the cases within 24–48 h. A less severe form is the development of abnormal behavior or of a psychotic reaction during treatment; most of these patients will survive but they are difficult to manage in an understaffed rural hospital. Encephalopathies are characteristically brutal events: we saw several patients in status epilepticus less than an hour after having had a normal conversation with them during the ward round. This danger is well known to all inhabitants of endemic villages, and results in a high level of anxiety when a diagnosis of HAT is made; it also irrationally discourages some persons from participating in active case-detection surveys, as they are afraid of being diagnosed and having to receive melarsoprol. Encephalopathy will usually occur at the end of the first series of injections, during the interval between the first and second series, or during the second series. It is rarely seen during the third series, and almost never thereafter.

In our patients, as in series from the 1950s, the frequency of encephalopathy increased in parallel with the CSF WBC count (Duggan and Hutchinson, 1951; Neujean and Evens, 1958; Pépin *et al.*, 1989a). For patients not receiving prednisolone and having CSF WBC counts of 6–19, 20–99, and $\geqslant 100\ \text{mm}^{-3}$, the frequency of encephalopathy was 7.7, 9.2 and 15.4% respectively. The overall frequency (11.4%) in the control group was higher than the 2–10% usually referred to in the literature. Albeit rarely, encephalopathy can occur and be fatal in patients with normal CSF treated with a single series or even with a single injection of melarsoprol (Richet *et al.*, 1959; Sina *et al.*, 1977), and there is, in our opinion, no justification for giving melarsoprol rather than pentamidine or suramin to this subgroup.

Rates of encephalopathy and of death during melarsoprol treatment reported in various publications are tabulated in Tables 1 and 2. It is difficult to extract precise information from the references cited as it is often unclear whether deaths during treatment were caused by reactive encephalopathy or by sleeping sickness itself, and what was the frequency of non-fatal encephalopathy. Reactive encephalopathy is clearly more common in rhodesiense than in gambiense sleeping sickness and has been seen in up to 18% of patients in some trials from East Africa. Both in gambiense and in rhodesiense patients there is considerable variability in the frequency of reactive encephalopathy, which is probably multifactorial: differences in the distribution of CSF WBC counts of the patients treated in a given center, variations in the treatment schemes, and maybe some genetic susceptibility or variation in the pathogenicity of the local trypanosomes.

Table 2 Efficacy and toxicity of melarsoprol in late-stage *T.b. rhodesiense* infection.

Country	Regimens used	Number treated	Failure rate (%)	Death rate (%)	Encephalopathy rate (%)	Reference
Tanzania	Various regimens	176	10	7	2	Apted, 1957
Burundi	Various regimens	55	—	18	≥18	Adriaenssens, 1960
Uganda	Graded doses	110	5	5	13	Robertson, 1963a, 1963b
Ethiopia	Graded doses	150	—	6	9	Hutchinson, 1971
Zambia	Graded doses	231	5	3	5	Buyst, 1975
Tanzania	Graded doses	109	—	12	18	Veeken *et al.*, 1989
Kenya	Various regimens	269	5	5	1.5	Wellde *et al.*, 1989

Within a given stratum of CSF WBC counts, patients in whose CSF trypanosomes have been found are more likely to develop reactive encephalopathy (Pépin and Milord, 1991). This is one of many arguments suggesting that reactive encephalopathy represents an auto-immune reaction rather than a direct toxic effect of the drug. Other arguments are the histopathological findings (Adams *et al.*, 1986; Haller *et al.*, 1986), the chronology of encephalopathies (if they were a direct effect of the drug, they should be seen more often during the third series than during the first), their prevention by steroids, and the finding that, before the availability of eflornithine, the patients who survived and had to be given additional doses of melarsoprol (to have a reasonable chance of being cured) never experienced a second bout of encephalopathy (Robertson, 1963a; Sina *et al.*, 1977; Pépin *et al.*, 1989a; Veeken *et al.*, 1989). The exact mechanism remains controversial (Jennings *et al.*, 1989; Pépin and Milord, 1991; Hunter *et al.*, 1992), but our epidemiological and clinical data suggest that the encephalopathy is probably triggered by the rapid release of trypanosomal antigens, resulting in either the formation and subsequent deposition of immune complexes or the binding of these antigens to brain cells which then become the targets of antibodies and/or T cells (Pépin and Milord, 1991). Other precipitating factors, reported anecdotally by several authors and which are difficult to relate to an auto-immune mechanism, are physical exertion and the use of alcohol during treatment; clearly patients should be kept in hospital throughout their treatment, even during the intervals between series. Clinically and histopathologically similar reactive encephalopathies were seen when arsenical drugs were used in the treatment of syphilis, but much less frequently (1 per 5000 to 1 per 63 000 injections), and were thought to represent some type of Jarish–Herxheimer reaction rather than direct toxicity of the drug (Call and Gunn, 1949).

In a large randomized trial, the concomitant administration of prednisolone significantly reduced the frequency of reactive encephalopathy in patients receiving melarsoprol for late-stage Gambian trypanosomiasis (Pépin *et al.*, 1989a). Prednisolone reduced the overall frequency of encephalopathy-related deaths from 6.2 to 2.8%. This was achieved by a reduction in the frequency of encephalopathy in every CSF WBC count stratum, and not by a reduction in the severity of encephalopathies, as 57% of episodes resulted in a fatal outcome. Among patients with encephalopathy, prognosis was especially poor for those who were comatose and for those who were febrile. Whether or not these findings can be extrapolated to rhodesiense HAT patients is unknown but, given the similarities between the two subspecies of the parasite and the higher risk of reactive encephalopathy in rhodesiense patients, we believe that they should be given prednisolone, until a study specifically addresses this issue. The only other randomized trial of prednisolone, often quoted by detractors of steroid coverage, involved a total of only 36 patients with rhodesiense HAT, of whom 18 received prednisolone (Foulkes,

1975), and thus had virtually no statistical power to detect even an enormous difference between the two treatment groups.

What can be done when a patient develops an encephalopathy even with prednisolone coverage? BAL (British anti-lewisite, dimercaprol) has been recommended for decades. The impression of most clinicians is that this drug is useless (Robertson, 1963a; Bertrand *et al.*, 1973; Apted, 1980), which is not surprising considering that the pathogenesis of reactive encephalopathy is probably auto-immune rather than a direct toxic effect of the arsenic. In Nioki (Zaire), 10 of 14 patients (71%) with reactive encephalopathy given BAL died compared to 17 of 33 patients (52%) for whom BAL was not available (Pépin and Milord, 1991). The most useful measures are probably high-dose parenteral steroids (intravenous dexamethasone or hydrocortisone) to decrease cerebral edema, and anticonvulsants (phenobarbital, diazepam or phenytoin). If available, hypertonic fluids such as mannitol should also be given to reduce cerebral edema. Subcutaneous adrenalin (1 mg every 2 h for 6 h, then every 4 h until the patient's condition improves) has been recommended by some authors (Sina *et al.*, 1982; Ginoux *et al.*, 1984b), by analogy with what was apparently used (with equivocal results) in patients who developed an encephalopathy during the treatment of syphilis with arsenical drugs (Call and Gunn, 1949). There is no controlled trial, but we gave it to our patients considering its relative lack of toxicity and its low cost. In the survivors, no sequelae are seen.

Polyneuropathy is the other common (10% of patients) and potentially severe adverse effect of melarsoprol (Nkanga *et al.*, 1988; Gherardi *et al.*, 1990). As steroids do not reduce its frequency (Pépin *et al.*, 1989a), it is probably a direct toxic effect of the arsenic, as seen with other heavy metals, and a recent post-mortem study showed that the arsenic is deposited more in the anterior horn of the spinal cord than in the distal peripheral nerves (Gherardi *et al.*, 1990). It manifests firstly as paresthesias with a "stockings and gloves" distribution. If not recognized and if melarsoprol is not withheld, the paresthesias will become more and more proximal and motor weakness will become obvious. Severe cases can end up being quadriplegic for months, until they die of a superinfection. Although there is not, to the best of our knowledge, any study to substantiate it, our impression, shared by other clinicians (Schneider, 1963), is that the administration of thiamine (100 mg three times daily) can significantly improve the symptoms of polyneuritis, presumably because there is some interaction between thiamine deficiency and arsenic in its pathogenesis. If symptoms subside with thiamine, melarsoprol administration can be resumed safely. Tremors are another adverse effect, frightening for the patient, but not predictive of encephalopathy. Tremors can often be improved with a β-blocker.

Febrile reactions with or without an encephalopathy are common and are presumed to represent a Jarish–Herxheimer reaction following the lysis of trypanosomes (Whittle and Pope, 1972). It has been recommended to

administer a single dose of either suramin or pentamidine before initiating melarsoprol in order to prevent this reaction (Marneffe, 1955; Robertson, 1963a; Buyst, 1975); this is widely followed despite the lack of evidence in the literature in support of this contention and the demonstration that febrile reactions are correlated with indications of CSF inflammation and not with parasitemia (Whittle and Pope, 1972). Clearly, this pre-treatment does not reduce the frequency of reactive encephalopathy (Sina *et al.*, 1977). It is important not to attribute the fever to melarsoprol too rapidly, but rather to look for infectious complications, the most common being aspiration pneumonia. Abdominal pains can be disturbing for some patients, but bear no consequence. Cutaneous reactions are more troublesome, though fortunately uncommon (1%), and can improve with steroids if the patient is not already on prednisolone coverage. Renal failure has been reported (Gherardi *et al.*, 1990), but this is essentially without clinical repercussion, and most rural hospitals cannot in any case measure creatinine or blood urea nitrogen levels. Liver toxicity is so rare that its relation with melarsoprol is doubtful. Arsenical enteritis, with life-threatening diarrhea, has been described by some authors (Jonchère *et al.*, 1953; Hutchinson 1971) but we have never seen a case among the 1500 patients that we have treated. Phlebitis is caused by the propylene glycol solvent, but is never important enough to preclude administration of all doses of melarsoprol, if the nursing staff is sufficiently skilled. Extravasation of the drug can cause a chemical cellulitis, which is painful but can be treated with local measures only. Electrocardiogram anomalies can appear or worsen during melarsoprol treatment, but it is unclear if rare cases of "sudden death" during treatment are related to an arrythmia or to a poorly documented rapidly progressive encephalopathy (Fouchet and Gateff, 1968; Buyst, 1975).

The teratogenicity of melarsoprol is unknown, but given the risk of untreated trypanosomiasis for the mother and the risk of congenital trypanosomiasis for the fetus, melarsoprol should not be withheld because of pregnancy. We have administered melarsoprol to several pregnant patients and have not noticed any adverse effect on the newborn.

2.4.5. *Availability and Cost*

Melarsoprol is sold under its trade name Arsobal® by Rhône Poulenc Rhorer Doma in Paris (20, avenue Raymon Aron, Tri 350, 92165 Antony Cedex, France). The current price is approximately FF25 per 5-ml ampoule. Thus a course of nine injections of melarsoprol for an adult will cost approximately US$45. This is very expensive for endemic countries, and international aid agencies will usually cover its cost. It remains much cheaper than a 14-day course of eflornithine.

The production of Mel W (Trimelarsan®), another trivalent arsenical, was discontinued 10 years ago. This drug was useful in children, as it could be

given intramuscularly. However, it was generally considered to be more toxic and less effective than melarsoprol, especially in rhodesiense HAT (Robertson, 1963c; Collomb *et al.*, 1964).

2.5. Eflornithine

2.5.1. *Chemistry and Mode of Action*

Ornithine decarboxylase is the enzyme that catalyzes the conversion of ornithine to putrescine, the first and rate-limiting step in the synthesis of putrescine and of the polyamines spermidine and spermine (Bacchi *et al.*, 1980). Polyamines are essential for the growth and multiplication of all eukaryotic cells; they affect nucleic acid synthesis and contribute to the regulation of protein synthesis (Pegg and McCann, 1982). Eflornithine (α-difluoromethylornithine, also known as DFMO) is a selective and irreversible inhibitor of ornithine decarboxylase which thus decreases putrescine and spermidine concentrations. It is a "suicide inhibitor", being a substrate of its target enzyme (McCann *et al.*, 1986). In animal models, its curative effect against trypanosomes can be reversed by the administration of putrescine, spermidine and spermine (Nathan *et al.*, 1981).

2.5.2. *Pharmacokinetics*

The pharmacokinetics of eflornithine have been well studied in human volunteers (Haegele *et al.*, 1981). After oral doses, peak plasma concentrations were reached on average 4 h after ingestion. The mean half-life was 3.3 h. Renal clearance represented 83% of drug elimination, most of this being unchanged drug. Bioavailability of an orally administered 10 mg kg^{-1} dose was estimated at 54%. Similar results were obtained in pharmacokinetic studies in AIDS patients with PCP (Sahai and Berry, 1989).

An earlier report on three patients demonstrated that eflornithine had reasonable CNS penetration (Taelman *et al.*, 1987). In a more extensive study of 63 patients in Zaire, the mean CSF/plasma ratio of eflornithine measured at the end of a 14-day course of intravenous administration was 0.91 in adults and 0.58 in children less than 12 years of age (Milord *et al.*, 1993). This excellent CSF penetration probably contributes to a large extent to the efficacy of eflornithine, whereas melarsoprol's efficacy is explained by its highly trypanocidal properties which more than compensate for its poor CSF penetration. In this trial from Zaire, children less than 12 years old had a mean steady state serum eflornithine concentration half that of adults given the same 400 mg kg^{-1} daily dose, and only one-third the CSF level of adults. This can probably be explained mostly by children having a higher renal clearance of the drug than adults, and militates in favor of children

receiving a higher dose per kilogram of body weight than adults, or of calculating their daily dose using body surface area as is done with numerous other drugs. Relapses following eflornithine occurred mostly among patients whose CSF eflornithine levels at the end of the 14-day treatment were around or below 50 nmol ml^{-1}. Interestingly, CSF eflornithine levels and CSF/serum ratios were higher among arsenical refractory cases than among newly diagnosed cases, presumably because chronic trypanosomal meningo-encephalitis results in a severely impaired blood–brain barrier (Milord *et al.*, 1993).

2.5.3. *Efficacy*

The first field trials reported in the mid-1980s already showed that eflornithine was a very efficient treatment of late-stage gambiense trypanosomiasis in several locations in West and Central Africa (Van Nieuwenhove *et al.*, 1985; Doua *et al.*, 1987; Pépin *et al.*, 1987; Taelman *et al.*, 1987; Kazyumba *et al.*, 1988; Eozenou *et al.*, 1989). It was initially used in patients with arseno-resistant HAT, for whom eflornithine was an almost miraculous development, similar to that which melarsoprol represented in the late 1940s. These first trials also showed that its toxicity, discussed below, was acceptable. Shortly thereafter, eflornithine was given also to new cases.

We recently published the results of a large open trial, during which various regimens of eflornithine were given to 100 patients with arseno-resistant HAT and to 107 new cases (Milord *et al.*, 1992). During treatment, trypanosomes disappeared from the CSF of all 87 patients in whom they had been found before eflornithine, and the mean CSF WBC count dropped sharply from 186 mm^{-3} pre-treatment to 21 mm^{-3} at the end of treatment. More importantly, this study confirmed the remarkable long-term efficacy of eflornithine in gambiense trypanosomiasis, as only 9% of patients were subsequently found to relapse. Treatment failures were more common among new cases than among patients given eflornithine for a post-melarsoprol relapse, which was presumably related to higher CSF eflornithine levels in the latter group. Clearly, there was no cross-resistance between melarsoprol and eflornithine, in contrast to what had been suggested by animal studies (Bacchi *et al.*, 1990).

As with melarsoprol, relapses were more common among patients with a pre-treatment CSF WBC count higher than 100 mm^{-3}. Relapses were also more common among patients who were given eflornithine only orally, not an unexpected finding given the 54% bioavailability of orally administered eflornithine (Haegele *et al.*, 1981) and the impossibility of further increasing the dose because of the osmotic diarrhea it induces. Relapses were more common among children less than 12 years old than among adults; although this was partially confounded by the mode of administration (more children received oral eflornithine), this finding probably indicates that children should be given doses of intravenous eflornithine higher than the 400 mg

kg^{-1} day^{-1} used in adults, as they have lower serum and CSF eflornithine levels (Milord *et al.*, 1993). The standard treatment regimen that emerged from this and other trials is 100 mg kg^{-1} intravenously every 6 h for 14 days. Giving 200 mg kg^{-1} every 12 h for the same duration seems less effective (Milord *et al.*, 1992), but the data are not totally convincing. Adding oral eflornithine for 3 weeks after the initial intravenous phase is probably useless, according to data from several treatment centers available to the manufacturer. This is not surprising considering that after 14 days of intravenous eflornithine, CSF inflammation has subsided considerably (Pépin *et al.*, 1987; Milord *et al.*, 1992) and thus CSF eflornithine levels obtained during oral administration must be very low. Oral eflornithine should be used only in situations where venous access is problematic, such as congenital trypanosomiasis (Pépin *et al.*, 1989c). It should be pointed out that the logistics of eflornithine administration are difficult for rural hospitals dealing with a large number of cases. The drug has to be given intravenously every 6 h even at night; a typical 60-kg patient will require not only 168 vials for a total of 0.34 kg of eflornithine (2 g per vial) but also many liters of intravenous fluids to dilute the drug.

The minimum duration of treatment necessary for a cure is unknown. Animal studies have suggested that at least 14 days are needed (Bacchi *et al.*, 1987), but their relevance to human trypanosomiasis is debatable, as in murine *T. b. brucei* infection eflornithine monotherapy is not very effective. Trials are currently being initiated to evaluate the efficacy of a 7-day regimen.

Eflornithine is probably a suboptimal treatment for trypanosomiasis patients who happen to be infected with HIV. Of the four HIV-seropositive patients that we treated with eflornithine, two subsequently relapsed, one died shortly after treatment, and the fourth died at home 2 years after eflornithine, probably of AIDS (Milord *et al.*, 1992; Pépin *et al.*, 1992b). These poor results are not unexpected considering that eflornithine is trypanostatic rather than trypanocidal, and that animal studies have shown that a normal immune response is necessary to achieve a cure (DeGee *et al.*, 1983; Bitonti *et al.*, 1986b). Obviously the number of observations is small but, until more information is available, we would treat HAT patients known to be infected with HIV with melarsoprol rather than eflornithine. However, we do not recommend systematic HIV testing of HAT patients, as sleeping sickness remains essentially a health problem of rural communities where the prevalence of HIV is, at the present time, rather low.

There has been only one published trial of eflornithine in arseno-resistant *T. b. rhodesiense* trypanosomiasis, in Kenya. All three patients given 400 mg kg^{-1} day^{-1} subsequently relapsed (Bales *et al.*, 1989). Similar results have been seen in a dozen more patients elsewhere (Schechter and Sjoerdsma, 1987). More recently, the World Health Organization (WHO) has tried to organize a trial with 800 mg kg^{-1} day^{-1} but very few patients have been enrolled.

Eflornithine has also been found to be effective in AIDS patients with PCP who do not respond, or have severe adverse reactions, to trimethoprim-sulfamethoxazole (Sahai and Berry, 1989; Paulson *et al.*, 1992), but who are not mechanically ventilated upon initiation of eflornithine. Again the drug seems to be acting slowly and the survival rate in patients who received less than 14 days' treatment was poor. A comparative study of eflornithine vs. cotrimoxazole as first-line therapy of PCP had to be stopped prematurely because of poor results in the eflornithine arm (Smith *et al.*, 1992); it seems unlikely that further trials will be organized unless eflornithine is used in combination with another antimicrobial drug.

2.5.4. *Toxicity*

Our earlier trial suggested significant toxicity, with 5 of 26 patients dying during treatment (Pépin *et al.*, 1987). However, in retrospect, this was probably related more to the desperate condition of several of these patients than to inherent toxicity of the drug. In the larger trial, only 4 of 207 (2%) patients died during or shortly after treatment; three of them were children less than 2 years of age and the last one was a woman whose level of consciousness deteriorated during treatment and who was infected with HIV (Milord *et al.*, 1992).

The most frequent adverse effect associated with eflornithine is bone marrow suppression. Half of the patients will develop leucopenia, but which is not severe enough to result in opportunistic infections. More than 40% of patients will also develop anemia, which is fortunately reversible and rarely severe enough to require blood transfusions (Milord *et al.*, 1992). Thrombocytopenia could not be documented in our hospital, but has been seen frequently among AIDS patients with PCP (Sahai and Berry, 1989; Paulson *et al.*, 1992).

Convulsions were seen in nine of our patients (4%), most of them arseno-resistant cases. These are absolutely different from convulsions seen in the course of a melarsoprol-induced encephalopathy. Generalized seizures occurred shortly after the first or second dose of eflornithine, with a post-ictal stupor lasting at most a few hours. Eflornithine was resumed the next day, usually without recurrence of convulsions. Patients with convulsions had higher CSF eflornithine levels than other patients (Milord *et al.*, 1993), and seizures probably represent a direct toxic effect of the drug rather than an auto-immune reaction. Higher frequencies (8–18%) of convulsions have been reported in other series (Schechter and Sjoerdsma, 1987; Kazyumba *et al.*, 1988). It seems from currently available literature that seizures have not been reported among patients given eflornithine for PCP. This could suggest that some kind of interaction between eflornithine and the parasite is involved in the pathogenesis of seizures, but a more likely explanation is that, because of the CSF inflammation, HAT patients end up having much higher CSF

concentrations of eflornithine than PCP patients. Other neurological adverse effects were rare in our series (one patient each): logorrhea, auditory hallucinations, insomnia, psychosis. Half of the patients given oral eflornithine will develop osmotic diarrhea, which will subside rapidly upon cessation of the drug. Alopecia and hearing loss are rare.

Eflornithine has contragestational effects in rodents: it is abortive in early pregnancy and organ-specific developmental deficits have been noted when it is administered during the later stages of gestation (Fozard, 1987). Our experience in HAT is limited: one woman aborted at 2 months, another one delivered at 9 months just after the first dose of eflornithine and a third, who was treated during the eighth month of pregnancy, delivered normally 1 month later. Although melarsoprol may be safer for the fetus, its use will expose the mother to the risk of reactive encephalopathy.

2.5.5. *Availability and Cost*

Eflornithine is currently sold by Marion-Merrell-Dow under the trade name Ornidyl®. A 2-week course of intravenous eflornithine costs approximately US$150. The future of the drug is not clear, as Marion-Merrell-Dow has stated that it is not interested in making it available after 1993. WHO is trying to find another manufacturer. Obviously all this could change should other indications for eflornithine emerge but this seems unlikely at the present time since the results of studies in patients with AIDS-related PCP are not encouraging.

2.6. Nifurtimox

2.6.1. *Chemistry and Mode of Action*

Nifurtimox is a 5-nitrofuran that has been used since the mid-1970s in the treatment of American trypanosomiasis (Chagas disease) (Brener, 1979). It inhibits trypanothione reductase, resulting in the production of superoxide and peroxide, trypanothione being the major polyamine-containing analog of glutathione in trypanosomes, which acts as a cellular protectant against free radicals (Fairlamb *et al.*, 1985; Docampo and Moreno, 1986; Fairlamb, 1990a). Nifurtimox may also cause DNA breaks.

2.6.2. *Pharmacokinetics*

Initial pharmacokinetic studies showed that, after oral administration, serum levels of nifurtimox remained very low and peaked after 1–3 h, followed by a rapid decline so that no nifurtimox was detected after 24 h (Medenwald *et al.*, 1972). This suggested either that nifurtimox is poorly absorbed or that it

undergoes extensive metabolism. A more recent study confirmed these findings. Among seven healthy volunteers given a single dose of 15 mg kg^{-1}, peak levels were seen on average 3.5 h (range 2.25–5.5 h) after ingestion. The mean elimination half-life was 2.95 h (range 2.0–5.4 h) (Paulos *et al.*, 1989). Again, serum concentrations of nifurtimox were low, possibly as a consequence of an important pre-systemic first-pass effect. The drug is almost completely metabolized by the liver, and this raises the question of the antiparasitic effects of the metabolites. Only 0.5% of the drug is excreted unchanged in the urine. Because of its short half-life, nifurtimox is usually administered every 6–8 h.

2.6.3. *Efficacy*

There have been few trials of nifurtimox in patients infected with *T. b. gambiense* and none in those with *T. b. rhodesiense* (Janssens and De Muynck, 1977; Moens *et al.*, 1984; Pépin *et al.*, 1989b, 1992a). In gambiense trypanosomiasis, results have been inconsistent. In southern Sudan, cure rates of 80% (among a large group of 115 patients) have been reported in an abstract but never published, so that it is impossible to evaluate the quality of the post-treatment follow-up (Van Nieuwenhove and Declerq, 1989). In north-west Zaire, 12 of 15 patients treated with 12.5–15 mg kg^{-1} for 60 days were apparently cured, but the CSF IgM levels following treatment were abnormal and progressively rising in all nine patients for whom this assay was performed, suggesting that they may have relapsed later (Moens *et al.*, 1984). Despite clear evidence of antitrypanosomal activity (disappearance of trypanosomes from the CSF and decrease of the CSF WBC count during nifurtimox administration), long-term results were much less encouraging in two trials in central Zaire, approximately 800 km from the site of the other trial. Using 15 mg kg^{-1} for 60 days in arseno-resistant cases, 13 of 19 patients (68%) with at least one follow-up visit relapsed within the following year, as did 9 of 25 patients (36%) given 30 mg kg^{-1} day^{-1} for 30 days (Pépin *et al.*, 1989b, 1992a). We are aware of two other trials made in Zaire with comparable if not higher failure rates, the results of which have not been published (H. Bruneel, personal communication; G.L. Kazyumba, personal communication).

We are not sure of the explanation for these discrepant results between Zaire and Sudan. As mentioned, completeness of follow-up could be involved, as well as the frequency and severity of CNS involvement among study patients, since there are no data on the CSF penetration of nifurtimox. It may very well be that there are regional variations in susceptibility of *T. b. gambiense* to nifurtimox. This is well described for *T. cruzi* (Brener, 1979; Gutteridge, 1985), and is apparently related to the fact that strains from a given geographical area usually belong to a single type, as defined by their isoenzymes patterns, and that type III strains are highly resistant to nifurtimox

(and to benznidazole) (Andrade *et al.*, 1985; Filardi and Brener, 1987). This variability in susceptibility to nifurtimox was also seen in animals experimentally infected with *T. cruzi* (Haberkorn and Gonnert, 1972). Until further trials are done in other parts of Africa, it seems obvious that, considering its modest efficacy and substantial toxicity, nifurtimox should be used only in patients who have relapsed after both melarsoprol and eflornithine, or for arseno-resistant cases when eflornithine is not available.

It is worth mentioning that three other nitrofurans have been experimented with in the treatment of HAT in the 1960s and the 1970s: nitrofurazone, furaltadone, and levo-furaltadone (Evens *et al.*, 1957; Apted, 1960; Adriaenssens, 1962a, b; Ruppol and Burke, 1977). None of them has survived these preliminary trials, being too toxic (polyneuropathy and other neurological adverse effects, hemolytic anemia in subjects with glucose-6-phosphate dehydrogenase deficiency, myocardial depression, intractable vomiting) and only moderately effective (cure rates of 30–50%).

2.6.4. *Toxicity*

Most patients given nifurtimox will be anorexic and will experience significant weight loss. More worrying is the occurrence of neurological adverse effects. A cerebellar syndrome (ataxia, nystagmus, tremor and adiadococinesia) was seen in several of our patients, which was fortunately reversible upon cessation of nifurtimox. Seizures, coma, confusion, and choreiform movements were also seen less frequently (Pépin *et al.*, 1989b, 1992a). These neurological symptoms were more common with the 30 mg kg^{-1} daily for 30 days regimen than with 15 mg kg^{-1} daily for 60 days. The symptoms probably represent a direct toxic effect of the drug rather than an auto-immune reaction triggered by the release of trypanosomal antigens; they were not attenuated by the empirical administration of prednisolone, they have been described in patients with Chagas disease (less frequently, presumably because the standard doses are lower, 8–10 mg kg^{-1} for adults) (Castro and Diaz de Toranzo, 1988), and animal studies have also clearly shown that rats and dogs given high doses of nifurtimox (60–400 mg kg^{-1}) develop central nervous system toxicity (Hoffmann, 1972). Jaundice, nausea, vomiting, rash and fever have also been noted in a few patients. We have not seen polyneuropathy, but this has been reported in patients with Chagas disease, as have chromosomal aberrations in children given nifurtimox (Gorla *et al.*, 1989).

2.6.5. *Availability and Cost*

Nifurtimox is very difficult to obtain outside countries endemic for Chagas disease. It is produced by Bayer, and sold under the trade names Lampit®

or Bayer 2502®. It has to be ordered from Bayer Argentina (Bayer Argentina SACIFI y de M, Division Farma, Departemento Medico, Gral. Rivas 2466, 1417 Buenos Aires, Argentina), as European and American offices of the same company do not keep the drug in stock.

3. THE TREATMENT

3.1. *T. b. Gambiense*: Early-stage

For patients infected with *T. b. gambiense* and considered to be in early stage (i.e. no trypanosomes in the CSF, CSF WBC count less than 6 WBC mm^{-3}, no somnolence), we prefer the combination of suramin and pentamidine, because it has little toxicity and is probably more effective than either drug alone. Pentamidine isethionate (4 mg kg^{-1} base) is given intramuscularly on days 1, 3, 5, 13, 15 and 17 and suramin (20 mg kg^{-1}, up to a maximum of 1 g) is given intravenously on days 1 and 13.

Our second choice would be intramuscular pentamidine alone at 4 mg kg^{-1} every alternate day for a total of seven injections; this is especially interesting considering that, at the present time, national control programs can obtain pentamidine free of charge. An attractive alternative is diminazene 7 mg kg^{-1} every alternate day for three injections. Should large and carefully executed studies show this regimen to be as effective, and to have as little toxicity, as some authors claim, it could become the first choice, because of its low price and short duration. Finally, we would consider suramin monotherapy as a fourth choice, using the dosing schedule outlined later for Rhodesian HAT (see Section 3.4.). It is more expensive than pentamidine, more toxic and, probably, less effective.

We do not recommend using melarsoprol for these patients, as reactive encephalopathy can occur, albeit rarely, and we see no justification for exposing them to this risk. Of course eflornithine would be effective but, given its high cost, it should be reserved for late-stage patients, especially those who are resistant to melarsoprol.

3.2. *T. b. Gambiense*: Late-stage

If cost is not a problem, eflornithine (100 mg kg^{-1} intravenously every 6 h for 14 days) should be the first choice. It is as active and certainly less toxic than melarsoprol. Higher doses (i.e. 150 mg kg^{-1} every 6 h) should be given to children. Taking into consideration data available so far on toxicity and efficacy, it may be reasonable to give to new cases slightly higher doses (125 mg kg^{-1} every 6 h) than those given to cases relapsing after melarsoprol.

This having been said, the vast majority of hospitals on the African continent treating cases of HAT will not be able to afford eflornithine at US$ 150 per patient. For this reason, melarsoprol will remain the first line treatment for most patients with late-stage gambiense trypanosomiasis. Pre-treatment preparation should include deworming (mebendazole), antimalarials (e.g. chloroquine for 3 days) and a single dose of pentamidine or suramin 24–48 h before the first injection of melarsoprol. Examination of the stools should be performed to rule out concomitant strongyloidiasis or amebiasis, which should be treated before steroids are administered. French authors recommend nutritional support and multivitamins before treatment. We see no reason for giving vitamins, except maybe thiamine, hoping that this will reduce the frequency of melarsoprol-induced polyneuropathy. Certainly in many African hospitals it is not possible to give even normal meals to patients and HAT patients and their families will have to provide their own food, so we certainly would not delay treatment for nutritional reasons. Prednisolone should be given to all patients receiving melarsoprol, starting on the day of admission (24 h before the pre-treatment with suramin or pentamidine), at 40 mg daily (1 mg kg^{-1} daily for children). This should be continued throughout treatment and rapidly tapered over 3 days (30 mg, 20 mg, 10 mg) after the last injection of melarsoprol. We would also give an antihistaminic (promethazine or diphenhydramine, 50 mg daily) on the same days as melarsoprol: we are not sure that this reduces the frequency of allergic reactions, but at least it sedates the patient who is normally rather nervous when receiving melarsoprol. Patients should remain in hospital throughout the treatment, including the 1-week intervals between series.

We do not think there is any advantage in giving more than nine injections of melarsoprol. We would give 2 series of 3 injections to patients with 6–20 WBC mm^{-3} in the CSF, and 3 series of 3 injections to those with more than 20 WBC mm^{-3}. The standard maximum daily dose used to be 3.6 mg kg^{-1} up to 200 mg, which represented 5.6 ml. Now that melarsoprol is supplied in ampoules of 5 ml, we would use 5 ml (180 mg) as the maximum daily dose. There has been no controlled trial comparing graded dosing to regimens starting directly with the maximum dose. As mentioned earlier, there is probably a trade-off between a higher risk of encephalopathy (with the full dose on the first injection) and a higher risk of relapse (with graded doses). Now that, with eflornithine, there is an excellent second-line treatment for patients who relapse after melarsoprol, we feel that graded doses should be used. Given the lack of pharmacokinetic data and of comparative trials, it is difficult to choose between the various regimens of graded dosing. We believe it is more logical to increase the dose progressively from the first to the second to the third series, rather than to increase it from the first through the third injection of each series, as the penetration of melarsoprol in the CSF presumably decreases over time in parallel with the decreasing CSF inflammation. We would thus give 1.8 mg kg^{-1} day for the first series, and

3.6 mg kg^{-1} day^{-1} for the second and third. We would retain the traditional 1-week interval between series.

3.3. *T. b. Gambiense*: Relapses

Patients who relapse following treatment with pentamidine or suramin, or both, should be treated with either eflornithine or melarsoprol. The failure rate with the latter two drugs will not be higher than when they are used as the first treatment. Patients who relapse following melarsoprol should be treated with eflornithine and vice versa. For the rare patient who relapses twice, that is after having received both melarsoprol and eflornithine, there are at least two options: firstly, a combination of melarsoprol (12 injections of 3.6 mg kg^{-1}) with high-dose eflornithine (800 mg kg^{-1} day^{-1} for 14 days) and secondly nifurtimox (25–30 mg kg^{-1} day^{-1} for 30 days), alone or in combination with melarsoprol.

3.4. *T. b. Rhodesiense*: Early-stage

Apted (1980) recommended the following course: after a test dose of 200 mg, suramin should be given intravenously as 1 g injections on days 1, 3, 6, 14 and 21. This is similar to the schedule recommended by WHO (1986): 5 mg kg^{-1} on day 1, 10 mg kg^{-1} on day 3, and 20 mg kg^{-1} on days 5, 11, 17, 23 and 30. Given the very long half-life of suramin, it is not necessary to give more than five injections at the full dose. Pentamidine should not be used as it is less effective than suramin, and melarsoprol should be avoided with these patients as it may induce reactive encephalopathy.

3.5. *T. b. Rhodesiense*: Late-stage

Given the poor response of *T.b. rhodesiense* patients to eflornithine, there is no other option here than melarsoprol. As with *T.b. gambiense* patients, we recommend prednisolone coverage. Pre-treatment with suramin should also be given empirically. Given the higher frequency of encephalopathy, we think that an even more gradual increase in dosing should be used. In the absence of comparative trials, we would follow the recommendations of Apted (1980), who had considerable experience, as follows. (i) After pre-treatment with suramin (with 5, 10 and 20 mg kg^{-1} on days 1, 3 and 5), give melarsoprol in four series: 0.36 mg kg^{-1}, 0.72 mg kg^{-1} and 1.1 mg kg^{-1} on days 7, 8 and 9; 1.8 mg kg^{-1} on days 16, 17 and 18; 2.2, 2.9 and 3.6 mg kg^{-1} on days 25, 26 and 27; 3.6 mg kg^{-1} on days 34, 35 and 36 (maximum daily dose = 5 ml or 180 mg). (ii) For patients in good general condition, a more rapid schedule

can be used: after pre-treatment with suramin (5 mg kg^{-1} on day 1 and 10 mg kg^{-1} on day 3), give melarsoprol in three series: 1.4, 1.8, 2.2 mg kg^{-1} on days 5,6 and 7; 2.5, 2.9 and 3.3 mg kg^{-1} on days 14, 15 and 16; 3.6 mg kg^{-1} on days 23, 24 and 25 (maximum daily dose = 5 ml or 180 mg).

3.6. *T. b. Rhodesiense*: Relapses

Patients who relapse after melarsoprol should be given a second course of melarsoprol. Since these strains are obviously less susceptible to melarsoprol and since reactive encephalopathy is probably an auto-immune reaction triggered by the release of trypanosomal antigens, these patients should be given the maximum dose at the first injection, to maximize their chances of cure. We would also give them the maximum number of injections, that is, 3 series of 4 injections or 4 series of 3 injections.

Patients who relapse after a second course of melarsoprol are in trouble. Totally empirically, we would give them a third course of maximum doses of melarsoprol (12 injections of 3.6 mg kg^{-1}), this time combined with 14 days of double-dose eflornithine (200 mg kg^{-1} every 6 h). Those who relapse again could be offered nifurtimox with melarsoprol, but this would be purely experimental. Nitrofurazone and levofuraltadone, which, albeit very toxic, resulted in the cure of 30–50% of these patients, have never been commercially produced and are not available.

4. THE FUTURE

4.1. New Drugs

Apted wrote in 1980: "When it is considered that suramin has been in use for the treatment of HAT since 1920, pentamidine since 1941 and melarsoprol since 1949, there is remarkably little firm evidence that resistance has developed to any of these first-line drugs". That remains true in 1993, and it is a blessing considering that only one new drug (eflornithine) has appeared, that the nitrofurans including nifurtimox have proved inadequate, that diminazene remains in theory a veterinary product only, and that the production of Mel W has been stopped.

The sleeping sickness market for drug companies is negligible in comparison with the tremendous cost of developing a new drug for human use. Eflornithine was developed mostly by chance, as its manufacturer first hoped that it would be effective as an anticancer drug, and later thought that it might be useful in PCP. Marion-Merrell-Dow has apparently several other inhibitors of polyamines synthesis (such as α-monofluoromethyldehydroornithine methyl

ester [Bacchi *et al.*, 1987] and MDL73811, an inhibitor of S-adenosylmethionine decarboxylase [Bitonti *et al.*, 1990; Byers *et al.*, 1991] which are more active than eflornithine against trypanosomes, but will not be studied any further and, as mentioned earlier, diminazene was never approved for human use for the same commercial reasons.

We are much impressed by recent attempts at rational design of new trypanocidal drugs and we hope that such efforts, using the extraordinary tools of modern molecular biology, will one day give us the perfect trypanocidal drug: very effective, non-toxic, cheap, well absorbed orally, and with good CSF penetration. But researchers interested in trypanosomiasis should be realistic: the only way that a new drug could be approved for human trypanosomiasis in the next 10 or 20 years is for this drug to have activity against other parasites causing diseases with a better commercial potential. Chagas disease, with 10–20 million persons infected in countries richer than those endemic for HAT, is the first one that comes to our minds, as there is not yet any active drug in the chronic phase of *T. cruzi* infection. PCP, a major public health problem nowadays in most industrialized countries, is the second obvious area of interest: it certainly is remarkable that eflornithine and pentamidine are active drugs in both pneumocystosis and African trypanosomiasis and that, in an animal model of PCP, diminazene and several other veterinary trypanocidal drugs were the only antiparasitic drugs with good efficacy (Walzer *et al.*, 1988), suggesting that these two protozoa share a number of metabolic pathways.

The screening in animal models of new drugs developed for other protozoal infections may thus be more profitable. For instance, benznidazole, a drug developed for Chagas disease, has shown some activity in the *T. b. brucei* murine model (Zweygarth *et al.*, 1990), maybe not enough to be useful in monotherapy but it has a potential for synergism with eflornithine and this certainly warrants additional investigations. Trimethoprim-sulfamethoxazole, an antibiotic useful in PCP, but also in other protozoal infections (toxoplasmosis, malaria, isosporiasis), was unfortunately found to have no activity against *T. b. brucei* in a murine model (F.W. Jennings, personal communication). Atovaquone, a naphthoquinone (566C80), has recently been found to be an efficient treatment of PCP, toxoplasmosis and malaria; it is not toxic, is well absorbed after oral administration, and penetrates the CSF (Falloon *et al.*, 1991; Kovacs *et al.*, 1992); certainly it is worth trying on a few dozen mice infected with *T. b. brucei*. Novel analogs of pentamidine are being tested in animal models of PCP (Jones *et al.*, 1990; Tidwell *et al.*, 1990a, b) and, should one of these reach the stage of human trials, it should also be tested in the murine model of trypanosomiasis.

4.2. Better Use of Currently Available Drugs

As it is clear that currently available drugs will be around for decades, some effort should be made to improve their use, using modern epidemiological

concepts and new laboratory tools allowing a better understanding of their pharmacokinetics.

Melarsoprol regimens were empirically developed more than 40 years ago, and have remained unchanged ever since. A randomized trial comparing various methods of graded dosing, and including for gambiense trypanosomiasis an arm with the standard full doses of melarsoprol, should be a priority. Given the presumably better CSF penetration of melarsoprol at the beginning of treatment, it seems logical to start with lower doses in the first series, and to increase the daily dose during the second and third series, in order to achieve the same CSF melarsoprol levels. Another option would be to give much lower daily doses of melarsoprol, but every day for 2–4 weeks, without any drug-free interval. The minimal active concentration of melarsoprol should be determined as precisely as possible by studies *in vitro* and in animals, and pharmacokinetic studies should be performed to help define which regimens could result in CSF melarsoprol levels remaining above this threshold for long enough. Carefully designed and executed clinical trials must then be performed to confirm these deductions. Any reduction in the total dose of melarsoprol administered would not only decrease toxicity but also the cost, allowing more resources to be spent on case-finding and vector control.

Thirty years after its development for veterinary use, we believe that the efficacy and toxicity of diminazene should be precisely documented by studies on a large number of patients receiving close attention during and after treatment and having laboratory tests performed regularly. Diminazene is cheap, widely available, probably better tolerated than pentamidine, and possibly more effective. It seems obvious that priority should be given to this easy task rather than to the study of experimental drugs which are years and tens of millions of dollars away from human use.

The widespread use of pentamidine because of the AIDS epidemic has generated new knowledge on its pharmacokinetics. The CSF penetration of pentamidine, albeit poor, has been documented recently (Bronner *et al.*, 1991), and is in line with trials made 50 years ago which showed that pentamidine could cure 50–80% of patients with a CSF WBC count higher than 20 mm^{-3} (Lourie, 1942; Duggan and Hutchinson, 1951). This information should be used to design new regimens that could give a good cure rate for patients with mild anomalies of the CSF, thus avoiding for this subgroup the toxicity and cost of melarsoprol. Should reasonable CSF levels of pentamidine be obtained with new regimens, one wonders if synergism could be obtained by combining pentamidine with low-dose melarsoprol.

Obviously some additional work needs to be done with eflornithine. The minimum duration of treatment necessary for a cure is unknown. A multicenter trial comparing a 7-day course of eflornithine with the standard 14-day regimen is currently being organized by the manufacturer and WHO. We agree that this is a reasonable thing to do, but are afraid that a 20–25% failure

rate might be seen with the 7-day arm because, so far, any variation from the 14-day regimen of 100 mg kg^{-1} every 6 h has resulted in higher rates of treatment failure: changing to 200 mg kg^{-1} every 12 h, giving the drug to new cases rather than arseno-resistant patients, giving it to children, giving it to HIV-infected subjects. If this is so, a short course of a higher dose (600–800 mg kg^{-1} day^{-1}) of eflornithine could be studied, but this regimen would be almost as expensive as the current 14-day course and we believe that combination therapy is much more promising.

4.3. Combination of Drugs

The better understanding of the role of trypanothione in trypanosomes has stimulated investigations of combinations of drugs. Trypanothione, a spermidine–glutathione conjuguate (N1,N8-bis[glutathionyl]spermidine), represents over 80% of glutathione in trypanosomes (glutathione is essential in maintaining cellular resistance to oxidant stress) and is thought to be unique to Trypanosomatidae (Fairlamb *et al.*, 1985; Fairlamb, 1990a, b). It is the target of arsenical drugs such as melarsoprol, to which it binds irreversibly to form a compound called Mel T, blocking the hydrogen acceptor sites. Ornithine decarboxylase inhibitors such as eflornithine are effective at least partially through a decrease in the production of spermidine, and thus of trypanothione. Pentamidine and diminazene also reduce the synthesis of trypanosomal polyamines, through the inhibition of S-adenosylmethionine decarboxylase (Bitonti *et al.*, 1986a). Nifurtimox is an inhibitor of trypanothione reductase, an enzyme that normally reduces trypanothione disulphide.

Several combinations of drugs have been shown to have synergistic activity against *T. b. brucei* in murine models. Of all these, the combination of eflornithine and melarsoprol is the most attractive, as these two drugs are effective in monotherapy even in patients with late-stage disease. The combination of a short course of eflornithine and a few days of low-dose melarsoprol could have many advantages: cheaper and as effective as the 14-day course of eflornithine, less toxic than the standard course of melarsoprol. According to Jennings (1988a, b, 1990), who demonstrated synergism in the mouse model, it may be preferable to give the melarsoprol at the end of the eflornithine course, when the polyamines have already been depleted and when small quantities of melarsoprol will be sufficient to bind the remaining trypanothione. Such a trial should be given the utmost priority should the currently planned trial with short courses of eflornithine monotherapy show an unacceptable failure rate. Combinations of nifurtimox and melarsoprol could also be attempted in melarsoprol-resistant rhodesiense sleeping sickness, as it was demonstrated 15 years ago that other nitrofurans gave better results when melarsoprol was administered concomitantly even in melarsoprol-resistant cases (Ruppol and Burke, 1977).

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Plasmodium Species Infecting *Thamnomys rutilans*: a Zoological Study

Irène Landau and Alain Chabaud

Biologie Parasitaire, Muséum National d'Histoire Naturelle, 61 Rue Buffon, 75231 Paris cedex 05, France

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1. INTRODUCTION

Most of our knowledge of malaria, and of many human diseases, derived from the discovery and use of cheap, easily bred, animal models.

With malaria, two species of *Plasmodium* have provided most of the information: *P. gallinaceum*, described by Brumpt in 1935, adapted to chickens and transmitted through *Aedes aegypti* and, later, *P. berghei*, described by Vincke and Lips in 1948, adapted to white mice and rats; its complete experimental life cycle, through *Anopheles quadrimaculatus*, was elucidated by Yoeli *et al.* (1964) and Yoeli and Most (1965).

The years between 1935 and 1980 were filled with extensive work and important discoveries: exoerythrocytic meronts* in birds (Raffaele, 1936; James and Tate, 1937) and in mammals (Shortt and Garnham, 1948; Shortt *et al.*, 1951); amongst much other work were the numerous publications on bird malaria by Corradetti and co-workers (Corradetti *et al.*, 1963; Corradetti, 1937, 1955) and Huff (see review by Huff, 1963) and on the circadian rhythms of merogony and gametocyte infectivity by Hawking *et al.* (e.g. 1968, 1972).

During the last decade, important developments in immunology and molecular and cell biology led to the almost complete abandonment of this type of research, with some undesirable consequences because natural infections may differ considerably from the behaviour of parasites in culture and in experimental hosts such as, in the case of rodent malaria, the white mouse — as will become apparent later in this review.

It is important to investigate thoroughly the biology of each species and subspecies, because the biological and metabolic characteristics of the various stages may differ from one parasite to another.

The present review deals with the biology of the species of *Plasmodium* which infect the murid rodent *Thamnomys rutilans* in the Central African Republic (CAR; République Centrafricaine), which were first discovered in 1965 (Landau and Chabaud, 1965).

2. BIOGEOGRAPHY AND SYSTEMATICS

2.1. The Discovery of the Murid Malaria Foci

The first species of rodent *Plasmodium* transmissible to laboratory rodents was discovered in Katanga (in the then Belgian Congo, now Zaire), where

* Throughout this review, the terms meront and merogony have been used instead of schizont and schizogony.

Vincke (1946a, b) found sporozoites in the salivary glands of *An. dureni millecampsi*, a mosquito feeding mainly on rodents.

Vincke and Lips (1948) captured two *Grammomys surdaster*, an arboreal rodent living in the forest galleries around Elizabethville, parasitized by a species of *Plasmodium*. Blood passaged to white rats and mice produced an infection, and the parasite thus isolated was named *P. berghei*.

Following these results, Vincke systematically inoculated white mice with crushed salivary glands from *An. dureni millecampsi* and succeeded in isolating several strains. Finally, Vincke (1954) subinoculated blood from various wild rodents to mice and thus demonstrated the infection in *Grammomys surdaster*, *Praomys jacksoni* and *Leggada bella*.

In 1952 Rodhain described a second species, *P. vinckei*, isolated from the salivary glands of the same species of mosquito.

Bruce-Chwatt and Gibson (1955) inoculated to white mice pools of blood from 2500 rodents of many species trapped in Nigeria, and found in one mouse, inoculated with blood from a *Praomys tulbergi*, a parasite closely related to *P. vinckei*. Unfortunately the strain was lost, and the finding was considered to be an exceptional occurrence.

It was at the time commonly thought that the Katanga focus was unique, because of the very particular conditions prevailing in the restricted biotope of the high altitude forest galleries, where the temperature can drop at night to below 0°C and where there is a close association between vertebrate hosts and an anopheline vector feeding almost exclusively on rodents. This notion was confirmed by the work of Yoeli *et al.* (1964), who demonstrated that transmission of *P. berghei* could occur only at temperatures (18–21°C) lower than those necessary for the transmission of other known species of *Plasmodium* (25–28°C).

In 1965, in a completely different biotope (the fringes of the primary central African forest) and in another rodent species (*Thamnomys rutilans*), the first low altitude focus was discovered at La Maboké, 120 km west of Bangui in the CAR (Landau and Chabaud, 1965). The mean temperature in that locality is 25°C and the altitude is 600 m; *An. dureni* has never been recorded there.

Adam *et al.* (1966) captured numerous *Thamnomys rutilans* in forest galleries near Brazzaville (Congo Republic) and found a new focus of rodent malaria at an altitude of 300 m, where the average annual temperature is 25°C.

Examination of many other terrestrial and arboreal rodents, none of which was infected, captured in these two localities indicated that *Thamnomys rutilans* was the principal, or only, natural mammalian reservoir of the parasites. *Thamnomys rutilans* is mainly arboreal, seldom descending to the ground, and builds nests at a height of about 2 m (Genest-Villard, 1972). It rarely enters traps set on the ground, in which many other rodents may be caught. This was the main reason why, in Nigeria, Bruce-Chwatt and Gibson

(1955), while attempting to discover new species of *Plasmodium*, trapped 2500 rodents with little success. These authors probably used traps set on the ground, because about 1500 of the animals caught were the essentially terrestrial *Praomys* (personal communication, quoted by Killick-Kendrick, 1978).

Thamnomys rutilans is widely distributed at the edge of the primary forest, around the Congo river forest block. Its abundance varies according to the locality; very large populations exist in the CAR and the Congo Republic. In 1967 Killick-Kendrick went to Ilobi in Nigeria, the locality where Bruce-Chwatt and Gibson (1955) had worked, set traps in trees 2 m from the ground, and caught six parasitized *Thamnomys rutilans* (Killick-Kendrick *et al.*, 1968). Ilobi is situated at an altitude of 100 m, and temperatures are high throughout the year.

Finally, in Cameroon, in a locality approximately equidistant from the foci in the CAR, Nigeria and Congo Republic, Bafort (1977) captured seven rodents with malaria: six *Thamnomys rutilans* and one *Hylomyscus*.

2.2. Foci in the Central African Republic and the Congo Republic

The two localities that we studied in detail were La Maboké, the Paris Natural History Museum experimental station in the central African forest region of the CAR, and the forest galleries around Brazzaville in the Congo Republic.

2.2.1. Plasmodium *Species*

Three species may coexist in the same host and locality: *P. yoelii*, *P. vinckei* and *P. chabaudi*. However, *P. chabaudi* was never found in Katanga, where numerous strains of the first two species were isolated, nor was it present in the 18 *Thamnomys* examined by Killick-Kendrick *et al.* (1968) in Nigeria; however, it has been isolated from Cameroon.

It is noteworthy that, during surveys made in March–April 1965 and 1967, all adult *Thamnomys rutilans* in the CAR were parasitized, while fewer than 40% of those in the Congo Republic were infected.

More recently (April 1988), during a visit to La Maboké, we noted intense destruction of the primary forest and a sharp decrease in the infection rate (to about 50%) in *Thamnomys rutilans*. N'ganga Lingolo, a forest gallery near Brazzaville where infected animals had been captured between 1966 and 1970, had been destroyed.

2.2.2. *Rodents*

At La Maboké, thanks to Francis Petter and Huguette Genest-Villar, who trapped many rodents and made them available to us, and the help of the

Babinga Pygmies who knew how to find the nests of *Thamnomys rutilans* and who caught all sorts of rodents with great dexterity by hand, we examined hundreds of rodents belonging to 30 species; only *Thamnomys rutilans* was found to be infected.

In the Congo Republic, although fewer rodents were examined, the result was the same.

In western Nigeria a single rodent (*Praomys tulbergi*) of 2500 examined was found to be infected, while six of 18 *Thamnomys rutilans* captured by Killick-Kendrick (1973) were infected. The single *Praomys tulbergi* had probably been infected by chance, perhaps while climbing a tree.

In Katanga, where there is no *Thamnomys rutilans*, malaria parasites have several rodent hosts (Vincke, 1954): *Grammomys surdaster*, the most commonly infected species (7 of 61), *Praomys jacksoni* (5 of 99), and *Leggada bella*, in which *P. berghei* was seen, but the number of specimens examined was not stated.

2.2.3. *Vector*

In Katanga the close relationship between *An. dureni* (feeding mainly on rodents) and the vertebrate hosts was studied by Vincke and his collaborators (Vincke, 1946a, b, 1954; Vincke and Lips, 1948).

The vector of the murid *Plasmodium* in the lowland localities is still unknown. Development in the vector and experimental transmission in the laboratory have been accomplished at 24°C (Landau and Killick-Kendrick, 1966a, b).

In the Congo Republic and the CAR, the vector is likely to be a sylvatic species of *Anopheles* with feeding behaviour closely related to the arboreal habitat of *Thamnomys rutilans*; many African terrestrial rodents, which are not found infected in nature, are susceptible to experimental infection (*Mastomys*, *Praomys*, *Hybomys*, *Hylomyscus*, *Leggada*).

In a survey conducted at La Maboké in March–April 1967 (Landau, 1973), blood films and liver biopsies from 42 *Thamnomys rutilans* were examined: blood films from 24 contained malaria parasites, and five of the rodents had relatively high parasitaemia (1–3%); exoerythrocytic meronts were found in the liver biopsy of one individual. Transmission was, therefore, probably occurring at that time.

2.3. Systematics

Although it may be difficult, in the blood of the rodents, to differentiate morphologically between the strains from different localities, clear morphological and biological differences can be seen when the life cycles are reproduced in the laboratory (Landau and Killick-Kendrick, 1966b), leading

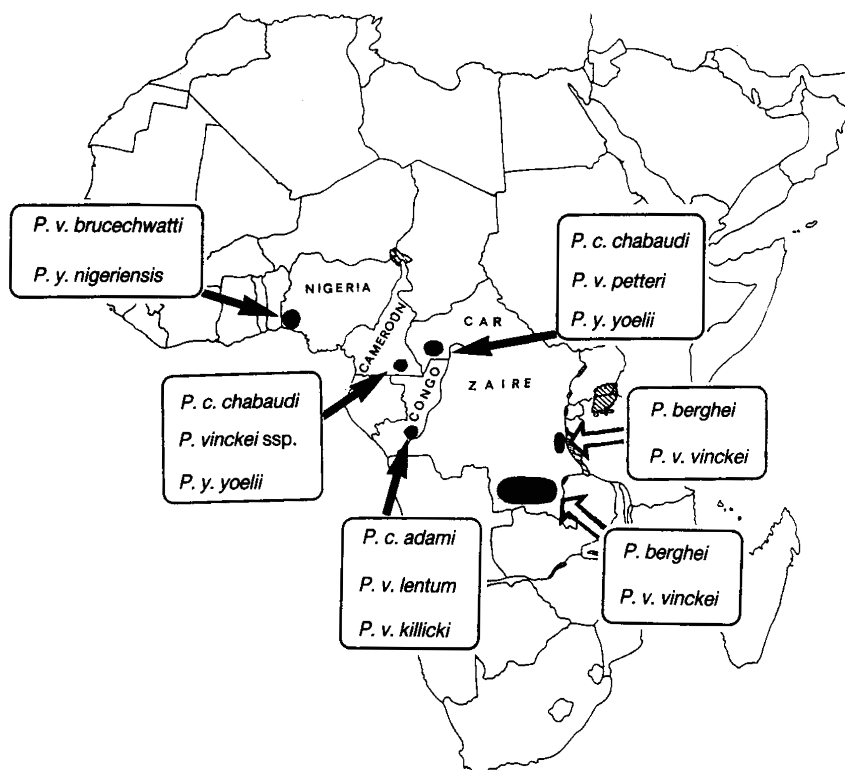


Figure 1 The foci of murine malaria in Africa. White arrows indicate high altitude foci, black arrows show those at lower altitudes.

to the concept of *vicariance** among the murid *Plasmodium* species (Landau, 1966).

Studies by the Edinburgh school (Beale *et al.*, 1978) also revealed isoenzymatic differences between strains of different geographical origins.

All localities where murid *Plasmodium* has been found border what used to be the Congo forest block. The Nigerian locality is now the most isolated, separated from the rest of the forest block by a wide deforested zone and the ecological barrier of the Cross river. Most localities represent different biotopes and in each the parasites have undergone more or less pronounced speciation (Figure 1).

As laboratory studies progressed and new strains were isolated, the systematics of the murid *Plasmodium* species has been progressively modified;

* *Vicariance* refers to the replacement of one species or subspecies by another closely related form in similar ecological niches, separated by a past or present ecological barrier.

the presently accepted nomenclature was detailed by Killick-Kendrick (1974) and Landau and Boulard (1978).

The species and subspecies of rodent malaria which we recognize are listed below:

1. In Katanga: *P. vinckei vinckei* Rodhain, 1952; *P. berghei* Vincke and Lips, 1948.
2. In CAR: *P. chabaudi chabaudi* Landau, 1965; *P. yoelii yoelii* Landau and Killick-Kendrick, 1966a; *P. vinckei petteri* Carter and Walliker, 1975.
3. In the Congo Republic: *P. chabaudi adami* Carter and Walliker, 1976; *P. yoelii killicki* Landau, Michel and Adam, 1986b; *P. vinckei lentum* Landau, Michel, Adam and Boulard, 1970.
4. In Nigeria: *P. yoelii nigeriensis* Killick-Kendrick, 1973; *P. vinckei brucechwatti* Killick-Kendrick, 1975.
5. In Cameroon: *P. chabaudi chabaudi* Landau, 1965; *P. yoelii yoelii* Landau and Killick-Kendrick, 1966b; *P. vinckei lentum* Landau, Michel, Adam and Boulard, 1970; *P. vinckei* subsp. Lainson, 1983.

Differences between subspecies, revealed when their life cycles were studied in the laboratory, will not be detailed here; however, two points are worthy of note:

1. *P. berghei* and its homologue *P. yoelii* in the lowland localities were at first considered as two subspecies of *P. berghei*; they were raised to specific rank by Killick-Kendrick (1974) on the basis of biological and isoenzymatic differences. Nevertheless it remains unquestionable that they are closely related.
2. Isoenzyme studies confirmed this nomenclature and revealed that, in Cameroon, there was an overlap between the distribution of *P. vinckei lentum*, extending from the CAR, and *P. vinckei* subsp., restricted to Cameroon (Lainson, 1983).

3. COURSE OF INFECTION AND BIOLOGY OF GAMETOCYTES

3.1. Course of Infection

The course of infection by each of the rodent malaria parasites of the central African lowlands in its natural host, *Thamnomys rutilans*, differs considerably from that in other rodents.

The infection is chronic in *Thamnomys rutilans*, lasting throughout the host's life, but it is not always patent and even subinoculation of blood to white mice may sometimes fail to detect parasitaemia (Landau, 1992).

In experimentally infected rodents the infection does not become chronic, and radical cure occurs after a variable length of time.

Liver meronts were found in wild-caught *Thamnomys rutilans* up to 8 months after their capture and transfer to the laboratory in Paris; these meronts were the chronic, slowly evolving type (Landau and Chabaud, 1968; Landau *et al.*, 1968a).

Meronts with some of these characteristics were obtained experimentally in the liver of various rodents under specific conditions. They were found in *Steatomys* sp., a rodent with a deficient thermoregulatory mechanism, when it was kept at 18°C (Landau and Michel, 1970), and in *Thamnomys gazellae*, in which a fatty liver condition was induced either by ethionine injections (in accordance with the findings of Dunn *et al.*, 1972) or by feeding the rodents on a methionine-deficient diet (Landau *et al.*, 1975). These methods resulted in delayed maturation of meronts and fluctuating parasitaemia (as seen in natural infections), but the effects were transient, unlike the situation in naturally infected *Thamnomys rutilans*.

Inoculation of sporozoites to laboratory bred *Thamnomys rutilans* also induced a long-lasting and fluctuating parasitaemia, which may have resulted from the development of "chronic" liver meronts. However, inoculation of blood infected with *P. vinckei petteri* to *Thamnomys rutilans* was also followed by a chronic infection, and other factors such as latency of merozoites (see below) may be involved in the mechanism of chronicity.

It appears, at all events, that "chronic" meronts ("hypnoschizonts"), comparable to those known in some other haemosporidians such as *Leucocytozoon* (see Desser *et al.*, 1968), may be considered as analogous to the dormant trophozoites of *Isospora*, which have been described in the reticulo-endothelial cells of birds by Grulet *et al.* (1986) and Boulard *et al.* (1987), and the hypnozoites of *P. cynomolgi* (see Krotoski *et al.*, 1980) and *P. vivax* (see Krotoski *et al.*, 1982).

It is difficult to follow directly the course of natural infections because parasites are usually very scanty. When blood from *Thamnomys rutilans* is subinoculated to mice, the periodicity of merogony in the three species is approximately 24 h but the meronts mature at about 15.00 in *P. vinckei petteri* and at midnight in *P. chabaudi chabaudi*; maturation is asynchronous in *P. yoelii yoelii* (see Landau, 1992).

The development of gametocytes also cannot be studied directly in the natural host, in which sexual stages are very scanty in the peripheral blood, whereas they are abundant in laboratory rats, mice, etc.

3.2. Morphological Development of Gametocytes

In general, work on the biology of gametocytes has been based on investigations into their infectivity to *Anopheles*, or simply on the ability of the microgametocyte to "exflagellate".

Gametocyte morphology appears to undergo age-related transformations

Table 1 Nuclear characteristics of gametocytes of the malaria parasites of rodents at different developmental stages.

Stage	0	I	II	III
Size of nucleus relative to size of parasite cell	1/3	2/3	1/2	1/3
Nucleus[a]	G	G	H	H
Relative staining intensity of nucleus and cytoplasm[b]	N < C	N = C	N > C	N > C

[a] G, granular; H, homogeneous colloid.
[b] N, nucleus; C, cytoplasm.

(Landau *et al.*, 1979). For convenience, four morphological types of gametocyte have been defined: 0, I, II and III. These types occur in succession and all intermediate stages can be observed. The differentiation was based on microgametocytes, because their characteristics are easier to define than those of females. The subspecies *P. yoelii yoelii* was studied in more detail but the characteristics of each developmental stage appeared to be the same for all species of what we called the "vivax" group (Landau *et al.*, 1976).

Schematically, during the course of normal ageing, the microgametocytes, which are at first large (types 0 and I), reaching 10.5 μm in diameter, become smaller, denser and increasingly chromophilic (types II and III). The nucleus is at first relatively small (type 0), but it enlarges to fill three-quarters of the parasite's volume (type I) and then becomes condensed and progressively smaller (types II and III); nuclei of stages 0 and I are granular, while those of stages II and III are homogeneous and colloidal in appearance.

The main nuclear differential characteristics are summarized in Table 1. We found, when examining blood films, that the larger gametocytes were often concentrated at the "tail" of the film, while smaller forms were more uniformly distributed.

3.3. Chronology of Gametocyte Development

The chronology of gametocyte development cannot be established directly. However, a rough idea of the timing can be obtained from miscellaneous observations:

1. Killick-Kendrick and Warren (1968) demonstrated infective gametocytes (type 0 or I, see below) of *P. yoelii yoelii* 72 h after the injection of sporozoites to mice. The pre-erythrocytic phase of this species in the white mouse lasts approximately 48 h; this implies that it takes about 24 h for the hepatic merozoite to develop to the type I gametocyte stage.
2. Type II gametocytes of *P. yoelii nigeriensis* were seen approximately 27 h, and type III approximately 30 h, after the rupture of hepatic meronts (Landau *et al.* 1979).

3. A pre-gametocyte is morphologically equivalent to an old trophozoite. Therefore, assuming a similar rate of development, it must take at least 18 h for it to reach this stage.

Based on these few observations, a tentative chronology of gametocyte development can be proposed, as follows: merozoite (type 0), 18 h; type I, 18 + 3 = 21 h; type II, 21 + 6 = 27 h; type III, 27 + 3 = 30 h.

In synchronized *P. berghei* infections, Mons *et al.* (1985) estimated the minimum developmental time of gametocytes, from merozoite to exflagellating young male gametocyte (pre-gametocyte in our nomenclature), to be 26 h, which is compatible with our estimate.

3.4. Occurrence of Gametocytaemia

In naturally infected *Thamnomys rutilans*, gametocytes were seldom seen. A laboratory-bred specimen was inoculated with *P. yoelii yoelii* and its blood was examined at intervals of 6 h from days 7 to 10. Parasitaemias remained low (below 1%) and the maximum number of gametocytes seen in any one blood film was 22. Gametocytes of types II and III were the most frequently seen, types O and I being probably retained in the small capillaries, and no periodicity could be demonstrated.

In experimentally infected white mice, on the contrary, gametocytaemias are frequently intense. Many factors are involved in determining gametocytaemia, some of which are listed below:

1. Species and strain of parasite. In white mice, *P. yoelii* and *P. vinckei* produce abundant, continuous gametocytaemia. With *P. chabaudi*, two waves of gametocytaemia are seen: a first wave of short duration with scanty gametocytes on the fourth day after infection, and a second abundant wave starting on the eighth day. Within the species *P. yoelii*, the subspecies *P. y. nigeriensis* produces many more gametocytes than does *P. y. yoelii*.
2. Vertebrate host. The proportion of gametocytes in the blood of the African murid *Hybomys univittatus* was considerably higher than that in the white mouse, even when the parasitaemias were comparable.
3. Virulence. In a highly virulent strain such as *P. yoelii nigeriensis*, the first wave of gametocytaemia occurred between days 2 and 3 after inoculation of cryopreserved blood; in the less virulent strains *P. yoelii yoelii* 17X and 646X, the first wave appeared later, at days 4 or 5. The period of time after which gametocytes appear in the blood may vary with the size of the inoculum and the magnitude of initial parasitaemia.

3.5. Infectivity and Age of Gametocytes

The "exflagellation" test used by some authors is not a valid test of the infectivity of gametocytes, as the process may occur in the blood of mice totally uninfective to *Anopheles*.

Experimentation has led us to the conclusion that infective gametocytes belong to types 0 and I and that types II and III are not infective (Landau *et al.*, 1979). The infectivity of gametocytes of types 0 and I was confirmed by observations demonstrating that no infection was obtained in *Anopheles* when the blood meal contained no type 0 or I gametocytes. Analysis of gametocyte infectivity is made difficult by the fact that the young stages, being larger, appear to be retained in the small capillaries and are not easily found in blood films. Thus, the problem of gametocyte infectivity is connected with the feeding process of *Anopheles*, which will be discussed in the next section.

3.6. Ingestion of Gametocytes by the Vector

The composition of the capillary blood ingested by a female *Anopheles* feeding on a mouse may be very different from that of blood collected from the tip of the same rodent's tail (Landau *et al.*, 1979).

When the infectivity was high, a comparison between the gametocyte picture in blood taken simultaneously from the tip of the tail by the usual procedures and by a feeding *Anopheles* revealed an interesting phenomenon: early in the course of infection, when infectivity was at its maximum, there was a considerable discrepancy between the number of gametocytes of types 0 and I in the mosquito's blood meal and that in the rodent's tail blood. In the mosquito, type 0 and I gametocytes were abundant, while they were scanty in the tail blood.

It was suggested that, when they feed, *Anopheles* take blood from capillaries of small diameter, while cutting the tip of the mouse's tail releases blood from much larger vessels. Young, healthy gametocytes of types 0 and I, being larger than the older forms, would be retained mechanically in smaller calibre capillaries; presumably the suction pressure exerted by the feeding mosquito frees them.

In order to test this hypothesis, Petit (1985) inoculated inert microspheres 8–24 μm in diameter (Sephasorb®) into rats and studied their distribution in mosquitoes that had fed on the rats. Microspheres 12 μm in diameter were more numerous in the blood meal than in blood taken from the heart or the ocular sinus. It is conceivable that infective gametocytes, being approximately this size, may also be taken up by mosquitoes in greater numbers than would be expected from the examination of a blood film.

3.7. Crisis and Gametocyte Infectivity

The occurrence of a crisis separates the course of the infection into two stages: the first when gametocytes are infective, the second when they are no longer infective. Crisis, an abrupt decrease of parasitaemia associated with the onset of anaemia, occurs when parasitaemias are between 20 and 45%. After the crisis, numerous signs of haemolysis are seen: a reduction in the number of circulating erythrocytes and the sudden appearance of altered or lysed erythrocytes and extracellular parasites and cellular debris, which increases in amount over several days. Reticulocytosis becomes intense.

Gametocytes are abundant but are greatly altered. They undergo profound morphological degeneration; the four types of gametocytes can still be identified but they are vacuolated, smaller, more condensed and more chromophilic than before. The pigment is often clumped.

The series of events accompanying the crisis has been detailed by Bastien *et al.* (1987). Before the crisis the mouse harbours a normal malarial infection, comparable to that of naturally infected animals; during and after the crisis there is anarchic multiplication of the parasites, bearing no similarity to the development of a natural infection.

In practice, gametocyte infectivity in laboratory white mice is reduced by the crisis phenomenon. *P. yoelii nigeriensis* gametocytes are infective until days 3 or 4 of the infection, and those of *P. yoelii yoelii* until the fifth or sixth days. These infective periods may be shorter when infections are very virulent, or prolonged when they are more attenuated. Some infected animals (about 5%) do not become visibly ill and do not have a clearly defined crisis. In such mice parasitaemias remain relatively low, reticulocytosis develops early and becomes pronounced, and gametocyte infectivity is prolonged.

In *P. chabaudi* infections in the white mouse, the first gametocytes could be seen 4 days after inoculation of infected blood, but they were scanty and the rate of infection of *Anopheles*, if fed at this time, was very low. The second wave of gametocytes was abundant and usually began at day 8, at the time of, or just after, the crisis. Such gametocytes were not infective.

In white rats, *P. chabaudi* infections were less virulent and crisis occurred later than in mice. Watier *et al.* (1990) showed that gametocytaemia was also more abundant and that the smaller the inoculum, the later the crisis occurred.

With *P. vinckei*, as with *P. yoelii*, gametocyte production is continuous and numbers rise progressively. Montalvo-Alvares (1987) studied the influence of various factors such as the parasitaemia, the time of inoculation of blood to the mice used as gametocyte producers, and the time at which the *Anopheles* were fed. Infectivity was found to be related only to the parasitaemia and to the time of onset of the crisis.

In conclusion, an abrupt fall in infectivity occurs at the time of crisis in all three of the rodent malaria species studied.

Bastien *et al.* (1987) showed that the serum of mice, infected with *P. yoelii nigeriensis*, taken at the time of crisis had a strong, dose-dependent inhibitory effect *in vitro* on gametocyte infectivity. Crisis may occur so swiftly that a specific immunological phenomenon can be excluded; thus, the possible presence in the serum of a factor that is not produced by an immunological process must be considered.

3.8. Merogony and Gametocyte Infectivity

While attempting to identify the possible inhibitory factor, proposed in the preceding section, by means of membrane feeding experiments, Motard *et al.* (1990) found that serum showing slight haemolysis had, *in vitro*, a strong inhibitory action. Similar inhibition was observed when mouse haemoglobin or ox haematin was added to the serum.

These observations drew attention to the possible role of haemolysis, which occurs both at the time of crisis and at the rupture of mature intraerythrocytic meronts. Motard *et al.* (1990) demonstrated that, in infections with the highly synchronous parasite *P. vinckei petteri*, there was a significant decrease of gametocyte infectivity at the time of rupture of meronts. In a series of experiments, *Anopheles* were fed on infected mice before, during and after meront rupture. In all cases the number of oocysts developing in the mosquitoes was much higher in those fed before and after rupture of the meronts than it was in those fed during the process.

3.9. Recovery of Infectivity by Gametocytes After Transfer to Naive Mice

Landau *et al.* (1979) showed that non-infective gametocytes of *P. yoelii nigeriensis*, in the blood of mice collected near the time of crisis, regained their infectivity when the blood was inoculated intravenously into an uninfected mouse. A similar recovery of infectivity after the transfer of gametocytes from crisis blood into normal serum was observed by Dearsley *et al.* (1990), working with *P. berghei*.

Work by Carter and Gwadz and their colleagues (see particularly Gwadz, 1976 and Carter and Chen, 1976) showed that the further development of *P. gallinaceum* microgametocytes in the vector could be blocked by immunizing the donor vertebrate host with microgametes. This phenomenon was reversible if gametocytes were transferred into non-immune serum, when they became infective again.

Some of these observations are comparable to those described above for rodent malaria: blockage of infectivity but not of gametocyte production, and the regaining of infectivity after transfer to normal serum. However, the

suppression of infectivity in *P. gallinaceum* differs in being caused by specific immunological factors, while inhibition of the infectivity of gametocytes of rodent malaria at the time of crisis is due to a non-specific factor (or factors).

These factors have not yet been identified, but as mentioned above, it is unlikely that they are circulating antibodies because of the precocity and abruptness of the phenomenon (which occurs as early as the third to fifth days of infection with *P. yoelii nigeriensis*). The massive haemolysis occurring at the time of crisis may bring about the release of toxic substances from the erythrocytes, the parasites, or the large amount of circulating debris.

Rosenberg *et al.* (1984) proposed the absence of a substrate indispensable for the maturation of gametocytes, which disappears when parasitaemias are high and particularly when there is haemolysis. In contradiction to this, Bastien *et al.* (1987) argued that the inhibitory serum is active *in vitro* even when it represents only 20% of the medium in which the parasitized erythrocytes are suspended.

Several authors (quoted in the reviews by Carter and Gwadz, 1980 and Sinden, 1983) have mentioned the possible involvement of an unknown toxic factor; others, from experiments *in vitro*, have suggested that the phenomenon is due to the production of cytokines by the host (Dearsley *et al.*, 1990; Mendis *et al.*, 1990). The possible involvement of liberated haemoglobin has already been referred to.

3.10. Comments of the Validity of Extrapolating from Results Obtained *in vitro* to the Situation *in vivo*

Studies on the biology of gametocytes are very often performed *in vitro* using cultures of infected blood, or of parasites suspended in artificial nutrient media, or by a combination of both techniques.

Although very interesting results are often obtained, such experiments cannot account for the complexity of gametocyte behaviour in the vertebrate host. The concept of gametocyte infectivity, particularly, cannot be separated from the many factors that facilitate or impair transmission *in vivo*.

Mons *et al.* (1985) and Janse *et al.* (1985) challenged both our morphological analysis of gametocyte development in the mouse and our observations on the differences of the gametocyte picture in the mosquito's blood meal and in blood taken from the tip of the mouse's tail. These workers took blood from the heart or tail of mice, and from the stomach of fed mosquitoes, and evaluated *in vitro* the numbers of exflagellating gametocytes and ookinete yield, finding no difference between these various sources of blood.

We recently examined, *in vivo*, the strain of *P. berghei* (ANKA) used by these authors and found the morphological development of the microgametocytes to be identical to that which we observed with *P. yoelii* in the mouse. We think that, by using *in vitro* techniques, these authors suppressed the very

factors that determine *in vivo* the differences in gametocyte distribution within the host, such as serum inhibitors and the age and size of gametocytes. We think that one should be very careful in extrapolating from results obtained *in vitro* to the situation existing *in vivo*.

4. THE VESICULAR SYSTEM OF HEPATIC MERONTS

Seureau *et al.* (1980) described in *P. yoelii yoelii* one of the essential features of the exoerythrocytic meronts of species of *Plasmodium* parasitizing mammals: a network of vesicles within the meront cytoplasm.

This vesicular system comprises several components (Figure 2): a zone of synthesis, the granular endoplasmic reticulum; a transport system consisting of numerous vesicles of differing size; and active material represented by flakey granules within the vesicles.

The usual time taken by exoerythrocytic meronts of *P. yoelii yoelii* in the rat to mature is 45–53 h. Seureau *et al.* (1980) described the normal hepatic meront, as seen by electron microscopy, 35 and 43 h after sporozoite inoculation, as follows (Figure 3):

At 35 h sections of meronts showed numerous profiles of granular

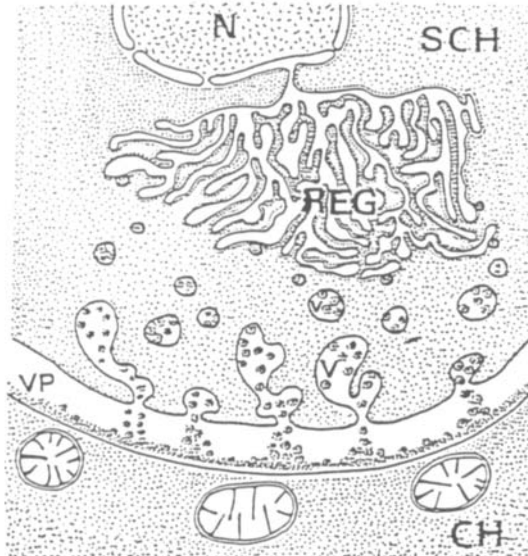
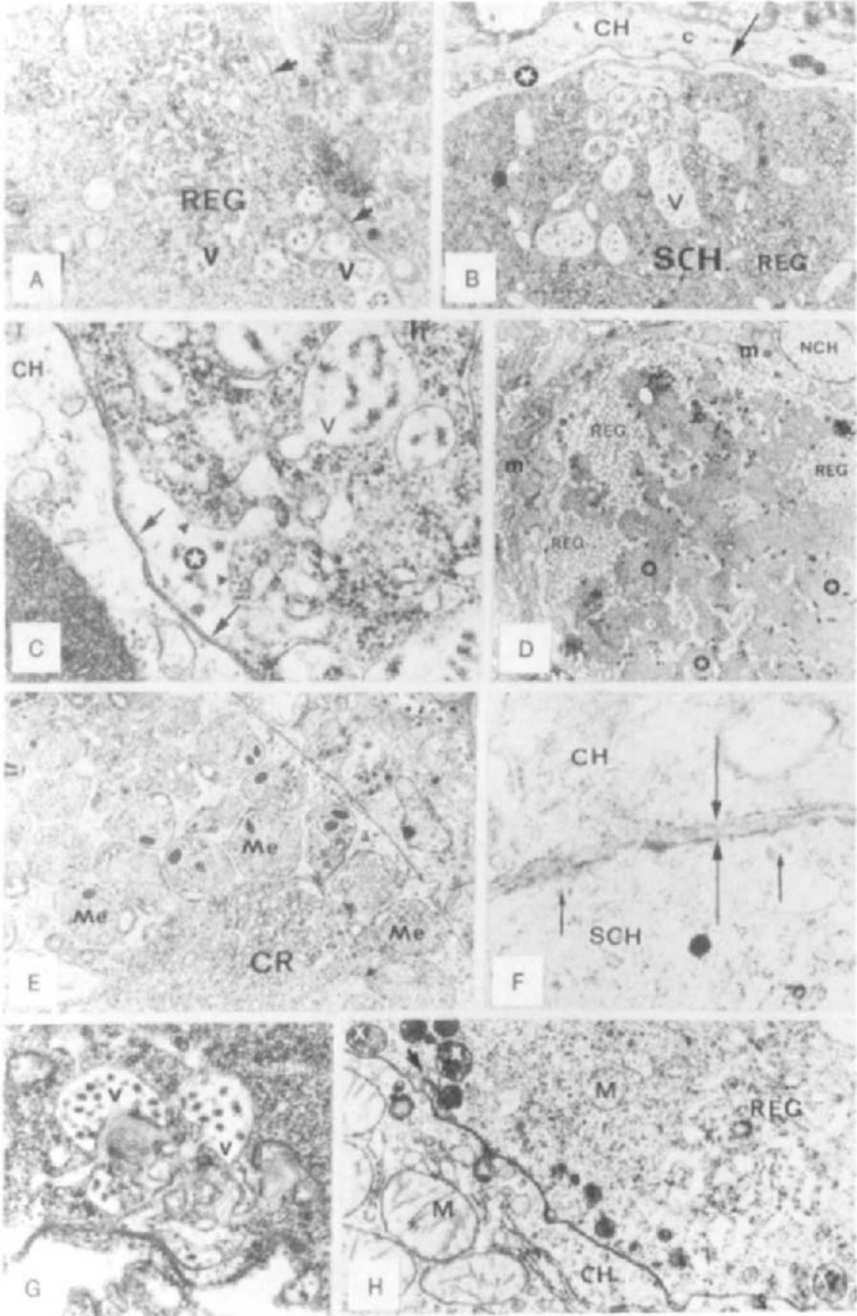


Figure 2 Schematic representation of the vesicular system in hepatic meronts of *P. yoelii*. CH, host cell; N, nucleus; REG, granular endoplasmic reticulum; SCH, meront (= schizont); V, transport vesicle; VP, parasitophorous vacuole. (From Boulard, 1987.)


REG
V
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endoplasmic reticulum scattered throughout the cytoplasm and a small number of vesicles containing sparse granules. At the periphery of the meront the plasmalemma was still in contact with the parasitophorous vacuole membrane of the host hepatocyte (Figure 3A).

At 43 h, the granular reticulum was still plentiful. The plasmalemma and the parasitophorous vacuole membrane had become separated, forming a continuous and well-defined space surrounding the parasite (Figure 3B). The vesicles, produced by the granular endoplasmic reticulum, had become very numerous and packed with granules. Many came into contact with the plasmalemma and were seen to open outside the parasite, expelling the granular material into the parasitophorous vacuole; the granular material became concentrated against the vacuole wall (Figure 3C).

In meronts nearing maturity the vesicles tended to aggregate and become confluent, separating isolated cytoplasmic islands of differing size (Figure 3D). On reaching maturity, the meronts developed an extensive system of rough endoplasmic reticulum, which became interwoven in some places, and cisternae. The vesicles became very abundant and fused, their membranes forming the plasmalemma of the developing merozoites (Figure 3E).

During growth of the parasite, the host hepatocyte became progressively

Figure 3 Electron micrographs of the vesicular system of hepatic meronts of *P. yoelii*. (A) Part of a 35-h-old meront in the liver of a rat showing granular endoplasmic reticulum (REG), scanty vesicles (V), and the zone of contact between meront and host cell (arrows) (× 40 000). (From Landau *et al.*, 1982.) (B) Meront, 43 h old, in the liver of *Thamnomys gazellae*. Note the confluent vesicles (V) and the continuous space (asterisk) between the parasite (SCH) and the parasitophorous vacuole membrane (arrow); c, cytoplasm of host cell (CH); REG, granular endoplasmic reticulum (× 6600). (C) Part of the periphery of a 43-h-old meront in rat liver showing fusion of the vesicles (V) with the plasma membrane of the parasite and discharge of vesicular contents into the parasitophorous vacuole (asterisk). The material discharged accumulates near the membrane of the parasitophorous vacuole (arrows); CH, host cell (× 76 000). (From Seureau *et al.*, 1980.) (D) Almost mature meront, showing proliferation of the granular endoplasmic reticulum (REG) and its labyrinthine appearance, and the splitting of the cytoplasm into cytomeres (asterisks). Alterations of the host hepatocyte include the more lightly staining nucleus (NCH) and the hypertrophied mitochondria (M) surrounding the parasitophorous vacuole (× 6600). (From Boulard, 1987.) (E) Part of a maturing meront showing individual merozoites (Me) surrounding areas of residual cytoplasm (CR) (× 20 000). (From Boulard, 1987.) (F) Part of a meront in rat liver after treatment of the host with a single dose of primaquine (30 mg kg^{-1}), showing additional membranes around the parasite (long arrows) and the remains of disorganized vesicles (short arrows). CH, host cell; SCH, meront (× 40 000). (From Boulard *et al.*, 1983.) (G) Part of a 65-h-old meront in the liver of a rat which had been fed on a methionine-deficient diet for 22 days, showing membrane whorls associated with a vesicular area (V) (× 84 000). (From Landau *et al.*, 1982.) (H) Part of a 43-h-old meront in a rat treated with a single dose of mefloquine (50 mg kg^{-1}), showing altered vesicles containing non-granular material (X) and regions of exudation into the parasitophorous vacuole (arrow). Note the normal appearance of the mitochondria (M) and the endoplasmic reticulum of the host cell (CH); REG, rough endoplasmic reticulum of the parasite (× 2700). (From Boulard *et al.*, 1986.)

altered, while neighbouring cells remained unchanged. Nuclei of the parasitized cells became hypertrophied and less dense. The cytoplasm also lost its density and its glycogen reserves, the endoplasmic reticulum became disorganized showing irregular dilatations or disaggregation of the cisternae, and the mitochondria became swollen and electron-lucent. Finally, the glycogen entirely disappeared and the host cell cytoplasm itself became electron-lucent and vacuolated (Figure 3B and 3C).

Hepatic meronts of other mammalian malaria parasites have been found to have similar vesicular systems. Meis *et al.* (1981) confirmed the observations of Seureau *et al.* (1980) with *P. berghei*, and Boulard *et al.* (1982) described similar structures in *P. falciparum*.

Although Bafort (1971) did not describe an organized vesicular system, he did observe a villous configuration at the border of hepatic meronts of *P. vinckei vinckei* and noted the presence of peripheral vesicles containing granules which he thought were the product of digestion of nutritive material obtained from the host cell.

On the contrary, Seureau *et al.* (1980), Landau *et al.* (1982) and Boulard (1987) considered that the vesicular system represented an exocytosis system and that the granules were discharged from the meront into the parasitophorous vacuole. They suggested a dual function for the system:

1. Digestion and destruction of the host cell components. Secretion, probably enzymatic, appears to play a part in the feeding process of the parasite on the host hepatocyte. Glycogen, for example, disappears completely from the hepatocyte during growth of the parasite. Lysis of the host cell was one of the more obvious effects of enzyme production; the destruction of the host cell components was proportional to the development of the vesicular system and, if this was prevented or disrupted by external factors, the host cell retained its normal appearance.
2. Participation in maturation of the meront. The partition of the cytoplasm into cytomeres and the formation of merozoites followed the confluence of the numerous vesicles found inside the cytoplasm.

The endoplasmic reticulum and the vesicular system are very sensitive indicators of the meront's development, reflecting alterations in the meront after drug treatment with primaquine (Boulard *et al.*, 1983) (Figure 3F), by WR 225–428, a primaquine derivative (Peters *et al.*, 1984), by mefloquine (Boulard *et al.*, 1986) (Figure 3H), or by injection of the host with ethionine and methionine deprivation (Landau *et al.*, 1982) (Figure 3G).

5. BIOLOGICAL RHYTHMS

Classically, clinicians used to differentiate between the two groups of human malarias on the basis of the periodicity of the clinical symptoms, the bout of

fever corresponding to the rupture of the meronts: that is, quartan fever (72 h) for *P. malariae*, and tertian fever (48 h) for *P. vivax*, *P. ovale* and *P. falciparum*. This indicates the significance of the biological rhythms of these parasites.

Early workers, such as Stauber (1939), were very interested in the periodicity of erythrocytic merogony of avian malaria parasites and its alteration by factors such as varying the light and dark periods to which the host was exposed. In mammals, except for the work of Hawking and his colleagues (Hawking *et al.*, 1968, 1972), these aspects of the host–parasite relationship have been little investigated. Although the human malarias are relatively well synchronized, direct experimental work is obviously unacceptable. *In vitro* models exclude the host factor, which is of great importance. Rodent or avian models are the most suitable for this type of study; the synchronicity of *P. chabaudi*, for example, is well known. However, even with this species, it is impossible to obtain perfect synchronicity of all stages.

Studies on the separation of merozoites from all other stages of the parasite, for example by freezing and thawing infected blood (Montalvo-Alvarez *et al.*, 1988), in order to be able to inoculate this stage alone, are, in our opinion, useful approaches to this type of research.

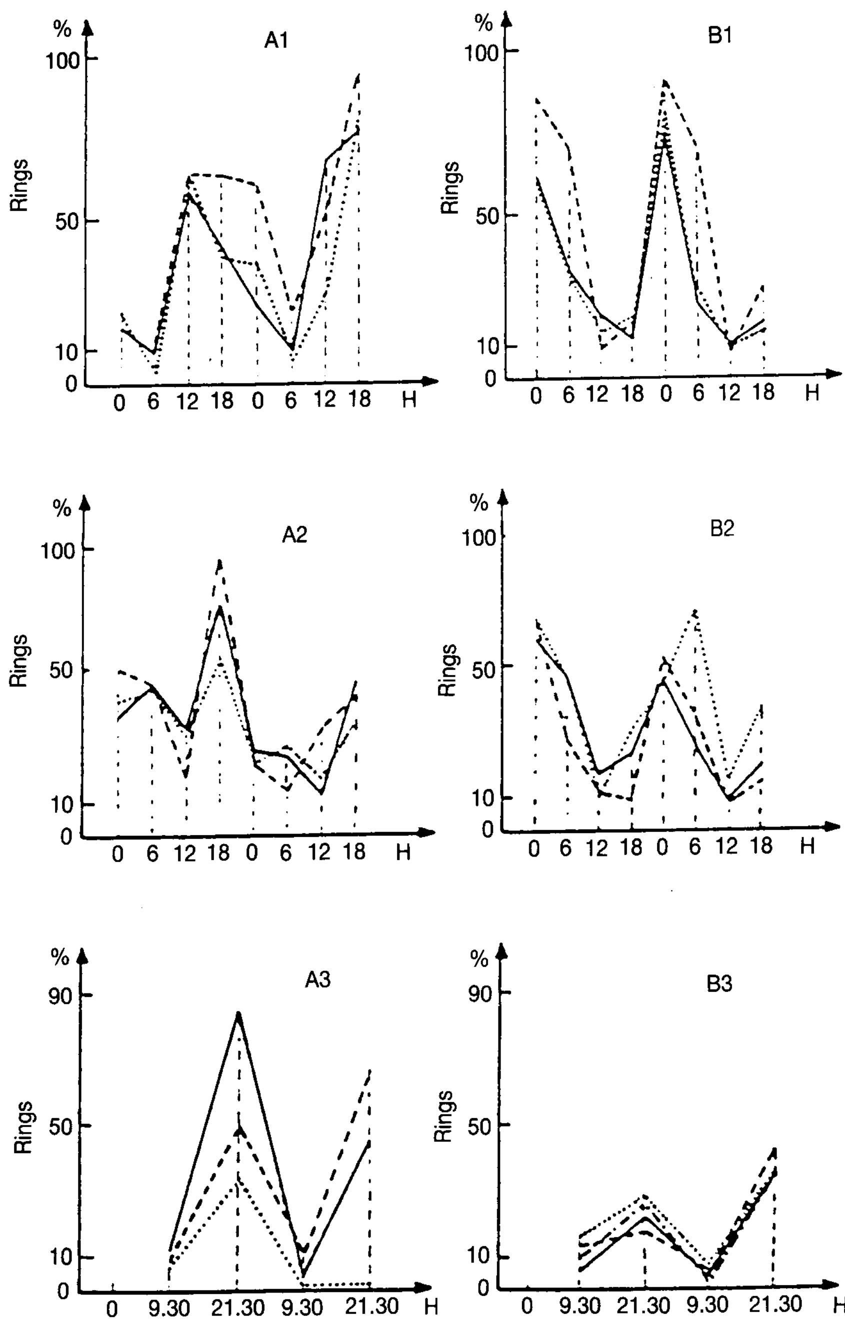
5.1. Resistance of Merozoites to Freezing and Thawing

Experiments with *P. yoelii nigeriensis* by Landau and Chabaud (1980) showed that, when infected blood was frozen and thawed rapidly in a medium containing glycerol and then inoculated to uninfected mice, parasitaemia was initiated by a single stage which was thought to be the merozoite.

Investigations on the effect of freezing and thawing procedures on the blood stages and the merogonic rhythm of malaria parasites were carried out by Montalvo-Alvarez *et al.* (1988), who considered the subspecies *P. vinckei petteri* to be the best model because of its synchronicity and its morphological characteristics that allowed easy differentiation of the various stages. This identification is essential because meronts are sequestered in the deep capillaries and the rhythm of merogony can be studied only indirectly by, for example, determining the percentage of ring-infected erythrocytes.

In a series of experiments, summarized below, the periodicity of merogony of *P. vinckei petteri* was investigated by varying the time of inoculation and the type of inoculum — fresh or frozen and thawed blood.

Mice were inoculated at 09:00 and at 21:00 with frozen or fresh blood (Figure 4). In the mice receiving frozen blood at 09:00 the peak number of ring forms, expected at 12:00 occurred at either 12:00 (Figure 4, graph A1, first peak) or at 18:00 (graph A1, second peak, and graph A2). This discrepancy was probably related to the number of previous merogony cycles in the donor animal. When these cycles were numerous, the cycle tended to


A1
B1
A2
B2
A3
B3
%
Rings
100
50
90
10
0
0 6 12 18 0 6 12 18 H
0 9.30 21.30 9.30 21.30 H

resume the natural rhythm it has in *Thamnomys rutilans*, in which merogony occurs at 15:00. In the mice receiving frozen blood at 21:00, the peaks occurred at the expected time except in one mouse (of six), in which the peak of ring forms occurred 6 h late (graph B2), which again can be interpreted as a progressive reversion to the natural timing of the cycle (which in nature appears to depend at least in part on the circadian rhythm of the host, and in the laboratory tends to revert to the natural timing).

In mice receiving fresh blood, the rhythm of merogony in the receptor mice remained the same as it had been in the donor mice (graphs A3, B3). Thus, it can be assumed that (i) the merozoite is the main surviving stage after freezing and thawing infected blood, and (ii) the merozoites penetrate immediately into the recipient's red blood cells.

The destruction of intracellular parasites probably occurs after thawing. The blood which is inoculated into the recipient mouse contains the glycerol added to the medium before freezing, and is therefore hypertonic; thus the erythrocytes are damaged before injection, and the only parasites capable of survival are the merozoites which were already free in the plasma or were about to be liberated by the rupture of mature meronts.

5.2. Circadian Rhythm of Merogony

The process of freezing and thawing infected blood thus made it possible to improve the synchronicity of experimental infections.

Cambie *et al.* (1990) devised a series of experiments to investigate the biological rhythms of the three species of rodent malaria that coexist in the *Thamnomys rutilans* captured at La Maboké, *P. vinckei petteri*, *P. yoelii yoelii*, and *P. chabaudi chabaudi*.

The rhythm of development according to the time of inoculation and the circadian rhythm of the host was studied with each species by recording at regular time intervals the percentage of ring-form parasites compared with other stages.

5.2.1. Plasmodium chabaudi chabaudi

It has been shown by David *et al.* (1978) that the timing of merogony of *P. chabaudi* varies when the circadian rhythm of the infected mice is inverted. Cambie *et al.* (1990) confirmed this, and showed that the timing of merogony

Figure 4 *Plasmodium vinckei petteri*: course of development expressed as the percentage of ring forms in blood films. *X* axis, time of making blood film; *Y* axis, percentage of rings. Each curve represents an individual mouse. A1 and A2, frozen and thawed blood inoculated at 09:00; B1 and B2, frozen and thawed blood inoculated at 21:00; A3, fresh blood inoculated at 09:00; B3, fresh blood inoculated at 21:00. (From Montalvo-Alvarez *et al.*, 1988.)

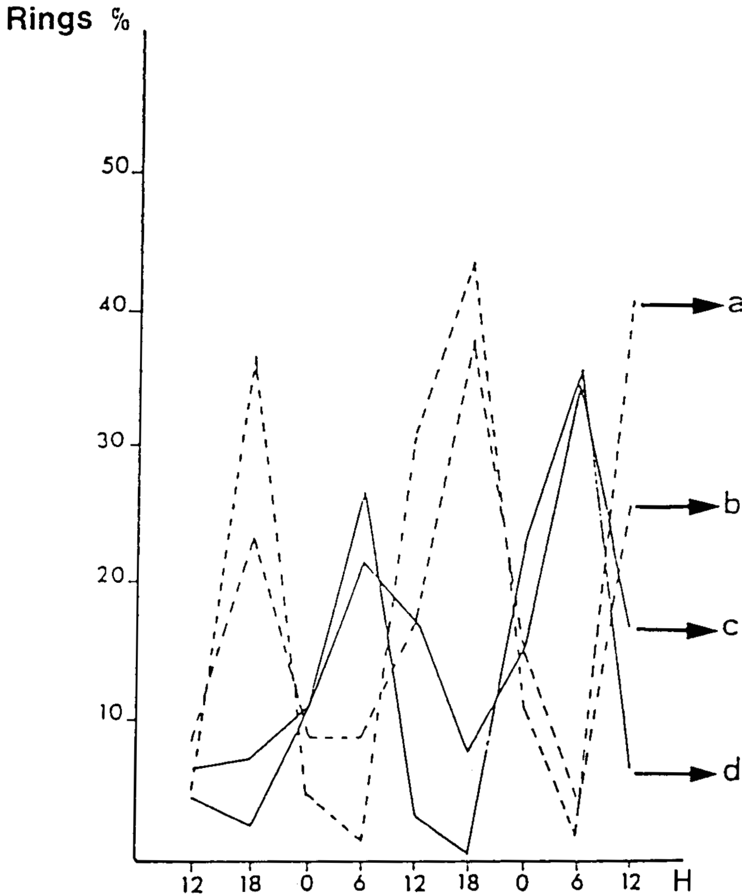


Figure 5 *Plasmodium chabaudi chabaudi*: percentages of ring forms in peripheral blood at intervals of 6 h during a 48-h period in mice with a normal (—) or an inverted (---) circadian rhythm, inoculated at different times of day. Each curve represents the mean value for five mice; a and c, inoculated at 06:00; b and d, inoculated at 18:00. Note that, whatever the time of inoculation, merogony occurred around midnight in mice with a normal circadian rhythm and around noon in mice with an inverted rhythm. (Modified from Cambie, 1991.)

is independent of the time of inoculation and is strictly governed by the circadian rhythm of the host (Figure 5).

5.2.2. Plasmodium vinckei petteri

The work of Montalvo-Alvarez *et al.* (1988), showing that the timing of merogony was governed by the time of inoculation, was confirmed. Experi-

ments with mice maintained with standardized normal or inverted circadian rhythms showed that, in the laboratory, merogony of *P. vinkei petteri* was independent of the host's rhythm.

5.2.3. Plasmodium yoelii yoelii

P. yoelii yoelii is generally considered to be an asynchronous subspecies. An attempt to induce synchronicity, by using frozen and thawed blood to initiate an infection, failed. Although, in some mice, peak percentages of ring forms were seen at intervals of 24 h, the margin between the maximum (29%) and the minimum (6%) percentages was relatively small.

Thus, this series of experiments showed that each species possesses marked individual characteristics. Merogony of *P. yoelii yoelii* is less synchronous than that of the other two species; the maximum proportion of ring forms is only 30%. In *P. chabaudi chabaudi*, synchronicity is more marked, with a maximum of 50% of ring forms; the timing of merogony is strictly dependent on the circadian rhythm of the host. In *P. vinckei petteri*, synchronicity is very pronounced (the peak proportion of ring forms is 70%), but the timing of merogony is independent of the host's circadian rhythm: it depends on the time of inoculation.

5.3. Biology of Merozoites

While studying the duration of prepatency of *P. yoelii nigeriensis*, when merozoites were inoculated into white mice at different times of the day, we found that when they were inoculated at midnight the infection became patent more rapidly than when merozoites were inoculated at 16:00 or 09:00 (Landau and Chabaud, 1980). This implies that development of the merozoites inoculated at the two latter times was delayed in comparison with that of those inoculated at midnight.

Our observations indicated an approximate delay of 8 h following inoculation at 16:00 and of 15 h after inoculation at 09:00. This suggests that extracellular merozoites (which, we think, are the only viable stages in frozen and thawed blood; see Section 5.1) were capable of penetrating into erythrocytes of the host only at a pre-determined time in the circadian cycle — midnight in the case of *P. yoelii nigeriensis*. This implies that merozoites remain latent in the blood and wait until the appropriate time to penetrate the red blood cells. A series of experiments was conducted to investigate this.

Donor mice were inoculated with frozen and thawed blood and, to evaluate the length of time for which infective merozoites persisted in the blood without invading erythrocytes, blood samples were taken at various times during the 24 h preceding the first merogony, frozen and thawed and inoculated intraperitoneally to receptor mice (Table 2). It was assumed that

Table 2 Demonstration of latent period before inoculated extracellular merozoites invade erythrocytes (Cambie *et al.*, 1990).

	Subinoculation time[a]	*P. y. yoelii*						*P. c. chabaudi*					*P. v. petteri*				
		1st experiment			2nd experiment												
A	15:00	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+
B	17:00	+	+	+	+	+	+	+	+	−	−	−	+	+	−	−	−
C	19:00	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−
D	00:01	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
E	08:00	+	+	+	+	+	+	+	+	−	−	−	+	−	−	−	−
F	12:00	+	+	+	+	+	+	+	+	+	−	−	+	−	−	−	−
G	18:00	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−

[a] Donor mice were inoculated with frozen and thawed blood at 12:00. Blood was collected from them at the times shown, frozen and thawed (a process which only extracellular merozoites survive) and subinoculated into three or five recipient mice. +, recipient became infected; −, recipient did not become infected.

the development of infection in the recipient mice indicated the presence of latent merozoites in the blood of the donor mice.

5.3.1. Plasmodium chabaudi chabaudi

At least one mouse of each batch developed parasitaemia. The number of infected mice increased progressively until midnight (batch D), decreased at 08:00 (batch E), and then increased again (batches F and G).

In batches, A, B, C, D and E, inoculated with blood taken from donor mice less than 24 after inoculation, parasitaemia must have been initiated by merozoites originally inoculated to the donor mice. In batches F and G, which received blood more than 24 h after the inoculation of the donor mice, merozoites responsible for the development of parasitaemia may also have been products of the first merogony.

In conclusion, the very characteristic rhythm of *P. chabaudi chabaudi* can be accounted for only by the hypothesis that most merozoites enter red blood cells between midnight and 08:00. In this respect, *P. chabaudi chabaudi* from CAR behaves similarly to *P. yoelii nigeriensis* from Nigeria. However, the apparent disappearance of *P. chabaudi chabaudi* merozoites from the blood of the donor mouse soon after inoculation and their reappearance at midnight remains to be explained. Our hypothesis is that merozoites may be sequestered during part of the day and then be released around midnight, at the time of penetration into erythrocytes.

5.3.2. Plasmodium vinckei petteri

There was a sharp decrease in the infectivity of the blood from the donor mice between the third and the seventh hours after inoculation, although a small number of merozoites persisted in the circulation for 24 h. At 18:00 on the second day merogony had occurred in the donor mice (at 12:00) and their blood was again highly infective. Thus, the majority of merozoites appeared to penetrate rapidly into red blood cells, between 3 and 7 h after inoculation.

5.3.3. Plasmodium yoelii yoelii

Numerous merozoites of this species remained in the circulating blood during at least 24 h. In batches F and G (Table 2), merozoites may have been produced by the first merogony. This indicates that merozoites of this subspecies may penetrate into red blood cells at all times of the day.

The three different patterns of development are summarized in Table 3. In *P. vinckei petteri*, almost all merozoites disappear rapidly from the blood; they enter erythrocytes immediately after their inoculation, whatever the time of day, and synchronicity is very pronounced. In *P. chabaudi chabaudi*, most merozoites enter erythrocytes at a particular time, depending on the

Table 3 Summary of relationship between synchronicity of merogony and latency of extracellular merozoites (modified from Cambie *et al.*, 1990).

	Synchronicity	Merozoite latency	Merozoite penetration
P. y. yoelii	+	+++	Progressive
P. c. chabaudi	++	++	At midnight[a]
P. v. petteri	+++	+	Immediate

[a] In animals kept under normal diurnal rhythm of light and dark periods.

circadian rhythm of the host (midnight when the host is kept in the light from 08:00 to 20:00, noon when the host is exposed to light from 20:00 to 08:00); synchronicity is relatively pronounced. In *P. yoelii yoelii* merozoites remain in the plasma for at least 24 h after inoculation, entering the erythrocytes progressively throughout that period; consequently, synchronicity of subsequent development is poor.

6. MEROZOITE LATENCY, ASYNCHRONISM AND DRUG RESISTANCE

6.1. Merozoite Latency

A given proportion of merozoites, which varies with the different species or subspecies, has delayed entry into erythrocytes; such merozoites have been termed "latent" merozoites (Landau *et al.*, 1990).

In order to estimate the maximum duration of latency of merozoites, blood from donor mice infected with *P. yoelii nigeriensis* was frozen and thawed 12 h after inoculation — before merogony could have occurred — and inoculated to recipient mice. Blood was then taken 12 h later from the recipient mice, frozen and thawed, and inoculated to other mice; the experiment was repeated until subinoculation failed to result in parasitaemia.

It was hypothesized that each inoculum contained two merozoite populations: X and Y. Population Y invaded erythrocytes and developed into trophozoites which were killed when frozen and thawed. Population X, the latent merozoites, remained extracellular and thus survived freezing and thawing and could initiate an infection in the recipient mice. Latent merozoites of *P. yoelii nigeriensis*, derived from the original inoculum, could be demonstrated in the recipient mice for three passages at intervals of 12 h (i.e. for 1.5 days) (A. Beauté-Lafitte, V. Caillard, I. Landau and A. Chabaud, unpublished observations). However, considering the extent of dilution of the original blood inoculum at each passage and the relatively small proportion of latent merozoites, it would not be surprising if some

merozoites of *P. yoelii nigeriensis* remained latent for much longer than 1.5 days. The same experiments performed with *P. vinckei petteri* and *P. chabaudi* showed that parasites of these species could be passaged only once.

6.2. The Relationship Between Merozoite Latency, Asynchronism and Drug Resistance

Our interpretation of the experiments described above (Section 6.1) is based on the assumption that extracellular merozoites are the only surviving stage after freezing and thawing, which is difficult to demonstrate directly. Nevertheless, several indications support this hypothesis:

1. It is most unlikely that intracellular parasites freed when erythrocytes are damaged (by freezing and thawing) could continue their development.
2. Electron microscopical observations have revealed degeneration of trophozoites in frozen and thawed blood; the best preserved stages were the mature meronts (unpublished observations).
3. Merogony of *P. vinckei petteri* occurs about 24 h after inoculation of frozen and thawed blood, which is approximately the normal duration of the blood cycle from merozoite to mature meront.
4. In experiments involving successive blood passages, if some parasite stages old enough to complete merogony in less than 6–18 h (i.e. before the next freezing) remained viable, blood frozen 6 h later should be less infective than that frozen after 18 h. In fact, the reverse is true (about 1 in 60 merozoites infective at 6 h compared with about 1 in 100 at 18 h (see Section 6.1), which is in accordance with the hypothesis of progressive penetration of merozoites into erythrocytes.

The overall experimental results are, in fact, difficult to interpret unless it is true that the merozoite is the major stage resistant to freezing and thawing. Even if this assumption were not strictly correct, the only other resistant stages that could account for the results obtained would be those developing very close to the time of merozoite liberation (i.e. mature meronts), and therefore our conclusions would remain valid: infective latent stages persist for at least 36 h.

The persistence of latent merozoites of *P. yoelii yoelii* and their penetration into erythrocytes at all times of day account for the characteristic asynchronicity of this species. Knowing that merozoites are resistant to chloroquine, and that the only naturally chloroquine-resistant species of rodent malaria is *P. yoelii*, it is logical to consider that there is a relationship between these three factors: merozoite latency, asynchronism and drug resistance.

7. CHRONOTHERAPY

Chronotherapy, the timing of drug treatment to achieve maximum therapeutic effect, can be made more precise by experimental work based on knowledge of the biology and circadian rhythms of the various rodent malaria parasite species described in the preceding sections (Landau *et al.*, 1991).

Chronotherapeutic research is logically preceded by study of the pharmacokinetics of the drug under investigation, in normal and infected mice. Preliminary unpublished investigations by F. Verdier, G. Cambie, I. Landau and H. Ginsburg have shown that chloroquine, given during the day or the night, reached similar levels in the blood, after comparable lengths of time, in uninfected mice and in mice with a low parasitaemia (as used in our experiments).

The species best adapted to chronotherapeutic studies is *P. vinckei petteri*, because infections with this subspecies resulting from inoculation of frozen and thawed blood are particularly synchronous. The timing of the merogonic cycle depends on the time of inoculation and each stage can be obtained at a convenient time of day by adopting the appropriate time of inoculation.

For convenience, four blood stages of the parasites were defined, as follows:

1. Ring. Small form with a peripheral nucleus and a large vacuole surrounded by a thin band of cytoplasm, with no visible pigment.
2. Young trophozoite. Larger, but occupying less than one-third of the volume of the erythrocyte, with a smaller vacuole, more abundant cytoplasm, and fine pigment granules (if any).
3. Mid-term trophozoite. Occupying one-third to one-half of the erythrocyte, with scattered fine pigment granules and a smaller vacuole than previously.
4. Old trophozoite. Large, occupying more than half the volume of the erythrocyte, with dense chromophilic cytoplasm and abundant pigment.

The duration of the stages is 3 h for the rings and 6 h each for the young, mid-term and old trophozoites. The subsequent stages, merogony and merozoite formation, occur mostly in the deep capillaries and cannot be accurately assessed by inspection of tail blood films.

Determination of the stage or stages most sensitive to a particular drug was attempted indirectly, by assessing the pre-patent period (from infective inoculation to 1% parasitaemia) in mice given the drug at various times (and hence targeted at particular stages) (Landau *et al.*, 1990); and directly, by assessing the parasite stages present in peripheral blood following administration of the drug (Cambie *et al.*, 1991).

7.1. Chloroquine

7.1.1. *Length of the Pre-patent Period*

When mice were infected at 12:00, the times at which the various stages predominated during the following 24 h were as follows: 15:00 for rings, 18:00 for young trophozoites, midnight for mid-term trophozoites, and 06:00 for old trophozoites, followed by merogony and merozoite formation at 12:00.

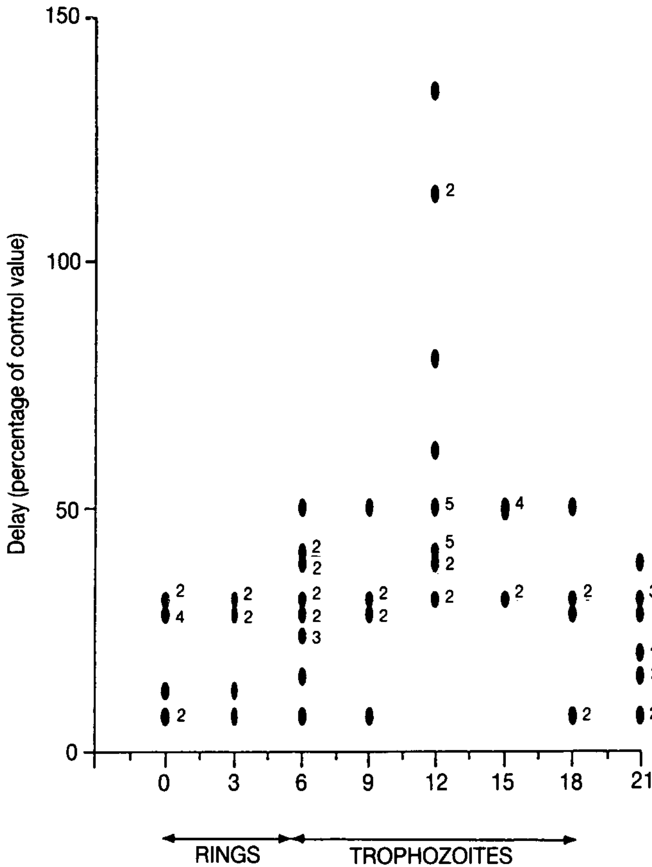


Figure 6 Delay in development of 1% parasitaemia in mice inoculated with frozen and thawed blood containing *Plasmodium vinckei petteri* and subsequently given chloroquine (5 mg kg^{-1} subcutaneously) at the times shown. The numbers beside some points indicate the number of mice sharing the same value. (From Landau *et al.*, 1990.)

A single injection of chloroquine diphosphate (5 mg kg^{-1} base) was given subcutaneously to batches of infected mice, following inoculation of frozen and thawed blood at 12:00, at midnight (9 mice), 03:00 (6 mice), 06:00 (14 mice), 09:00 (6 mice), 12:00 (18 mice), 15:00 (6 mice), 18:00 (6 mice) and 21:00 (14 mice). The control, untreated batch comprised 15 mice.

To judge the effect of treatment, the number of days between subinoculation and development of 1% parasitaemia in each mouse was compared with the equivalent figure for the control mice. The pre-patent period was longest in mice treated 12 h after inoculation (see Figure 6), when mid-term trophozoites formed the majority of the population — suggesting that this stage was the least resistant to the drug (Landau *et al.*, 1990).

7.1.2. *Alteration of the Parasitic Pattern*

Single subcutaneous injections of chloroquine diphosphate (5 mg kg^{-1} base) were given to mice with parasitaemias between 1 and 8%. The development of the parasitaemia and the percentage of each parasite stage was followed for 48 h, and the direct effect of treatment on each stage was observed (Table 4) (Cambie *et al.*, 1991).

Chloroquine had an immediate inhibitory effect on mid-term trophozoites; it did not prevent growth of rings or young trophozoites, nor did it prevent the development of old trophozoites into meronts and the subsequent liberation of merozoites and their entry into erythrocytes. The effect of removing one brood of mid-term trophozoites was to modify the subsequent rhythm of merogony, which was then entrained by the predominant surviving stages.

These observations also suggested that the mid-term trophozoites were the stage most sensitive to chloroquine. Because of this, and because drug treatment at that stage interfered with the subsequent rhythm of merogony, it appeared to be of interest to determine whether two consecutive drug treatments, aimed at consecutive broods of mid-term trophozoites, would be even more effective.

Amongst the many drug schedules tested, the best result (i.e. the longest pre-patent period in subinoculated mice) was obtained when chloroquine was first injected at the time when mid-term trophozoites were first predominant and the second injection of drug was given 18 h later, when mid-term trophozoites had again become predominant, the cycle having been delayed 6 h following the first chloroquine injection (Table 5) (Cambie *et al.*, 1991).

7.1.3. *Chloroquine in Humans*

Data obtained with mice were shown to be relevant to human patients infected with *P. falciparum* by Landau *et al.* (1992). Thirty-one patients presenting with falciparum malaria at a dispensary in Madagascar were

Table 4 *Plasmodium vinckei petteri:* effect on parasitaemia and subsequent development of a single injection of chloroquine (5 mg kg^{-1}, subcutaneously) at different times during the erythrocytic merogony cycle[a].

Experiment A

Time	15:00	18:00	00:01	06:00	12:00	18:00	00:01	06:00
Parasitaemia (%)	8	11.4	10.9	7.8	5.5	2.7	1.6	2.8
Rings	*432*	366	44	39	*110*	30	32	45
Trophozoites								
Young	304	*638*	286	172	132	*57*	46	118
Mid-term	0	114	**748**	546	198	113	*32*	106
Old	64	23	22	**23**	110	70	50	*11*

Experiment B

Time	00:01	06:00	12:00	18:00	00:01	06:00	12:00
Parasitaemia (%)	1.95	0.8	0.24	0.14	0.18	0.24	0.38
Rings	2	0	2	1	1	0	1
Trophozoites							
Young	24	1	1	3	10	2	4
Mid-term	**138**	30	5	2	5	16	10
Old	36	**49**	17	7	2	6	24

Control

Time	12:00	18:00	00:01	06:00	12:00	18:00
Parasitaemia (%)	6.8	19.4	22.1	18.4	24.8	49.8
Rings	367	310	0	0	1055	1891
Trophozoites						
Young	177	1494	66	0	496	2736
Mid-term	0	116	1706	405	25	348
Old	140	20	443	1438	893	0

[a] All mice were inoculated at 12:00 and treated 5 days later, at the time of maximum ring production (15:00) in experiment A (illustrating a delayed drug effect) and at the time of maximum mid-term trophozoites (00:01) in experiment B (immediate effect). Parasite counts were made just before treatment (first column) and then at the times shown. The development of each brood of parasites can be followed along the diagonals (upper left to lower right) of each set of counts, as indicated by the *italicized* figures in experiment A. The two pairs of numbers indicating the effect of the drug in experiments A and B are printed in **bold** type. For the sake of comparability between experiments, each parasite count is presented as the product of the percentage of each stage and the percentage parasitaemia. (Data from Cambie *et al.*, 1991 and Caillard *et al.*, 1993.)

Table 5 *Plasmodium vinckei petteri:* pre-patent periods in mice receiving blood from donors previously given two injections of chloroquine aimed at either mid-term or young trophozoites (data from Cambie *et al.*, 1991).

No. of Mice	Time of treatments First Day	Time	Second Day	Time	Target stage	Interval between treatments (h)	Mean Pre-patent period (days)
9	1	00:01	1	18:00	Mid-term trophozoites	18	9.5
4	None (controls)				—	—	4.0
10	1	06:00	2	06:00	Old trophozoites	24	6.8
3	None (controls)				—	—	4.3

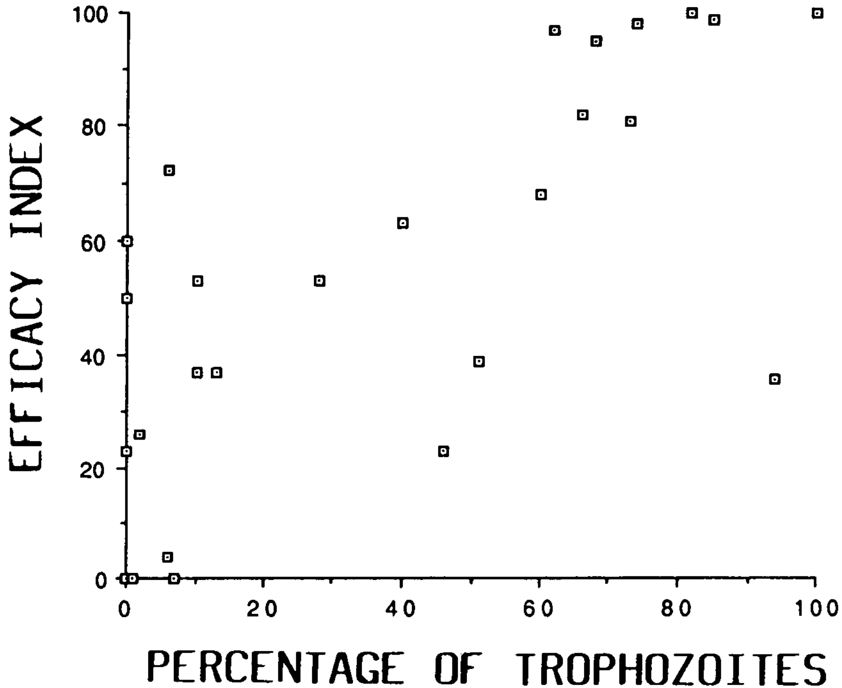


Figure 7 Effect of proportion of trophozoites in the blood on the efficacy of chloroquine treatment of *Plasmodium falciparum* malaria. Parasitaemia was determined by microscopical examination of blood films on admission of patients to the clinic before drug treatment (A) and on the following day (B). The efficacy of drug treatment was computed using the formula $Y = 100(A-B)/A$ and plotted against the percentage of trophozoites present at admission. (From Landau *et al.*, 1992.)

followed in order to find out if the effect of chloroquine differed according to the stage of parasite present in the blood (rings or trophozoites) at the beginning of treatment. The routine chloroquine therapy was 10 mg kg^{-1} on days one and two and 5 mg kg^{-1} on the third day. As with *P. vinckei petteri* in mice, the trophozoites of *P. falciparum* (equivalent to the mid-term trophozoites of *P. vinckei petteri*) were the most susceptible stage to chloroquine treatment.

7.2. Arteether

Experiments similar to those performed with chloroquine and *P. vinckei petteri* were conducted with arteether, an artemisinine derivative (Caillard *et al.*, 1993). The lowest subcurative dose showing an effect on parasitaemia was found to be 2.2 mg kg^{-1}. When the drug was dissolved in an organic solvent, such as alcohol, and subsequently diluted in water, the effect on the parasites was immediate and the sensitive stages were easily identified.

Both tests previously used with chloroquine were performed: duration of pre-treatment period in subinoculated mice and analysis of blood stages present after treatment of mice on day 5 of the infection. The results of both tests showed that rings and young trophozoites were the stages most susceptible to arteether. The drug had no effect on merozoites and little effect on mid-term trophozoites.

Drug dissolved in coconut oil (Miglyol 840®) showed more, though slower, activity. Assuming the drug was active 3–21 h after its injection, the same stages were found to be sensitive as with the alcoholic/aqueous solution — the rings and young trophozoites.

7.3. Quinine

V. Caillard, A. Beauté-Lafitte, A. Chabaud, H. Ginsburg and I. Landau (unpublished observations) showed that quinine activity was maximal when mid-term trophozoites of *P. vinckei petteri* predominated, but destruction of the parasites did not occur until they reached the meront stage.

7.4. Conclusions

It would be advantageous, for economic reasons and in order to avoid undesirable side effects and to reduce the risk of developing drug resistance, to be able to diminish the frequency of drug administration to human malaria patients. In order to increase efficacy, it would be logical to adapt the treatment to the stage present in the blood. Derivatives of artemisinine could

be given when ring forms predominate, as recommended by Jiang *et al.* (1982), chloroquine at the time when trophozoites predominate, and pyrimethamine–sulfadoxine when the larger parasites are sequestered in the capillaries (Rieckmann *et al.*, 1987).

Thus, treatment of malaria could be diversified according to the circumstances, with chronotherapy being taken into consideration in the design of therapeutic schedules.

8. SUMMARY AND GENERAL CONCLUSIONS

The only known malaria parasites of murid rodents are geographically restricted to six localities in the Congo forest basin in Africa. Two foci are situated at high altitude in Katanga (Zaire), the other four are in lowland forest in the Central African Republic; the principal rodent host is the arboreal species *Thamnomys rutilans*.

Two or three species of *Plasmodium* coexist in the same rodent species, and vicariant forms (see p. 54) occur in several localities. In each locality, the rodents harbour *P. vinckei* and either *P. berghei* or *P. yoelii*. A third species, *P. chabaudi*, is inconstantly found. Species or subspecies from the different localities are characterized by morphological differences, by isoenzymatic analysis, and by important biological features.

The life cycle of these parasites can be completed in experimentally infected mosquitoes, only at low temperatures for the parasites from Katanga but at higher temperatures for the lowland forest species.

The natural hosts harbour chronic infections, while experimentally infected rodents, including *Thamnomys gazellae*, have more or less acute infections.

This review has dealt more particularly with the three *Plasmodium* species from *Thamnomys rutilans* in the Central African Republic: *P. yoelii yoelii*, *P. chabaudi chabaudi* and *P. vinckei petteri*.

8.1. *Biology of Gametocytes*

The age of gametocytes can be determined by their morphology, thus enabling information to be gained about their developmental cycle, longevity and infectivity.

The period during which gametocytes in white mice are infective seems to be very fleeting and to be controlled by the following factors: (i) age — only young gametocytes are infective; (ii) size — young stages are larger than older ones, and appear to be retained in the fine capillaries from where they are dislodged by the suction exerted by feeding *Anopheles*; they may escape microscopical detection since blood films are usually prepared with blood from the larger vessels in the mouse's tail; (iii) course of infection —

gametocytes lose their infectivity at the approach of the crisis (anarchic multiplication of the parasites which begins as parasitaemias reach 20–45%); (iv) parasitaemic profile — gametocytes transitorily lose infectivity just after the occurrence of merogony.

The loss of infectivity (at crisis or merogony) is reversible; gametocytes recover their infectivity when transferred to uninfected mice. An inhibitory factor is present in the plasma, but cannot be immunological in origin because it appears very early in the course of infection.

8.2. *Ultrastructure of Hepatic Meronts*

One of the first characteristics to be observed in this stage is the vesicular system, which consists of zones of synthesis (the rough endoplasmic reticulum) and a transport system of vesicles containing a flakey, granular substance. We believe that this vesicular system plays a part in nutrition and maturation of the meront, and in the eventual destruction of the host hepatocyte. The vesicular system is particularly sensitive to attack by drugs, its morphological alterations being detectable before those of other parasite organelles.

8.3. *Chronicity of Infections*

Infections are chronic in *Thamnomys rutilans* but not in experimentally infected rodents. Exoerythrocytic meronts with the morphological characteristics of chronic, slowly developing stages could be found in the livers of naturally infected, wild-caught *Thamnomys rutilans* up to 8 months after their capture.

8.4. *Rhythm of Merogony and Biology of Merozoites*

The rhythm of merogony of *P. vinckei petteri* differed in mice inoculated with fresh or with frozen and thawed infected blood. Erythrocytes, and consequently the intracellular parasites, are destroyed by freezing and thawing procedures and only merozoites, and perhaps the mature meronts, survive. When fresh infected blood was inoculated, the rhythm of the infection in the donor mouse was maintained in the recipient. When the inoculum of infected blood had been frozen and thawed, the rhythm in the recipient mouse depended on the time of inoculation: merogony always occurred 24 h after inoculation, corresponding to the time required for the inoculated merozoites to develop into mature meronts.

Inoculation of frozen and thawed blood enhanced the synchronicity of the parasites, and revealed clear differences between species. Merogony of *P. chabaudi chabaudi* was entrained by the circadian rhythm of the host. Whatever the time of inoculation of infected blood, the meronts ruptured and the merozoites penetrated into erythrocytes at midnight if mice were illuminated from 08:00 to 20:00, and at noon if the light period was from 20:00 to 08:00.

Merogony of *P. vinckei petteri* depended on the time of inoculation, and occurred 24 h after the inoculation of frozen and thawed blood. Most merozoites penetrated into erythrocytes immediately after inoculation. *P. yoelii yoelii* was less synchronous. Merozoites progressively entered erythrocytes, and merogony occurred throughout the day. With *P. yoelii nigeriensis*, from Nigeria, merozoites appeared to be capable of penetrating erythrocytes mainly around midnight. Infection began earlier when the infective inoculum was given at midnight than when it was given at 16:00, and even earlier when the inoculation was given at 09:00.

8.5. *Merozoite Latency, Asynchronicity and Drug Resistance of* P. yoelii

Penetration of erythrocytes appeared to be more or less delayed; infective merozoites of *P. yoelii nigeriensis*, in particular, seemed capable of remaining latent for a long time, 36 h and probably more. As merozoites are chloroquine resistant and *P. yoelii* subspp. are the only spontaneously chloroquine-resistant rodent malaria species, there is probably a relationship between latency of merozoites, asynchronism, and chemoresistance.

8.6. *Chronotherapy*

Following inoculation of frozen and thawed blood infected with *P. vinckei petteri*, mice developed highly synchronous infections. It was thus possible to determine the effect of chloroquine against each parasite stage. The mid-term trophozoite was the most sensitive stage to chloroquine treatment, which had an immediate effect when this stage was predominant. Other stages were unaffected but could subsequently be destroyed if sufficient chloroquine remained in the plasma at the time they reached the mid-term trophozoite stage.

When two successive doses of a drug were given, the first eliminated the sensitive stages, thus modifying the life cycle rhythm which was subsequently entrained by the least damaged stage, which became predominant. The second dose should be timed to take account of the new rhythm. In the case of chloroquine, the elimination of mid-term trophozoites by the first drug dose shifted the phase cycle by at least 6 h; the second dose showed the best activity when given 18 h later, when mid-term trophozoites were again predominant.

Similar investigations with arteether and quinine showed that the sensitive stages for these compounds were rings and young trophozoites, and mid-term trophozoites, respectively.

Results obtained with chloroquine in the rodent model were applicable to human infections; a survey of patients in Madagascar showed the sensitive stage of *P. falciparum* to be the trophozoite; chloroquine was more effective when this stage was predominant at the time of treatment than at any other phase of the blood cycle.

8.7. *Envoi*

These studies on the malaria parasites of the tree rat *Thamnomys rutilans* are a good example of what zoology can offer to medicine. These murid species of *Plasmodium* have been the subject of hundreds of publications, and we have been studying them for more than 25 years, but the subject still seems to be barely touched.

It appears that each of the subspecies isolated from the known foci has marked biological particularities; we still need more information about the various geographical strains. For all of the problems investigated in this work, it was necessary to select a specific strain or to make comparisons between different species or subspecies. More knowledge of these parasites would lead to their becoming even better tools to help solve the important, and sometimes very worrying, problems that remain concerning human malaria.

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Metacercarial Excystment of Trematodes

Bernard Fried

Department of Biology, Lafayette College, Easton, Pennsylvania 18042, USA

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1. INTRODUCTION

With the exception of the schistosomes, most digeneans enter the vertebrate host when encysted metacercariae (cysts) in or on a second intermediate host are swallowed by a vertebrate (definitive) host. The encysted metacercariae then excyst usually in the host intestine aided by physicochemical conditions in that site. Excystation is accomplished by some combination of external (extrinsic) factors from the host (e.g. enzymic, temperature, pH, bile salts, CO_2 tension, reductants, or other) and internal (intrinsic) factors from the parasite (e.g. secretions, muscular activity, or other).

An extensive literature is available on metacercarial excystation. Erasmus (1972) reviewed the literature from about 1930 to 1970 and described extrinsic and intrinsic factors involved in excystation; he provided coverage of the pioneering studies by Dixon (1966) on excystation of *Fasciola hepatica* and discussed the need for excystation procedures to obtain digenean larvae for studies on trematode morphology and cultivation.

Lackie (1975) reviewed metacercarial excystation emphasizing activation phenomena associated with the release of organisms from their cyst envelopes. She provided tabular data on excystation in 20 species from 14 families. In this review papers already discussed by Lackie are given minimal or no coverage.

Smyth and Halton (1983) reviewed excystation in both fasciolid and non-fasciolid digeneans with emphasis on the former; they also reviewed metacercarial encystment, an understanding of which is important to appreciate events that occur during excystation. Sommerville and Rogers (1987) examined the nature and action of host signals involved in the excystation process and tabulated excystation requirements in 13 species of digeneans from eight families. Sukhdeo and Mettrick (1987) examined mainly the nature of metacercarial behavior during the excystment process, an area that for the most part has been neglected.

The purpose of this review is to examine the pertinent excystation literature from 1975 to 1993. In this review information on *in vitro* excystation has been organized according to families of digeneans in the classification scheme of LaRue (1957). Emphasis in this review is on *in vitro* (mainly chemical) excystation, but the limited studies on *in vivo* excystation are also covered. Most work on *in vivo* excystation is usually incidental to bionomic and life history studies on digeneans. Since no previous review

deals specifically withuses of excysted metacercariae, that topic is covered herein. The relative role of extrinsic versus intrinsic excystation is discussed and finally some consideration is given to possible future research on excystation.

2. AVAILABILITY OF ENCYSTED METACERCARIAE

Encysted metacercariae can be obtained from naturally or experimentally infected second intermediate hosts. Many animals, including snails, bivalves, aquatic insect larvae, crustaceans, frogs, fishes, and reptiles, can serve as second intermediate hosts. Numerous snail first intermediate hosts release cercariae that encyst on vegetation or a substratum in the field or on the sides and bottoms of containers in the laboratory. Encysted metacercariae can be removed from intermediate hosts or from the surfaces of laboratory containers and used for *in vivo* or *in vitro* excystation studies. Encysted metacercariae can be stored at 4°C in Locke's 1 : 1 solution up to a year with little or no loss of viability (Fried and Wilson, 1981; Fried and Perkins, 1982; Fried and Emili, 1988). Storage of cysts is important where severe weather may preclude the possibility of obtaining larval trematodes in the wild.

Encysted metacercariae can be obtained from investigators who work with particular species of digeneans. I have sent cysts of *Echinostoma caproni* and *E. trivolvis*, *Zygocotyle lunata*, *Parorchis acanthus* and *Philophthalmus hegeneri* to colleagues in the USA and abroad. I usually ship cysts by express mail in plastic vials filled with Locke's 1 : 1 solution and advise recipients to store the cysts in Locke's 1 : 1 solution at 4°C until use.

Snails infected with larval trematodes can also be sent by mail and if the material is received within 7 days the snails are usually alive. Snails should be wrapped in several layers of moist paper towels followed by several layers of dry paper towels and shipped in a cardboard container.

Numerous biological supply houses furnish snails that are infected with cercariae or encysted metacercariae. Infected physids and lymnaeids may be available from Carolina Biological Supply Co., Burlington, NC, and Wards Natural Science Inc., Rochester, NY. The marine Biological Lab (MBL), Woods Hole, MA may be able to supply *Ilyanassa*, *Thais* and *Urosalpinx* snails which provide a good source of cercariae and encysted metacercariae of marine trematodes. Jones Biological Co., Long Beach, CA is a supplier of the marine snail *Cerithidea californica* which has at least 15 species of larval trematodes (see Martin, 1972 for details) including cercariae and encysted metacercariae of heterophyids, philophthalmids, echinostomatids, parorchids, strigeids, microphallids and other digeneans.

3. BIOLOGY OF ENCYSTED METACERCARIAE

Cercariae of numerous digeneans encyst in or on a second intermediate host (e.g. *Clonorchis sinensis* in fish; *Echinostoma trivolvis* in snails; *Paragonimus westermani* in crabs) to produce cysts (encysted metacercariae). Other cercariae encyst in the wild on vegetation or objects such as clam and snail shells, and in the laboratory on glass, cellophane, polyethylene tubing or plastic dishes (e.g. *Fasciola hepatica*, *Philophthalmus hegeneri*, *Parorchis acanthus*, *Zygocotyle lunata*). The process of encystment is complex, variable from species to species, and not fully understood. Usually, prior to encystment, the cercaria shows increased activity, the suckers often attach to tissue of the intermediate host or a substratum; the tail decaudates from the body and cystogenous material is released from cystogenous glands to form part or all of the cyst wall; in a cercaria that encysts in or on a host, the host may contribute secretions and/or tissues to cyst wall formation.

The shape of metacercarial cysts is variable from one species to another and may be spherical (e.g. *Echinostoma trivolvis* and *Sphaeridiotrema globulus*), ovoidal (e.g. *Parorchis acanthus*), flask-shaped (e.g. *Philophthalmus hegeneri*), or hemispherical (e.g. *Fasciola hepatica* or *Zygocotyle lunata*). A specialized structure called the ventral plug (mucus plug) or ventral lid may be present in a specific area of the cyst wall (e.g. *Zygocotyle lunata*, *Fasciola hepatica*). During excystation the ventral plug is dissolved or the ventral lid opens to allow for release of the larva from the metacercarial cyst. Mechanisms involved in the dissolution of the ventral plug or dehiscence of the ventral lid are not well understood.

Some cercariae, particularly those that encyst on vegetation or on a substratum, usually encyst within a few hours and are immediately infective to their definitive hosts (e.g. *Zygocotyle lunata*, *Fasciola hepatica*). Other cercariae (e.g. *Echinostoma trivolvis*, *Echinostoma caproni*), which encyst in the tissues of an intermediate host, may take up to a day for the cyst to become infective to the definitive host (Anderson and Fried, 1987). Cysts of *E. trivolvis* that have not yet hardened, cannot be excysted *in vitro* in the Fried and Roth (1974) medium (see Section 5 and Anderson and Fried, 1987). Some cercariae, for example, strigeids, have an extended post-cercarial development period, and the resulting metacercaria is considerably more advanced in development than the cercaria. The development time for transformation of various species of cercariae to infective metacercariae has been reviewed by Dönges (1969).

The structure of encysted metacercariae is variable from one species to another. In *Fasciola hepatica* the encysted metacercaria consists of an inner and outer cyst each with additional layers (Dixon and Mercer, 1964; Dixon, 1965). The outer cyst has an external layer of tanned protein and an underlying fibrous layer of mucoprotein. The inner cyst has a complex mucopolysaccharide layer that can be subdivided into three layers and an

additional layer (layer IV) of laminated or keratinized protein. A region of layer IV is specialized to form the ventral plug.

Metacercarial cysts that form on vegetation or on a substratum usually have more layers than those that encyst in intermediate hosts. The free-encysting metacercaria of *Zygocotyle lunata* has five layers (Robbins *et al.*, 1979) and that of *Cloacitrema narrabeenensis* has four layers (Dixon, 1975). The cysts of *Echinostoma trivolvis* and *Sphaeridiotrema globulus*, both of which encyst in snail intermediate hosts, have three layers (Gulka and Fried, 1979; Huffman, 1986). Some metacercariae (e.g. *Posthodiplostomum nanum* and *Clinostomum tilapiae*) have only a single layer of parasite origin and do not contain a capsule of host origin (Asanji and Williams, 1973). In other species the host contributes substances to the cyst wall, for example collagen in *Echinostoma trivolvis* (see Gulka and Fried, 1979), and fibroblasts in *Ornithodiplostomum ptychocheilus* and in *Uvulifer ambloplitis* (see Spellman and Johnson, 1987). Proteins and mucopolysaccharides are usual components of cyst walls, but their distribution within cyst layers varies from one species to another (Fried *et al.*, 1978b; Gulka and Fried, 1979; Huffman, 1986). Transmission electron microscopy shows that different layers vary in electron density depending upon the species, but usually the innermost layer is lamellated and the lamellae are made up of keratinized proteins (Robbins *et al.*, 1979; Gulka and Fried, 1979; Huffman, 1986). Cyst layers are often difficult to enumerate by light microscopy alone and unequivocal determination of the layers often requires examination by transmission electron microscopy.

Numerous histologic, histochemical, and ultrastructural studies on many species of encysted metacercariae show that considerable specific variation exists in both the number of cyst layers and their chemical composition. Because of this it is not surprising that numerous methods have been described for the *in vitro* excystation of metacercarial cysts of different species.

The generalization about cyst complexity as a function of free encystment in the environment versus encystment in an intermediate host may need re-examination as more cysts are studied by transmission electron microscopy. Lou *et al.* (1992) did ultrastructural studies on the cysts of four species of *Paragonimus* obtained from the gills and muscles of various freshwater and brackish crustaceans. Whereas one species (*Paragonimus iloktsuenensis*) had two distinct layers, three species (*P. westermani*, *P. miyazakii* and *P. ohirai*) had five distinct layers.

4. *IN VIVO* EXCYSTATION

Numerous studies on the life cycles and bionomics of trematodes have described cysts in various phases of excystation in the intestine of the host.

Krull and Price (1932) observed empty cysts of *Diplodicus temperatus* in the frog rectum with cyst walls that had not been affected by enzymes, suggesting that excystment was active. Willey (1941) observed encysted and excysted metacercariae of *Zygocotyle lunata* in the cecum of the rat within 15 h post-feeding of cysts. He did not find excysted metacercariae in the upper intestine and suggested that excystation occurred in the cecum. In a subsequent *in vivo* excystation study on *Z. lunata* in the domestic chick, Fried *et al.* (1978b) showed that encysted metacercariae excysted in the lower ileum within 2 h post-feeding of cysts. Following excystation larvae entered the ceca and established therein. It is not known if entry of the excysted metacercariae into the ceca is by active or passive means.

Relatively few studies have been designed specifically to study metacercarial cysts *in vivo*. Such studies require a large number of parasite-free experimental hosts maintained on carefully monitored diets since the presence of food in the gut will influence excystation. Cysts of most species are small (<0.5 mm) and encysted or excysted metacercariae are difficult to find in the gut; many excysted metacercariae are transparent and can be confused with the intestinal villi at necropsy.

Early studies on excystation have indicated that the duodenum is a prime site for excystation: *Clonorchis sinensis* by Faust and Khaw (1927); *Parorchis acanthus* by Stunkard and Cable (1932); *Cryptocotyle lingua* by Smyth (1962). Although the site of excystment has been established for some species, little quantitative work has been done on the rate and percentage of excystment *in vivo*.

Asanji and Williams (1974) reported excystment of *Clinostomum tilapiae* and *Posthodiplostomum nanum* in the stomach of experimentally infected avian hosts, whereas excystation of *Parorchis acanthus*, *Posthodiplostomum* sp. and *Posthodiplostomoides leonensis* occurred in the duodenum of birds. Differences in the site of excystation were related to the structure of the cyst wall and the final site occupied by the worms. Parasites that excysted in the stomach and established in either the mouth or esophagus (*Clinostomum tilapiae*) or just posterior to the pylorus (*Posthodiplostomum nanum*) had single or bilayered cyst walls. Those that excysted in the duodenum (*Parorchis acanthus*, *Posthodiplostomum* sp. and *Posthodiplostomoides leonenses*) and subsequently established posteriad to the duodenum had multilayered cyst walls. Asanji and Williams (1974) showed that at least for some species of avian digeneans there is a correlation between excystation site and complexity of the cyst wall.

Fried and Kletkewicz (1987) examined *in vivo* excystation of *Echinostoma trivolvis* in unfed, day-old domestic chicks. Chicks were fed 400 cysts each and necropsied at various time intervals from 15 min to 24 h post-feeding of cysts. Excystation occurred within 30 min in the lower ileum. Some excysted metacercariae were seen in the upper ileum at 0.5 h. Viability of cysts that were retained in the gizzard for various periods of time was tested by treating

cysts in the Fried and Roth (1974) medium (see Section 5). Cysts removed from the gizzard after 3 h were not viable as determined by *in vitro* excystation. Most cysts removed from the gizzard within 2 h post-feeding excysted in the artificial medium within 10 min. Cysts retained in the gizzard for more than 3 h were adversely affected by the acidic environment of that organ or by other factors such as mechanical disruption. Intestinal emptying is very rapid in the domestic chick (about 1–1.5 h) and will influence the rate and percentage of excystation in this host.

Radlett (1979) studied *in vivo* excystation of *Notocotylus attenuatus* in the domestic chick and found excysted metacercariae in the lower intestine and juvenile worms in the cecum within 4 h post-infection. He fed charcoal to the chicks which was used as a tracer. The charcoal reached the duodenum in 0.5 h and the cecum in 2.25 h. The metacercariae were slower in their passage, being recovered from the duodenum and cecum at 1 and 4 h, respectively. The first excysted juvenile worm was recovered from the lower intestine at 2.25 h. Young worms were found in the proximal region of the cecum nearest to the ileocecal junction by 4 h post-infection. No excysted worms were found in the rectum. Radlett suggested that excysted metacercariae probably moved actively into the cecal opening and were not carried there by peristaltic activity.

Graczyk and Shiff (1993b) examined *in vitro* (see Section VI) and *in vivo* excystation of *Notocotylus attenuatus*. Identification of species in the genus *Notocotylus* is confusing and it is not known if the species used by Graczyk and Shiff was identical to that of Radlett (1979). Graczyk and Shiff did not find excysted metacercariae of *N. attenuatus* in the lower intestine of experimentally infected ducks, but found viable cysts in the duck's cecum at 3 days post-infection. The cyst of *Notocotylus attenuatus* is resistant to chemical and physical treatment and *in vitro* studies (see Section 6) showed that the cyst could only be excysted when cationic detergents were used as a pre-treatment followed by conventional enzymic treatments. Cysts of this species are multilayered and contain unique structures in the mucoid envelope including a funnel-shaped cavity and a narrow tube. During *in vitro* excystation, and only in the presence of cationic detergents, does disruption of the cyst begin in the area of the funnel-shaped cavity. Apparently this cyst is not appreciably altered *in vivo* until it enters the avian cecum where changes equivalent to the effects of *in vitro* cationic detergents probably occur.

Surgical procedures have been used to provide information on factors associated with *in vivo* excystation. Wykoff and Lepes (1957) ligated rabbit bile ducts and still obtained excystation of *Clonorchis sinensis* in the duodenum. The results suggested that metacercariae of *C. sinensis* did not require exposure to bile for excystation.

Encysted metacercariae of *Echinostoma caproni* surgically implanted into the small intestine of ICR mice produced adults suggesting that these metacercariae can excyst *in vivo* without passage through the stomach (Chien

et al., 1993). Fried and Emili (1988) showed that metacercariae of this species can excyst *in vitro* in the absence of acid pepsin pre-treatment.

Surgical implantation of encysted metacercariae into the peritoneal cavity of several vertebrates has clarified the role of active versus passive excystation in some digenean species. The peritoneal cavity is devoid of intestinal enzymes and can be used to examine active excystation. Earlier studies (reviewed by Dixon, 1966) showed that metacercariae of *Fasciola hepatica* excyst in the sheep and mouse peritoneal cavity, possibly in response to stimulation from low CO_2 concentration; the results suggested that excystment in this species is an active process not requiring digestive enzymes.

Asanji and Fried (1990) used the mouse peritoneal cavity to examine excystation of various species of digeneans. For some species excystation occurred as well in warm saline as in the mouse peritoneum, for example *Clinostomum tilapiae* and *Posthodiplostomum nanum*. These species probably rely mainly on intrinsic factors that are stimulated by elevated temperature. One species studied, *Parorchis acanthus*, never excysted in warm saline or in the mouse peritoneum indicating that this species is dependent at least in part on extrinsic enzymes. *Fasciola hepatica* could be excysted in the mouse peritoneum, but not in warm saline and reasons for this are not clear (Asanji and Fried, 1990).

Prior to the development of a successful *in vitro* excystation medium for *E. trivolvis*, metacercariae of this species were excysted *in vivo* in the domestic chick; thousands of cysts were fed to chicks which were killed 24 h later to obtain newly excysted juveniles for subsequent chick chorioallantoic transplantation studies (Fried *et al.*, 1968). With the advent of simple procedures for the *in vitro* excystation of echinostomes (see Section 5), the *in vivo* technique for obtaining newly excysted juveniles is no longer recommended.

5. PREPARATION OF EXCYSTATION MEDIUM

Two media are used often to excyst metacercariae of various non-fasciolid digeneans. The first medium was developed by Fried and Roth (1974) to excyst *Parorchis acanthus* metacercariae and has since been used for the following species: *Echinoparyphium flexum* by Fried and Grigo (1975); *Cotylurus strigeoides* by Fried *et al.* (1978a); *Sphaeridiotrema globulus* by Fried and Huffman (1982); *Fascioloides magna* by Fried and Stromberg (1985); *Cyathocotyle bushiensis* by Fried and Ramundo (1987); and *Echinostoma caproni* and *Echinostoma trivolvis* by Fried and Emili (1988).

The second medium was developed for excystation studies on *Himasthla leptosoma* by Irwin *et al.* (1984) and has since been used for the following species: *Microphallus abortivus* by Saville and Irwin (1991a); *Microphallus*

primas by Saville and Irwin (1991b); *Parapronocephalum symmetricum* by Irwin *et al.* (1989) and *Zygocotyle lunata* by Irwin *et al.* (1993).

The first medium (hereafter referred to as the Fried and Roth medium) is made up as follows. Prepare a 0.5% trypsin (1–250, pig pancreas, USB, Cleveland, Ohio) plus 0.5% bile salts mixture in Earle's Balanced Salt Solution; add sufficient 7.5% $NaHCO_3$ to adjust the pH of the medium to 8.0± 0.2. Filter the medium and distribute the filtrate in 10- or 20-ml aliquots in plastic disposable tubes. This medium can be used fresh or can be stored at −20°C for up to 2 years with no apparent loss of activity. If frozen, defrost the medium in warm water and filter it before use. Add cysts in a minimal amount of saline to 2–3 ml of the medium in 3.5-cm diameter Petri dishes. Place the dishes in an incubator at 37–42°C for up to 2 h. Monitor excystation at convenient intervals (every 15 or 30 min). Remove excysted metacercariae to saline for subsequent use in studies on morphology, cultivation, biochemistry, immunology, etc. See Section 6 for various uses of chemically excysted metacercariae.

The second medium (hereafter referred to as the Irwin *et al.* medium) is made up as follows. For solution A prepare a 5 ml bicarbonate saline solution (0.8% w/v sodium chloride and 1.5% w/v sodium bicarbonate) containing 0.8% w/v sodium taurocholate and 0.3% w/v trypsin. Make up solution B which consists of 5 ml of 0.02 M HCl containing 0.8% w/v L-cysteine. Mix solutions A and B just before use. This mixture should be prepared fresh. Tests on the encysted metacercariae are usually done in watch glasses maintained in a water bath at 40–42°C.

6. *IN VITRO* EXCYSTATION

6.1. General

The following section considers studies on the *in vitro* excystation of digeneans since about 1975. The arrangement of the section is based on the list of families of Digenea provided by LaRue (1957). Whenever possible uses of excysted metacercariae are given at the end of each subsection. The appendix on pp. 127–135 lists studies (arranged in alphabetic order by species) on *in vitro* excystation from about 1975 to 1993.

6.2. Strigeidae

Lackie (1975) reviewed the study by Voge and Jeong (1971) on excystation of *Cotylurus lutzi* tetracotyles from *Biomphalaria glabrata* snails. Excystation of *Cotylurus lutzi* is active requiring only treatment in Earle's BSS at an

elevated temperature of 41–42°C. Magnus and Johnson (1985) also showed active excystation of the tetracotyles of *Cotylurus flabelliformis* from the lymnaeid snail, *Stagnicola elodes*. The other species of *Cotylurus* studied are more fastidious requiring both extrinsic and intrinsic factors for excystation. Thus, *Cotylurus strigeoides* tetracotyles from physid snails (Fried *et al.*, 1978a), *C. variegatus* from the swim bladder and pericardium of *Perca fluviatilis* (see Thompson and Halton, 1982) and *C. erraticus* from the pericardium and heart of rainbow trout, *Salmo gairdneri* (see Mitchell *et al.*, 1978) require trypsin and bile salts in an alkaline medium at about 41°C for *in vitro* excystation.

Excysted metacercariae of *Cotylurus* species have been used for *in ovo* and *in vitro* cultivation studies. Voge and Jeong (1971) cultivated excysted tetracotyles of *C. lutzi* in NCTC 135 supplemented with 50% chicken serum and obtained adults that produced non-viable eggs. Basch *et al.* (1973) added upper chicken intestine mucosal extract to the Voge and Jeong (1971) medium and obtained *C. lutzi* adults that produced eggs capable of embryonation and hatching. Excysted tetracotyles of *C. strigeioides* showed some post-metacercarial development on the chick chorioallantois, but better development occurred, including the production of ovigerous adults, in several defined culture media supplemented with natural products (Fried *et al.*, 1978a). Magnus and Johnson (1985) also cultivated excysted tetracotyles of *C. flabelliformis* to ovigerous adults in defined media supplemented with natural products.

Mitchell *et al.* (1978) used *in vitro* excysted tetracotyles of *C. erraticus* for various purposes. They cultured this organism *in vitro* to the ovigerous adult stage (eggs were not viable) in defined medium supplemented with serum. They also provided scanning electron microscopical observations on the excysted metacercariae and histological and histochemical studies on the developing metacercariae. Halton and Mitchell (1984) used *C. erraticus* excysted metacercariae to demonstrate the uptake of thymidine during *in vitro* cultivation.

Histochemical and thin-layer chromatographic analyses were done on neutral lipids in the excysted metacercariae of *Cotylurus strigeoides* by Fried and Butler (1977). The histochemical study showed the presence of Oil Red O positive neutral lipids in the metacercarial excretory system and their TLC studies showed release of neutral lipids from the excretory pore.

Excystation of two species of strigeids in the genus *Apatemon* has been reported by Blair (1976) and Kearn *et al.* (1989). Requirements for both species included acid pepsin pre-treatment followed by alkaline bile-trypsin at elevated temperatures. In these species it appears that the contents of the cyst are under internal pressure. When treated in pepsin the layers of the cyst wall peel back from one end and when cysts are transferred to the alkaline trypsin-bile medium, one pole of the cyst ruptures and the worm is forcibly expelled.

Graczyk and Shiff (1993a) described excystation of *Cotylurus cornutus* tetracotyle cysts from infected *Physa acuta* snails in Maryland, USA. Pretreatment in an acid medium was essential for excystation of this species. Both acid pepsin or acid saline were effective but optimum excystation occurred after 15 min in acid pepsin followed by 2 h treatment at 41.5°C in the Fried and Roth medium (see Section 5). The rate of excystation was rapid reaching 78% by 1 h and 84% after 2 h. Storage of metacercarial cysts up to 16 weeks at 4°C in sterile Locke's 1 : 1 did not alter cyst viability.

6.3. Diplostomatidae

Lackie (1975) reviewed the earlier studies on excystment of *Bolbophorus confusus* and *Posthodiplostomum minimum*. Whereas *P. minimum* required only treatment in acidified pepsin at an elevated temperature, *Bolbophorus confusus* needed acidified pepsin followed by alkaline trypsin.

Schroeder *et al.* (1981) studied excystation of *Neascus pyriformis* cysts from the skin and fins of cyprinid fishes and found that optimal excystation occurred following two treatments: (i) low pH (2.0) with acidified Locke's; and (ii) 0.5% trypsin plus 0.5% sodium cholate at pH 7.4 at 41°C. Spellman and Johnson (1987) reported optimal excystment of *Uvulifer ambloplitis* metacercariae from the sunfish, *Lepomis cyanellus* was obtained following three treatments: (i) 0.5% pepsin at pH 2.0 for 0.5 h; (ii) the reductant, 0.2% sodium dithionite at pH 7.4 for 10 min; and (iii) 0.5% bile salts at pH 7.4 for 2 h. For *U. ambloplitis* the synergistic effect of bile salts and trypsin was not a prerequisite for optimal excystation in contrast to that of *Neascus pyriformis* reported by Schroeder *et al.* (1981). Wittrock *et al.* (1991) used histochemical and ultrastructural procedures to examine encysted metacercariae of these diplostomatids. Numerous histochemical tests on the cyst walls of both species gave identical staining results. However, ultrastructural studies showed some differences in the two species of cysts. The parasite portion of the capsule of *U. ambloplitis* consisted of two layers, an outer dense and an inner light layer, whereas the parasite component of the cyst of *N. pyriformis* was composed of three layers. Subtle ultrastructural differences in cyst walls may account for different excystation requirements of closely related species.

Relatively few studies have been done on excysted metacercariae of diplostomatids. Ferguson's (1940) classical study on *Posthodiplostomum minimum* metacercariae was the first to report significant post-metacercarial development of a trematode cultivated *in vitro*. Fried (1970) cultivated excysted metacercariae of *P. minimum minimum in ovo* and described the histopathologic effects of this parasite on the chick chorioallantois.

Some species of diplostomatids are free (unencysted metacercariae) in host tissue and do not require chemical excystation for activation. These organisms provide good material for both *in vitro* and *in ovo* cultivation studies. Use of

free diplostomatid metacercariae in cultivation studies has been described by Kannangara and Smyth (1974), Irwin and Saville (1988), and Leno and Holloway (1986).

6.4. Cyathocotoylidae

Lackie (1975) reviewed the earlier study by Erasmus and Bennett (1965) on excystation of *Cyathocotyle bushiensis* and *Holostephanus luhei*. For both species excystation required acid pepsin pre-treatment followed by an alkaline trypsin-bile treatment at 37°C.

Fried and Ramundo (1987) examined excystation of the metacercarial cysts of *Cyathocotyle bushiensis* from *Bithynia tentaculata* snails. The encysted metacercariae excysted in the Fried and Roth medium (see Section 5) in the absence of acid pepsin pre-treatment. Cysts pretreated in acid pepsin for more than 2 h and then treated in the Fried and Roth medium did not excyst showing the adverse effects of prolonged treatment in acid pepsin. Storage of cysts in Locke's 1 : 1 at 4°C was studied. The percentage of cysts that excysted in the Fried and Roth medium after storage was 94% at 1 week, 62% at 4 weeks, and 15% at 8 weeks. Excysted metacercariae were used for *in ovo* and *in vitro* cultivation studies and post-metacercarial development was achieved in both sites. Best development of excysted metacercariae occurred in a medium of NCTC 135 supplemented with 50% chick serum in which worms increased their body area 10 times and became ovigerous by day 8 post-inoculation.

6.5. Clinostomatidae

Lackie (1975) reviewed Hemenway's (1948) early study on excystation of *Clinostomum* sp. in which cysts were treated in acid pepsin for 1 h followed by alkaline trypsin for 15 min at 37°C. It is doubtful if trypsin is essential for excystation of *Clinostomum* sp. since Fried *et al.* (1970) were able to excyst the metacercariae of *C. marginatum* from naturally infected frogs in acid pepsin at 40°C. These large cysts, commonly known as yellow grubs, can be excysted mechanically in Ringer's solution at 22°C (Fried and Foley, 1970).

Excystation of *Clinostomum* species is probably related to their site in the upper esophagus–buccal cavity region of avian hosts; these cysts are usually not found posterior to the proventriculus and following excystation in the acid pepsin environment of the proventriculus, the excysted metacercariae migrate to the mouth region. Reasons for such an anteriad migration are not known.

Studies on excystation of *Clinostomum* species in the 1980s and 1990s have been reported by two separate groups. Kalantan and Arfin (1988) obtained

cysts of *C. complanatum* from the skin and musculature of cyprinodont fishes, *Aphanius dispar*, and excysted them in 1% acidified pepsin (pH 2.3–3.5) in Tyrode's at 42°C. Larson and Uglem (1990) obtained encysted metacercariae of *C. marginatum* from yellow perch, *Perca flavescens*, and excysted them mechanically in cold Ringer's solution.

Excysted metacercariae of *Clinostomum* spp. have been used in various developmental studies. Fried and Foley (1970) achieved development of the excysted metacercariae of *C. marginatum* on the chick chorioallantois and showed by light microscopy developmental changes from the spinose tegument of the excysted metacercaria to the aspinose one in worms grown on the chick chorioallantois or in the mouth of domestic chicks. Further developmental changes in the tegument of the metacercaria of *Clinostomum complanatum* during *in vivo* development in the buccal cavity of the domestic chick were reported by Abidi *et al.* (1988).

Larson and Uglem (1990) obtained development of excysted metacercariae of *C. marginatum* on the chick chorioallantois, but reported better development of these metacercariae in the mouse coelom. Uglem and Larson (1987) examined facilitated diffusion and active transport systems for glucose in excysted metacercariae of this species.

6.6. Brachylaimidae

Excystation studies have been done on *Leucochloridium variae* cysts obtained from the broodsacs (highly modified sporocysts) of naturally infected *Succinea ovalis* snails (Fried, 1973; Lewis, 1974). Excystation was achieved by pretreating cysts in 1% acidified pepsin for 15 min followed by treatment in 1% alkaline trypsin for 30 min at 37°C. The pH of the acidified pepsin or alkaline trypsin was not critical to obtain large numbers of excysted metacercariae (Fried, 1973). Excysted metacercariae of this species inoculated into 3-day-old fertile domestic chick embryos produced ovigerous adults on the chick chorioallantois within 2 weeks post-inoculation. Most brachylaimid metacercariae are free, that is, unencysted in their snail hosts, and do not require additional treatment for *in vitro* or *in ovo* studies (see Fried and Stableford, 1991 and Fried, 1978 for further details).

6.7. Bucephalidae

Excystation *in vitro* of *Bucephaloides gracilescens* from whiting fish (*Merlangius merlangus*) occurred in two phases (Johnston and Halton, 1981). The first phase, a passive one, required 0.5% acid pepsin (pH 2.0) digestion for 1 h to remove most of the outer capsule of the cyst. The second phase, an active one, was triggered by a rise in the environmental pH to 7.2 which stimulated

muscular activity in the metacercaria resulting in excystation within 5 min. Various types of bile salts, although not necessary for excystation, increased the rate and percentage of excystation. The location of the cyst in the fish host played a role in subsequent excystation. Cysts from the orbit and nasal regions of the fish host had thicker walls than those from the auditory capsules and cranial fluid of the brain and required longer treatment in pepsin for optimal excystation. Trypsin, reducing agents, and a CO_2 atmosphere played no apparent role in the excystation process.

Halton and Johnston (1983) cultivated excysted metacercariae of *B. gracilescens* in several defined media supplemented with natural products. Ovigerous adults, but with non-viable eggs, were obtained at 18°C in NCTC-135 supplemented with chicken serum and a gas phase of air. Halton and Johnston (1983) used autoradiography to demonstrate the uptake of labeled tyrosine and thymidine into the reproductive system of cultured worms.

6.8. Echinostomatidae

Lackie (1975) and Fried (1978) reviewed the earlier work by Howell (1968, 1970) on the chemical excystation of metacercariae of *Echinoparyphium serratum*. Considerable work has been done on excystation of echinostomes, mainly in the genera *Echinostoma*, *Echinoparyphium*, *Himasthla* and *Acanthoparyphium*, since those earlier reviews.

Fried and Butler (1978) obtained encysted metacercariae of *Echinostoma trivolvis* (referred to as *E. revolutum* in their study) from experimentally infected physid snails. They showed that the metacercariae could excyst in the Fried and Roth medium (see Section 5) in the absence of acid pepsin pre-treatment and that reductants or special gas phases were not needed. Excystation did not occur in the absence of either the bile salt or the trypsin; the alkaline pH and the high temperature were necessary for a high rate and per cent of excystation within a physiological optimal time. Fried and Emili (1988) compared excystation of *E. trivolvis* with an Egyptian species of 37-collar-spined echinstome, *Echinostoma caproni* (referred to as *E. liei* by several authors; see Christensen *et al.*, 1990 for clarification of the nomenclature). Encysted metacercariae of *E. caproni* were obtained from experimentally infected *Biomphalaria glabrata* snails and were excysted in the Fried and Roth medium (see Section 5). The rate and percentage of excystation of *E. caproni* was higher than that of *E. trivolvis* under the identical conditions used to excyst the latter species. The results of the studies on *E. trivolvis* and *E. caproni* showed that both extrinsic and intrinsic factors are involved in the excystation of these echinostomes and there is a synergistic effect between bile salts and trypsin. Mohandes and Nadakal (1978) obtained metacercariae of *Echinostoma malayanum* from the kidneys of naturally infected *Indoplanorbis exustus* snails. They excysted this echinostome in a medium of 0.5%

trypsin + 0.5% sodium cholate in Hank's BSS adjusted to pH 8.1 with 7.5% $NaHCO_3$ at a temperature of 42°C. An acid pepsin pre-treatment was not essential for a high percentage of excystment, but did enhance the rate of excystment. Likewise, the reductant, sodium dithionite, although not essential for excystment, did increase the rate and percentage of excystment.

Howell (1968, 1970) obtained encysted metacercariae of *Echinoparyphium serratum* from the pericardial tissue of naturally infected freshwater snails *Isidorella brazieri*. Howell's (1968) chemical excystation procedure involved an acid pepsin pre-treatment, followed by treatment in a 0.02 M sodium dithionite reductant solution; the final treatment consisted of an excystation medium of 0.3% trypsin plus 0.05% sodium cholate in Hank's BSS at pH 7.3 at 38 ± 1°C. The per cent excystation within 10 min was about 75%. Howell's (1970) later paper was concerned with detailed factors involved in excystment of this species. He also found that acid pepsin pre-treatment was not obligatory for excystation and amended some of his excystation procedures published in the 1968 paper. Excystation requirements for another species of *Echinoparyphium*, *E. flexum*, were less fastidious than that of *E. serratum*. Fried and Grigo (1975) obtained *E. flexum* from the kidney and pericardium of naturally infected physid snails and achieved a high rate and per cent of excystation in the Fried and Roth medium (see Section 5) without acid pre-treatment, reductant, or special gases.

Irwin *et al.* (1984) studied excystment of *Himasthla leptosoma* metacercariae from cockles (*Cardium*) and mussels (*Mytilus*). The cysts were treated in a medium containing 0.8% sodium taurocholate, 0.3% trypsin and 0.8% L-cysteine at 41°C (henceforth, referred to as the Irwin *et al.* medium, see Section 5). This treatment resulted in intense metacercarial activity (activation) and after 20 min the larva emerged through the cyst wall (emergence). Ultrastructural studies showed that the juvenile fluke emerged through a small area of the cyst wall that was devoid of a layer of lamellae present elsewhere towards the innermost surface. The appearance of ruptured cyst walls indicated that they had been softened by the excystment medium (extrinsic factors). However, intense activity of the organism during excystation and the possible release of metacercarial enzymic factors also suggested an active role of the organism (intrinsic factors) during excystation.

Kirschner and Bacha (1980) used encysted metacercariae of *Himasthla quissetensis* to study *in vitro* excystation of this echinostome. Cysts were obtaining following Laurie's (1974) method of artificially inducing encystment of cercariae emitted from *Ilyanassa obsoleta* snails. In the wild, cysts of this species form in the tissues of various marine bivalves. Excystment of this species involved enzymic weakening (extrinsic factors) of the cyst wall followed by vigorous muscular movements (intrinsic factors) of the activated metacercaria. Maximum excystment occurred following low pH treatment (Earle's BSS at pH 2.5); the reductant, sodium dithionite (0.015 M); treatment in 0.3% trypsin plus 0.05% bile salts in Earle's BSS adjusted to pH 7.8

with $NaHCO_3$ at 41°C. It was not determined if lytic substances released by activated larvae while still within the cysts contributed to the excystation process.

LeFlore and Bass (1982) examined *in vitro* excystation of *Himasthla rhigedana* metacercariae. Cercariae of this species emerge from naturally infected *Cerithidea californica* snails and encyst on the bottom and sides of glassware in the laboratory. Maximum excystation was obtained following pre-treatment of cysts in 0.015 M sodium dithionite for 7 min and then treatment in a complete medium of 0.2% sodium taurocholate plus 0.5% trypsin in Hank's BSS at pH 7.8 (made alkaline with $NaHCO_3$) for 1 h at 42°C. LeFlore and Bass (1982) found that acid pre-treatment was not a prerequisite for excystation of this echinostome, but the synergistic action of trypsin and sodium taurocholate was necessary. This finding was different than that of Kirschner and Bacha (1980) who found that some excystation of the related species, *H. quissetensis*, could occur in trypsin alone. Some differences in excystation patterns may be expected since *H. rhigedana* encysts in the open on a substratum whereas *H. quissetensis* ordinarily requires marine bivalves for encystment. Bass and LeFlore (1984) examined *in vitro* excystation of *Acanthoparyphium spinulosum* metacercariae from the radular tissue of naturally infected *Cerithidea californica* snails. Optimal excystation of this echinostome involved pre-treatment in 0.5% pepsin in Hank's BSS at pH 2.0 (HCl) for 1 h; the reductant, 0.015 M sodium dithionite, for 10 min and then incubation in a complete medium, 0.2% sodium taurocholate plus 0.5% trypsin in Hank's BSS at pH 7.8 (with $NaHCO_3$) for 1 h at 42°C. For this echinostome there was no synergism between trypsin and bile salts; in fact, trypsin had an inhibitory effect on excystation.

The excysted metacercariae of echinostomes have been used for *in ovo* and *in vitro* cultivaton studies; for light, histochemical and electron microscopical examinations; for pheromonal behavioral studies; for analyses of excretory–secretory products; and for immunological studies.

Various studies have examined the *in ovo* cultivation of *Echinostoma trivolvis* in domestic chick embryos (Fried and Butler, 1978; Fried and Pentz, 1983; Wisnewski *et al.*, 1986). These studies have provided basic methods for the *in ovo* cultivation of this species and also examined growth and development of this organism to the vitellinogenesis stage. In spite of numerous studies, this organism has not yet been cultivated to the ovigerous adult stage in chick embryos. Attempts to cultivate this organism from excysted metacercariae to ovigerous adults in various *in vitro* culture media (i.e. defined media supplemented with either serum, hen's egg yolk, or chick mucosal extracts) have produced organisms which show some post-metacercarial development, but success in cultivation to the ovigerous adult stage has not been achieved (Butler and Fried, 1977; Fried and Kim, 1989). Scanning electron microscopical observations were made on the excysted metacercariae

of *E. trivolvis* and compared with those of adults grown in domestic chicks and on the chick chorioallantois (Fried and Fujino, 1984). Considerable differences were seen in the tegument, papillae, collar spines, and tegumentary spines of excysted metacercariae compared to adults grown in chicks or *in ovo*. Worms grown *in ovo* or in chicks showed similar ultrastructure. Considerable growth and differentiation of this species occurred from the excysted metacercarial stage to the adult.

Excysted metacercariae of *E. trivolvis* were used to study the histochemical localization of hydrolytic enzymes (Fried *et al.*, 1984). Comparisons were made with the cercarial stages of this echinostome and fundamental differences in the localization of various enzymes in the major systems were noted in cercariae versus excysted metacercariae. Pheromonal behavior studies were done using excysted metacercariae and adults of *E. trivolvis* (see Fried *et al.*, 1980). Newly excysted metacercariae did not pair in an *in vitro* bioassay, whereas immature and mature adults obtained from experimentally infected domestic chickens showed significant pairing. The neutral lipid excretory–secretory products from excysted metacercariae (mainly free fatty acids) were quite different from those in adults (mainly free sterols and triacylglycerols). Neutral lipids are involved in aggregational responses in adult echinostomes (Fried *et al.*, 1980). Fried and Emili (1988) studied the comparative biology of excystation between *E. trivolvis* and the related allopatric species *E. caproni*. Differences in the rate and per cent excystation were seen in these two closely related species. Information on viability after storage of these encysted metacercariae at 4°C in Locke's 1 : 1 for up to 1 year was also given. Only subtle differences were seen in the structure of the cysts and excysted metacercariae of the two species by both light and electron microscopy (Fried and Emili, 1988; Irwin and Fried, 1990). An attempt to observe differences in the excysted metacercariae of these two echinostomes fixed and stained by a new acetocarmine procedure showed only slight differences in measurements of some organs (Fried and Manger, 1992).

Recent studies on the excysted metacercariae of *E. caproni* suggest that this organism is less fastidious than that of *E. trivolvis* and can be grown to the ovigerous adult stage in the chick chorioallantois or the allantoic sac (Fried and Rosa-Brunet, 1991; Rosa-Brunet and Fried, 1992; Chien and Fried, 1992). *E. caproni* excysted metacercariae developed to adults in the allantoic sac and were capable of producing eggs with viable miracidia (Chien and Fried, 1992).

Excysted metacercariae of *E. caproni* have been used in immunological studies by Simonsen and coworkers at the Danish Bilharziasis Laboratory. Excysted metacercariae were obtained following chemical excystation of the cysts in a trypsin and sodium taurocholate medium modified after Fried and Butler (1978). These excysted metacercariae were used in immune assays since they excreted considerable amounts of antigen (Simonsen *et al.*, 1990). Excysted metacercariae which had never been exposed to mouse host

antibodies became coated with antibodies in mouse immune serum. Andresen *et al.* (1989) used newly excysted metacercariae as a source of antigen; they noted that some surface antigen was associated with the tegument of the excysted metacercariae. In an earlier study, Christensen *et al.* (1986) found that when newly excysted metacercariae were transplanted duodenally into resistant mice hosts, these larvae were rejected. They concluded that the newly excysted metacercariae were a target of the host immune response.

Excysted metacercariae of echinostomes in genera other than *Echinostoma* have been used for histochemical and ultrastructural studies. Various light level enzyme histochemical procedures were used by LeFlore and Bass (1983a) to visualize systems, particularly nervous, tegumentary, excretory and alimentary, of excysted metacercariae of *Himasthla rhigedana*. Light and scanning electron micrographs showing excysted metacercariae of *Acanthoparyphium spinulosum* emerging from cysts were presented by Bass and LeFlore (1984). Irwin *et al.* (1984) provided light, scanning and transmission electron micrographs of encysted and excysted metacercariae of *Himasthla leptosoma*. A detailed description of structural changes during excystation at the light and electron microscopical level was given in that study.

6.9. Acanthostomidae

Members of the Acanthostomidae have large circumoral spines near the mouth and the function of these spines is poorly understood. McDowell and James (1988) studied the functional morphology of circumoral spines in the excysted metacercariae of *Timoniella imbutiforme*. Cysts of *T. imbutiforme* were obtained from infected goby fish, *Pomatoschistus microps*. Metacercariae were excysted by digesting the cyst wall in 1% acid pepsin at pH 5 for 30 min followed by a 10-min treatment in 1% alkaline trypsin at pH 9 at 38°C. Details of the excystation process were not given. Scanning and transmission electron microscopy of the metacercariae suggested that the circumoral spines may assist in excystment and that their abrasive action may damage the cyst wall. Probably extrinsic and intrinsic factors are involved in excystation of *T. imbutiforme*.

6.10. Fasciolidae

Wikerhauser (1960) treated *Fasciola hepatica* cysts with 0.5% acidified pepsin for 2–3 h, followed by a 1% $NaHCO_3$ rinse and a final treatment of 0.4% trypsin plus 20% ox bile at 38°C. The per cent excystation within 2–3 h following the trypsin-bile treatment was 80%. Dixon (1966) described excystation in *F. hepatica* as an active process occurring in two phases, activation and emergence. Activation was initiated by a high CO_2 concentration and

reducing conditions at a temperature of 39°C. Whereas the CO_2 need only be applied for 5 min, exposure to the reducing agent (sodium dithionite or cysteine) should be about 30 min. The emergence phase was stimulated by 10% sheep bile. For a more detailed discussion of the pre-1970 studies on excystation of *Fasciola hepatica* see Lackie (1975).

A simple and effective procedure that eliminates the need for gassing with CO_2 or adding a reductant is that of Sewell and Purvis (1969). In this procedure cysts are placed in a vessel that can be capped tightly. To the cysts add 0.05 N HCl at 39°C. Then add an equal volume of a solution containing 1% $NaHCO_3$, 0.8% NaCl and 20% ox bile at 39°C. Cap the vessel immediately to avoid loss of the CO_2 that is produced. Incubate at 39°C for 4–5 h at which time the rate of excystation should be 70–80%. Fried and Butler (1979) reported an excystation procedure that achieved a high rate of excystation of *F. hepatica* metacercariae within a physiologically optimal time. In this procedure cysts are placed in a vial with 5 ml of 0.05 N HCl and an equal volume of a solution containing 0.8% NaCl plus 1% NaOH is added. The vial is capped and incubated at 39°C for 10 min, then 70 mg of sodium dithionite crystals are added. Thirty minutes later 10 ml of a 2% solution of sodium taurocholate are added and the vial is incubated at 39°C for 3 h. The per cent excystation should be about 75%. Hanna (1980) has also provided a useful technique for excystation of *F. hepatica*. Cysts are placed in 0.5% pepsin in Hank's BSS (pH 2.0) at 37°C for 20 min to remove the outer cyst walls; the cysts are then washed in Hank's BSS (pH 7.2) and incubated at 37°C in a medium containing 5 ml of 0.02 N HCl plus 1% $NaHCO_3$ plus Na taurocholate (50 mg) + L-cysteine HCl (40 mg). A high rate and per cent excystation should occur within 2 h. Refinements of the Hanna (1980) procedure can be found in papers by Tielens *et al.* (1981) and Reddington *et al.* (1984).

Fasciola gigantica excystation was studied by Hanna and Jura (1976) who found that excystation for this species was similar to that of *F. hepatica*. Their medium was similar to that described by Hanna (1980).

Fascioloides magna has been excysted in the Fried and Roth medium (see section 5; also Fried and Stromberg, 1985).

Excysted metacercariae of *Fasciola hepatica*, *F. gigantica* and *Fascioloides magna* have been used for *in vitro* and *in ovo* cultivation studies, histological, histochemical, ultrastructural, and immunological studies.

Davies and Smyth (1978) cultivated excysted metacercariae of *F. hepatica* to immature adults; the worms failed to become ovigerous and worm growth was retarded compared to *in vivo* development in mice. Fried and Butler (1979) cultivated the excysted metacercariae of *F. hepatica* to pre-ovigerous adults on the chick chorioallantois. They also used Oil Red O histochemical procedures to detect neutral lipids in the calcareous corpuscles of the excretory system. Thin-layer chromatographic analysis showed also that excysted metacercariae incubated in a non-nutrient medium released free fatty acids.

Scanning electron microscopy of the excysted metacercariae of *F. hepatica* was reported by Bennett (1975) and Køie *et al.* (1977). Bennett and Threadgold (1973) examined chemcially excysted metacercariae by transmission electron microscopy. Some differences in ultrastructure were seen between metacercariae and adults. Transmission electron microscopy (TEM) confirmed the presence of earlier light microscopical observations on excretory (calcareous) concretions and lipid droplets in the excretory system of the metacercariae. Bennett and Threadgold (1973) were the first workers to use TEM to examine ultrastructural changes during the transformation of excysted metacercariae to young adults.

Fry *et al.* (1985) described a new technique to isolate encysted metacercariae of *F. hepatica* by density gradient centrifugation. Subsequent excystation of these metacercariae was greater than 80%. Anderson and Fairweather (1988) excysted *F. hepatica* using Hanna's (1980) procedure for *F. gigantica*. Excysted metacercariae of *F. hepatica* were used to test the action of the fasciolicide diamphenethide (DMP) *in vitro*. Damage to excysted metacercariae was rapid with the tegument having been stripped of most of its ventral surface after 1 h; after 2 h little recognizable structure remained on the tegument. Apparently the anthelmintic DMP is effective in its mode of action on the surface of excysted metacercariae of *F. hepatica*.

Studies on the immunology of *F. hepatica* excysted metacercariae have been reported. Davies and Goose (1981) showed that newly excysted metacercariae injected intraperitonially into sensitized (immune) rats were surrounded quickly by host peritoneal cells. They showed transmission electron micrographs of peritoneal cells, particularly eosinophils, adhering to the metacercarial tegument in sensitized rats. Davies *et al.* (1979) in an earlier study found that antigens from cultured excysted metacercariae were not immunogenic.

Hanna (1980) showed that newly excysted metacercariae of *F. hepatica* exposed to immune sheep serum were coated with IGG which adhered specifically to the surface of the glycocalyx. Fry *et al.* (1985) developed a technique for the intraperitoneal injection of excysted metacercariae into rodent hosts for immunology studies. Reddington *et al.* (1984) showed that large numbers of excysted metacercariae of *F. hepatica* injected intraperitoneally into rats may produce small flukes in the rat liver which erroneoulsy can be thought to be associated with a challenge infection.

Hanna and Jura (1976) examined the requirements for *in vitro* excystment of *F. gigantica* and concluded that they were: (i) temperature of 37°C; (ii) presence of CO_2 and bile; and (iii) anaerobic conditions. They suggested that bile may serve to increase the permeability of the cyst wall to CO_2. Activation in this species probably involves CO_2 and low redox potential in the gut of the host; according to Hanna and Jura (1976) bile is less important than CO_2 in stimulating excystment of *F. gigantica*. Hanna and Jura (1977) later showed that excysted metacercariae of *F. gigantica* exposed to immune bovine serum

were coated with IGG which adhered specifically to the surface of the glycocalyx.

Fried *et al.* (1986) used the Fried and Roth medium (see Section 5) to excyst metacercariae of *Fascioloides magna* and carried out scanning electron microscopy of encysted and excysted metacercariae; comparisons with the ultrastructure of *F. hepatica* metacercariae were also reported.

6.11. Psilostomatidae

Lackie (1975) reviewed the study by Macy *et al.* (1968) on excystation of *Sphaeridiotrema globulus* in which excystation was achieved by treating metacercariae in acid pepsin followed by alkaline trypsin at an elevated pH; bile salts were not essential for excystation. Fried and Huffman (1982) obtained cysts of *S. globulus* from naturally infected *Goniobasis virginica* snails and obtained excystation in the Fried and Roth medium (see Section 5) at 41°C; in contrast to the study by Macy *et al.* (1968), acid pepsin was not a prerequisite for excystation.

Light microscopic observations during excystation of *S. globulus* are similar to those of other intestinal digeneans and are described herein as representative. Within snail tissue cysts of *S. globulus* are usually cemented to each other in a calcareous-like matrix (see Figure 1 in Fried and Huffman, 1982). In other species of digeneans, adhesion of one cyst to another may be by fibrocytes or amebocytes of host origin (see Figures 9–11 in Anderson and Fried, 1987). Encysted metacercariae of *S. globulus* or *Echinostoma* spp. usually show an outer and inner cyst by light microscopy. Within a few minutes after the cysts are placed in the Fried and Roth medium (see Section 5) at 37–42°C, the outer cyst swells and moves away from the inner cyst; the larva within the inner cyst becomes activated and rotates rapidly within the cyst (see Figure 3 in Fried and Huffman, 1982). Partial or complete dissolution of the outer cyst occurs (see Figures 4 and 5 in Fried and Huffman, 1982). The larva emerges from an opening in the inner cyst and is then free in the medium (excystation) leaving behind the inner cyst (see Figures 5 and 6 in Fried and Huffman, 1982).

Excysted metacercariae of *S. globulus* have been used for *in vitro* and *in ovo* cultivation studies. Berntzen and Macy (1969) described the *in vitro* cultivation of excysted metacercariae to ovigerous adults in a defined medium supplemented with hen's egg yolk in the presence of a complex gas mixture. Fried and Huffman (1982) showed that excysted metacercariae of *S. globulus* cultivated on the choriallantois (CAM) of chick embryos at 41°C developed into ovigerous adults by day 4 post-implantation. Huffman *et al.* (1984) reported that excysted metacercariae cultivated on the CAM produced hemorrhagic and cellular responses in that site similar to the ulcerative hemorrhagic enteritis caused by adults of *S. globulus* in natural and

experimentally infected avian hosts. The excysted metacercariae induced a significant beta-hemolysis response after 8-h incubation at 42°C on blood agar plates (Tabery *et al.*, 1988). Excysted metacercariae of this species probably contain hemolysins that cause the release of hemoglobin from erythrocytes (Tabery *et al.*, 1988).

6.12. Philophthalmidae

Lackie (1975) reviewed the Fried and Roth (1974) study on chemical excystation of *Parorchis acanthus*. Cysts of this philophthalmid were obtained from cercariae released from naturally infected *Thais lapillus* snails. The cercariae encysted on the inner surface of laboratory glassware and the metacercariae were excysted in the Fried and Roth medium (see Section 5). *P. acanthus* was the first of six trematode species excysted in this medium without the need of acid pepsin pre-treatment. Asanji and Williams (1985) reported optimal excystation of this species when cysts were treated in 1% acid pepsin for 1.5 h at 39°C followed by 1% alkaline trypsin at 39°C for 3 h. They also reported on the effects of age and other environmental factors on subsequent excystation of *P. acanthus*.

Cloacitrema michiganensis adults also live in the lower intestine, rectum, and cloaca of avian hosts in microhabitats similar to that of *Parorchis acanthus*. LeFlore and Bass (1983b) examined excystation of *C. michiganensis* cysts. Cercariae released from *Cerithidea californica* snails encysted on the inner surface of laboratory glassware and provided cysts for this study. Optimal excystation of this species occurred in an alkaline trypsin-bile salts medium at 41°C.

Adults in the genus *Philophthalmus*, unlike the closely related parorchids, live in the Harderian gland, conjunctiva, nictitating membrane and orbit of the eyes of their avian hosts. *In vivo*, the cysts are excysted by the warmth of the host environment, usually in the mouth cavity of the avian host. Excysted metacercariae migrate to the eye via the median slit of the dorsal palate, enter the nasolacrimal ducts, and then the orbit (Danley, 1973). Excystation *in vitro* can be achieved by treating cysts in warm saline or water as reported for *P. gralli* by Cheng and Thakur (1967) and *P. hegeneri* by Fried (1981). Some species differences in excystation requirements were noted in that different temperature optima were required for maximal excystation of each species. These philophthalmids required no enzymic treatment and elevated temperature alone induced active excystation. No information is available on possible intrinsic secretions emitted by larval eyeflukes stimulated by a rise in temperature.

Excysted metacercariae of philophthalmids have been used in developmental studies. *P. hegeneri* metacercariae inoculated into the median slit in the mouth cavity of domestic chicks migrated to the nictitating membrane

and developed therein into ovigerous adults in that site (Fried, 1962a). Monometacercarial infections were incapable of producing ovigerous adults and showed stunted growth in chicks' eyes compared to worms obtained from multiple-metacercarial infections (Fried, 1962a). Excysted metacercariae of *P. hegeneri* were cultivated to ovigerous adults on the chick choriallantois in the first successful *in ovo* cultivation study of a trematode (Fried, 1962b). LeFlore *et al.* (1986) used histochemical methods to localize the nervous system in excysted metacercariae of *Cloacitrema michiganensis*. Their observations on whole excysted metacercariae clearly showed ganglia, commissures, and three pairs of nerve cords in the larvae.

6.13. Paramphistomatidae

Lackie (1975) reviewed Lengy's (1960) study using crude mouse gut extract for 20 h to excyst *Paramphistomum microbothrium*. Fried *et al.* (1978b) removed encysted metacercariae of *Zygocotyle lunata* from the surface of laboratory glassware and obtained optimal excystation following a three-stop procedure: (i) pre-treatment in 1% acidified pepsin in Earle's BSS; (ii) subsequent treatment in the reductant, 0.02 M sodium dithionite; and (iii) final treatment in an excystation medium of 1% sodium glycocholate plus 1% trypsin in Earle's BSS adjusted to pH 8.8 with tris buffer. All treatments were done at 41°C. Irwin *et al.* (1993) have achieved successful excystation of this species in the Irwin *et al.* medium (*see Section 5*).

Gill and Bali (1988) removed cysts of *Paramphistomum cervi* maintained on polyethylene sheets in the laboratory and excysted the metacercariae using acid pepsin for 3 h followed by alkaline trypsin + 20% sheep bile for 3 h at 37°C.

Excysted metacercariae of *Z. lunata* were cultivated on the chick chorioallantois and produced distinct histopathological lesions in that site (Fried and Nelson, 1978). Irwin *et al.* (1991) used scanning and transmission electron microscopy to examine the tegument of excysted metacercariae of *Z. lunata* and noted distinct ultrastructural differences in metacercariae compared to adults grown in mice.

6.14. Notocotylidae

Metacercarial cysts of *Parapronocephalum symmetricum* from *Littorina saxatilis* snails were treated in an excystation medium containing trypsin and bile salts at 41°C (Irwin *et al.*, 1989). The metacercariae emerged from the cysts about 30 min after treatment. Prior to metacercarial emergence, the cyst wall extended outward at one end resulting in a pear-shaped cyst. The extensions occurred adjacent to the anterior region of the metacercaria, and

transmission electron microscopy showed that at the points the structure of the inner cyst layer had changed from a compact to a disaggregated form. Interestingly, this effect was not seen in cysts whose metacercariae were removed mechanically prior to treatment with the excystation medium. Irwin *et al.* (1989) suggested that the disaggregation of the inner cyst layer represented a weakening process caused by enzyme released from the anterior region of the metacercaria. Final escape, through the weakened inner layer and the two outer layers, was eventually achieved by physical efforts of the activated metacercaria. This study provided evidence for the active role of the metacercaria in the excystment process of this species.

Graczyk and Shiff (1993b) studied excystment of *Notocotylus attenuatus* metacercariae from *Physa acuta* snails. A sequence of three events was needed to obtain optimal excystment: (i) pre-treatment of the cysts for 2 h in an aqueous solution of the cationic detergent *N*, *N'*, *N'*-polyoxyethylene (10)-*N*-tallow-1,3-diaminopropane (EDTA-20) and this was an absolute prerequisite for subsequent excystation; (ii) a second treatment for 15 min in 1% HCl acid-1% pepsin (pH 2); and (iii) final treatment in the Fried and Roth medium (see Section 5). All treatments were done at 41–42°C. The per cent excystation following the three-part treatment was 72% by 1 h. The authors suggested that the cationic detergent acted by dissolving lipoproteins and lipids in the cyst wall. This cationic detergent, which is undoubtedly not present in the host gut, produces a distinct process of *in vitro* excystation that probably differs from that which occurs in an avian host.

6.15. Plagiorchiidae

Bock (1989) used an enzyme-free medium to show the active release of *Plagiorchis species* 1 metacercaria from its cyst. The medium consisted of 0.05% sodium cholate, 0.015 M $NaHCO_3$, 0.015 M NaCl and 0.05 M sodium phosphate at pH 7.5; excystation was studied at 22°C. During excystation an opening was formed in the two-layered cyst wall presumably by the release of cecal fluid from the activated larva and internal pressure created by the contraction of the cyst wall upon metacercarial hatching. These events led to a rapid expulsion of the metacercaria through the opening in the cyst wall. The cyst could be penetrated at any place where the larva released its cecal contents. Following excystation the hatching medium showed significant phosphatase and proteinase activities. The medium was also capable of dissolving inner walls of empty cyst envelopes. Bock (1989) suggested that proteinase(s) represented the hatching enzyme(s) of this metacercaria because only proteolytic activity was detectable in the pH range optimal for excystment. He also suggested a possible relationship of thiol proteinase(s) detected in the hatching medium to the hemoglobin-digesting proteases from the intestinal ceca of *Schistosoma mansoni* and *S. japonicum*. Bock's (1989) study

provided evidence for an intrinsic enzyme responsible for the release of the larva from its cyst.

Lowenberger and Rau (1993) modified Bock's (1989) medium to study excystation of *Plagiorchis elegans* metacercariae from experimentally infected *Aedes aegypti* mosquitoe larvae. They reported optimal excystation in a medium consisting of 0.05% bile salts (sodium cholate: sodium deoxycholate, 1 : 1), 0.015 M $NaHCO_3$ and 0.015 M NaCl at pH 7.5. Optimal excystation was achieved at 37°C.

6.16. Mesocoeliidae

Mesocoeliids are plagiorchiid-like trematodes that encyst in the hemocoel of isopods; adults are found in poikilothermous vertebrates such as lizards and toads. Fashuyi (1986) examined *in vitro* excystation of *Mesocoelium monodi* cysts removed from *Oniscus* and *Armadillidium* isopods. Excystation occurred in various media at 22°C including 0.025% chymotrypsin, chicken and toad bile, duodenal and intestinal extracts of toads, lizards and rats, and also in human urine at 36°C. Pre-treatment with enzymes or other reagents was not needed. During excystation the organism became activated and then escaped through an aperture at one end of a simple one-layered ovoidal cyst. Excystation in this species was considered to be an active process in which the excystment medium probably stimulated the metacercaria to increase its muscular activity and also to produce secretions which dissolve an escape aperture at one end of the cyst wall. The rest of the cyst wall remained intact following metacercarial emergence from the cyst. Evidence for the production and release of secretions by the activated larva was not given.

6.17. Microphallidae

Lackie (1975) reviewed the earlier work on microphallid excystation. Since her review there have been excystation studies on eight species of microphallids. Although some microphallids excyst in the presence of warm saline others require complex enzymic treatments. Fujino *et al.* (1977) excysted the metacercariae of *Microphalloides japonicus* from marsh crabs, *Helice tridens*, in 0.85% saline at 37°C. Davies and Smyth (1979) obtained cysts of *Microphallus similis* from the digestive gland of naturally infected *Carcinus maenas* crabs and excysted the metacercariae by pre-treatment in 0.5% acid pepsin in Hank's solution (pH 2) for 30 min followed by treatment in 0.5% trypsin in Hank's saline (pH 7.0) for 10–20 min at 38°C. Irwin (1983) excysted metacercariae of *Maritrema arenaria* from the intestinal wall of *Balanus balanoides* by treating the encysted metacercariae in alkaline Hank's BSS containing 0.3% sodium taurocholate plus 0.5% trypsin. Dunn *et al.* (1990) obtained

cysts of *Gynaecotyle adunca* from the soft tissues of naturally infected *Uca pugilator* crabs and achieved excystation by treating the cysts in 0.05% trypsin in Hank's BSS at pH 7.2 at 40°C. The rate of excystation was increased in the presence of saturated CO_2 conditions. Interestingly, cysts of the related microphallid, *Probolocoryphe uca,* from the soft tissues of *Uca* crabs, would excyst in Hank's BSS at 40°C (Dunn *et al.*, 1990).

Saville and Irwin (1991a) obtained encysted metacercariae of *Microphallus abortivus* from the marine snail host, *Hydrobia ulvae*. Metacercariae were excysted in the medium of Irwin *et al.* (see Section 5). This microphallid cyst is unique in that it can be breached by the activated metacercaria at either pole of the elliptical cyst. Transmission electron microscopy of encysted metacercariae whose larvae had been forcibly expelled before exposure to excystment medium showed that the structure of the two outer cyst layers (the cyst wall consists of four layers in this species) was affected in a similar way to that of intact cysts that had been exposed to excystment medium. However, the two inner cyst layers retained their original structure and appeared to be unaffected by the medium. Therefore, structural changes observed in the inner layers of intact cysts following exposure to the excystment medium had resulted from the activity of the enclosed larvae or their metabolic products (active excystation). The authors concluded that *M. abortivus* presented a novel escape mechanism in that it softened both poles of an ellipsoidal cyst and afforded itself a choice of escape apertures. Saville and Irwin (1991b) excysted metacercariae of *Microphallus primas* from tissues of the shore crab, *Carcinus maenas*. Excystation was done following the procedure of Irwin *et al*, (see Section 5), but details of the excystation process in this species were not given. Ramasamy and Panicker (1991) obtained encysted metacercariae of *Microphallus madrasensis* from the ovary and hepatopancreas of sand crabs, *Emerita astatica*, and excysted them by treatment in 0.85% saline at 40°C.

Excysted metacercariae of microphallids have been used for histochemical and *in vitro* cultivation studies. Davies and Smyth (1979) cultivated excysted metacercariae of *Microphallus similis* to the ovigerous adult stage using simple media and also made light microscopical observations on gametogenesis, vitellogenesis and egg production in developing metacercariae. Irwin (1983) detected the presence of acid phosphatase on the surface of excysted metacercariae of *Maritrema arenaria*. Dunn *et al.* (1990) cultivated *in vitro* excysted metacercariae of *Gynaecotyle adunca* and *Probolocorphye uca* to ovigerous adults in simple media. Microphallids are progenetic and their cultivation requirements are less fastidious than non-progenetic digeneans (Smyth, 1990). Saville and Irwin (1991a) provided light, scanning, and transmission electron microsopical observations on the metacercaria of *M. abortivus* during the excystment process. Saville and Irwin (1991b) inoculated excysted metacercariae of *M. primas* into the allantoic sac of fertile hens' eggs. Ovigerous adults were obtained *in ovo* and eggs from these worms were

used to establish the life cycle of this parasite in the laboratory. Ramasamy and Pannicker (1991) used their excystation procedure to remove the larvae of *Microphallus madrasensis* from the cysts and then studied the chemical nature of cysts devoid of larvae.

6.18. Troglotrematidae

Studies (reviewed by Lackie, 1975) on excystation of *Paragonimus westermani* by Yokogawa *et al.* (1960), showed that a temperature of 40°C plus a balanced salt solution with an alkaline pH of 9.0 were optimal for active excystation of this species.

Recent excystation studies on *Paragonimus westermani* have used cysts from freshwater crabs, *Eriochier japonicus*; and studies on *P. ohirai* have used cysts from freshwater crabs, *Sesarma dehaani*. Yamakami and Hamajima (1988) excysted the metacercariae of *P. westermani* following treatment for 10 h at 40°C in an RPMI 1640 medium supplemented with 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. Fujino and Ishii (1990) excysted metacercariae of this species in 0.07% HCl followed by Ringer's solution containing 0.1% $NaHCO_3$ at 39°C. Excystation in the absence of extrinsic enzymes suggests that the process is active in *P. westermani*.

Recent studies (Fujino *et al.*, 1989a; Fujino and Ishii, 1990; Ikeda and Oikawa, 1991) on excystation of *Paragonimus ohirai* have used encysted metacercariae from freshwater crabs, *Sesarma dehaani*. Fujino *et al.* (1989a) achieved excystation by incubating the cysts overnight in alkaline Tyrode's solution at pH 8.0 at 37°C. Fujino and Ishii (1990) excysted *P. ohirai* by treating the metacercariae in 1% pancreatin plus 0.1% $NaHCO_3$ maintained at 39°C. The role of pancreatin was not determined but it may have been involved in the partial dissolution of the cyst wall. That excystation in this species is active and can occur in the absence of extrinsic enzymes, was demonstrated by Ikeda and Oikawa (1991) who achieved excystation by treating cysts overnight in Tyrode's solution (pH 8.0) containing 1000 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Fujino *et al.* (1989b) used light and electron microscopy to study excysted metacercariae of *P. westermani* in normal and abnormal hosts. Adults from abnormal hosts resembled the excysted metacercariae in general features, but ultrastructural studies showed differences in the composition of tegumental granules, and in the development of the excretory bladder and intestinal ceca. Hamajina *et al.* (1985) localized a thiol protease in the newly excysted metacercariae of *P. westermani* and showed that this protease hydrolyzed host collagen and hemoglobin. The thiol protease was detected in the gastrodermis of the excysted metacercariae by the indirect fluorescent antibody technique (IFAT). Fluoresence was seen over the epithelium, brush border, and luminal substances in the intestinal ceca; the tegument and

excretory bladder did not show fluorescence. Fujino and Ishii (1990) examined the gut of excysted metacercariae of both *P. westermani* and *P. ohirai* and compared its structure with that of adult worms from vertebrate hosts. The gut of excysted metacercariae in both species was tubular consisting of flattened epithelial cells with short cytoplasmic projections extending to form lamellae. Features consistent with adult gut morphology only occurred during post-larval development. Fujino and Ishii (1990) also reported some inter-specific differences in the newly excysted metacercariae of the two species. The gastrodermis of *P. westermani* was thicker than that of *P. ohirai*. Whereas autophagic vacuoles, glycogen particles, and unidentified vacuoles were usually seen in the gastrodermis of *P. westermani*, they were rarely found in *P. ohirai*.

Fujino *et al.* (1989a) used an immunocolloidal gold method to develop monoclonal antibodies against tegumental glycoproteins of newly excysted metacercariae of *P. ohirai*. The tegument of newly excysted metacercariae was filled with ovoid, moderately electron-dense granules (GO) and relatively few electron dense, round granules (G1). Most of the GO and G1 granules became labelled and gold particles also localized on the apical plasma membrane. The GO granules were specific to the newly excysted metacercariae, and did not occur in adults. It was concluded that antigen was associated with the glycocalyx of excysted metacercariae, but not present in adult worms. Ikeda and Oikawa (1991) used a monoclonal antibody (designated MS0Mab) to characterize immunobiochemically the tegumental glycocalyx of excysted metacercariae of *P. ohirai*.

6.19. Opisthorchiidae

Lackie (1975) reviewed the excystation studies on *Clonorchis sinensis* prior to the 1970s. The earlier studies used gastric and intestinal juices supplemented with bile at 37°C. Fujino *et al.* (1979) showed that excystation of *C. sinensis* is mainly active and can be achieved in a pancreatin plus $NaHCO_3$ medium at 27°C. The role of pancreatin and details of the excystation procedure were not given. Fujino *et al.* (1979) showed fundametal differences in the ultra-structure of newly excysted metacercariae compared to that of adults. Spines were present in excysted metacercariae but disappeared in adults possibly being absorbed into the tegument during post-metacercarial development. The tegument of excysted metacercariae was thinner and less lobulated than that of adults and fibrillar extensions from the surface of the tegument were not yet developed in metacercariae. Choi *et al.* (1981) used SDS-polyacrylamide gel electrophoresis to examine protein components in the excysted meta-cercariae of *C. sinensis*. They found two bands of protein of approximately 97 000 and 178 000 molecular weight which were unique to the excysted metacercariae.

6.20. Heterophyidae

The pre-1970s work on heterophyids has been reviewed by Lackie (1975) and considers the use of enzymic treatment supplemented with bile salts at 40°C to excyst the metacercariae of *Cryptocotyle lingua* and *Metagonimus yokogawai*.

Køie (1992) used fine needles to excyst (mechanical excystation) the metacercariae of *Pygidiopsis ardeae* Køie, 1990 from experimentally infected three-pointed sticklebacks, *Gasterosteus aculeatus*. The encysted metacercariae underwent a period of maturation up to 20 weeks in the fish intermediate host. Marked changes in the ultrastructure of the tegument of the metacercariae were noted during the period of maturation. The most significant change was the development of tegumental microvilli to increase the absorptive surface. The tegumental surface of the infective metacercariae was identical to that of adults grown in natural or experimentally infected definitive hosts.

7. EXTRINSIC FACTORS

7.1. General

Extrinsic factors are those outside the metacercarial cyst that play a role in excystation. They are factors in the physicochemical environment of the host during *in vivo* excystation or in the culture medium used to study *in vitro* excystation. The most widely discussed extrinsic factors are temperature, gas phase and pH, oxidation–reduction potential and osmotic pressure, enzymic treatments, and bile.

7.2. Temperature

For some digeneans temperature appears to be the major extrinsic factor inducing metacercarial excystation. Thus, in *Philophthalmus gralli* (see Cheng and Thakur, 1967) or *P. hegeneri* (see Fried, 1981) cysts maintained in distilled water or saline will excyst at temperatures ranging from 30–54°C. Likewise, tetracotyles in the genus *Cotylurus* may require an elevated temperature of 41–42°C in the presence of a balanced salt solution for optimal excystation, for example *Cotylurus lutzi* by Voge and Jeong (1971); and *Cotylurus flabelliformis* by Magnus and Johnson (1985). For most digeneans that live as adults in homiothermous vertebrates, a temperature range of 37–42°C is optimal for excystation. In some species of avian digeneans the upper rather than the lower range is optimal. Thus, in *Parorchis acanthus* the rate of excystation in an alkaline trypsin-bile salts medium was 10, 80, and 100% at 37, 39 and 42°C, respectively (Fried and Roth, 1974). For

some metacercariae that excyst in poikilothermous vertebrates, for example *Plagiorchis species* 1 by Bock (1989), excystation was optimal at 22°C. The mechanism of how temperature influences excystation in the Digenea is not understood.

7.3. Gas Phase and pH

Although most *in vitro* excystation studies are done with air as the gas phase, there is ample evidence to suggest that CO_2 stimulates excystment in *Fasciola hepatica* and *F. gigantica* (see Dixon, 1966; Hanna and Jura, 1976; Fried and Butler, 1979). Information from *in vivo* excystation studies showed that feeding hosts 3% $NaHCO_3$ enhanced the infectivity of *Zygocotyle lunata* in rats (Bacha, 1964) and in chicks (Fried, 1970). The bicarbonate might have lessened the acidity of the stomach contents or caused increased gut peristalsis. Lackie (1975) suggested that CO_2 could have been released from the bicarbonate and served as a stimulus for enhanced excystation. Although the role of carbonic acid as the host signal for development of larval stages of nematodes has been studied (Petronijevic *et al.*, 1985), similar work on metacercariae is not available.

Some studies are available on the effects of pH on *in vitro* excystation, for example *Posthodiplostomum leonensis* by Asanji and Williams (1975); *Cotylurus variegatus* by Thompson and Halton (1982); *Zygocotyle lunata* by Irwin *et al.* (1993). In these species significant excystation occurred within a broad pH range of 5.5 to 8.5. However, not all digeneans show such a broad pH range for optimal excystation. Asanji and Williams (1975) showed that 80% of *Parorchis acanthus* metacercariae tested *in vitro* excysted between pH 7 and 8.

7.4. Oxidation–Reduction Potential and Osmotic Pressure

Redox values play a role in activation phenomena in parasites including excystation of digeneans. The mechanism of how reductants act in the excystation process is unclear. The two most widely used reductants in excystation studies are sodium dithionite and L-cysteine. The reductants are used mainly as a separate treatment between the pre-treatment (usually acid saline or acid pepsin) and the treatment medium (typically alkaline trypsin or alkaline trypsin-bile salts). Enhancement of excystment with reducing agents used as described above has been reported by Howell (1970) for *Echinoparyphium serratum*; by Mohandas and Nadakal (1978) for *Echinostoma malayanum*, and by Fried *et al.* (1978b) for *Zygocotyle lunata*.

Reductants have also proved effective when included in the excystation medium (*Fasciola hepatica* by Dixon, 1966; *Cryptocotyle lingua* by McDaniel,

1966; various species of digeneans by Asanji and Williams, 1975; *Fasciola hepatica* by Fried and Butler, 1979; *Zygocotyle lunata* by Irwin *et al.*, 1993).

Time of treatment in the reductant is important. Fried *et al.* (1978b) using *Zygocotyle lunata* and Kirschner and Bacha (1980) using *Himasthla quissetensis* found sodium dithionite to be effective for 1–10 min, but longer exposure resulted in reduced excystment and larval death. Asanji and Williams (1975) found that the redox potetial had a marked effect on excystment in *Parorchis acanthus*, *Posthodiplosomoides leonensis*, and *Posthodiplostomum* sp. with an optimum range of −20 to +40 mV.

Little information is available on the effects of osmotic pressure on *in vitro* excystation of digeneans. Asanji and Williams (1975) showed that *Posthodiplostomoides leonensis* and *Posthodiplostomum* sp. could excyst within a range of about 100–600 mosmolal, whereas the range for *Clinostomum tilapiae* was wider, about 100–1000 mosmolal.

7.5. Enzymic Treatments

For some digeneans acidified pepsin is a major extrinsic factor inducing metacercarial excystation (*Clinostomum marginatum* by Fried *et al.*, 1970; *Posthodiplostomum minimum minimum* by Fried, 1970). For these digeneans acidified saline is either not effective or effects a low percentage of excystation. Some digeneans require acid pepsin pre-treatment (*Leucochloridium variae* by Fried, 1973; *Timoniella imbutiforme* by McDowell and James, 1988) prior to subsequent treatment in an alkaline trypsin solution. In several studies where acid pepsin was compared as a pre-treatment versus acid saline, the rate and per cent excystation in an alkaline trypsin-bile salts medium was greater with the acid pepsin pre-treatment (*Fascioloides magna* by Fried *et al.*, 1986; *Cotylurus cornutus* by Graczyk and Shiff, 1993a). There is no question that the acid pepsin pre-treatment dissolves some component(s) of the thick outer cyst layer, especially in those cysts which are heavily invested with host tissue, for example *Bucephaloides gracilescens* by Thompson and Halton (1982).

However, in numerous studies on digeneans of homiothermic hosts, there is no absolute requirement for either acid pepsin or acid saline pre-treatment and optimal excystation occurs in an alkaline trypsin-bile salts medium (*Cyathocotyle bushiensis* by Fried and Ramundo, 1987; *Echinoparyphium flexum* by Fried and Grigo, 1975; *Echinostoma caproni* and *E. trivolvis* by Fried and Emili, 1988; *Parorchis acanthus* by Fried and Roth, 1974; *Fascioloides magna* by Fried and Stromberg, 1985; *Cotylurus strigeoides* by Fried *et al.*, 1978a; and *Sphaeridiotrema globulus* by Fried and Huffman, 1982).

The role of trypsin in the excystation of digenean metacercariae is poorly understood. Trypsin is often used in combination with crude bile salts or with sodium taurocholate. Whether trypsin acts mainly to dissolve cyst layers or

to effect in some unknown way the organism within the cyst is not known. The source and purity of trypsin is variable from study to study and such information is not always included in the published report.

Considerable discrepancies exist on the necessity of trypsin in studies on the same species or closely related species of digeneans. Thus, Fried *et al.* (1978b) found trypsin to be obligatory for optimal excystation of *Zygocotyle lunata*, but Irwin *et al.* (1993) showed that this species was capable of excysting in the absence of trypsin. Dunn *et al.* (1990) showed that *Probolocoryphe uca* excysted in the absence of trypsin whereas its presence was essential for the excystment of the related microphallid, *Gynaecotyle adunca*. Kirschner and Bacha (1980) reported excystation of *Himasthla quissetensis* in trypsin alone, whereas the related species *H. rhigedana* required the synergistic effect of bile-trypsin for optimal excystation (LeFlore and Bass, 1982). Bass and LeFlore (1984) reported that trypsin had an inhibitory effect on excystation in the echinostome, *Acanthoparphyium spinulosum*.

7.6. Bile

Bile is a complex substance made up of numerous components including water, mucins, proteins, bile pigments, conjugated and unconjugated bile salts, neutral lipids, phospholipids, and inorganic ions (Haselwood, 1978). Although Smyth and Haselwood (1963) stressed the importance of using pure rather than crude samples of bile salts, when this has been done the crude form, that is, sodium cholate ox bile, has promoted more rapid excystation than the refined bile salt, that is pure grade of sodium taurocholate (Thompson and Halton, 1982 on *Bucephaloides gracilescens* and Irwin *et al.*, 1993 on *Zygocotyle lunata*). These results have been attributed to the presence of traces of other bile salts, acids or possibly lipids in the crude extract.

Most studies on digeneans show that trypsin without bile salts may bring about some excystment, but larval activity and the rate of excystment is markedly reduced. Bile salts have a synergistic effect on the action of trypsin in most digenean studies. The mechanism of this synergism is not understood. Although there is no single model available to explain the action of bile salts on the metacercaria most authors (see reviews in Dixon, 1966; Lackie, 1975; Smyth and Halton, 1983; Sommerville and Rogers, 1987; Sukhdeo and Mettrick, 1987) suggest that bile salts enhance the muscular activity of the metacercaria thereby helping the larva escape from the cyst. Sukhdeo and Mettrick (1986) have speculated that following stimulation by bile salts the metacercaria of *Fasciola hepatica* recognizes the ventral plug region of the cyst via specific cues which coordinate larval activity and effect escape of the larva from the cyst. Sukhdeo and Mettrick (1986) also showed that glycine conjugated bile salts were more effective at stimulating the emergence of behavior of *F. hepatica* metacercariae than taurine conjugated bile salts;

optimal emergence of this metacercaria occurred after treatment with cholic acid conjugated to glycine. However, in another study (Fried and Butler, 1979), glycocholate was ineffective in stimulating excystment of *F. hepatica*, whereas taurocholate was optimal. Reasons for the divergent results in the two studies are not clear.

8. INTRINSIC FACTORS

Intrinsic factors include substances secreted by the metacercaria or activities, particulalry muscular, on the part of the metacercaria which may be involved in excystation. Intrinsic factors highlight the metacercaria compared to components of the medium or physicochemical factors in a host environment which effect excystment. Previous reviews (Lackie, 1975; Smyth and Halton, 1983; Sommerville and Rogers, 1987; Sukhdeo and Mettrick, 1987) have noted that several species of digenean metacercariae play an active role in the excystment process. This role usually takes the form of intense physical activity and/or the release of secretions (including enzymes) which may be involved in weakening or dissolving components of the cyst wall.

Release of substances that aid in excystment have been described by Irwin (1983) for *Maritrema arenaria*; Sukhdeo and Mettrick (1986) for *Fasciola hepatica*; Bock (1989) for *Plagiorchis species* 1. Irwin (1983) forcibly removed the larva of *Maritrema anenaria* from its cyst. Extrinsic enzymes had no affect on digesting the innermost wall of the vacant cysts. Transmission electron microscopy showed that in the process of excystment the inner layer of the cyst wall changed from a compact to a loose fibrous layer. Only cysts with viable metacercariae underwent this change. The study showed that significant changes in the inner layer of the cyst wall could not be attributed to the excystment medium. The softening of the inner cyst layer was attributed to intrinsic enzymes released by the metacercaria. A similar study done by Irwin *et al.* (1989) on *Parapronocephalum symmetricum* (see Section 6.14) showed that excystation was active in that species.

At least in *Fasciola hepatica* (see Sukhdeo and Mettrick, 1986) and in *Plagiorchis species* 1 (see Bock, 1989) an important function of the activation phase of excystation is the production and release of intestinal cecal secretions that are generated by an intrinsic behavioral pattern. Bock (1989) has provided evidence (see Section 6.15) for the role of an intrinsic thiol protease serving as a hatching enzyme in the metacercaria of *Plagiorchis species* 1. Information on biochemical characteristics of putative hatching factors from *Fasciola hepatica* metacercariae is not available, although Sukhdeo and Mettrick (1986) observed two major intrinsic behavior patterns in this species as follows: (i) the emptying of the intestinal ceca by peristaltic-like contractions, and (ii) coordinated body movement and sucker activity apparently

directed at disrupting the ventral plug. These factors are important in the release of the fasciolid larva from its cyst. Sukhdeo and Mettrick (1986) used videotape methodology to study intrinsic factors in *F. hepatica* metacercariae and it would be worthwhile to extend such work to non-fasciolids.

During excystation metacercariae often release lipophilic substances usually from the excretory pore. Johnston and Halton (1981) noted the periodic release of large quantities of oil-like droplets from the excretory pore of *Bucephaloides gracilescens* metacercariae during excystation; the oil-like droplets were not further characterized. Release of lipids from the excretory pore of *Cotylurus strigeoides* during excystation was detected by histochemical and thin-layer chromatographic methods (Fried and Butler, 1977; Fried *et al.*, 1978a). Neutral lipids have been detected in the excretory system of metacercariae of *Echinostoma trivolvis* by Butler and Fried (1977) and *Fasciola hepatica* by Fried and Butler (1979). Gracyzk and Shiff (1993a) have observed the release of refractile granules from the mouth and excretory pore of *Cotylurus cornutus* during excystation. In spite of numerous observations on the release of lipophilic material from either the alimentary or excretory systems of metacercariae, the role of these compounds in excystation is unclear.

Hotez *et al.* (1993) used gelatin gel electrophoresis to characterize the proteases from encysted metacercariae of *Echinostsoma trivolvis* and *E. caproni*. On SDS-gelatin gels at alkaline pH, the echinostome metacercariae showed greater protease activity than either rediae or adult worms. Two major protease activities were identifed from the metacercariae of *E. trivolvis*: a high molecular weight complex (about 400 kDa) and a collection of closely migrating proteases in the 66–97 kDa range. The metacercarial proteases of *E. trivolvis* catalyzed the hydrolysis of keratin, a component previously identifed in cyst walls of some digeneans. The metacercarial proteases are probably involved in excystation of *E. trivolvis*. Using histochemical localization procedures, Fried *et al.* (1984) found that excysted metacercariae of *E. trivolvis* contained less hydrolytic enzyme activity than cercariae, suggesting that these enzymes were used up during excystation. Excystation in this species also coincides with the release of excretory concretions that are visible in the inner cyst and not found in the adult stage (Fried and Butler, 1978; Fried *et al.*, 1984). The role of excretory concretions in excystation needs further study.

9. CONCLUDING REMARKS

This review covers the literature on *in vitro* excystation of metacercariae from about 1975 to 1993 and extends observations on this topic found in the reviews of Lackie (1975) and Sommerville and Rogers (1987).

The availability of metacercariae from naturally and experimentally infected hosts is described. Commercial suppliers that provide metacercariae are

listed. Methods for obtaining, handling, and storing metacercariae are described.

The structure of encysted metacercariae of numeorus species of digeneans has been discussed. The extreme variation in both structure and chemical composition of cyst layers from species to species has been documented. It is apparent that transmission electron microscopy studies will be needed to elucidate the ultrastructure of cysts from species in digenean families where such information is meager. Light microscopy alone is not sufficient to resolve the complex layers associated with spherical or hemispherical metacercarial cysts. For many species *in vitro* excystation is related to the complexity of the encysted metacercaria and the location *in vivo* where excystment normally occurs. This may not always be the case as evidenced by the recent study of Lou *et al.* (1992) on four species of *Paragonimus*. Although the four species all excyst in the duodenum of mammalian hosts the number of cyst layers in each species varied from two to five. Additional TEM studies will be needed to see how variable the number of cyst layers is in closely related digenean species.

Information on *in vivo* excystation is useful for a better understanding of *in vitro* excystation. Relatively few precise studies are available on *in vivo* excystation. Such studies require a large number of parasite-free experimental hosts maintained on carefully monitored diets. Cysts of most species are small and encysted or excysted metacercariae are difficult to find in the gut. Model studies on *in vivo* excystation are those of Fried and Kletkewicz (1987) and Graczyk and Shiff (1993b) and should be consulted prior to initiating research on this topic.

Detailed descriptions are provided for the preparation of excystation media. Numerous media are described for *in vitro* excystation of fasciolids, particularly *Fasciola hepatica*. The two methods used most frequently for the excystation of non-fasciolid metacercariae are described in detail. The first method, that of Fried and Roth (1974), has been used for the excystation of six species of digeneans and the second method, that of Irwin *et al.* (1984), for four species. These methods are recommended as starting points for investigators who wish to initiate excystation studies on non-fasciolid digeneans.

The major aspect of the review has been concerned with detailed descriptions of *in vitro* excystation of 50 species of digeneans from 19 families and extends the information on this topic found in Lackie (1975) and Sommerville and Rogers (1987). In addition to the mechanisms of excystation, coverage goes into the uses of excysted metacercariae, particularly in studies on light, scanning and transmission electron microscopy; growth development and differentiation of excysted metacercariae *in vivo*, *in vitro* and *in ovo*; and studies on behavior and immunology of excysted metacercariae. Extensive coverage of uses of excysted metacercariae does not appear in previous reviews on this topic.

There are no significant advances in our understanding of extrinsic excystation factors since the reviews of Lackie (1975) and Somerville and Rogers (1987).

There is no apparent absolute requirement for extrinsic factors in digenean excystation. In the absence of one enzymic factor another may substitute. Although bile is important as a stimulus for excystation, it is by no means a universal factor. Although acid pepsin or acid saline pre-treatments are essential for some digeneans, they are not needed for most. The synergistic effect of trypsin-bile is effective for most digeneans, but reasons for this synergism are not clear. Substances used for successful *in vitro* excystation are often impure, particularly crude enzyme and bile preparations. Yet attempts to use pure preparations usually result in nil or decreased excystation.

Mechanisms of how extrinsic factors work during excystation are not well known. A major factor discussed by Sommerville and Rogers (1987) is concerned with the effects of extrinsic factors on the cyst versus the larva within the cyst. Significant experiments to determine the effects of extrinsic factors on the cyst versus the organism within the cyst appear not to be available. In addition to the usual extrinsic factors, cationic detergents appear necessary for the *in vitro* excystation of *Notocotylus attenuatus* (see Graczyk and Shiff, 1993b) with the detergent apparently effecting lipids and lipoproteins in the cyst wall. Experiments to determine the mechanisms of extrinsic factors on excystation of digeneans will tax the ingenuity of the intellectually curious helminthologist.

Probably the most useful information on excystation within the past 10 years is concerned with intrinsic factors. This work is best exemplified by the studies of Irwin (1983) on *Maritrema arenaria* and Irwin *et al.* (1989) on *Parapronocephalum symmetricum* in which larvae were forcibly removed from their cysts to determine the effects of extrinsic versus intrinsic factors on the inner layers of the cyst wall; and the studies of Bock (1989) on *Plagiorchis species* 1 and Sukhdeo and Mettrick (1986) on *Fasciola hepatica* which showed the active role of secretions from the intestinal ceca of the metacercariae. Bock's (1989) analysis of the enzymic material from *P. elegans* implicating the role of thiol protease as a hatching enzyme should serve as an impetus for further studies on active excystation in the Digenea. The videotape methodology of Sukhdeo and Mettrick (1986) to study active excystation in *F. hepatica* should serve as an impetus for precise behavioral studies on other digeneans.

Lipophilic factors, often mentioned as concomitants of the excystation process, should be examined more closely. It should not be a problem to collect lipophilic excretory–secretory products during excystation and subject this material to microchemical analysis. Techniques for collecting, handling, and analyzing small amounts of lipids are well documented (Fried and Sherma, 1986; Fried, 1993).

Hotez *et al.* (1993) have provided useful information on the electrophoretic analysis of thiol preteases in echinostome metacercariae. This study should be extended to other species of digeneans.

In vitro excystation of metacercarial cysts[a].

Species	Location of metacercariae (final hosts)	Treatment	Authors
Acanthoparyphium spinulosum (Echinostomatidae)	Radular tissue of *Cerithidea californica* snails (black bellied plover, *Pluvialis sqaturola*; American avocet, *Recurvirostra americana*)	0.5% pepsin in Hank's BSS at pH 2.0 (HCl) for 1 h; the reductant, 0.015M sodium dithionite for 10 min; a complete medium of 0.2% sodium taurochlorate +0.5% trypsin in Hank's BSS at pH 7.8 ($NaHCO_3$) for 1 h at 42°C	Bass and LeFlore (1984)
Apatemon gracilis (Strigeidae)	Pericardial cavity of trout, *Salmo gairdner*, and eyes of stickleback, *Gasterosteus aculeatus* (birds)	Acid pepsin followed by alkaline trypsin + 0.3% sodium taruoglychocholate	Blair (1976)
Apatemon minor (Strigeidae)	Leeches, *Erpobdella octoculata* (ducklings)	1% acid pepsin at pH 2.0; 1% bile salts (ox bile) + 1% trypsin in Earle's BSS at pH 7 at 41°C	Kearn *et al.* (1989)
Bucephaloides gracilescens (Bucephalidae)	Tissues of gadoid fishes (intestine of monkfish, *Lophius piscatorius*)	0.5% pepsin at pH 1.5–2.0 (HCl) for 1 h; 0.3% sodium taurocholate in Hank's BSS at pH 7.2 for 10 min at 10°C	Johnston and Halton (1981)
Clinostomum complanatum (Clinostomatidae)	Skin and musculature of cyrpinodontid fish, *Aphanius dispar* (birds)	1% pepsin in acidified Tyrode's (HCl) at pH 2.3–3.5 at 42°C	Kalantan and Arfin (1988)
Clinostomum marginatum (Clinostomatidae)	Tissues of *Rana pipiens* (birds)	1.0% acid pepsin (HCl) at pH 2.3 at 40°C	Fried *et al.* (1970)
Clinostomum marginatum (Clinostomatidae)	Skin, musculature, peritoneum, and lymph spaces of *Rana pipiens* (birds)	Mechanical excystation in Ringer's at 22°C	Fried and Foley (1970)

Species	Location of metacercariae (final hosts)	Treatment	Authors
Clinostomum marginatum (Clinostomatidae)	Tissues of yellow perch, *Perca flavescens* (birds)	Mechanical excystation in cold Ringer's	Larson and Uglem (1990)
Cloacitrema michiganensis (Philophthalmidae)	Substratum; bottom and sides of laboratory glassware (shore birds)	0.5% trypsin + 0.2% sodium taurocholate in Hank's BSS at pH 7.8 ($NaHCO_3$) at 41°C	LeFlore and Bass (1983b)
Clonorchis sinensis (Opisthorchiidae)	Southern top-mouthed minnow, *Pseudorasbora parva* (man, carnivores)	1% pancreatin + 0.1% $NaHCO_3$ at 27°C	Fujino *et al.* (1979)
Cotylurus cornutus (Strigeidae)	Tissues of *Physa acuta* snails (birds)	1.0% HCl–1.0% pepsin in saline (pH 2.0) followed by 0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 7.8	Graczyk and Shiff (1993a)
Cotylurus erraticus (Strigeidae)	Pericardium and heart of rainbow trout, *Salmo gairdneri* (gull chick)	Hank's BSS with 0.5% trypsin + 0.2% sodium taurocholate at pH 7.5 at 41°C	Mitchell *et al.* (1978)
Cotylurus flabelliformis (Strigeidae)	Tissues of lymnaeid snail, *Stagnicola elodes* (birds)	Ringers at 41°C	Magnus and Johnson (1985)
Cotylurus lutzi (Strigeidae)	Tissues of snail, *Biomphalaria glabrata* (finches)	Earle's BSS at 41–42°C	Voge and Jeong (1971)
Cotylurus strigeoides (Strigeidae)	Tissues of *Physa heterostropha* snails (domestic chicks)	0.5% bile salts + 0.5% trypsin in Earle's BSS at pH 8.1 ($NaHCO_3$) at 41°C	Fried *et al.* (1978a)

Cotylurus variegatus (Strigeidae)	Swim bladder and pericardium of *Perca fluviatilis* (birds)	0.5% trypsin + 0.4% sodium taurocholate (ox bile) at pH 7.5 at 41°C	Thompson and Halton (1982)
Cyathocotyle bushiensis (Cyathocotylidae)	Tissues of *Bithynia tentaculata* snails (ducklings)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 ($NaHCO_3$) at 41°C	Fried and Ramundo (1987)
Echinoparyphium flexum (Echinostomatidae)	Kidneys and pericardium of physid snails (waterfowl)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 ($NaHCO_3$) at 41°C	Fried and Grigo (1975)
Echinostoma caproni (Echinostomatidae)	Kidneys and pericardium of *Biomphalaria glabrata* snails (birds and mammals)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 ($NaHCO_3$) at 41°C	Fried and Emili (1988)
Echinostoma malayanum (Echinostomatidae)	Kidneys of *Indoplanorbis exustus* snails (albino rats)	0.5% trypsin + 0.5% sodium cholate in Hank's BSS at pH 8.1 ($NaHCO_3$) at 42°C	Mohandas and Nadakal (1978)
Echinostoma trivolvis (Echinostomatidae)	Kidneys of snails and *Rana* tadpoles (birds and mammals)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.1 at 41°C	Fried and Butler (1978)
Fasciola gigantica (Fasciolidae)	Vegetation; cellophane in laboratory (herbivores)	Mix equal volumes of A and B before use. A contains 0.05N HCl; B contains 1% $NaHCO_3$ + 0.8% NaCl + 20% ox bile; add 2 mg ml^{-1} of L-cysteine HCl before use; incubate cysts for 2 h at 37°C	Hanna and Jura (1976)
Fasciola hepatica (Fasciolidae)	Cysts on moist filler paper at 4°C (herbivores)	Place cysts in vial with 5 ml of 0.05N HCl; add equal volume of a solution containing 0.8% NaCl + 1% NaOH; cap vial and incubate at 39°C for 10 min; add 70 mg sodium dithionite to vial; in 30 min add 10 ml of a 2% solution of sodium taurocholate; incubate at 39°C for 3 h	Fried and Butler (1979)

Species	Location of metacercariae (final hosts)	Treatment	Authors
Fasciola hepatica (Fasciolidae)	Cellophane strips (herbivores)	0.5% pepsin in Hank's BSS (pH 2.0) at 37°C for 20 min to remove the outer cyst walls; wash in Hank's BSS (pH 7.2); incubate at 37°C in a medium containing 5 ml of 0.8% NaCl + 5 ml of 0.02N HCl + 1.0% $NaHCO_3$ + sodium taurocholate (50 mg) + L-cysteine HCl (40 mg); excystation within 2 h	Hanna (1980)
Fasciola hepatica (Fasciolidae)	Cellophane strips (herbivores)	Remove outer cyst walls between glass slides. Activate cysts in Earle's BSS at 38°C for 1 h in 60% CO_2/40% N. Excyst metacercariae by adding 10% sterile sheep bile to the culture; excystment occurs in an apparatus which allows the newly excysted juveniles to escape from the bile-containing medium into one with a low bile content	Tielens *et al.* (1981)
Fasciola hepatica (Fasciolidae)	Cellophane strips (herbivores)	Treat cysts for 1 h in 0.5% pepsin + 0.5% trypsin + 2% HCl at 37°C in a shaking water bath; activate cysts at 37°C for 1 h in Hank's BSS (pH 7.3) with a gas phase of 60% CO_2/40% N_2. Excyst by placing cysts in a chamber with 80% Hank's BSS + 10% fetal calf serum + 10% sterile bovine bile	Reddington *et al.* (1984)
Fascioloides magna (Fasciolidae)	Substratum; lettuce experimentally (cattle, sheep, deer)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 ($NaHCO_3$) at 41°C	Fried and Stromberg (1985)

Gynaecotyle adunca (Microphallidae)	Tissue of *Uca pugilator* crabs (shore birds and some fish species)	0.5% trypsin in Hank's BSS at pH 7.2 at 40°C. Excystment rate increased in presence of saturated CO_2.	Dunn *et al.* (1990)
Himasthla leptosoma (Echinostomatidae)	Cockles, *Cardium*; mussels, *Mytilis* (gulls)	5 ml of bicarbonate saline (0.8% w/v NaCl and 1.5% w/v $NaHCO_3$) containing 0.8% w/v sodium taurocholate and 0.3% w/v trypsin to which an equal amount of 0.02M HCl containing 0.8% w/v L-cysteine is added just before use; incubate at 41°C	Irwin *et al.* (1984)
Himasthla quissetensis (Echinostomatidae)	Tissues of *Ilyanassa obsoleta* snails (gulls)	0.025% pepsin in Earle's BSS at pH 2.5 (HCl); then 0.3% trypsin + 0.5% bile salts in Earle's BSS at pH 7.8 ($NaHCO_3$) at 41°C	Kirschner and Bacha (1980)
Himasthla rhigedana (Echinostomatidae)	Glassware; substratum in marine environment (domestic chicks; curlews, *Numericus*)	0.015M sodium dithionite for 7 min; 0.3% trypsin + 0.05% bile salts (50% sodium cholate + 50% sodium deoxycholate) in Earle's BSS at pH 7.9 at 42°C	LeFlore and Bass (1992)
Leucochloridium variae (Brachylaimidae)	Broodsacs of *Succinea ovalis* snails (birds)	1% acid pepsin for 15 min followed by 1% alkaline trypsin for 30 min at 37°C	Fried (1973)
Maritrema arenaria (Microphallidae)	Gut wall of barnacles, *Balanus balanoides* (anseriform and charadriform birds)	Hank's BSS with 0.22% sodium taruocholate + 0.5% trypsin	Irwin (1983)
Mesocoelium monodi (Mesocoeliidae)	Hemocoel of *Oniscus* and *Armadillidium* (rainbow lizard, *Agama agama*, and African toad, *Bufo regularis*)	0.025% chymotrypsin in H_2O at pH 6.8 at 22°C; also natural products including chicken bile and human urine	Fashuyi (1986)

Species	Location of metacercariae (final hosts)	Treatment	Authors
Microphallus abortivus (Microphallidae)	Tissues of *Hydrobia ulvae* snails (marine birds)	0.8% NaCl + 1.5% $NaHCO_3$ + 0.3% trypsin + 0.8% sodium taurocholate; add equal volume of 0.02M HCl + 0.08% cysteine just before use; incubate at 41°C	Saville and Irwin (1991a)
Microphallus madrasensis (Microphallidae)	Ovary and hepatopancreas of sand crabs, *Emerita asiatica* (shore birds)	0.85% saline at 40°C	Ramasamy and Panicker (1991)
Microphallus primas (Microphallidae)	Tissues of shore crab, *Carcinus maenas* (shore birds)	0.8% NaCl + 1.5% $NaCO_3$ + 0.3% trypsin + 0.8% sodium taurocholate; add equal volume of 0.02M HCl + 0.08% cysteine just before use; incubate at 41°C	Saville and Irwin (1991b)
Microphallus similis (Microphallidae)	Digestive gland of naturally infected *Carcinus maenas* crabs (gulls, *Larus*)	0.5% pepsin in Hank's BSS at pH 2 for 0.5 h at 38°C; 0.5% trypsin in HBSS at pH 7 for 10–20 min at 38°C	Davies and Smyth (1979)
Microphaloides japonicus (Microphallidae)	Tissues and organs of marsh crabs, *Helice tridens* (rat, *Rattus norvegicus*)	0.85% saline at 37°C	Fujino *et al.* (1977)
Neascus pyriformis (Diplostomatidae)	Skin and fins of fathead minnows, *Pimephales promalus*; creek chubs, *Semotilus atromacularus*; common shiners, *Notropis cornutus* (birds)	Low pH treatment (pH 2.0) with acid Locke's (HCl); 0.5% trypsin + 0.5% sodium cholate at pH 7.4 at 41°C	Schroeder *et al.* (1981)
Notocotylus attenuatus (Notocotylidae)	Encysts on substratum (birds)	1st pre-treatment for 2 h in cationic detergent EDTA-20: water, 1:2 mixture for 2 h; 2nd pre-treatment for 15 min in 1% acid pepsin (pH 2); treatment in alkaline trypsin + bile salts (pH 8.0); all treatments at 41–42°C	Graczyk and Shiff (1993b)

Paragonimus ohirai (Troglotrematidae)	Crabs, *Sesarma dehaani* (carnivores, man)	Incubate overnight in Tyrode's solution (pH 8.0) at 37°C	Fujino *et al.* (1989a)
Paragonimus ohirai (Troglotrematidae)	Tissues of crabs (carnivores, man)	1% pancreatin + 0.1% $NaHCO_3$ at 39°C	Fujino and Ishii (1990)
Paragonimus ohirai (Troglotrematidae)	Tissues of crabs (man, carnivores)	Tyrode's solution at pH Ikeda 8.0 with 1000 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin for 12 h	Ikeda and Oikawa (1991)
Paragonimus westermani (Troglotrematidae)	Tissues of crabs, *Eriochier japonicus* (man, carnivores)	RPMI 1640 with 100 U ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomyin for 10 h at 40°C	Yamakami and Hamajima (1988)
Paragonimus westermani (Troglotrematidae)	Crabs, *Eriochier japonicus* (carnivores, man, dogs experimentally)	0.07% HCl then 0.1% $NaHCO_3$ in Ringer's saline at 39°C	Fujino and Ishii (1990)
Paramphistomum cervi (Paramphistomatidae)	Substratum; polyethylene sheets in laboratory (mice and rabbits experimentally; cattle, sheep, and deer)	Acid pepsin for 3 h; alkaline trypsin + 20% sheep bile for 3 h at 37°C	Gill and Bali (1988)
Parapronocephalum symmetricum (Notocotylidae)	Viscera of *Littorina saxatilis* snails (purple sandpiper, *Calidris maritima*)	Equal amounts of solution A containing 0.8% NaCl, 1.5% $NaHCO_3$, 0.8% sodium taurocholate + 0.3% trypsin with solution B containing 0.02% 1M HCl + 0.8% L-cysteine; incubate at 41°C	Irwin *et al.* (1989)
Parorchis acanthus (Philophthalmidae)	Substratum (aquatic birds)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 ($NaHCO_3$) at 41°C	Fried and Roth (1974)
Parorchis acanthus (Philophthalmidae)	Substratum (various waterfowl)	1% acid pepsin for 1.5 h at 39°C followed by 1% alkaline trypsin at 39°C for 3 h	Asanji, and Williams (1985)

Species	Location of metacercariae (final hosts)	Treatment	Authors
Philophthalmus gralli (Philophthalmidae)	Vegetation; on glassware in laboratory (various waterfowl)	Thermal activation with distilled water at 35–54°C	Cheng and Thakur (1967)
Philophthalmus hegeneri (Philophthalmidae)	Vegetation; on glassware in laboratory (various waterfowl)	Thermal activation with saline at 30–49°C	Fried (1981)
Plagiorchis elegans (Plagiorchiidae)	Insects, e.g. *Aedes aegypti* (birds)	0.05% bile salts (sodium cholate: sodium deoxycholate, 1:1) in 0.015M $NaHCO_3$ + 0.015M NaCl at pH 7.5 at 37°C	Lowenberger and Rau (1993)
Plagiorchis species 1 (Plagiorchiidae)	Aquatic insects, e.g. *Chaborus* and *Cloeon* (birds)	0.05% sodium cholate + 0.015M $NaHCO_3$ + 0.05M sodium phosphate; at pH 7.5 at 22°C	Bock (1989)
Probolocoryphe uca (Microphallidae)	Tissues of *Uca pugilator* crabs (shore birds)	Hank's BSS at 40°C	Dunn *et al.* (1990)
Sphaeridiotrema globulus (Psilostomatidae)	Tissues of *Goniobasis* snails; (various waterfowl)	0.5% trypsin + 0.5% bile salts in Earle's BSS at pH 8.2 with 7.5% $NaHCO_3$ at 41°C	Fried and Huffman (1982)

Timoniella imbutifome (Acanthostomatidae)	Infected gobies, *Timoniella imbutifome* and *Pomatoschistus microps* (rectum of bass, *Dicentrachus labrax*)	1% acid pepsin at pH 5.0 for 0.5 h, then 1% alkaline trypsin at pH 9.0 at 38°C	McDowell and James (1988)
Uvulifer ambloplitis (Diplostomatidae)	Tissues of green sunfish, *Lepomis cyanellus* (birds)	0.5% pepsin at pH 2.0 for 0.5 h; 0.2% sodium dithionite at pH 7.4 for 10 min, 0.5% bile salts at pH 7.4 for 2 h	Spellman and Johnson (1987)
Zygocotyle lunata (Paramphistomatidae)	Substratum; glassware in laboratory (aquatic birds and mammals)	1% acid pepsin (HCl) in Earle's BSS; the reductant, 0.02M sodium dithionite; 1% Na glycocholate + 1% trypsin in Earle's BSS at pH 8.8 at 41°C	Fried *et al.* (1978b)
Zygocotyle lunata (Paramphistomatidae)	Substratum; glassware in laboratory (aquatic birds and mammals)	5 ml of bicarbonate saline (0.8% w/v NaCl and 1.5% w/v $NaHCO_3$) containing 0.8% w/v sodium taurocholate and 0.3% w/v trypsin to which an equal amount of 0.02M HCl containing 0.8% w/v L-cysteine is added just before use; incubate at 41°C	Irwin *et al.* (1993)

[a] From studies done mainly between 1970 and 1993. For earlier work see Lackie (1975).

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The Minor Groups of Parasitic Platyhelminthes

Klaus Rohde

Department of Zoology, University of New England, Armidale NSW 2351, Australia

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1. INTRODUCTION

The major groups of parasitic Platyhelminthes, that is, the monogeneans, digenean trematodes and eucestodes, contain many species of great economic and/or medical importance. Consequently, they have been well studied and

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are well represented in textbooks, monographs and reviews. Much less attention has been paid to various "minor" parasitic platyhelminth groups. These include the Aspidogastrea (= Aspidobothrea = Aspidobothria), the Udonellidea, Amphilinidea, Gyrocotylidea and a number of taxa within the largely free-living Turbellaria. None of these groups has great economic or medical importance, but they have fascinated parasitologists and zoologists for various reasons. Thus, the Aspidogastrea have much simpler life cycles than other trematodes and may therefore be at the root of trematode phylogeny, but they have nervous systems and sensory receptors much more complex than known from any other platyhelminth. Udonellidea have been claimed to be turbellarians or to be related to various major parasitic platyhelminth groups, Amphilinidea are said to be neotenic eucestodes or a taxon well separated from the eucestodes, and Gyrocotylidea are thought to be modified monogeneans or cestodes. Among the parasitic turbellarians are species infecting the very archaic echinoderms, therefore probably representing very ancient parasites, and some have been claimed to be sister groups of the trematodes, cestodes or monogeneans.

This review aims to give a detailed and up-to-date account of the minor parasitic platyhelminth groups. An earlier review by the author (Rohde, 1972) on the Aspidogastrea should be consulted for early references that are not included in this review. A monograph by Dubinina (1982) concentrates on the taxonomy and biology of Amphilinidea and should be consulted for early references on this group. Williams *et al.* (1987) published a brief review article on Gyrocotylidea containing many references, which concentrated on aspects of reproduction, population ecology and phylogeny. Rieger *et al.* (1991) discussed light- and electron-microscopic aspects of Turbellaria, and in the present review I restrict myself to a discussion of those aspects of symbiotic turbellarians that may cast light on the phylogeny of parasitic Playhelminthes.

2. THE ASPIDOGASTREA

2.1. Development and Life Cycles

2.1.1. Aspidogaster conchicola

Information on the life cycle of this species (Figure 1) is mainly given in papers by Bakker and Davids (1973) and Huehner and Etges (1972a, 1977; see also Huehner, 1975). The second authors completed the life cycle experimentally in the North American freshwater snail *Viviparus malleatus*. Eggs did not hatch in freshwater, and infection attempts with six mechanically hatched larvae were unsuccessful. However, snails could be infected with

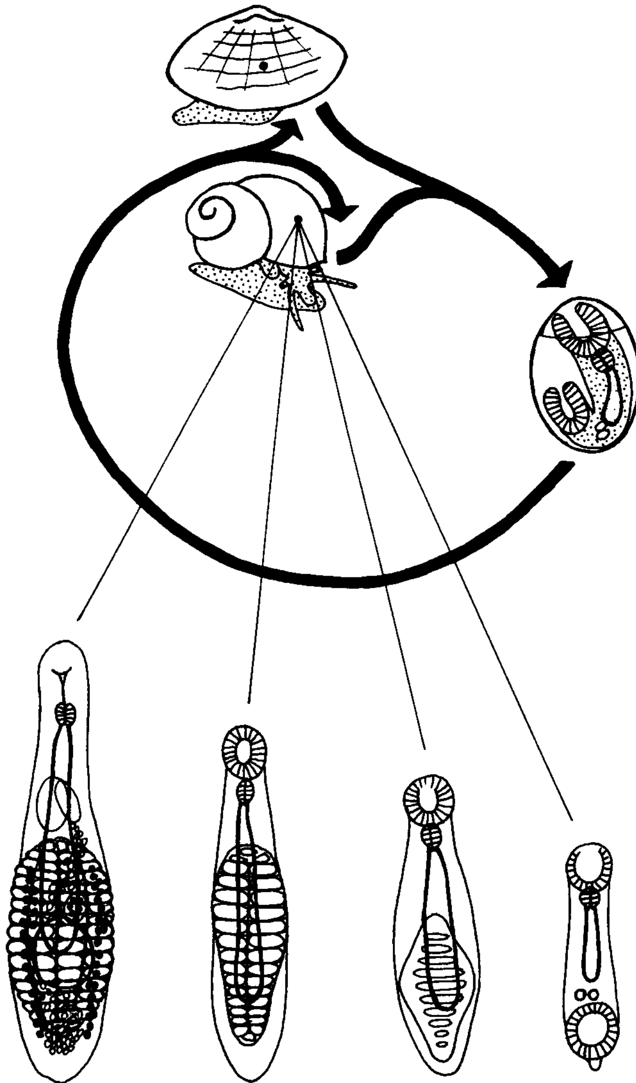


Figure 1 *Aspidogaster conchicola*, life cycle. Eggs containing larvae infect molluscs, in which worms mature. Note that vertebrates may also become infected by ingesting infected molluscs.

embryonated, unhatched eggs. From 42 experimentally infected snails that survived to examination, 664 worms were recovered, and three of the snails produced 13 fully grown, gravid worms. The life cycle was completed in 270 days at 20°C. During development, three growth phases could be distinguished:

1. worms <0.65–0.70 mm long, growth with only slight changes in body proportions (negative allometric growth of oral disc, positive allometric growth of ventral sucker), no alveolation of ventral sucker;
2. strong positive allometric growth and alveolation of ventral sucker;
3. worms >1.60–1.65 mm long, ventral sucker ceases positive allometric growth, lessening of negative allometric growth of oral disc until growth rates of body, oral disc and ventral sucker become equal at a worm length of 2.25 mm.

In alveolus formation, a transverse septum first divides the ventral sucker into two zones, subsequently 11–12 transverse alveoli rows develop and finally a medial longitudinal septum becomes evident along the first three to five alveoli, later extending further posteriorly. The two secondary, right and left longitudinal septa become visible as well.

A second freshwater snail, *Goniobasis livescens*, could also be successfully infected, but mortality of snails was high.

Bakker and Davids (1973) examined the question of whether unhatched larvae in eggs or hatched larvae infect mussels. They regularly sieved water from tanks in which mussels were kept and found that only eggs with fully embryonated embryos left the hosts. They observed hatching from most of the eggs within a day. Larvae became active and pushed off the operculum. At least 20 eggs, soon after shedding, were inserted into the inhalant opening of a mussel over 14 days; one worm was recovered from the kidney 3 months later. Twenty hatched larvae were inserted into the inhalant opening of another mussel but did not lead to infection. Twenty five embryonated eggs that had not hatched over several weeks also did not lead to infection. The authors traced the course of particles of about the same size as *Aspidogaster* eggs and concluded that infection occurs via the nephridiopore.

Numbers of hosts and parasites examined by Bakker and Davids are very small and some of their results (hatching) contradict those of Huehner and Etges (1977). Voeltzkow (cit. Rohde, 1972) considered it unlikely that larvae migrate via the kidney funnel, since the ciliary beat is directed outwards and since he never found young *Aspidogaster* in the kidneys near the opening. According to this author, a route via the intestine is more likely, since he found young worms in the intestine 8–14 days after infection.

Huehner and Etges (1981) observed encapsulation of *A. conchicola* in six out of seven unionid bivalve species found to be infected. Encapsulated worms were most common anterior to the pericardium and surrounded by inner fibroblastic and outer fibrocytic/fibrous walls, the walls containing acid and neutral mucins, phospholipids and reticulum fibres. Worms in the capsules were alive or moribund, the capsule probably contributing to the death of worms. According to the authors, the frequent occurrence of capsules anterior to the pericardium and dorsal to the digestive gland suggests that this route of infection is usually used by the worms.

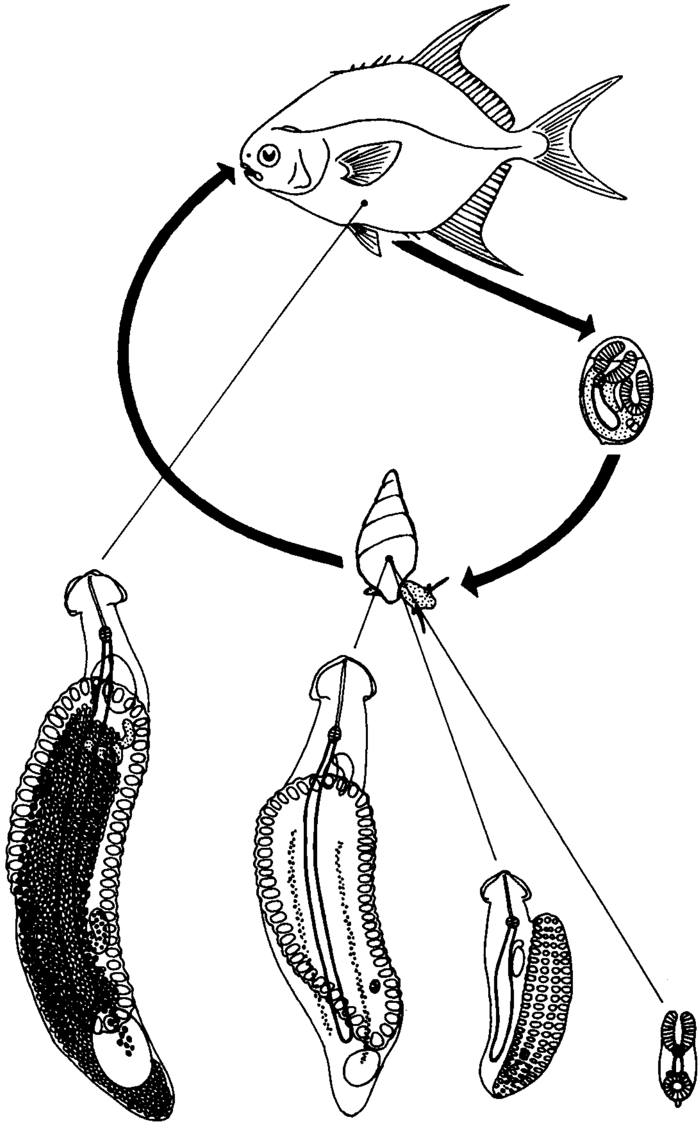


Figure 2 *Lobatostoma manteri*, life cycle. Snails ingest eggs containing larvae. Larvae hatch in the stomach and migrate into the digestive gland where they develop to pre-adults. Snails containing pre-adults are eaten by fish, *Trachinotus blochi*, where worms mature. (From Rohde, 1976.)

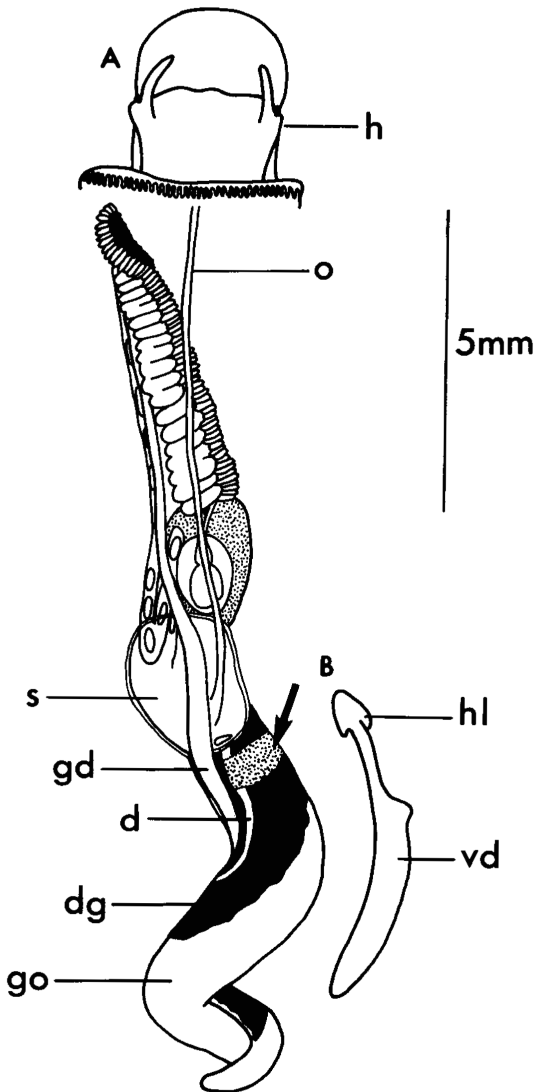


Figure 3 Diagram of internal organs of *Cerithium moniliferum* (A) and an extended *Lobatostoma manteri* (B). Drawn at same scale. Stippled area in digestive gland indicated by an arrow is the usual site of *Lobatostoma*. d, duct of digestive gland; dg, digestive gland; gd, gonoduct; go, gonad; h, head; hl, head of *Lobatostoma*; o, oesophagus; s, stomach; vd, ventral disc of *Lobatostoma*. (Modified from Rohde, 1973a.)

2.1.2. Lobatostoma manteri.

Rohde (1973a, b) worked out the complete life cycle of the species (Figure 2). Worms in the intestine of the carangid fish *Trachinotus blochi* lay eggs with fully developed larvae that are shed in the faeces. Snails of three species, *Cerithium (Clypeomorus) moniliferum* (Cerithiidae), *Peristernia australiensis* (Fasciolariidae) and *Planaxis sulcatus* (Planaxidae, Rohde, 1975) became infected by eating the eggs. Hatching occurs in the stomach of the snails. Larvae lack cilia, but have an "oral sucker" (according to Pearson, 1992 a "false" oral sucker), pharynx, single caecum, posterior sucker, two dorsal excretory bladder cells in front of the acetabulum, and a caudal appendage. In *Cerithium*, larvae migrate into the digestive gland and develop to pre-adults with fully developed genital organs and the full number of alveoli on the adhesive disc; young spermatozoa and egg cells develop but do not mature. A single juvenile worm is usually found in a cavity formed by the enlargement of the main duct and one or several (?) side ducts of the digestive gland of *Cerithium* (Figures 3, 4). In *Peristernia*, a larger snail, often several (up to six) worms are found in the stomach (Figure 4) or the main ducts of the digestive gland. Worms may creep between stomach and digestive gland. Fish become infected by eating snails. Attempts at experimental infection of four freshly hatched green sea turtles (*Chelonia mydas*) and 23 fish of nine species were negative, except for the recovery of one living worm 2 h after infection from a turtle, and of two living worms 1 day after infection from the teleost fish *Choerodon albigenea*. In nature, of the many fish of several species examined, only the snub-nosed dart, *Trachinotus blochi*, was found to be infected (14 of 17 fish with 1–25 worms), at Heron Island, Great Barrier Reef, where the study was undertaken. Juvenile fish of this species follow the incoming tide and feed on *Cerithium* in shallow water. They are uniquely adapted to feeding on this snail which fits exactly into their mouth, is prevented from slipping out of the mouth by a large tooth-like vomer, and is crushed between the enormously developed pharyngeal plates (Figure 5). Snail shells are very thick and need strong pressure for crushing. Strongly developed muscles, responsible for the "snub-nose" of the fish, move the pharyngeal plates during crushing.

Worms from fish died soon after transfer into sea water but could be kept alive for up to 13 days in frog's Ringer solution or dilute sea water (1 : 5) in which they laid eggs containing infective larvae. Worms from snails remained alive for weeks in dilute sea water (1 : 5), Tyrode or frog's Ringer solution (maximum 52 days in dilute sea water). These results indicate that worms from snails have a greater tolerance to osmotic pressure than worms from fish.

Three *Lobatostoma* from three *Trachinotus*, each with a single worm only, produced normal-looking eggs. However, eggs had the haploid chromosome number of seven chromosomes and no advanced blastula stages were found.

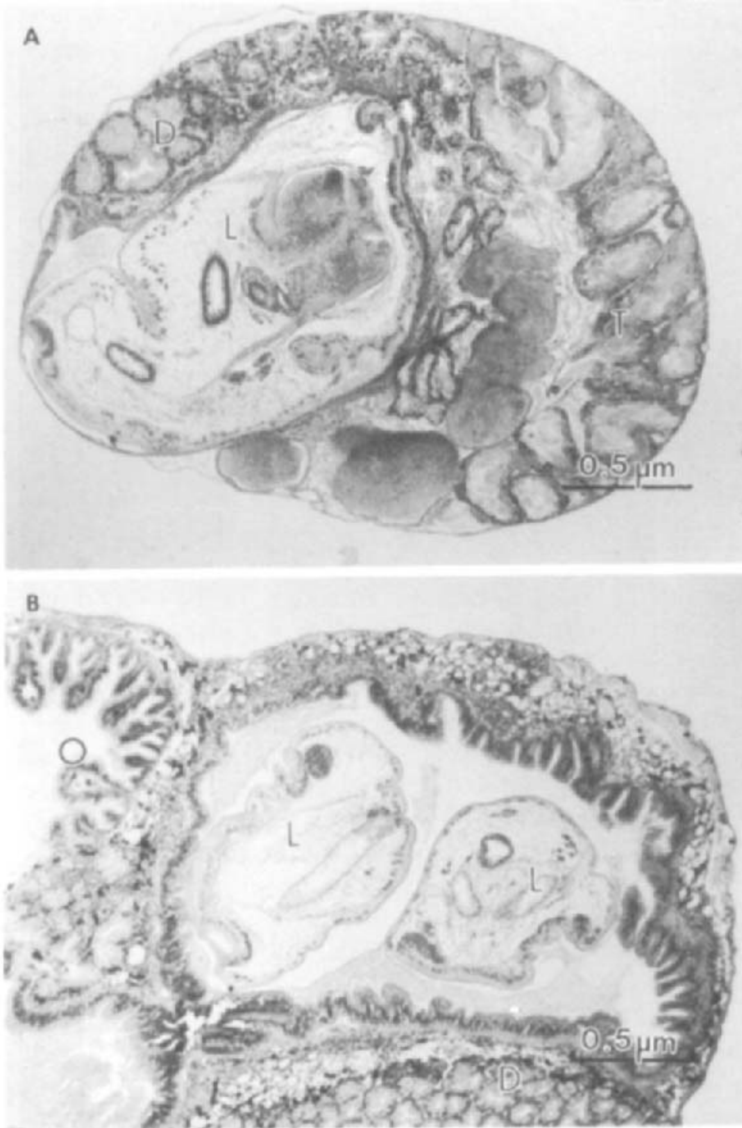


Figure 4 Sections through *Cerithium moniliferum* (A) and *Peristernia australiensis* (B) showing one *Lobatostoma* in digestive gland of *Cerithium* and two *Lobatostoma* in stomach of *Peristernia*. D, follicles of digestive gland, L, *Lobatostoma*; O, oesophagus; T, testes.

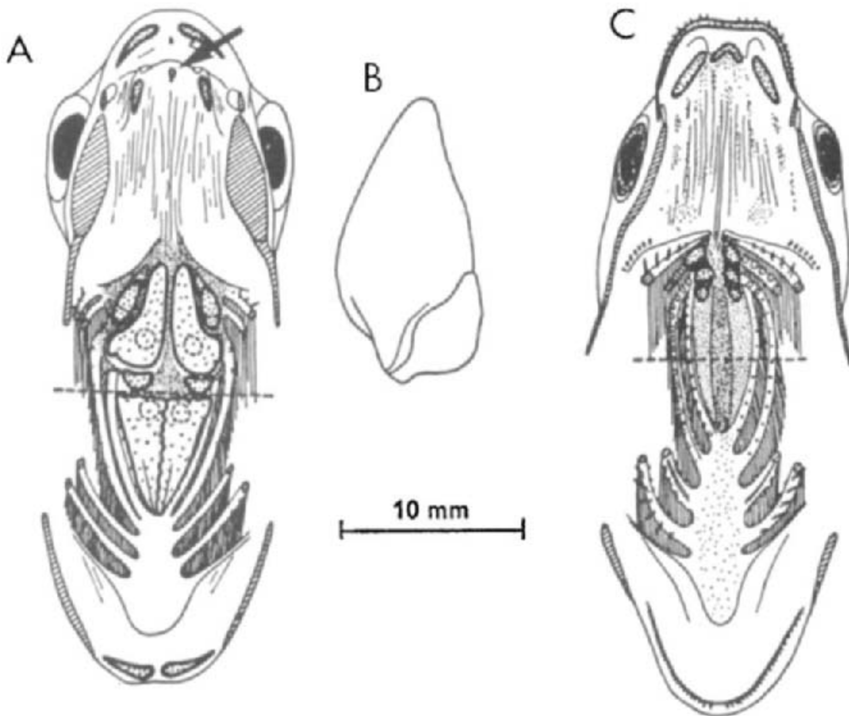


Figure 5 Mouth and pharyngeal cavities of juveniles of two species of Carangidae of equal size: *Trachinotus blochi* (A) and *Caranx melampygus* (C), opened along transverse interrupted lines. Tooth plates thick-lined, areas on pharyngeal plates of (A) which are sometimes thickened are indicated by circles of interrupted lines.(B) Outline of *Cerithium*. Note: The snail fits exactly into the mouth of *Trachinotus* and is crushed by the pharyngeal plates; a spine like vomer (arrow) prevents the snail from slipping out of the mouth. *Caranx* cannot feed on the snails and does not become infected with *Lobatostoma*. (Modified from Rohde, 1973a.)

Cleavage did not occur at all in most eggs, or stopped at the two- and perhaps (?) rarely at a several-cell stage. These results indicate that self fertilization does not usually occur.

Cleavage proceeds as follows: the zygote divides into three cells of approximately the same size that are variably arranged; sometimes one or all cells are separated by yolk. Each cell divides again to give rise to a large and a small cell, though the size difference is not always distinct. Further divisions lead to cell clusters that do not show any regular arrangement, and subsequently to a blastula that fills most of the egg. Later, the two excretory bladder cells become visible, and finally the posterior sucker, oral "sucker", pharynx and caecum. The excretory bladder cells always contain one large concretion each, probably waste material, which disappears after hatching. The larva lies

in the egg with its mouth end at the opercular pole and the posterior end bent forward at the other pole. Yolk fills most of the egg not occupied by the zygote or early embryo; at later stages of development, a nucleated membrane around the yolk and embryo becomes distinct.

According to Rohde (1975), larvae feed on digestive secretion and probably epithelial cells of the glandular follicles. The posterior sucker is used for adhesion to the epithelium and contributes to its erosion. In snails (*Cerithium* and *Planaxis*) heavily infected experimentally, the digestive follicles gradually disappear and the larvae live in cavities lined by a flattened epithelium, parts of which show secretory activity. In snails 47–66 days after infection, the cavities are fused and only small parts of the epithelium are still secretory. In experimentally infected *Planaxis* (as in naturally infected *Peristernia*, both larger snails than *Cerithium*), some worms were found in the stomach.

In larvae approximately 0.5–0.6 mm long, the posterior sucker begins to divide into alveoli and its anterior end grows forward; the anterior alveoli gradually increase in size and new alveoli are formed in the posterior undivided zone (Figure 6). The pharynx shows strongly negative allometric growth throughout, the anterior part of the body grows approximately isometrically until the body reaches a length of 0.05 mm, then ceases growth altogether, and grows with slightly negative allometry in worms longer than 1.5 mm. The posterior sucker grows with slight positive allometry until a body length of approximately 0.4 mm is reached, then grows with strong positive allometry and, in specimens longer than 1 mm, isometrically.

Worms in snails may be fully developed but not sexually mature. After

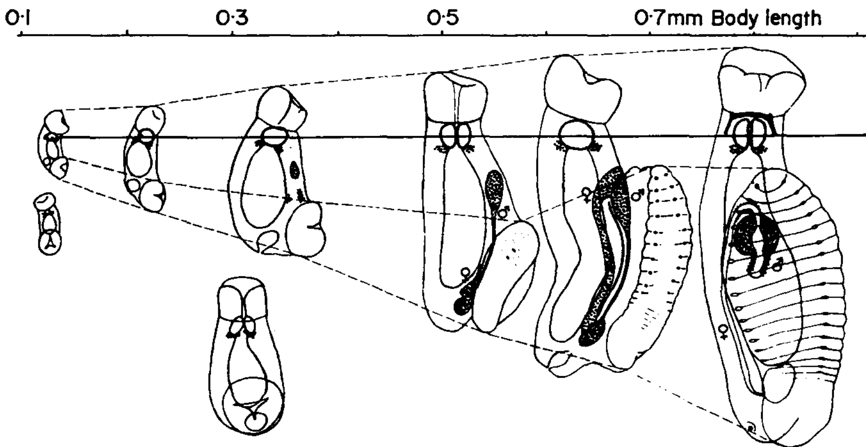


Figure 6 *Lobatostoma manteri*. Growth from 1-day-old larva to young worm with 35 marginal alveoli. (Modified from Rohde (1975), reproduced with permission of Pergamon Press.)

ingestion by fish, worms become slightly larger, but the increase in body size is apparently entirely or largely due to the large number of eggs in the uterus.

2.1.3. Cotylogaster occidentalis

Fredericksen (1980, see also 1973) gave a beautifully illustrated description of some aspects of the development of this species from naturally infected North American mussels, *Ligumia nasuta*. Juvenile worms first appeared in the mouth and oesophagus of the host, and during development moved gradually through the stomach into the intestine. Juveniles lose the ciliated patches characteristic of the larva (see below), but at first still have an undivided posterior sucker. Light microscopic and SEM studies revealed a zone of alveolar differentiation on the floor of the sucker, assuming a more posterior location with continuing differentiation, outlined by densely stained nuclei. The reproductive system begins development before alveoli are well differentiated.

2.1.4. Multicalyx cristata

Growth after maturity consists of addition of alveoli at the posterior end, leading to a shift in the relative position of the various internal regions (Thoney and Burreson, 1987, 1988).

2.2. Structure of the Larva

2.2.1. *General Morphology*

The larvae (cotylocidia) of *Lobatostoma manteri* (Aspidogastridae), *Multicalyx cristata* (Multicalycidae) and *Rugogaster hydrolagi* (Rugogastridae, system of Gibson and Chinabut, 1984) lack ciliated patches, but have a pharynx, single caecum and posterior sucker (Rohde, 1973a; Thoney and Burreson, 1987; Schell, 1973, respectively). Larvae of the first and third species also have a posterior appendage, not described for larval *Multicalyx*.

In contrast to larvae of the above three species, the cotylocidium of *Cotylogaster occidentalis* has altogether 14 ciliated patches arranged in a ventrally incomplete ring of eight at the end of the anterior quarter to third of the body, and a posterior circle of six (Fredericksen, 1978). This compares with a ring of four around the body and a posterior circle of six in *Multicotyle purvisi* (Rohde, 1972 and references therein). The larva of *Cotylogaster* differs from that of *Multicotyle* also in the number of flame bulbs, eight (2 (2 + 2)) in the former and six (2 (3)) in the latter species. Both species have a dorsal pair of excretory bladders in the posterior part of the body. Fredericksen (1978) also described cephalic gland cells, opening at the anterior end.

2.2.2. *Tegument and Gland Cells*

Larvae of *Cotylogaster occidentalis* (see Fredericksen, 1978) and *Lobatostoma manteri* (see Rohde and Watson, 1992a) have a tegument with short microvilli, so-called microtubercles, characteristic of all larval and adult Aspidogastrea examined to date (Rohde, 1972). For the cotylocidium of the first species, Fredericksen described goblet-like glandular cells using TEM. Secretory granules are only slightly electron-dense, highly irregular in shape, and do not appear to be membrane bound. The cells arise in the parenchyma, in clusters of two and probably more forming a pyriform body, and opening at the surface attached by means of desmosomes to the adjacent tegument.

2.2.3. *Nervous System and Sensory Receptors*

Timofeeva (1971) used staining for acetylcholinesterase to examine the nervous system of larval *Aspidogaster conchicola*. In its general pattern, the nervous system of this species closely corresponds to that described for larval *Multicotyle purvisi* by Rohde (1972 and further references therein) on the basis of serial sections impregnated with silver. A brain commissure gives rise to several anterior connectives anteriorly connected by a commissure, and to several posterior connectives, of which the ventral pair enters the posterior sucker to form a nerve ring. Connectives in the anterior half of the body are connected by several commissures. Timofeeva did not provide any information about the innervation of the pharynx and intestine, probably because such nerves could not be stained by the method used.

Fredericksen (1978) concluded from acetylcholinesterase localizations in larval *Cotylogaster occidentalis* that there is a large anterior concentration of nervous tissue, with posterior processes joining another concentration around the posterior end, but the nervous system was not reconstructed. Silver-nitrate treated whole mounts revealed approximately 60 presumptive sensilla appearing as raised dots, with an additional 20 appearing as rings. Transmission electron microscopy showed only one type of uniciliate receptor, occurring in clusters of three at the anterior end, and singly in more posterior parts of the body. Receptors have two electron-dense collars and a triradiate cross-striated rootlet.

Receptors of larval aspidogastrids belonging to two species have been examined in greater detail: those of *Multicotyle purvisi* (Rohde and Watson, 1990a, b, c, 1991b) and *Lobatostoma manteri* (Rohde and Watson, 1992a). Reconstruction of receptors of both species is based on sections (many of them serial) through all parts of the body of two and three larvae, respectively, and it is likely that all (or at least most) receptor types have been found. The first species has four types of presumptive non-ciliate receptors, seven types of ciliate receptors, an anterior pair of receptor complexes, and a pair of pigmented photoreceptors, whereas the second species has two types of non-ciliate and seven types of uniciliate receptors.

The following receptors of larval *Multicotyle* were described:

1. One pair of non-ciliate receptors in the mouth cavity with two to several electron-dense collars, a long cross-striated ciliary rootlet and some fibres diverging from the basal body;
2. four pairs of non-ciliate receptors in the ventral wall of the posterior sucker and in the tegument ventral to the sucker with many electron-dense collars and, diverging from the basal body, a large, round-ovoid ciliary rootlet that is cross-striated in its proximal and has many dense structures in its distal part;
3. two large non-ciliate bulbous receptors in the lateral surface tegument anterior to the sucker, with much dense material in their apical parts and some desmosome-like structures;
4. a single, unpaired receptor with a bulbous projection at the posterior end, containing many vesicles with dense inclusions and some long cytoplasmic processes;
5. uniciliate receptors with one electron-dense collar and an approximately 0.6 μm long cilium;
6. uniciliate receptors with two electron-dense collars and a cilium with much dense cytoplasm around the ring of peripheral microtubule doublets;
7. uniciliate receptors with two electron-dense collars and large reticulate extensions into the receptor;
8. uniciliate receptors with two electron-dense collars and a long cros-striated ciliary rootlet;
9. uniciliate receptors containing a cilium with numerous single microtubules and doublets;
10. uniciliate receptors with two elecron-dense collars and an at least 6 μm long cilium;
11. uniciliate receptors with two electron-dense receptors and an approximately 1.3 μm long cilium;
12. two anterior receptor complexes dorsal to the mouth cavity, each consisting of two dendrites, one forming a large liquid-filled cavity (approximately 7.5 × 3 μm in size) with at least 10 short cilia lacking ciliary rootlets, but possessing basal bodies and lamellate extensions of the ciliary membrane, the other penetrating the anterior wall of the cavity formed by the first dendrite and possessing a single cilium, star-shaped in cross-section (Figure 7);
13. paired pigmented photoreceptors, each consisting of one pigment cell and two receptor cells whose two rhabdomeres are located in the antero-lateral and postero-lateral parts of the pigment cup, respectively, each rhabdomere consisting of two bundles of microvilli diverging from a central dendritic mass that is connected by a narrow connection lined by dense material along the cell membrane to a perikaryon outside the ocellus (Figure 8).

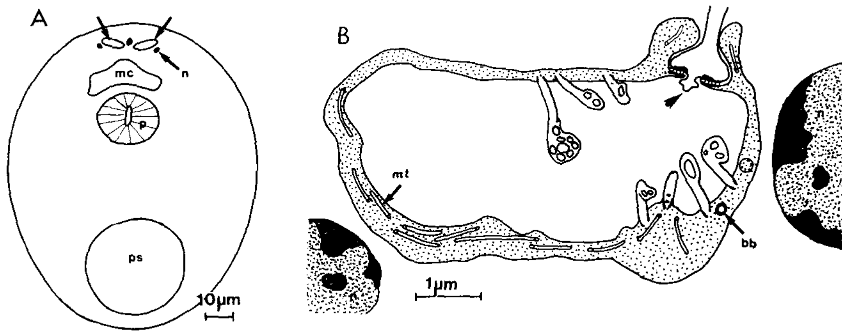


Figure 7 *Multicotyle purvisi.* Paired multiciliate receptor complexes of larva. (A) Diagram of larva showing position of receptor complexes (arrows). (B) Diagram of receptor complex. bb, basal body; mc, mouth cavity; mt, microtubule; n, nucleus; p, pharynx; ps, posterior sucker. Arrowhead in (B) indicates uniciliate receptor. (From Rohde and Watson, (1990c), reproduced with permission of Springer-Verlag.)

In larval *Lobatostoma*, uniciliate receptors differ in the number of electron-dense collars, length and shape of the cilium and absence or presence of a ciliary rootlet. The two types of non-ciliate receptors differ in the shape and structure of the ciliary rootlet (Figure 9).

The number of receptor types in *Lobatostoma* is smaller than that in *Multicotyle*, possibly due to the different modes of infection. In *Lobatostoma*, eggs are ingested by a snail, non-ciliated larvae hatch in the stomach and migrate into the adjacent digestive gland (see p. 151), whereas in *Multicotyle*, ciliated larvae hatch in freshwater and swim around until inhaled by snails; larvae then migrate to the kidneys (Rohde, 1972).

2.3. Structure of the Adult

2.3.1. *General Morphology*

Fredericksen (1972) gave an excellent description of *Cotylogaster occidentalis* including that of a histochemical method (by non-specific esterase localization) for demonstrating Laurer's canal. Juvenile *Lophotaspis interiora* were described by Hendrix and Short (1972), and Thoney and Burreson (1988) published excellent photographs of *Multicalyx cristata* and *M. elegans*. Bakker and Diegenbach (1974) reconstructed the musculature of the ventral disc of *Aspidogaster conchicola*, and, on the basis of muscle pattern and observation of movements of the worms, attempted to explain the functioning of the ventral disc. Hendrix and Overstreet (1977) described in some detail *Cotylogaster basiri* and *C. dinosoides* from fishes in the gulf of Mexico, Huber *et al.* (1975) described *Lophotaspis orientalis* from the freshwater mussel *Corbicula*

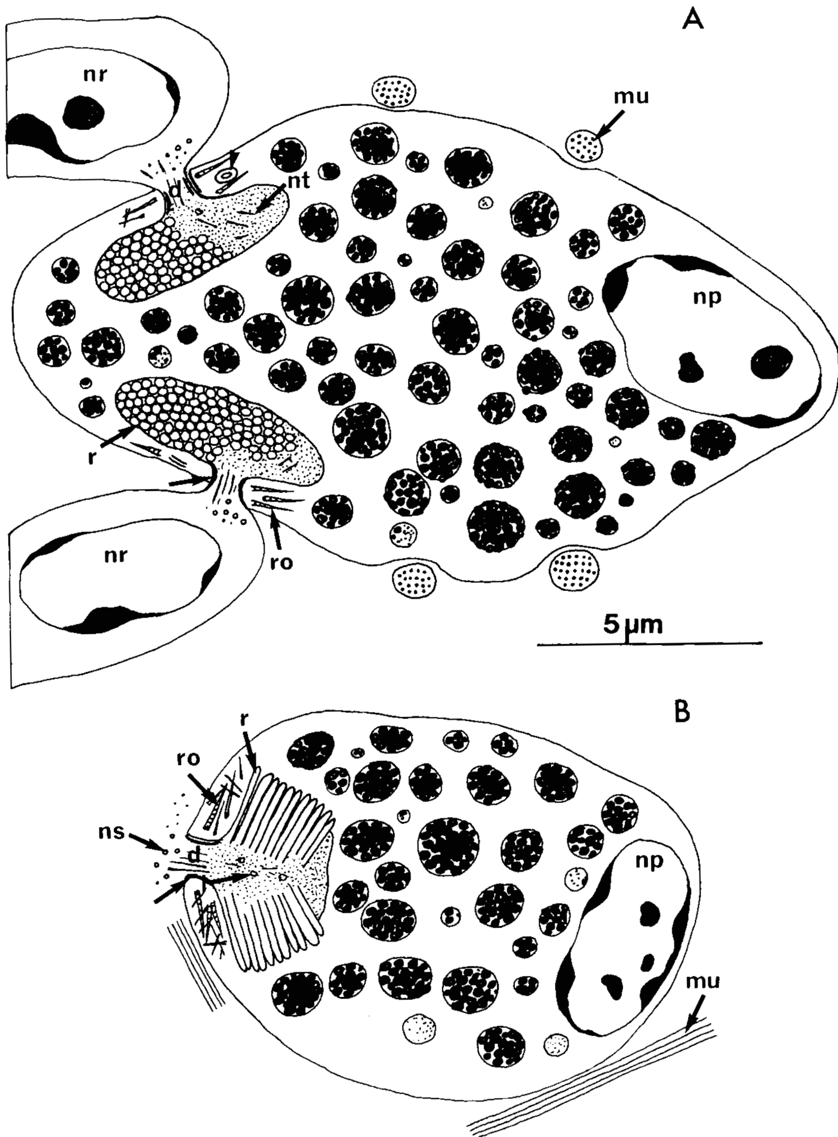


Figure 8 *Multicotyle purvisi*, larva. Photoreceptor. (A) Horizontal section. (B) Cross-section. d, dendrite; mu, muscle fibre; np, nucleus of pigment cell; nr, nucleus of receptor cell; ns, neurosecretion; nt, neurotubule; r, rhabdomere; ro, ciliary rootlet (?); arrows, thickenings of dendritic walls; arrowhead, circular body. (From Rohde and Watson (1991b), reproduced with permission of Springer-Verlag.)

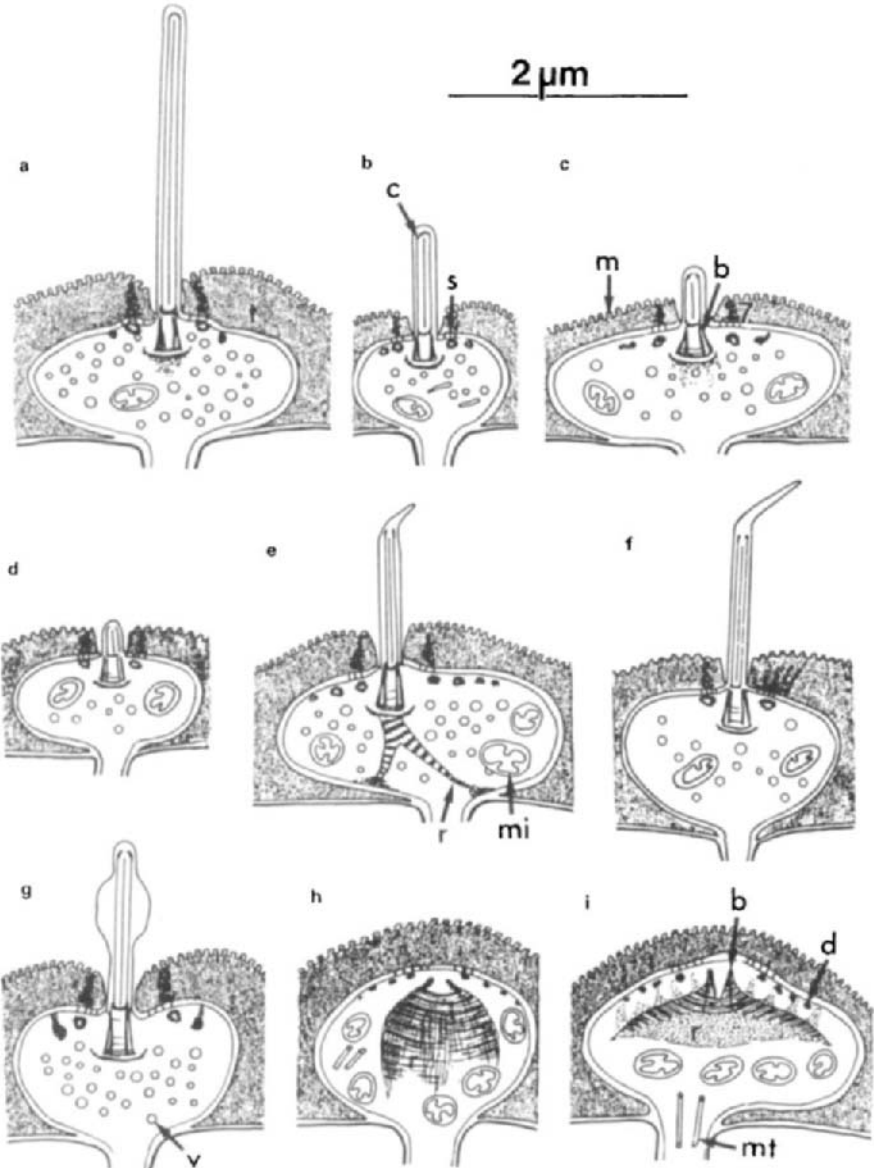


Figure 9 *Lobatostoma manteri*. Sensory receptors of larva. Uniciliate receptors with long cilium (a), with cilium of intermediate length (b), with short cilium on posterior body surface (c), with short cilium on *anterior* body suface (d), with ciliary rootlet (e), with bent ciliary tip (f), with widened middle part of cilium (g), non-ciliate receptor with large rootlet (h), and non-ciliate disc-like receptor (i). b, basal body; c, cilium; d, electron-dense collar; m, microtubercle; mi, mitochondrion; mt, microtubule; r, rootlet of cilium; s, septate junction; t, tegument; v, neurovesicle. (From Rohde and Watson (1992a), reproduced with permission of Pergamon Press.)

in China, and Lunaschi (1984) described *Lobatostoma jungwirthi* from the teleost *Cichlasoma facetum* in Argentina.

Histochemical localization of acid and alkaline phosphatases and carboxylic ester hydrolases in *Aspidogaster conchicola* was studied by Trimble III *et al.* (1971, 1972). Acid phosphatase was found in the ventral disc, intestine, testis and cirrus, and alkaline phosphatase in the ventral disc, tegument and tunica around the testis. The intestine contains a mixture of cholinesterase and type C esterases or a cathepsin. Hathaway (1979) described crystalline inclusions in the primary oocytes of the same species, and Ip and Desser (1984b) recorded a picornavirus-like pathogen from the cytoplasm of various cells in *Cotylogaster occidentalis*.

2.3.2. *Chromosomes*

Chromosome numbers of three aspidogastrid species have been determined, those of *Cotylogaster occidentalis*, *Cotylaspis insignis* (Lo Verde and Fredericksen, 1978) and *Lobatostoma manteri* (Rohde, 1973a). The first species has a diploid number of 12, (two pairs each of metacentric, submetacentric and acrocentric chromosomes), the second of 22, and the third of 14.

2.3.3. *Tegument, Sucker and Rugae*

According to Bailey and Tompkins (1971), the tegument of *Aspidogaster conchicola* is a syncytium with perikarya lying in the parenchyma, that is, it is a typical neodermis as known from all other Neodermata (major groups of parasitic Platyhelminthes). Circular and longitudinal muscle fibres, as well as myocytons, are located beneath the basal lamina. The tegument of the ventral disc has a thinner matrix, a thicker layer of muscle fibres, more mitochondria in the matrix and muscle fibres, and an additional, probably secretory cell type.

Rohde and Watson (1992b) examined the ultrastructure of the tegument, ventral sucker and rugae of *Rugogaster hydrolagi*, the first species not belonging to the Aspidogastridae thus examined (Figure 10). As in aspidogastrids, the tegument possesses surface microtubercles (short, regularly spaced microvilli), which, however, are longer than the ones of aspidogastrids. A unique adaptation, probably to the way worms are attached to the host, is the occurrence of processes of the basal lamina protruding into the tegument where they are anchored by hemidesmosomes, and of cross-striated processes of the basal lamina protruding into the underlying fibrous matrix. The worms live in narrow ducts of the rectal glands of chimaerid fishes, and they are so firmly attached that it is difficult to dissect them out of the host. Protrusions of the basal lamina may be adaptations to resist deformation by pressure exerted upon the worms.

As in other Neodermata, subtegumental perikarya are connected to the

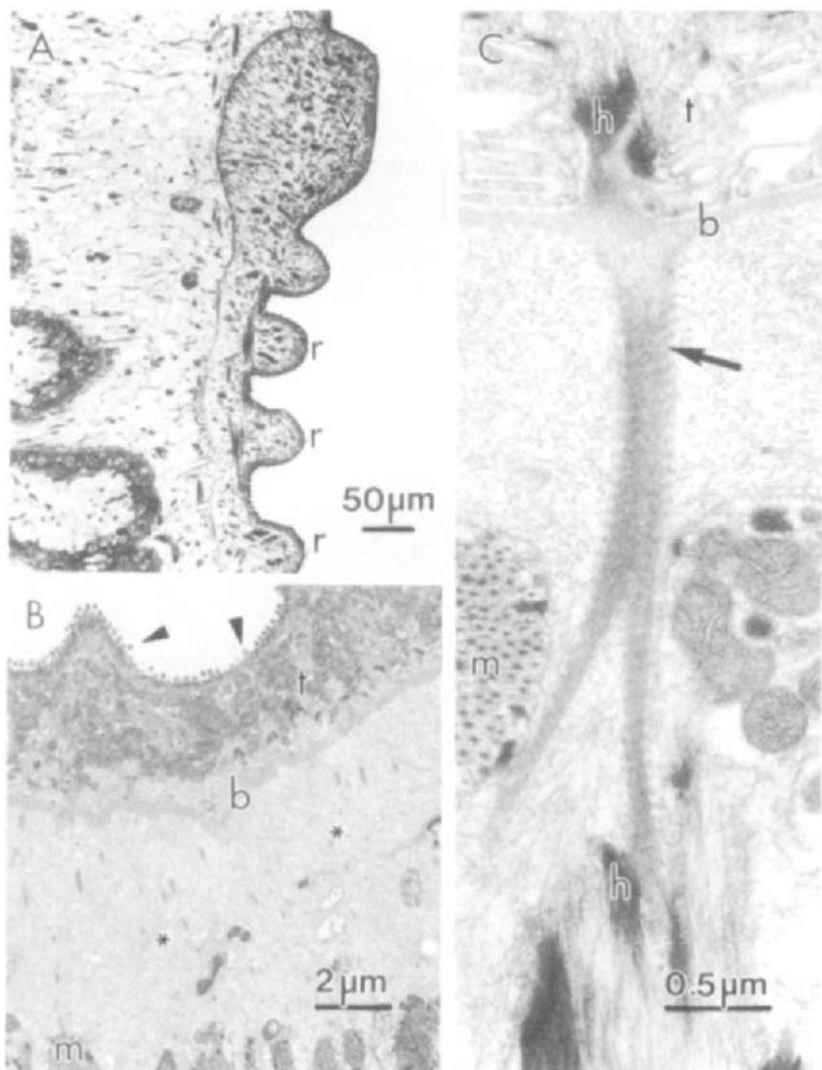


Figure 10 *Rugogaster hydrolagi*. (A) Longitudinal section through ventral sucker (v) and rugae (r). (B) Section through wall of ruga. Note microtubercles (arrowheads), tegument (t), basal lamina (b), muscle fibres (m) and thick fibrous layer (asterisks) below basal lamina. (C) Base of tegument and underlying tissue. Note cross-striated process (arrow), hemidesmosomes (h), tegument (t), basal lamina (b), and muscle fibre (m).

tegument by cell processes. The ventral sucker and rugae have a typical tegument with microtubercles, and the ventral sucker is internally lined by a basal lamina ("capsule") that extends posteriorly along the rugae. Hence, the ventral sucker plus the rugae must be considered to be homologous with the

ventral disc of the Aspidogastridae and the ventral sucker of Digenea. In adult specimens, the posterior extension of the basal lamina is discontinous. The sucker is rich in muscle fibres: transverse and longitudinal below the tegument and above the internal basal lamina, and predominantly dorsoventral between the tegument and the internal basal lamina. Most of the sucker is filled by a loose parenchyma. Common cell types in the parenchyma are (i) a type with Golgi complexes, some mitochondria and numerous vesicles (= subtegumental perikarya); (ii) myocytons; and (iii) parenchyma cells with numerous small vesicles and irregularly shaped inclusions of various sizes.

The rugae are thickenings of the body posterior to the ventral sucker, apparently developing from its posterior wall containing many muscle fibres. The fibrous matrix below the basal lamina increases in thickness in the troughs between the rugae (Figure 10).

2.3.4. *Alimentary Tract*

The ultrastructure of the alimentary tract of *Aspidogaster conchicola* was studied by Halton (1972). The mouth cavity, pharynx and oesophagus are lined by a neodermis (tegument) like that of the body surface, whereas the caecum is lined by an epithelium of tall columnar cells, many of which are specialized for both absorption and secretion. Other cells contain lipid droplets and their apical parts show varying degrees of cellular breakdown. It seems that the two cell "types" reflect different nutritional states of one cell type. Only a single type was observed by Hathaway (1972b) in the same species. Gentner (1971) showed that *Aspidogaster conchicola* and *Cotylaspis insignis* from *Amblema perplicata* and *Anadonta corpulenta* feed on the hosts' blood cells.

2.3.5. *Protonephridia*

The ultrastructure of the protonephridia of two species of aspidogastreans, that of the aspidogastrid *Lobatostoma manteri* (juvenile, 2.5 mm long) and the rugogastrid *Rugogaster hydrolagi* (adult), has been studied in detail by Rohde (1989b) and Watson and Rohde (1992b), respectively.

Flame bulbs of the first species have the structure typical of trematodes/monogeneans (Rohde, 1991). The weir is formed by two cells, a terminal cell which contributes the internal ribs, and a proximal canal cell which contributes the external ribs and two longitudinal cytoplasmic cords connected by a septate junction. Ribs are connected by a "membrane", apparently of extracellular matrix, and long cytoplasmic processes (leptotriches) arise from the internal and external ribs. Rootlets of the cilia forming the flame converge towards the nucleus of the terminal cell. Protonephridial capillaries have at least one junction, and their surface area is increased by lamellate evaginations of the surface cell membrane. Parts of the duct contain liquid-filled

spaces connected to the lumen of the ducts. The basal cell membrane forms many evaginations into the wall of the ducts, and there are lateral flames. The bladder wall is formed by an epithelium with perikarya deeply protruding into the lumen and with interconnected lamella at the surface. Round-ovoid

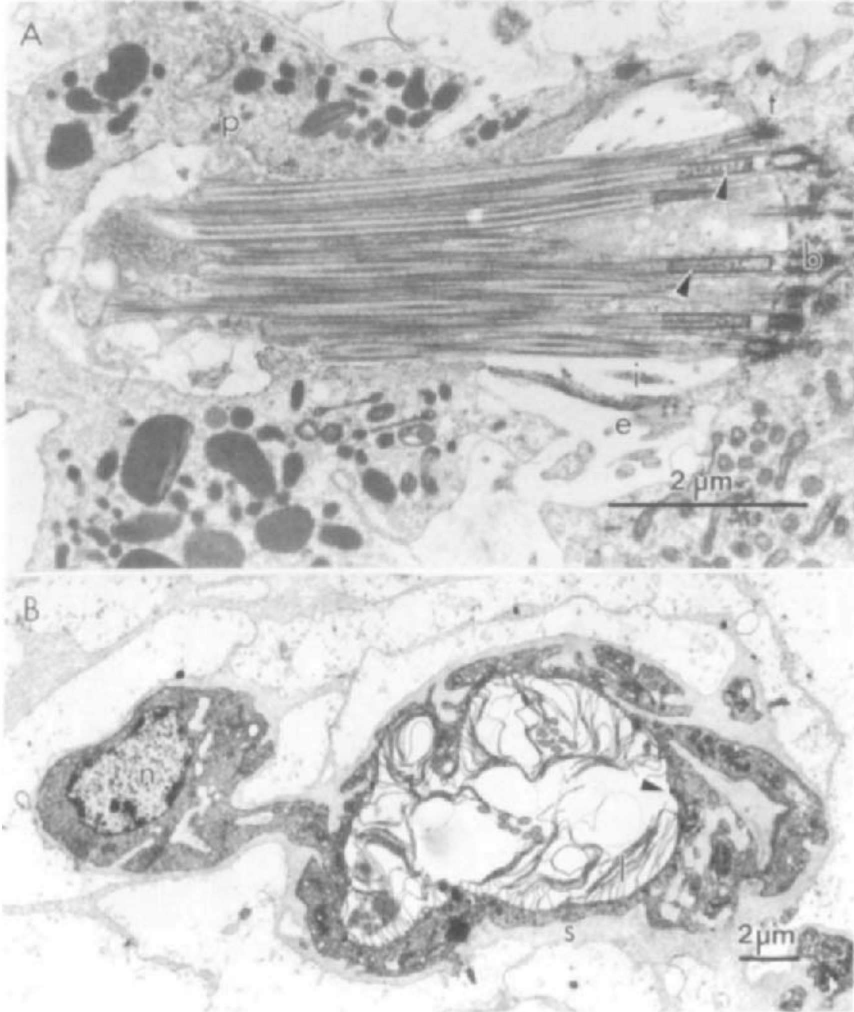


Figure 11 *Rugogaster hydrolagi.* (A) Longitudinal section through flame bulb. Note proximal canal cell (p) with prominent dense inclusions, and terminal cell (t). Note small rootlets of cilia, internal (i) and external (e) leptotriches, floccular material between cilia at their base and tip regions, reinforced region of cilia (arrowheads). (B) Cross-section through protonephridial capillary. Note nucleus (n) of canal cell, numerous lamellae (l), septate junction (arrowhead) and sheath (s) around capillary. (Originals N.A. Watson.)

vacuoles occur in the cytoplasm of the wall and in the lumen, and excretory bodies with concentric striations are common in the lumen. Muscle fibres extend along and around the wall. At its posterior end, the bladder wall is connected to the tegument lining a narrow terminal duct by a cell junction. A sponge-like body, consisting of a reticulum of electron-dense substance and spaces containing muscle fibres, extends along part of the terminal duct.

Rohde (1989b) suggested that the excretory bodies may have an osmoregulatory function, important for a parasite that lives in a region (the digestive gland and stomach of marine snails) of variable osmolarity. Alternatively, storage of the large number of excretory bodies in the very large excretory bladder may have the function to prevent damage to the host by a large volume of toxic excreted substances.

The flame bulbs of *Rugogaster* differ in two important aspects from those of *Lobatostoma* (Figure 11). Firstly, the ciliary membranes of the cilia of the flame extend beyond the end of the microtubules, forming a terminal network, with large quantities of floccular material found within the confines of the membranes. Such a network of membranes was not seen in *Lobatostoma* but is present in *Multicotyle purvisi* (Rohde, 1972, 1982). Secondly, the distal half of the flame is surrounded by cytoplasm of the proximal canal cell containing many electron-dense, apparently glandular, inclusions.

In *Rugogaster*, in a region close to the basal body, cilia of the flame have no central pair of microtubules and the peripheral doublets are incomplete. Further from the basal body, central pairs appear and the peripheral doublets are reinforced by additional spokes arising between pairs of doublets and also on the outside of the circle, between the a and b tubules. As in *Lobastostoma*, protonephridial capillaries contain many lamellae protruding into the lumen, and their walls contain septate junctions (Figure 11).

2.3.6. *Nervous System and Sensory Receptors*

Timofeeva (1971), using staining for acetylcholinesterase, reconstructed the nervous system of adult *Aspidogaster conchicola*, in its complexity resembling that of *Multicotyle purvisi* described by Rohde (1972 and further references therein). In both species, a well-developed brain commissure sends many nerves into the anterior end, anteriorly connected by a commissure; the ventral disc is innervated by several connectives connected by many commissures.

Esterase staining was also used to study the nervous system of adult *Lissemysia indica* (family Aspidogastridae) by Ramulu *et al.* (1980). The authors found a pair of cerebral ganglia connected by a cerebral commissure, three pairs of anterior and three pairs of posterior nerves, the latter connected by a number of commissures. The ventral disc is innervated by a complex pattern of connectives and commissures.

Halton and Lyness (1971) described the ultrastructure of sensory receptors in adult *Aspidogaster conchicola*. Uniciliate receptors were found in all parts

of the tegument. They have 9 + 2 axonemes, a basal body and an extensive trifid and fan-like rootlet system. The three electron micrographs of this receptor may indicate that two different types are illustrated, one with a single cilium and a single rootlet, one non-ciliate and with a trifid, fan-like rootlet, corresponding to receptor type II of *Cotylogaster occidentalis* and receptor type G of *Multicotyle purvisi* and *Lobatostoma manteri* described below. The second so-called receptor type in *Aspidogaster* referred to by Halton and Lyness is in fact the "marginal body", that is the terminal part of a glandular system (see p. 167).

Uniciliate receptors with long cross-striated rootlets in *Aspidogaster conchicola* and *Cotylaspis insignis* were briefly described by Allison *et al.* (1972).

Ip *et al.* (1982) examined the body surface of adult *Cotylogaster occidentalis* by means of scanning electron microscopy and found three morphologically different receptor types as follows:

1. type A receptors with a single cilium, occur throughout body and are arranged in a regular pattern around the marginal alveoli; a type A receptor is associated with each duct of the marginal organs;
2. type B receptors with a single cilium arising from a raised tegumental pedestal occur on the ventral disc;
3. type C receptors have a single cilium within a tegumental pit, only the ciliary tip is exposed.

Ip and Desser (1984a) examined the same species using transmission electron microscopy. They distinguished the following receptor types:

1. type I, uniciliate, with a free cilium, two electron-dense rings;
2. type II, non-ciliate, with disc-shaped, striated rootlet, several electron-dense collars, only on the alveolar ridges of the ventral disc;
3. type III, non-ciliate, with tripartite ciliary rootlet, several electron-dense collars;
4. type IV, non-ciliate, bipartite ciliary rootlet, two electron-dense collars;
5. type V, non-ciliate, elongate striated ciliary rootlet, dome-shaped apical junctional complex, possibly a stress-receptor.

Observation of uniciliate receptors of different types by SEM indicate that the type I receptor found by TEM may represent more than one type, differing in the length of the cilium, shape of the tegument around it, etc.

Based on large numbers of sections, many of them serial or semiserial, Rohde (1989a) and Rohde and Watson (1989a) described at least eight types of presumptive sense receptors in adult *Lobatostoma*, and Rohde (1990a) described at least eight types in adult *Multicotyle purvisi.* Receptors of the first species (Figure 12) differ in the presence or absence of a cilium, presence or absence of a ciliary rootlet, the length of the cilium, the shape and structure of the rootlet, the number of electron-dense collars, the location within the tegument and on certain parts of the body, the absence or presence

of "dense bars" and of tubules. In addition, there may be free nerve endings below the tegument. Some receptor types show a certain variability, for instance, G and H (Figure 12) almost certainly represent the same type of receptor, as indicated by the same position on the alveolar walls, but differ in the presence of a vertical rootlet (in H) in addition to the disc-like one also found in G.

In addition to receptors resembling those of *Lobatostoma, Multicotyle* possesses a uniciliate receptor with a cilium containing a thick layer of cytoplasm around the axoneme, a non-ciliate receptor with a branching rootlet, and a uniciliate receptor in a deep tegumental pit with a very short cilium.

Considering the large size of the worms examined (both adult *Lobatostoma* and *Multicotyle* several millimetres long), only small parts of which could be examined by transmission electron microscopy, it seems likely that both species have more receptor types than have been described. Thus, scanning electron microscopy of *Lobatostoma* revealed tegumental pits in the head region that may correspond to the pit receptor found in *Multicotyle*.

The considerable differences observed in receptors of different species of the same family (Aspidogastridae), was attributed by Rohde (1990b) to the great age of the group. It is unlikely that different receptors are adaptations to different hosts, since host specificity of aspidogastrids is known to be low (see p. 175).

Rohde (1989a) drew attention to the fact that the great variety of receptors in *Lobatostoma* is a counter-trend to "sacculinization" (= retrograde evolution in parasites leading to a reduction in the receptor-nervous system). The species does not have a free-living stage, and is very large relative to the size of its snail host. Rohde concluded that the most likely explanation for the presence of a large variety and large numbers (probably 20 000–40 000) of sensilla is avoidance of damage to the snail host. Without such an adaptation, parasites could easily commit suicide by causing the death of their host.

2.3.7. *Marginal Glands*

The so-called marginal bodies are the terminal parts of the secretory ducts of Aspidogastridae (Rohde, 1972). In *Lobatostoma manteri*, the glandular duct widens to form an ampulla which, in turn, opens through a narrow duct of a muscular papilla (Figure 13). Electron-microscopic examination showed that the ampulla is surrounded by strongly developed circular muscle fibres and some perikarya (Figure 14) (Rohde and Watson, 1989b). Its epithelium (or coagulated secretion ?) contains secretory granules and forms a well-developed reticulum, that is, a network of interconnected processes. In the muscular papilla, the even more strongly developed circular muscle fibres surround a very narrow duct. The duct of the papilla opens into a wider terminal duct lined by tegument with surface microtubercles, charcteristic of the tegument

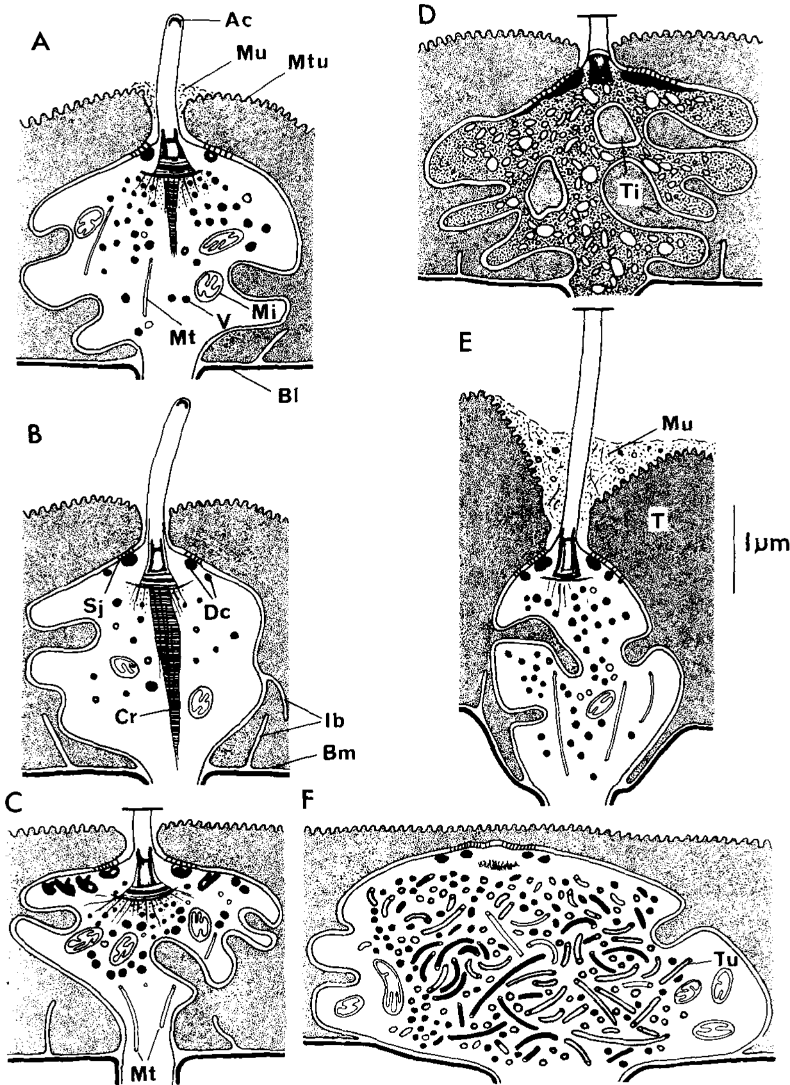
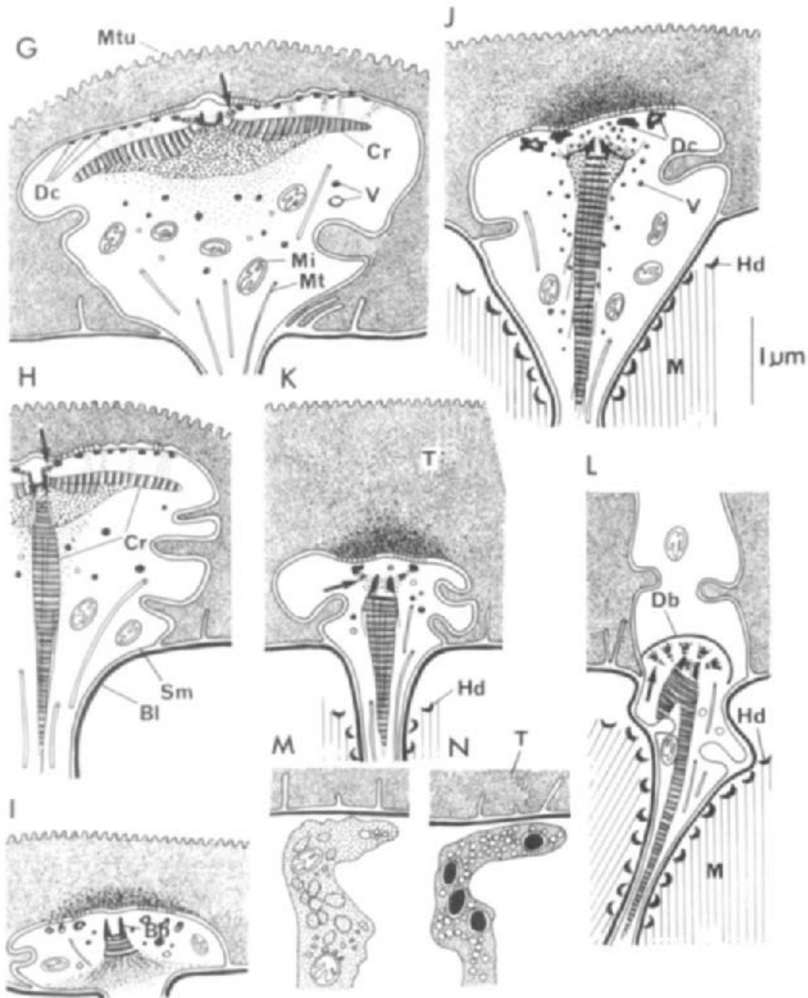


Figure 12 *Lobatostoma manteri*, pre-adult. Diagrams of presumptive sense receptors based on ultrathin sections, many of them serial, through various body parts of a single juvenile worm from the prosobranch snail *Cerithium (Clypeomorus) moniliferum* on the Great Barrier Reef. (A–E) Uniciliate. (F) possibly uniciliate. (G–N) non-ciliate. (A) one dense collar, short cilium (0.9–1.6 µm long) with electron-dense cap; ciliary rootlet missing or small. (B) Two dense collars, large ciliary rootlet, cilium of medium length (1.9 µm long). (C) At least three thick dense collars, fibres radiating out from the basal body. (D) dense cytoplasm with many inclusions and particularly deep tegumental invaginations. (E) Two or three dense collars, cilium at least 3.2 µm long. (F) Large, two dense collars, coiled tubules 50–80 nm in diameter. (G) Disc-shaped ciliary rootlet partly striated partly granular, many dense collars. (H) Disc-shaped plus vertical rootlet (modification of (G)). (I) At least three dense collars, granular material (rudimentary rootlet?) fanning out from base of basal body. (J) Two



thick dense collars, long ciliary rootlet. (K) Like (J) but smaller and one dense collar (modification of (J?). (L) Desmosome-like connections between basal body and "dense bar", upper part of ciliary rootlet with pairs of cross-bands of equal thickness, lower part with thick and thin cross-bands, in very deep invaginations of basal lamina. (M,N) Possible free nerve endings (but more likely connective tissue fibres). Ac, apical cap of cilium; Bl, basal lamina; Bm, basal tegumental membrane; Cr, ciliary rootlet; Db, dense bar; Dc, dense collar; Hd, hemidesmosome; Ib, invagination of basal tegumental membrane; M, muscle fibres; Mi, mitochondrion; Mt, microtubule; Mtu, microtubercle of tegument; Mu, mucoid layer; Sj, septate junction; Sm, sensillar membrane; T, tegument; Ti, tegumental invagination; Tu, tubule; V, vesicle. Arrows in (G), (H), (K) and (L) point at desmosome-like connections between basal body and dense collars or bars. (From Rohde, (1989a), reproduced with permission of Springer-Verlag.)

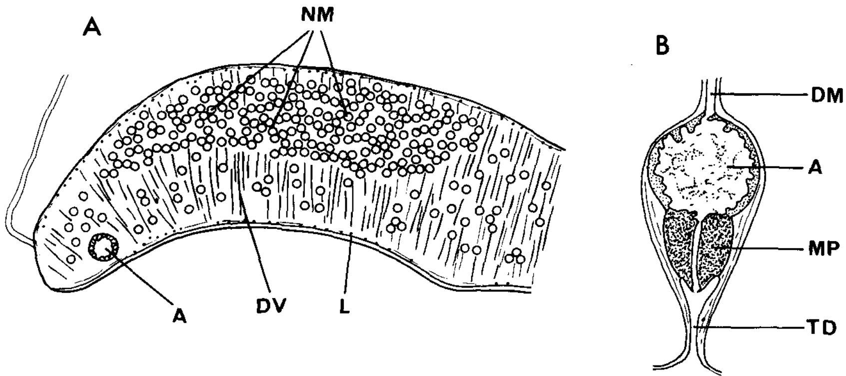


Figure 13 *Lobatostoma manteri* (A) Diagram of transverse section through marginal suckerlet (alveole) of ventral disc. (B) Diagram of sagittal section through marginal body. A, ampulla of marginal body; DM, duct of marginal gland; DV, dorsoventral muscles; L, longitudinal muscles, MP, muscular papilla; NM, nuclei of marginal gland; TD, terminal duct. (From Rohde and Watson (1989b), reproduced with permission of VEB Fischer-Verlag, Jena.)

of aspidogastreans. The gland cells are densely packed in the dorsal part of the marginal alveoli of the ventral disc (Figure 14). Gland cells are rich in granular endoplasmic reticulum, and secretory granules are round and electron-dense (Figure 14).

Timofeeva (1972), using light microscopy, found two types of unicellular gland cells in the alveoli of the "longitudinal medial rows" of *Aspidogaster limacoides*. The marginal alveoli contain the secretory ducts leading to the marginal bodies. The author suggests a function of the secretion in attachment and locomotion. According to Bakker and Diegenbach (1974) ducts from secretory cells open into the ampulla of the marginal organs of *Aspidogaster conchicola*: "From this point some muscle fibres run slantingly through the ampulla. The wall of the ampulla consists of two layers of muscle fibres . . . " They also refer to three retractor muscles attached to the exterior of the organ. The terminal duct is star-shaped, enabling the entire organ to protrude through it to the outside. The tegument extends into the terminal duct.

2.3.8. *Spermatogenesis and Vitellogenesis*

Mohandas (1983) reviewed early work on sperm and spermatogenesis of trematodes including the Aspidogastrea.

The ultrastructure of spermatogenesis of three species of Aspidogastrea has been studied: that of *Lobatostoma manteri* (Rohde *et al.*, 1991), *Multicotyle purvisi* (Watson and Rohde, 1991) and *Rugogaster hydrolagi* (Watson and Rohde, 1992a). The first two species belong to the Aspidogastrida

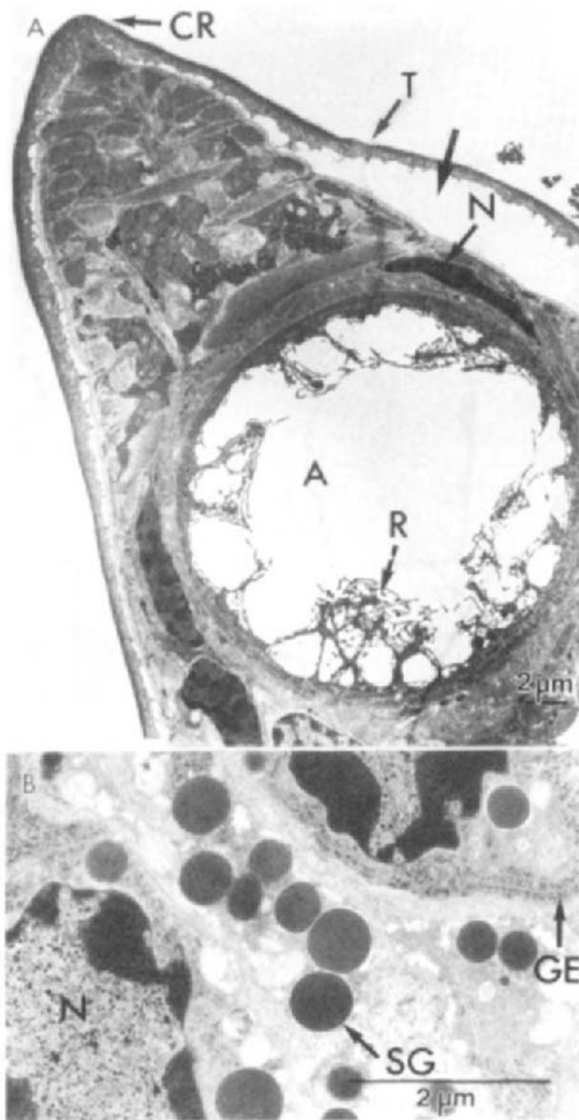


Figure 14 *Lobatostoma manteri*. (A) Ampulla of marginal body. (B) Marginal gland cells. A, ampulla; CR, crest of wall of suckerlet; GE, granular endoplasmic reticulum; N, nucleus; R, reticulum; SG, secretory granule; T, tegument. Large arrow indicates artefact (tegument detached from underlying tissue). (From Rohde and Watson (1989b), reproduced with permission of VEB Fischer Verlag, Jena.)

Aspidogastridae, the third to the Stichocotylida Rugogastridae (system of Gibson and Chinabut, 1984). All species have a 9 + "1" pattern of axonemes (9 peripheral microtubule doublets, one central complex rod). Two axonemes are incorporated in the sperm body and interrupt a surface row of microtubules along part of the sperm, and there are an elongate nucleus and mitochondrion (also found in *Aspidogaster conchicola* by Bakker and Diegenbach, 1973; see also Hathaway, 1971, 1972a, 1974). In *Lobatostoma* the sperm body possesses long lateral extensions (lobes) also seen under the light microscope (Figure 15), and many dense rod-like structures. A supporting rod extends underneath a specialized region consisting of alternating thin and thick transverse rows of irregular dense patches, and with surface ridges around (all or) most of the surface of the sperm. Primary spermatocytes in the prophase of the first meiotic division have synaptonemal complexes, and are rich in mitochondria. In early spermiogenesis, mitochondria are arranged around the surface of the nucleus, and a dense layer appears at one pole of the nucleus, close to an apposed dense layer at the cell membrane in which a row of microtubules develops. A striated intercentriolar (= central) body develops close to the nucleus from a larger electron-dense area with less distinct striations (Figure 15). The fully developed intercentriolar body is located perpendicular and close to the surface of the nucleus. Two flagella grow out from the basal bodies close to the intercentriolar body in almost opposite direction to each other, their cross-striated rootlets extending along the surface of the nucleus. At a later stage of development, rootlets and flagella become more parallel with the intercentriolar body, and the nucleus and fused mitochondria migrate into the median process. Flagella are incorporated into the median process. The outgrowing spermatozoa are connected to the cytoplasm of the cytophore by dense arching membranes. Finally, ciliary rootlets are resorbed and the spermatozoa are pinched off close to the basal bodies. Light-microscopic observations showed that spermatids undergo torsion leading to a screw-like shape of part of young sperm. A thin anterior process resembles an acrosome of certain spermatozoa, possibly (but not yet shown to be) supported by the dense rod (Figure 16).

The testis wall is formed by a cell layer on a basal lamina and contains some muscle fibres.

Mature sperm of *Multicotyle purvisi* lack the lateral extensions, but are otherwise similar to those of *Lobatostoma*. In contrast, spermatogenesis seems to differ in several respects. A "zone of differentiation", consisting of intercentriolar body, centrioles, ciliary rootlets and microtubules was not observed. Instead, spermatid components are assembled within the common cytoplasmic mass before spermatids grow out. Microtubules, mitochondrion, nucleus and axonemes including their basal body regions, migrate into the spermatid before it is pinched off. The basal region has an unusually complex structure. Investigations are underway to show whether the aberrant spermatogenesis is perhaps a pathological effect.

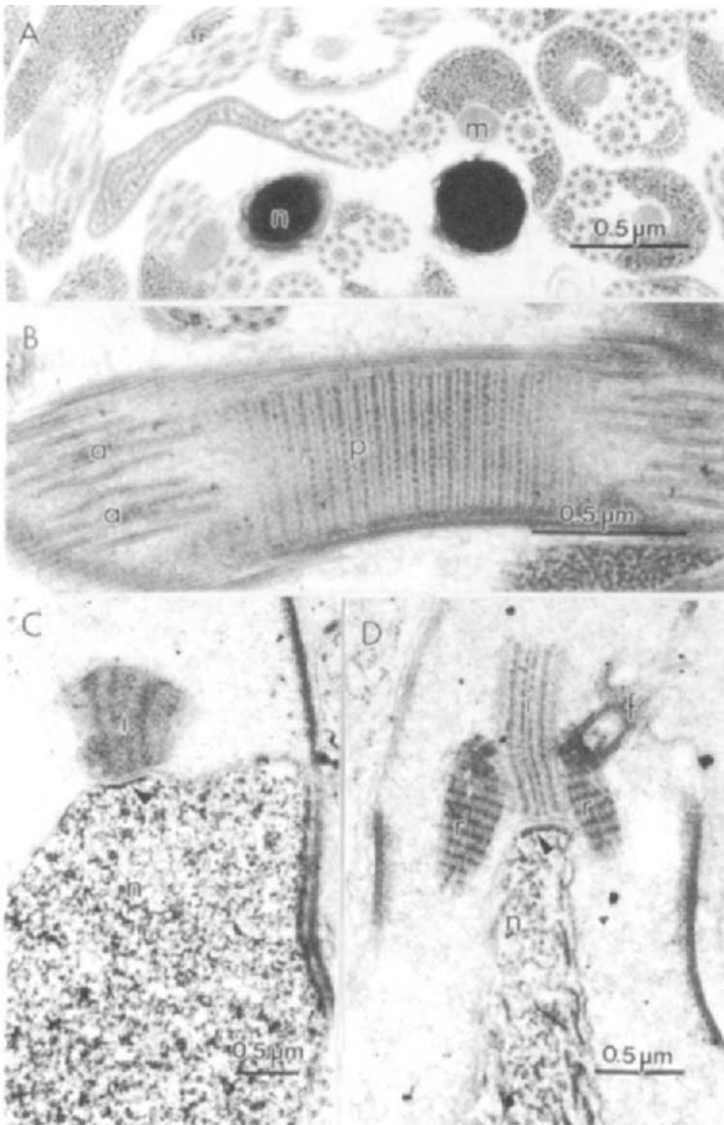


Figure 15 *Lobatostoma manteri*. Ultrastructure of sperm and spermiogenesis. (A) Mature sperm, cross-sections. (B) Mature sperm, longitudinal section through "pad" (p) and axoneme (a). (C) Early spermiogenesis. Note developing, intercentriolar plate (i). (D) Spermiogenesis. Note intercentriolar plate (i), ciliary rootlets (r), flagellum (f), and nucleus (n) with dense layer (arrowhead) close to intercentriolar plate. (Modified from Rohde *et al.*, 1991.)

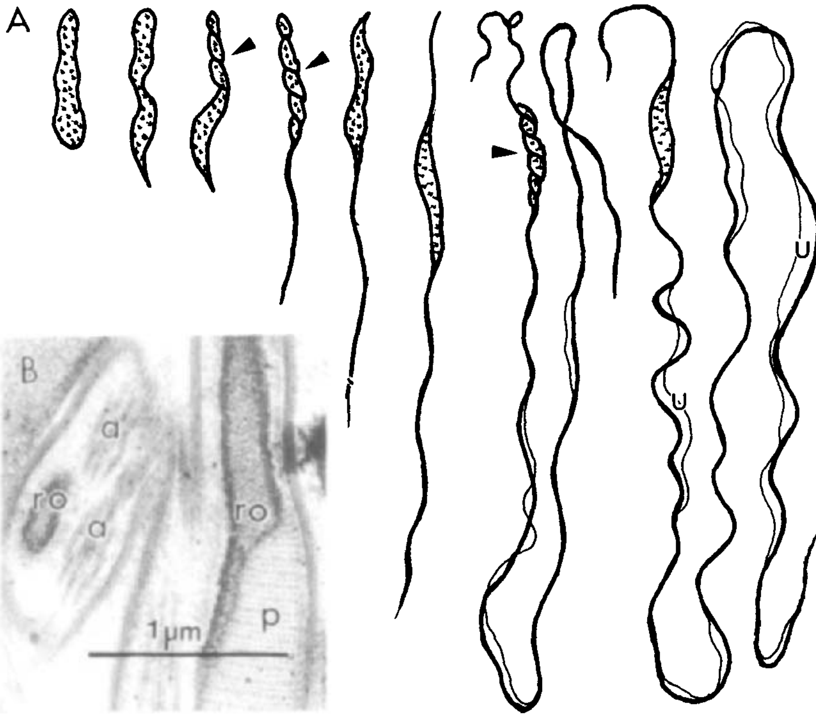


Figure 16 *Lobatostoma manteri.* (A) Free-hand drawings of developing spermatozoa. Note undulating membrane (u) and screw-like shape of parts of spermatids and young sperm due to torsion (arrowheads). (B) Longitudinal and oblique sections through sperm. Note dense rod (ro), "pad" (p) and axonemes (a).

Mature sperm of *Rugogaster hydrolagi* is similar to that of the other two species. It lacks lateral extensions. Spermiogenesis corresponds to that of *Lobatostoma*, that is, a zone of differentiation develops with a row of microtubules along the cell membrane, and an adjacent dense region along the nuclear membrane. An intercentriolar body and basal bodies appear between the microtubules and the nucleus. Free flagella grow out in opposite directions, the cell membrane protrudes and lengthens into a median cytoplasmic process into which the nucleus and mitochondrion move; flagella rotate and eventually fuse with the process. A dense region develops in the spermatid shaft some distance from the proximal end but it is not present in the mature sperm. The spermatid elongates and is eventually pinched off from the residual body at the level of the arching membranes, after rootlets and intercentriolar body have disappeared. One difference between *Rugogaster* and *Lobatostoma* is the rosette with central cytophore in the early spermatid phase of the former but not the latter species. Importantly, one residual cytoplasmic mass was seen to contain more than 40 proximal spermatid ends

in one ultra-thin section. Examination of several semi-thin sections revealed clusters of primary spermatocytes with 10–12 per group visible in one plane, while squashes of whole testes revealed in excess of 50 spermatids in a group. Thus the number of spermatids per group is assumed to be 64, not 32 as in other trematodes.

Vitellogenesis, for the first time in an aspidogastrean, was described for *Rugogaster hydrolagi* by Rohde and Watson (1991a). Vitelline follicles occur in large numbers and are surrounded by a basal lamina and fibrous matrix. Cytoplasmic processes of peripheral perikarya (nurse cells) extend between the vitelline cells and into them. Immature vitelline cells are relatively poor in cytoplasm and yolk granules but rich in mitochondria, rough endoplasmic reticulum, and Golgi complexes. During maturation, vitelline cells acquire more cytoplasm and large vacuoles with many dark grana, as well as free ribosomes, rough endoplasmic reticulum, lipid droplets, and mitochondria. At later stages of maturation and in mature yolk cells irregularly shaped inclusions and large interconnected spaces containing much α-glycogen are present. Intact yolk cells are incorporated into the egg.

Small yolk ducts are ciliated and have intraepithelial nuclei separated by lateral cell membranes.

2.4. Phylogeny, New Taxa, Host Specificity and Geographical Variation

Phylogenetic relationships of aspidogastrean families were discussed by Gibson (1987), Brooks *et al.* (1989) and Pearson (1992). Timofeeva (1975) emphasized the morphological similarity between aspidogastrids and trematodes, due to the common origin of both groups.

Several new genera of Aspidogastrea have been described after 1972. Schell (1973) described the interesting *Rugogaster hydrolagi* from the rectal glands of a chimaerid fish, *Hydrolagus colliei* on the Pacific coast of North America (Washington State). The larva resembles that of other aspidogastreans, but the adult is markedly different. The body posterior to the small, rudimentary ventral sucker bears many transverse rugae (for their electron-microscopic structure see p. 161), and it has two caeca and multiple testes. On the basis of these characters, Schell established a new family, Rugogastridae, for the species. The same species was subsequently recovered from a chimaera, *Chimaera* sp., off the coast of southeastern Australia by Rohde *et al.* (1992b).

Gibson and Chinabut (1984) described *Rohdella siamensis* gen. et sp. nov. (Aspidogastridae: Rohdellinae subfam. nov.) from freshwater fishes, *Osteochilus melanopleurus* and *Barbus daruphani*, in Thailand. It differs from all known aspidogastreans in that the male and female ducts unite to form a hermaphroditic duct.

Agrawal and Sharma (1990) described *Pseudoaspidogaster betwai* gen. et.

sp. nov. (Aspidogastridae: Paraaspidogasterinae subfam. nov.) from the freshwater fish *Tor tor* in the Betwa River, Jhansi, India. According to the authors, the new genus differs from the Aspidogasterinae and Cotylaspidinae in having a ventral disc with a single longitudinal row of alveoli. It differs from the Macraspinae in the presence of two testes and a different arrangement of the vitellaria. The illustrations are not of sufficient quality to permit evaluation of the validity of the genus.

The new genera *Texanocotyle* (for *T. pogoniae*) and *Laterocotyle* (for *L. padreinsulae*) from the teleost fish *Pogonias cromis* in the Gulf of Mexico, erected by Simpson and McGraw (1979), were synonymized with *Cotylogaster basiri* Sidiqqi and Cable and *C. dinosoides* Hendrix and Overstreet, respectively, by Hendrix and Overstreet (1983).

Among the newly described species are several species of the genus *Lobatostoma*: *L. jungwirthi* from the freshwater fish *Geophagus brachyurus* in Brazil (Kritscher, 1974); *L. hanumanthai* from *Trachinotus blochi* in the Bay of Bengal (Narasimhulu and Madhavi, 1980); *L. anisotremum* from the marine teleost *Anisotremus scapularis* of the coast of Chile (Oliva and Carvajal, 1984); *L. platense* from the marine teleost *Trachinotus glaucus* from the Rio de la Plata, Uruguay (Mañe-Garzón and Spector, 1976); *L. manteri* from the marine teleost *Trachinotus blochi* on the Great Barrier Reef, Australia (Rohde, 1973a); and *L. veranoi* from the marine fish *Menticirrhus ophicephalus* off the coast of Chile and Peru (Oliva and Luque, 1989). Of particular interest is the finding of a species of a genus otherwise known only from marine teleost fishes in a freshwater fish. The possibility cannot be excluded that some of the "species" are in fact geographical subspecies of one wide-ranging species. Geographical variation of aspidogastreans has been demonstrated by Rohde *et al.* (1992a) for *Rugogaster hydrolagi*, and it has been well studied in monogeneans (Rohde *et al.* 1992a and references therein).

Other new species are *Cotylogasteroides barrowi* from freshwater mussels in Ohio (Huehner and Etges, 1972b); *Aspidogaster africanus* from a freshwater fish in the Sudan (Saoud *et al.*, 1974); *A. tigarai* from *Puntius* sp. in India (Dandotia and Bhadauria, 1977); *Cotylogaster dinosoides* from the marine teleost *Pogonias cromis* in the Gulf of Mexico (Hendrix and Overstreet, 1977); *Lissemysia ocellata* from the freshwater snail *Vivipara bengalensis* at Raipur, India (Ramachandrula and Agarwal, 1984); and *L. agrawali* from the freshwater fish *Puntius ticto* at Goralchpur, India (Singh and Tewari, 1985).

Earlier descriptions of new taxa not included in Rohde (1972), are that of *Trigonostoma callorhynchii* from the bile ducts of the chimaera *Callorhynchus callorhynchus* off the coast of Argentina (Szidat, 1966), and that of *Lissemysis pandei* from the freshwater fish *Puntius sarana* at Gorakhpur, India (Rai, 1970). Thoney and Burreson (1988) revised the family Multicalycidae and synonymized *Trigonostoma callorhynchi* Szidat with *Multicalyx elegans* (Olsson), and *Multicalyx multicristata* Parukhin and Tkachuk with *Multicalyx*

cristata Faust and Tang. Timofeeva (1973) synonymized *Aspidogaster amurensis* Achmerov with *A. conchicola*, and only two species of freshwater aspidogastrids, *A. conchicola* and *A. limacoides* are listed for the Soviet Union by Bychowskaya-Pavlovskaya (1987).

Rohde (1972) had emphasized the low degree of host specificity of aspidogastreans, both for the mollusc and the vertebrate hosts. Recent host records for various species of Aspidogastrea support this view. Huehner and Etges (1971) reported the gastropod *Goniobasis livescens* as a new host for *Aspidogaster conchicola* in North America, and Nagibina and Timofeeva (1971) reported five species of freshwater fishes (Cyprinidae: *Blicca björkna*, *Abramis brama*, *A. sara*, *Leuciscus idus* and *Rutilus rutilus*) as hosts of *Aspidogaster limacoides* in the lower parts of the Volgograd reservoir. The bivalve *Dreissena polymorpha* contained immature and the bivalve *Sphaerium* mature worms, which were also recovered from the Caspian molluscs *Cardium* sp. and *Adacna* sp. The authors also refer to an earlier report by Akhmerov that *Aspidogaster amurensis* (a synonym of *A. conchicola*, see above) occurs in the fishes *Mylopharyngodon piceus*, *Ctenopharyngodon idella* and *Cyprinus carpio*, and in the bivalve *Cristaria plicata* in the Amur River. Hendrix and Overstreet (1977) reported *Cotylogaster basiri* from various marine teleosts (*Archosargus probatocephalus* (Sparidae), *Micropogonias undulatus* (Sciaenidae), *Menticirrhus americanus* (Sciaenidae), *Trachinotus carolinus* and *T. falcatus* (Carangidae)) in the Gulf of Mexico. *Cotylogaster dinosoides* was recovered, by the same authors in the same locality, from only one host fish, the sciaenid *Pogonias cromis*, but the authors found *Lobatostoma ringens* in a new bivalve host, *Donax roemeri protracta*, and *Multicalyx cristata* in three new elasmobranch hosts, *Pristis pectinata* (Pristidae), *Dasyatis sayi* (Dasyatidae), and *Cephaloscyllium ventriosum* (Scyliorhinidae). In a table, Hendrix and Overstreet (1977) list altogether 14 teleost species of eight families as hosts for *Lobatostoma ringens* (also recorded by Gomes and Fabio (1976) from Brazil), and according to Rohde (1973a, 1975), three snail species serve as hosts for *L. manteri* at Heron Island, Great Barrier Reef. Rees (1970) reported *Lobatostoma ringens* from *Stenostomus chrysops* at Bermuda, and Bray (1984) found immature *Lobatostoma* sp. in *Octopus vulgaris*, *Cotylogaster basiri* in *Rhabdosargus sarba*, and *Multicalyx cristata* in *Sphyrna lewini* and *Odontaspis taurus* off the coast of Natal, South Africa.

Thoney and Burreson (1986) examined 16 species of elasmobranchs (most of them in small numbers) on the Atlantic coast of the United States and found *Multicalyx cristata* in two of them: *Myliobatis freminvillei* and *Rhinoptera bonasus*. Adamczyk (1972) recovered *Aspidogaster conchicola* from 18 of 62 *Anodonta anatina* and from 29 of 65 *A. cellensis* in Poland, Duobinis-Gray *et al.* (1991) examined 219 bivalve molluscs of 10 species and seven genera from 17 localities in Kentucky Lake and found nine species infected with *A. conchicola*, and Huehner and Etges (1981) examined 334 North American

bivalves of 13 species and found 143 specimens of seven species infected with this species.

Hendrix *et al.* (1985) published a list of records of freshwater aspidogastrids and their hosts in North America. They reported 17 new bivalve hosts for *Aspidogaster conchicola*, 12 new bivalve hosts for *Cotylaspis insignis*, five new bivalve hosts for *Cotylogaster occidentalis*, and one new bivalve host for *Lophotaspis interiora*.

That there are possible exceptions to the wide host ranges of aspidogastreans is indicated by the observation of Hendrix and Overstreet (1983) that there is only one known vertebrate host, the teleost *Pogonias cromis*, for *Cotylogaster dinosoides*.

Geographical variation in aspidogastreans was recorded by Rohde *et al.* (1992b) in *Rugogaster hydrolagi.* Specimens from the North Pacific differ from specimens off the southeastern Australian coast in the number of rugae, but no other differences that would justify establishment of a new species for the Australian specimens were found.

3. THE AMPHILINIDEA

3.1. Egg Formation, Development and Life Cycles

For early studies of embryogenesis, see Dubinina (1982). In *Amphilina japonica*, the egg lacks an operculum, but has an anterior filament that differs morphologically and chemically from the egg shell (Bazitov and Lyapkalo, 1980). Yolk cells are broken down in the common vitelline duct and, consequently, mature eggs do not contain yolk cells. First cleavage is equal, but later cell divisions are unequal and asynchronous. At the 45– to 48–cell stage, cleavage is complete.

According to Coil (1987a, 1991), in *Austramphilina elongata* formation of the egg shell takes place in the ootype. Secretions of Mehlis' gland and yolk cells meet to form a thin shell around the egg cell and one or more yolk cells. Loss of granules of the yolk cells begins in the ootype and continues into the proximal uterus. The shell becomes thicker with increasing size of the egg, but the origin of the shell material is not clear. A double shell, appearing at about the mid-point of development, appears to be formed by the larva. *Amphilina bipunctata*, examined by Coil (1987b), differs in the mode and histochemistry of formation of the egg shell. Whereas the shell of *A. elongata* is PAS positive, that of *A. bipunctata* is PAS negative. There also are differences in the contributions of the developing embryo to the egg shell.

Rohde and Georgi (1983) worked out the life cycle of *Austramphilina elongata* (Figure 17). The adult lives in the body cavity of Australian freshwater turtles, *Chelodina longicollis* (and other species). Round,

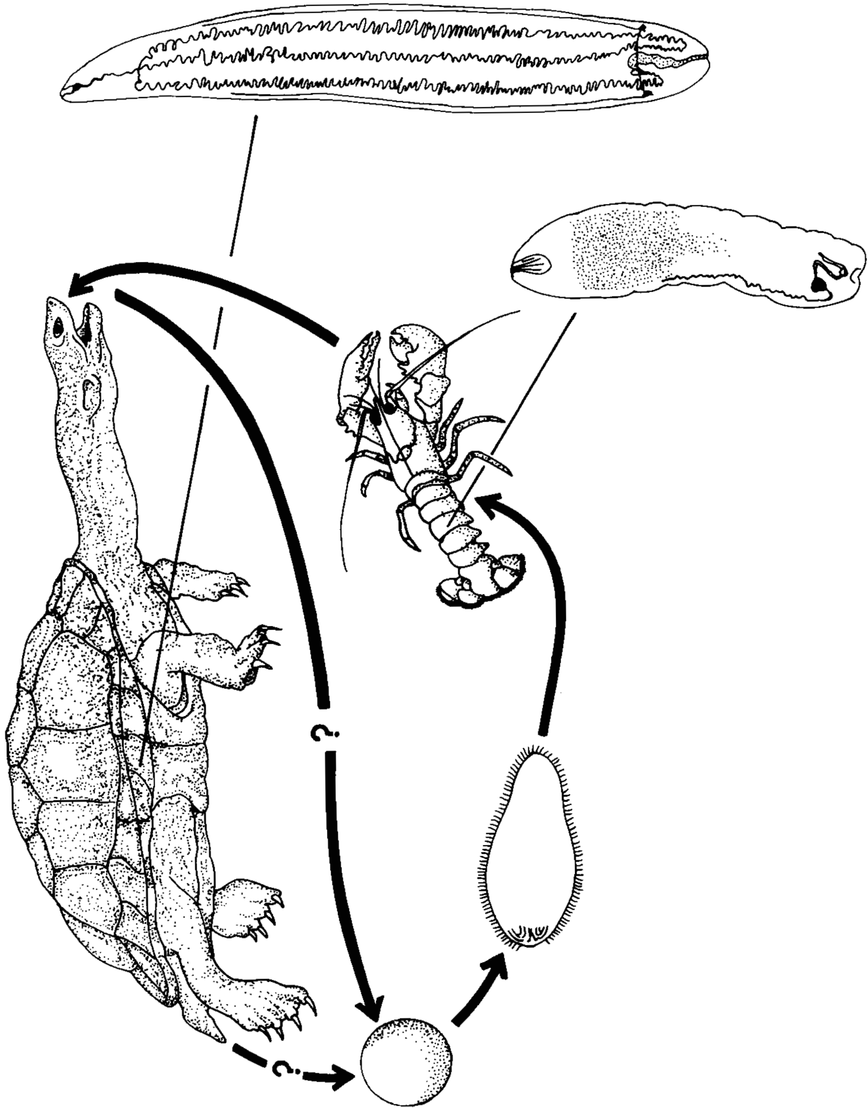


Figure 17 *Austramphilina elongata*, life cycle. Adults live in the body cavity of long-necked turtles, *Chelodina longicollis*. They lay eggs that escape by an unknown route (possibly via the lungs and trachea) from the host. Ciliated larvae hatch in freshwater and penetrate through the exoskeleton of juvenile crayfish, *Cherax destructor*, where they grow up to infective juveniles. Crayfish are eaten by turtles, the parasites penetrate through the oesophageal wall and migrate along the trachea into the coelom.

non-operculate eggs are laid. Ciliated larvae hatch in freshwater and penetrate through the exoskeleton of juvenile crayfish, *Cherax destructor* and of freshwater shrimps, *Parataya australiensis* and *Atya* (= *Atyoida*) sp., shedding their ciliated epidermis. Only in the first host, experimentally infected, did development to the infective stage occur. Larvae penetrate through the cuticle where it is thinnest — that of the gills and the junctions between the abdominal segments, on the ventral side.

Penetration of larvae through the crayfish exoskeleton is brought about by joint action of the hooks and penetration glands. During penetration, larvae are bent in a U-like fashion in such a way that anterior and posterior ends are in contact with the same piece of body surface of the crayfish. The hooks attach first, working back and forth continuously while the front end is brought to the same spot. At this stage the intermediate host becomes agitated, indicating that entry does not go unnoticed. The front end of the larva enters first, the hook end last. Mechanical action of the hooks is indicated by a sometimes strong bending of the hook, as if under strong mechanical pressure. All or most sense receptors except the subtegumental ones are probably lost during penetration (Rohde and Watson, 1989c). Type I secretion (see p. 183) was found in the host around the larvae, much type I, II and III secretion was still present in the glands of the juveniles even 8 days after penetration. Some juveniles become encapsulated by host tissue 24 h after penetration (Figure 18B); such juveniles have a much thinner tegument than younger, not encapsulated worms. During development, the tegument becomes rapidly thicker and develops numerous surface folds (Figure 18C). Parts of the surface lack a tegument, possibly indicating damage by the host. Occasionally, long branching tegumental processes protruding into the host were seen (Figure 18A). In juveniles 8 weeks after infection, clusters of a type of receptor not present in infective larvae were seen: non-ciliate, with an electron-dense collar and numerous microtubules (Figure 18C). The approximate maximum length of formalin-fixed juveniles was 1 mm at 43 days after infection, 2 mm at 75 days, and 3 mm at 90 days. It is not known at which size worms become infective to turtles, but juveniles used for successfully infecting turtles from naturally infected crayfish had a length of about 7 mm. They were usually found between the abdominal muscles near the anus, indicating that migration in the intermediate host had occurred. Turtles could be experimentally infected by feeding infective juveniles from crayfish, or crayfish containing the juveniles to them. Juveniles penetrate through the oesophageal wall, apparently after creeping up from the stomach where digestion of the crayfish has occurred, and then migrate along the trachea into the body cavity where they mature.

Gibson *et al.* (1987) recovered juveniles ("larvae") of the species *Nesolecithus africanus* from female freshwater prawns, *Desmocaris trispinosa*, in Nigeria. Worms have well-developed reproductive organs (apparently further developed than in infective juveniles of *Austramphilina elongata* from crayfish), but mature specimens were not present in the prawns.

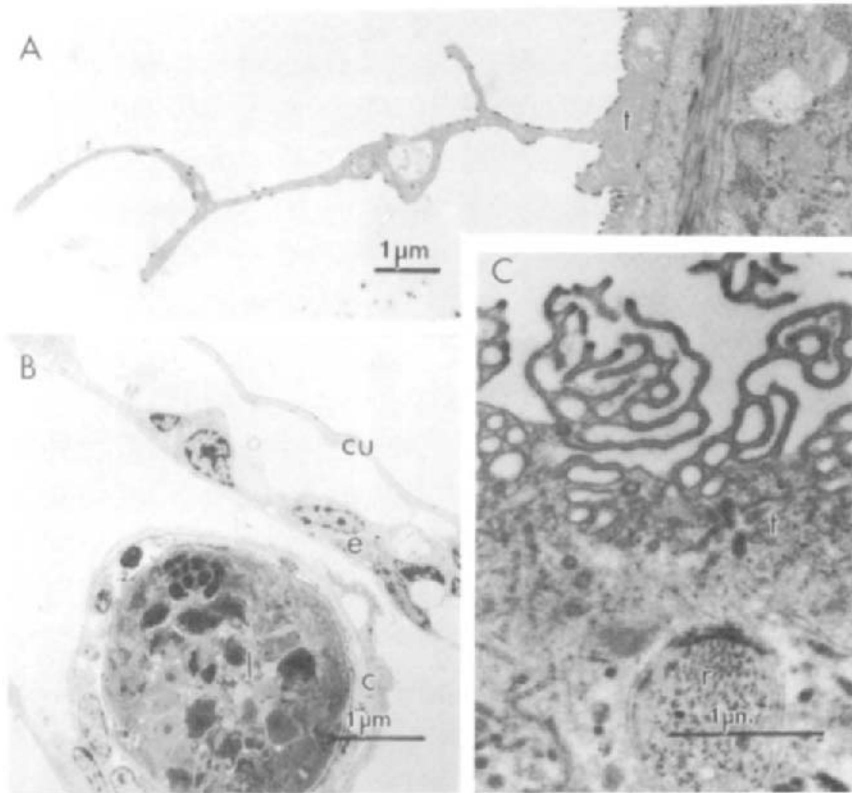


Figure 18 *Austramphilina elongata*. Larva after penetration into crayfish. (A) Tegument (t) of larva 8 days after infection in crayfish. Note long tegumental process. (B) Larva (l) encapsulated by host tissue. c, capsule; cu, cuticle of gill filament; e, epidermis of gill filament. (C) Tegument (t) of non-encapsulated juvenile 8 weeks after infection; r, receptor. (Modified from Rohde and Watson, 1989c.)

3.2. Structure of the Larva

The light- and electron-microscopic structure of larval *Austramphilina elongata* was described by Rohde and Georgi (1983), Rohde and Garlick (1985a, b, c, d), Rohde (1986, 1987), Rohde *et al.* (1986), Rohde and Watson (1987, 1988a, 1989c) (for light-microscopic descriptions of eggs and larvae of several species, and for early references see Dubinina, 1982).

3.2.1. *General Morphology*

The larva of *Austramphilina elongata* (160–210 × 60–79 µm) is ciliated and only the posterior end bearing a circle of posterior sense receptors and the

invagination through which the hooks protrude, and some small epidermal patches and glandular as well as excretory openings, lack cilia (Figure 19) (Rohde, 1986). Three flame bulbs on each side of the body open into protonephridial capillaries which join to form one duct on each side with postero-lateral nephridiopores. Capillaries and ducts lack lateral flames.

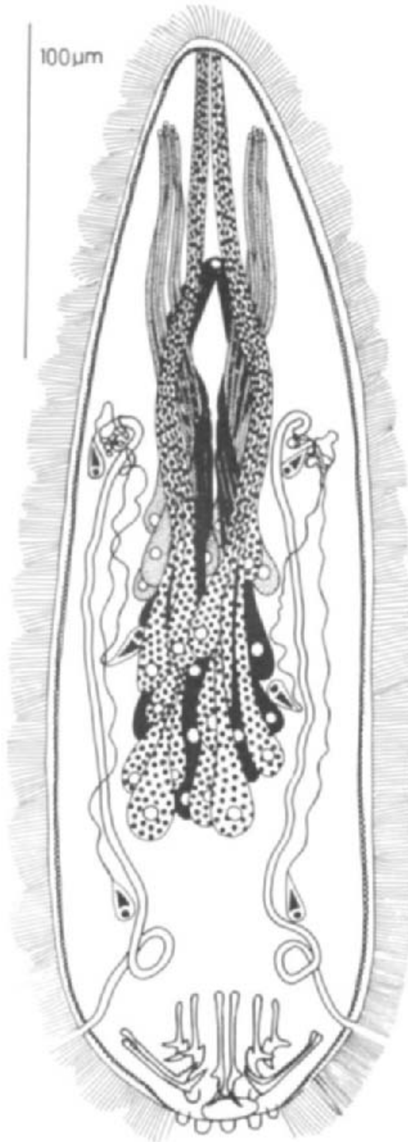


Figure 19 *Austramphilina elongata.* Diagram of larva. (From Rohde (1986) reproduced with permission of Springer-Verlag.)

There are three types of frontal glands. Twelve (exceptionally 11) type I gland cells have a dorsal position and open through discrete ducts into an anterior invagination of the tegument. In free-swimming larvae, the invagination does not appear to communicate with the environment, it is covered by epidermis. The perikarya of 10 type II cells lie ventral to the type I cells; their ducts descent ventrally in the anterior third of the body and open on a mid-ventral papilla some distance behind the anterior end. Ten type III cells are located ventrally in front of the type I and II cells. Five ducts open through five openings on each side near the anterior end of the body. Anterior to the

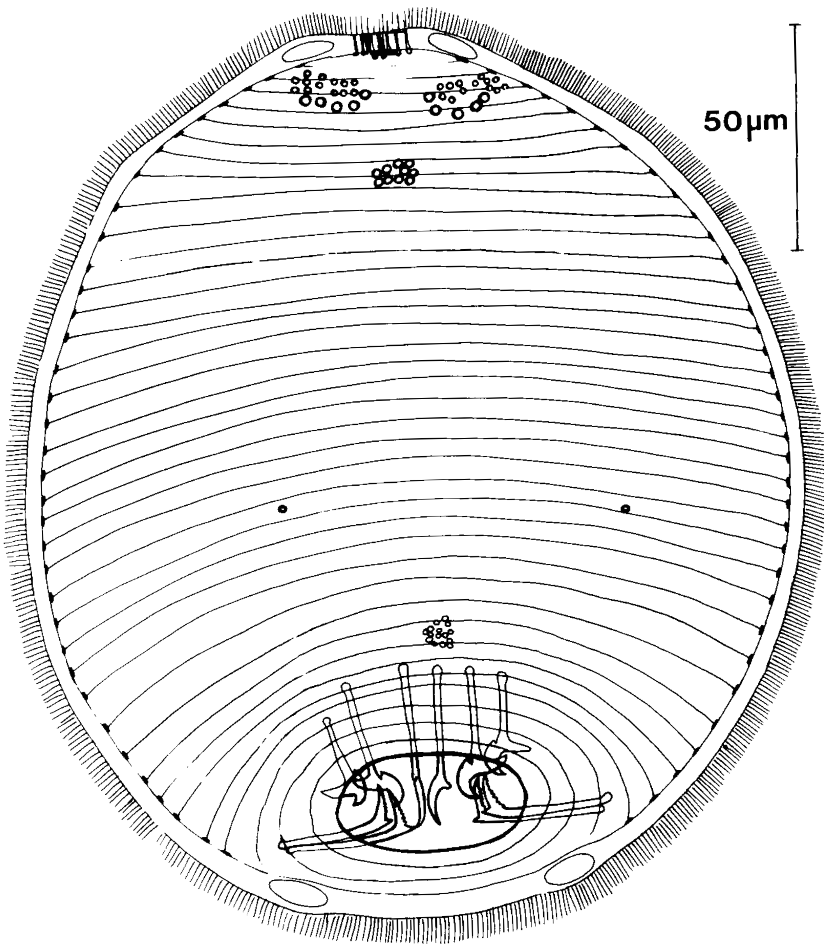


Figure 20 *Austramphilina elongata*. Diagram of larva stained with $AgNO_3$ to show sensilla, hooks and circular muscle bands. (From Rohde and Georgi (1983), reproduced with permission of Pergamon Press.)

openings of type II cells, one or two ventral cells with large vacuolar contents were observed, apparently opening mid-ventrally.

The larva possesses five pairs of hooks — one pair of median hooks with a long handle and sickle-shaped blade, two pairs of submedian halberd-shaped hooks with shorter handles, of which the more median pair is usually longer, and two pairs of lateral hooks with long handles and serrate blades. Eight large papillae are arranged in a circle around the posterior end. Silver impregnation showed clusters of small papillae (Figure 20), as well as transverse bands. In one larva, 37 dorsal, and in another, approximately 42 ventral bands were counted (Rohde and Georgi, 1983). These bands are muscular in nature, as shown by electron microscopy.

3.2.2. *Epidermis, Tegument and Parenchyma*

The epidermis of *Austramphilina elongata* is syncytial with nuclei located in the surface epidermis itself (Figure 21) (Rohde and Georgi, 1983). Cilia have a single horizontal rootlet, and axonemal microtubules in the ciliary tips terminate in a regular pattern as follows (Figure 22): doublet 1 and the

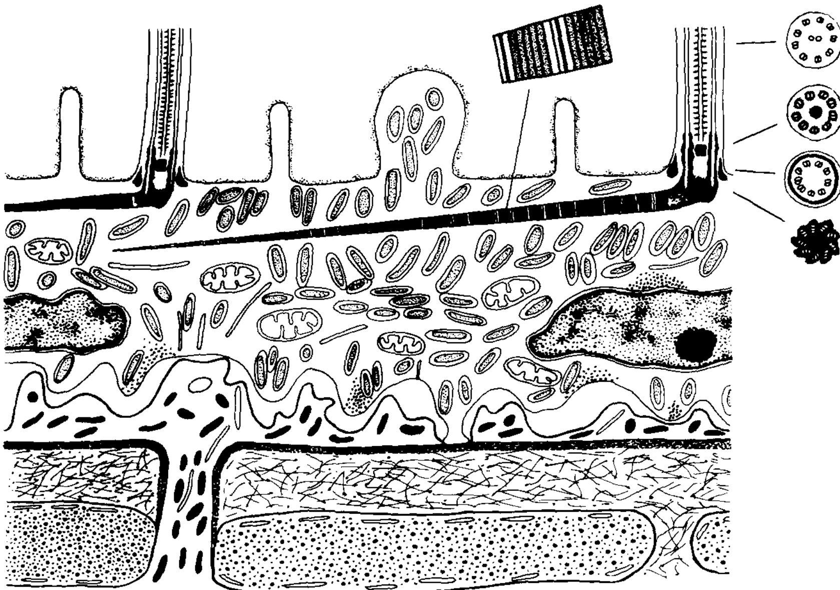


Figure 21 *Austramphilina elongata*. Diagram of larval epidermis, tegument, underlying fibrous matrix and muscle fibres. Note epidermal syncytium, cilia with a single horizontal rootlet, microvilli and knob-like projections, and ovoid bodies in the epidermis attached to underlying tegument (neodermis) by interdigitations, tegument connected to subtegumental perikaryon by cell process. (From Rohde and Georgi (1983), reproduced with permission of Springer-Verlag.)

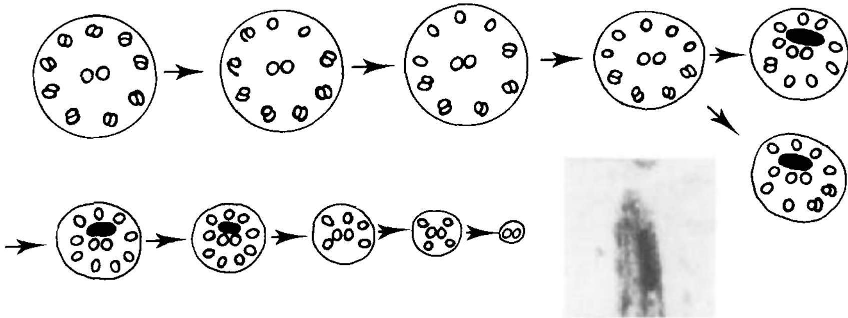


Figure 22 *Austramphilina elongata*. Diagram of series of cross-sections through tip of epidermal cilium, and an electron migrograph of a longitudinal section through ciliary tip. Note dense body and microtubules closely arranged around it. (From Rohde (1986), reproduced with permission of Springer-Verlag.)

doublets adjacent to it (2, 3, 9, 8) lose their microtubule B first and close in on the central pair in a spiral fashion, enclosing an electron-dense rod-like structure; gradually, all peripheral doublets lose their microtubule B, and microtubules A gradually disappear as well; the central pair of microtubules extends almost to the tip of the cilium. The only variability found in this pattern was a slight variability in the sequence of disappearance of microtubules B.

The surface of the epidermis including that of the microvilli and of the knob-like projections (which are not present in all specimens) is covered by a thin mucoid layer (Figure 21). Mitochrondria, ovoid inclusions and invaginations by the basal cell membranes are common. The epidermis is attached to the underlying tegument by means of interdigitations of epidermis and tegument. The syncytial tegument also contains invaginations by the basal cell membrane and ovoid inclusions which, however, are more electron dense than the epidermal ones. It is connected to subtegumental perikarya by branching cell processes with microtubules in their periphery. In the posterior region bearing the circle of large papillae and hooks, the tegument, connected to the adjacent epidermis by desmosomes, forms the surface layer. The electron-dense ovoid inclusions of the tegument were not observed in the perinuclear cytoplasm; they appear first in the cytoplasmic processes leading to the tegument.

Large fluid-filled intercellular spaces extend between the cells in the interior of the body. Densely packed germ cells fill much of the middle third of the body. They possess nuclei with large round nucleoli, numerous free ribosomes and mitochondria, and well-developed Golgi complexes. Nuclear envelopes contain large numbers of pores. Also found were some necrotic cells in the anterior part of the body.

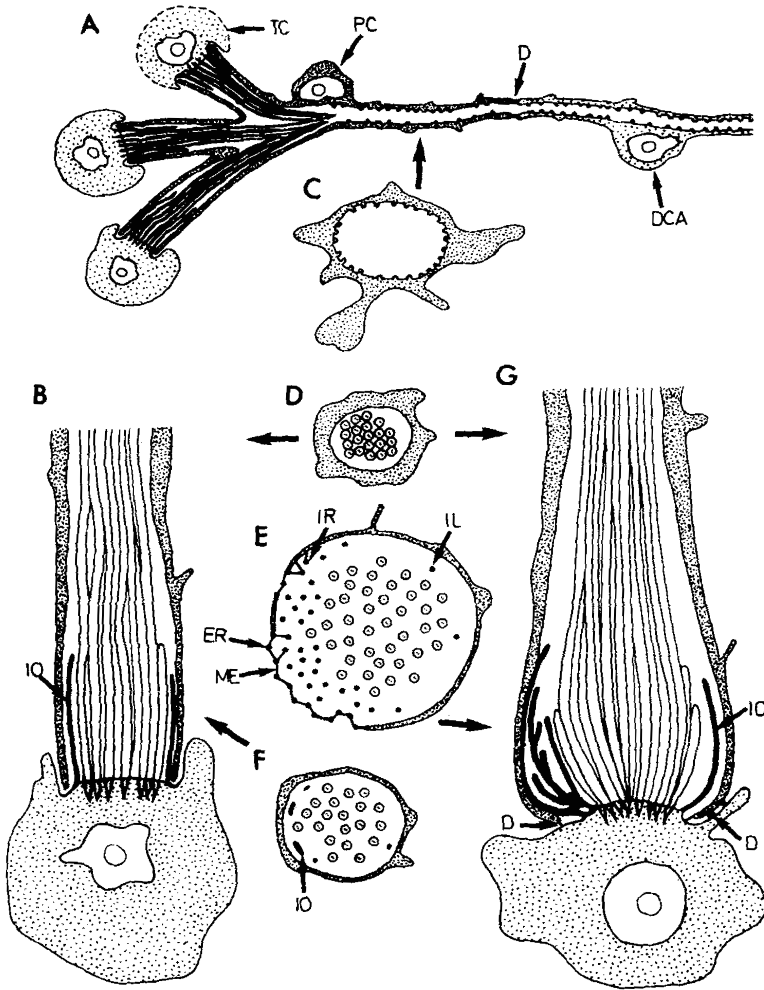


Figure 23 *Austramphilina elongata*. Diagrams of developing protonephridium. (A) Ciliary tufts of three terminal cells extending into the capillary of a proximal canal cell at early stage of development. Note that terminal cells are not in one plane. (B) Longitudinal section through the terminal cell and cytoplasmic cylinder of a proximal canal cell. (C) Cross-section through the capillary of a proximal canal cell. (D) Cross-section through tips of a flame cell and cytoplasmic cylinder of a proximal canal cell. (E) Cross-section through a developing weir at a more advanced stage of development. (F) Cross-section through the base of a terminal cell and cytoplasmic cylinder of a proximal canal cell at an early stage of development. (G) Longitudinal section through a terminal cell and cytoplasmic cylinder of a proximal canal cell at a more advanced stage of development. D, desmosome; DCA, distal canal cell; ER, external rib; IO, inner outgrowth; IR, internal rib; ME, "membrane"; PC, proximal canal cell; TC, terminal cell. (From Rohde and Watson (1988a), reproduced with permission of Springer-Verlag.)

3.2.3. *Ultrastructure and Development of Protonephridia*

Two cells, a terminal cell and a proximal canal cell, contribute to the filtration apparatus, the weir, of adult, juvenile and larval *Austramphilina* (Rohde and Watson 1987, and unpublished observations). The weir consists of internal and external longitudinal rods (ribs) connected by a "membrane" of probably extracellular matrix, and with many internal and some external leptotriches, that is, long cytoplasmic outgrowths. The internal ribs are continuous with the terminal, the external ones with the proximal canal cell. Cilia forming the flame are densely packed and have cross-striated and branching vertical rootlets. Axonemal doublets lose one microtubule and decrease in number towards the tip of the cilia. The nucleus of the terminal cell is located basal to the flame, the cytoplasm around it contains numerous mitochondria, some Golgi complexes, and many ribosomes. Neither the weir nor the proximal canal contains a septate junction. In the cell body of embryonic terminal cells some axonemes were seen. The wall of the proximal capillary forms numerous short stubby microvilli. Protonephridial ducts near the nephridiopores in the larva are in contact with the tegument by a junction, the excretory openings are simple pores in the tegument without any special structures, such as muscle fibres, valves or sensory receptors. The so-called "star-cell" described by Rohde and Garlick (1985d) may be an abnormal flame bulb.

The development of the flame bulbs was studied by electron microscopy in embryos of *Austramphilina elongata* by Rohde and Watson (1988a), the first such study in any parasitic or free-living platyhelminth. Ciliary tufts (flames) of three terminal cells are enclosed by cytoplasmic cylinders of a single proximal canal cell (Figure 23). The cylinders are joined, forming a single capillary (proximal canal) which is joined to the capillary of a distal canal cell by a junction. Internal outgrowths of the terminal cells grow into the space between the cylinders and the outermost cilia. During development, more cilia grow out, the parts of the cylinders closest to the terminal cells bulge out to accommodate the additional cilia, and at points of contact between the inner outgrowths and the cylinders external ribs are formed, connected to the internal ribs formed from the inner outgrowths by means of thin "membranes", apparently of extracellular matrix. During development and in fully developed protonephridia, the cytoplasmic cylinders are connected to the terminal cells by junctions but do not fuse with them.

Xylander (1992a) found a similar structure of flame bulbs and protonephridial capillaries in *Amphilina foliacea*. Lateral flames were not seen in any canal cells by light- and electron-microscopy. Many internal but few external leptotriches are present in the flame bulb, and the proximal canal lacks microvilli.

3.2.4. *Sensory Receptors*

Examination of many ultrathin serial sections revealed the presence of at least six types of sensory receptors in larval *Austramphilina* (Figure 24) (Rohde and Garlick (1985a, b, c; Rohde *et al.* 1986):

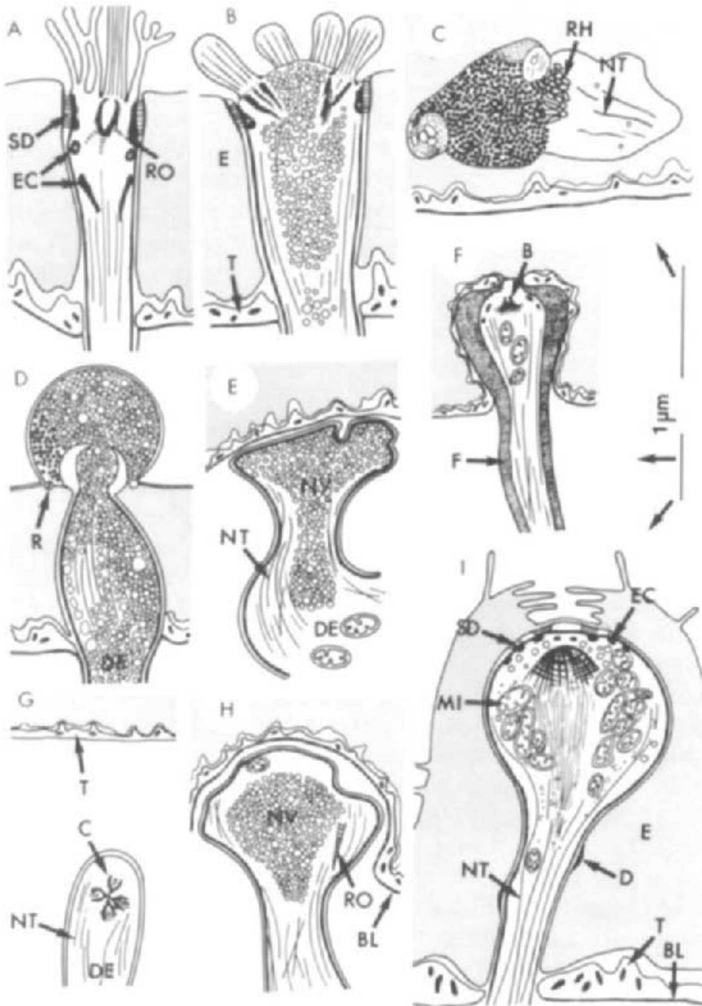


Figure 24 *Austramphilina elongata*. Sensory receptors of larva. (A) Unicilate microvillate receptor. (B) Multiciliate receptor. (C) Presumptive photoreceptor (?). (D) Free dendrite. (E) Subtegumental nerve ending. (F) Anterior thick-walled receptor. (G) Centrioles in anterior dentrite. (H) Tegumental nerve ending. (I) Posterior bulbous receptor. B, basal body; BL, basal lamina; C, centriole; D, hemidesmosme; DE, dendrite; E, epidermis; EC, electron dense collar; F, fibrous matrix; MI, mitrochondrion; NT, neurotubule; NV, neurovesicles; R, ring around base of free dendritic process; RH, rhabdomere; RO, rootlet; SD, septate desmosome; T, tegument. Upper scale bar for (A)–(C). (From Rohde *et al*. (1986), reproduced with permission of Pergamon Press.)

1. uniciliate receptors with numerous branched and interconnected microvilli, an electron collar consisting of several rings, and cross-striated rootlets diverging from the basal body of the cilium (Figure 24A);
2. multiciliate receptors with four (and possibly more?) club-shaped cilia and a single electron-dense collar (Figure 24B);
3. free nerve endings (terminal swellings of dendrites) containing numerous small vesicles, predominantly peripheral neurofilaments, and sometimes a cross-striated ciliary rootlet, projecting into the epidermis and surrounded by a sheath consisting of basal lamina and tegument (Figure 24H);
4. free nerve endings below the tegument, also containing small vesicles and neurofilaments but always lacking ciliary rootlets, (Figure 24E);
5. anterior dentrites containing single or clusters of centrioles, (Figure 24G);
6. eight large papillae arranged in a circle at the posterior end, each containing one terminal swelling of a thin dendrite with many mitochondria, a basal body from which a cross-striated ciliary rootlet diverges, a bundle of long non-striated filaments, and an electron-dense collar consisting of several thin rings (Figure 24I);
7. anterior non-ciliate dendrites penetrating deeply into the epidermis and surrounded by evaginations of the tegument, basal lamina and a thick layer of underlying fibrous matrix into the epidermis, and containing electron-dense collars, a basal body and a short ciliary rootlet (Figure 24F);
8. free bulbous nerve ending at the surface, at the end of a dendrite penetrating tegument and epidermis (observed only once) (Figure 24D);
9. a nerve ending (?) in the epidermis without electron-dense collars or a basal body, but containing microvilli-like structures (rhabdomere?) and flanked by a densely granular (pigment ?) body on one side (possibly a photoreceptor) (Figure 24C).

The sensory nature of types 5 and 9 is not certain. Centrioles in dendrites (type 5) may represent structures which play a role in development of the larva, and a connection of type 9 to nerves could not be demonstrated. Free tegumental and subtegumental nerve endings (types 3 and 4) may also be considered to be subtypes of one type of receptor. Type 8 was observed only once and may constitute an abnormality.

3.2.5. *Formation of Glandular Secretion*

Larvae of *Austramphilina elongata* possess three types of gland cells (see p. 183). Type I cells have round-ovoid, electron-dense secretory granules, type II cells contain long secretory granules with regular longitudinally arranged microtubules, and type III cells contain microtubules (appearing somewhat coiled) with an electron-dense core along part of their length (Figure 25) (Rohde, 1986). Rohde (1987) described the formation of the glandular

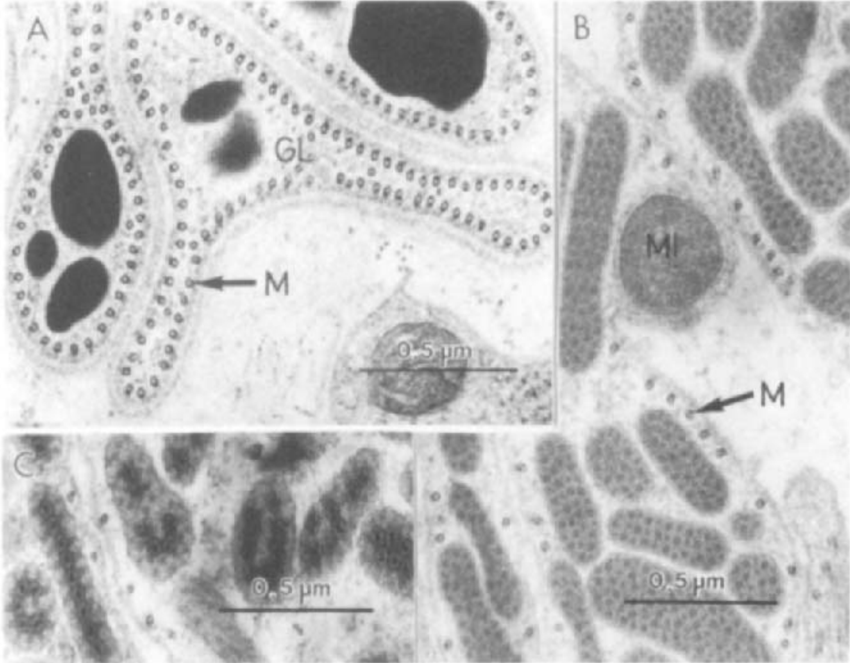


Figure 25 *Austramphilina elongata*. Secretion of anterior glands of larva in glandular ducts: type I(A), type II(B) and type III(C). GL, glandular duct; M, microtubule; MI, mitochondrion.

secretions (Figure 26): type I granules are formed by numerous Golgi complexes, type II granules are produced by Golgi complexes and the microtubules apparently condense in the cytoplasm of the secretory cells or in the granules themselves, and type III granules are formed by secretion derived from Golgi complexes and the microtubules aggregate around and migrate into the secretion; microtubules are at first hollow and the early secretory granules have a central electron-dense region.

3.3. Structure of the Juvenile and Adult

3.3.1. *Tegument*

Bazitov and Lyapkalo (1984) studied the ultrastructure and histochemistry of the body wall and parenchyma of *Amphilina japonica* and *A. foliacea*. The surface tegument lacks microtriches and is connected to subtegumental cells. It is rich in mucoproteins and sulphated acid mucopolysaccharides in the first, but contains only mucoproteins in the second species. The parenchyma contains muscle cells and amoebocytes.

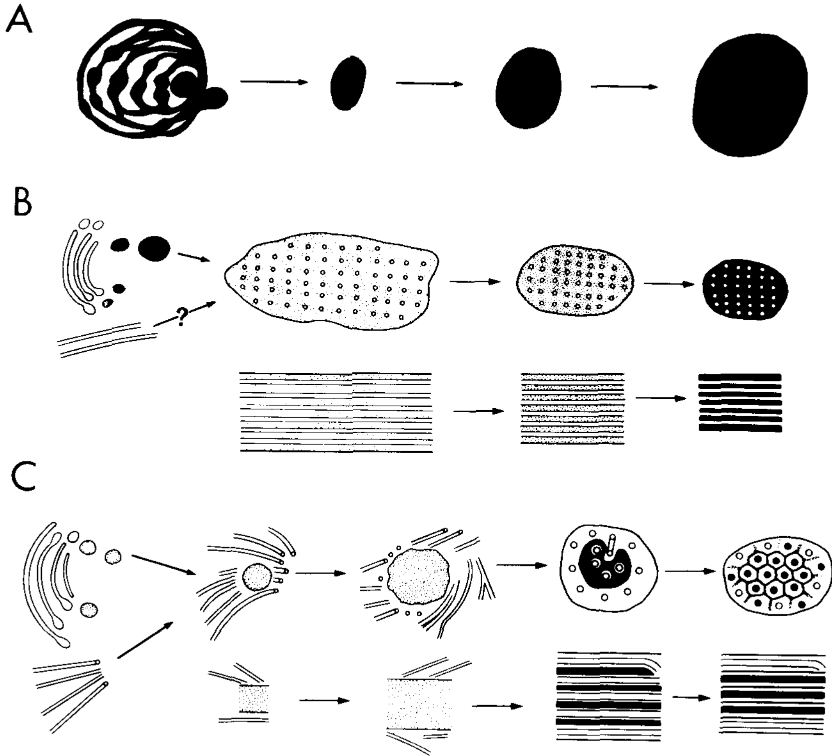


Figure 26 *Austramphilina elongata*, larva. Hypothetical formation of secretory granules type I(A), type II(B) and type III(C). (From Rohde (1987), reproduced with permission of Pergamon Press.)

Using SEM and TEM, Rohde and Watson (1990e) examined the tegument of juvenile *Austramphilina* from crayfish, of a size infective to turtles. They found a great variability in the surface structure. Large parts of the surface are formed by folds enclosing cavities, some of the folds may carry microvilli and large protuberances, and closed or open blisters apparently connected to tegumental cavities. "Cilia"-like structures (possibly thick tegumented protuberances) are present on parts of the surface. Deep interconnected surface folds found in younger juveniles (see Figure 18C) are absent. As in the neodermis (= tegument) of all Neodermata, the surface tegument, exceeding 10 μm thickness in some parts of the body, is syncytial and connected to subtegumental perikarya by cell processes. These processes possess incomplete peripheral rows of microtubules. Apparently, the tegument sloughs at the surface. Many glandular ducts containing electron-dense to light granular secretion open into tegumental cavities; they have one or two thick electron-dense rings below the septate junction connecting them to the tegument.

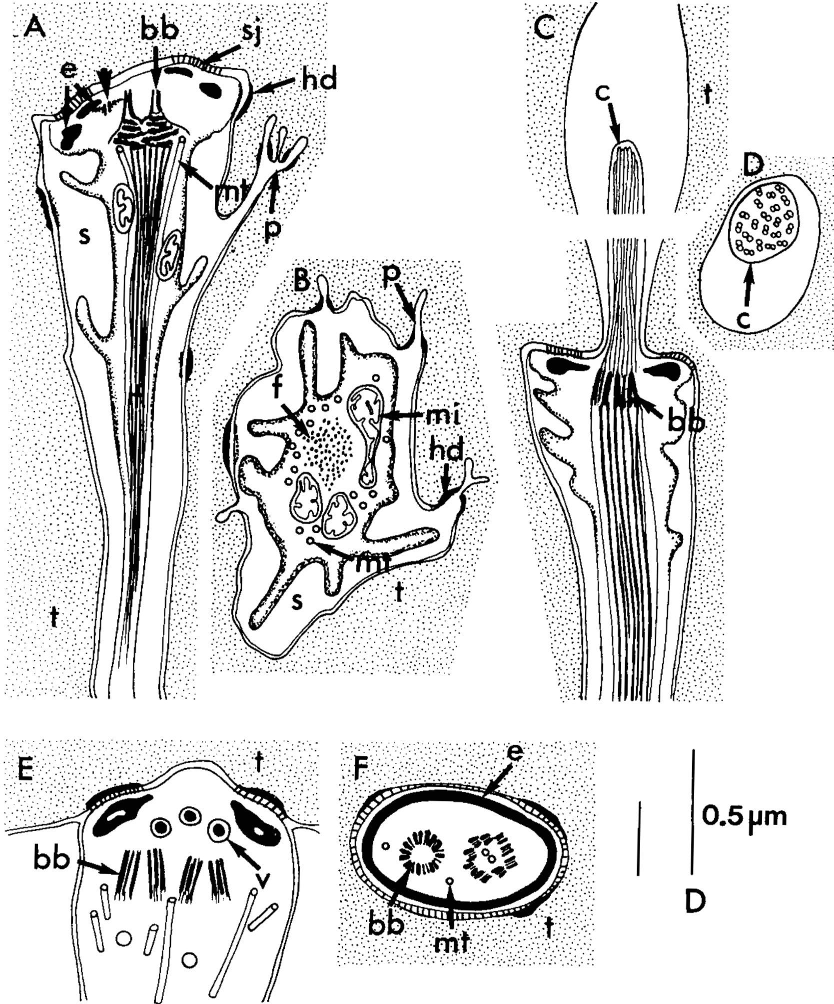


Figure 27 *Austramphilina elongata.* Receptors of juvenile, longitudinal and cross-sections. (A, B) Non-ciliate tegumental receptor. (C, D) Uniciliate tegumental receptor. (E, F) Non-ciliate receptor with two basal bodies below or in tegument. bb, basal body; c, cilium; e, electron-dense collar; f, filament; hd, hemidesmosomes; mi, mitochondrion; mt, microtubule; p, process of the sheath; sj, septate junction; t, tegument; v, vesicle. The arrowhead in (A) points at the desmosome-like connection between the basal body and the electron-dense collar. (From Rohde and Watson (1990d), reproduced with permission of Springer-Verlag.)

3.3.2. *Sensory Receptors*

Three types of receptors of juvenile *Austramphilina elongata* were described by Rohde and Watson (1990a). As characteristic of Neodermata, all receptors have electron-dense collars. One type is uniciliate, the cilium containing many irregulary arranged microtubule doublets and located in a deep tegumental pit, with a long non-striated rootlet (Figure 27C, D). Another type contains a basal body and long ciliary rootlet but no cilium and is located in the tegument (Figure 27 A, B), and a third type contains two basal bodies but no cilium nor rootlet and is located below or in the basal part of the tegument (Figure 27E, F). In view of the large size of the worm and the difficulty in examining all parts of a worm, it is likely that more receptor types exist. None of the receptors resembles those found in larval *Austramphilina* (see Figure 24).

3.3.3. *Spermatogenesis, Sperm Ducts and Vitellogenesis*

Light-microscopic studies of spermatogenesis of *Amphilina japonica* were made by Bazitov *et al.* (1979). The authors claim that cytophores are not syncytial. The number of cells in the cytophores increases from 16 to 32 to 64.

The ultrastructure of spermatogenesis and sperm of *Austramphilina elongata* was described by Rohde and Watson (1986a). Spermatogonia and spermatocytes form a layer several cells thick along the walls of the testis. The testis wall consists of a thin cytoplasmic layer based on a thick basal lamina and underlying fibrous matrix. In meiotic spermatocytes, chromosomal microtubules implanted in kinetochores were observed. Parts of spermatocytes are surrounded by a thin sheath of cytoplasm. A central cytophore with many interconnected channels connects the spermatid clusters. At the beginning of spermiogenesis a row of microtubules appears along the distal part of the cell membrane, two basal bodies are arranged at a right angle to each other, and a median process grows out near them. Next, a striated central (intercentriolar) body appears between the centrioles, as well as cross-striated ciliary rootlets (Figure 28A, B). Chromatin condenses along the nuclear envelope near the centrioles. Two outgrowing flagella terminate in bulbous swellings. The flagella, first growing in opposite directions to each other, rotate and assume a position parallel with each other (Figure 28A). The nucleus elongates and mitochondria apparently fuse, and nucleus and mitochondrion extend into the median cytoplasmic process after it has reached a considerable length. Now the flagella fuse with the central median process between the attachment zones on each side of the spermatid outgrowth. A short time before sperm separate from the residual cytoplasm, ciliary rootlets and central body are resorbed and the centrioles lie some distance from each other in the proximal end of the outgrowth where an electron-dense collar becomes evident. The cytoplasm of late spermatids contains axonemes, some

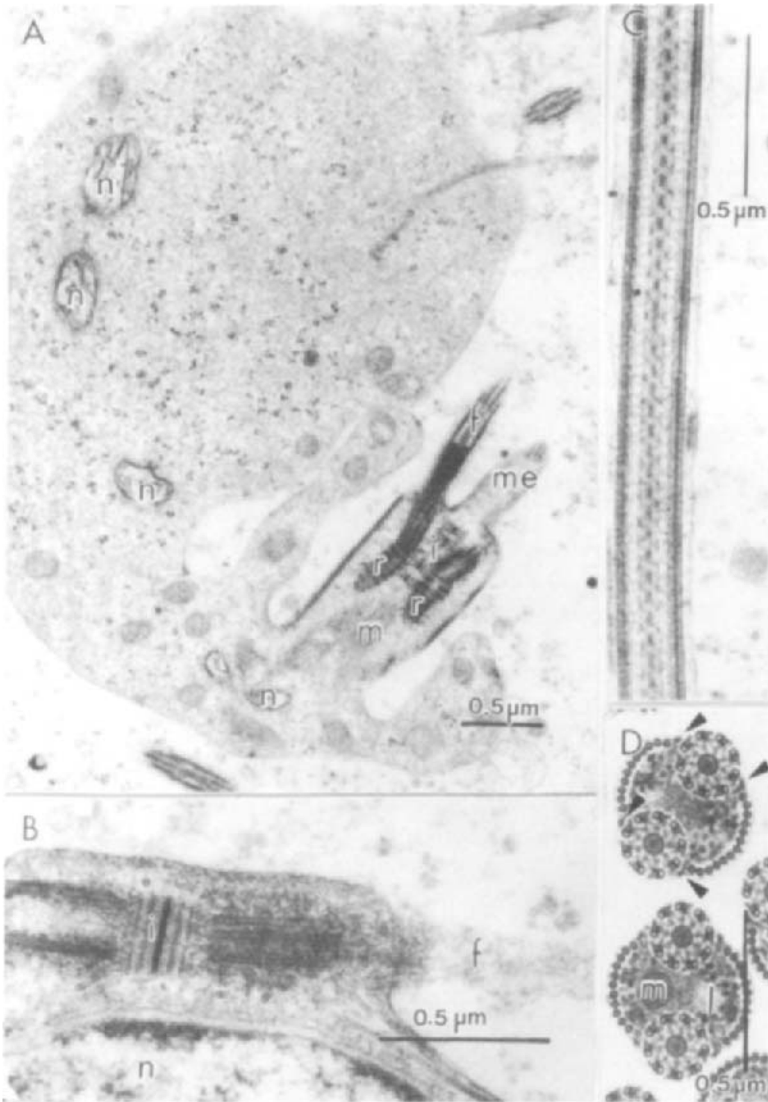


Figure 28 *Austramphilina elongata*, spermiogenesis and sperm. (A) Outgrowth of median cytoplasmic process (me) at right and flagella (f). Note long nucleus (n). (B) Section through plane of two basal bodies with flagella (f) and intercentriolar (central) body (i), ciliary rootlets (r) and mitochondria (m); n, nucleus. (C) Longitudinal section through axoneme. Note double-helical structure of central rod. (D) Cross-sections through mature sperm. Note rows of peripheral microtubules interrupted by the two axonemes, four attachment zones (arrowheads), mitochondrion (m) and lamellated body (l). (Modified from Rohde and Watson, 1986a.)

in channels partly lined by microtubules, apparently in a regular pattern. Mature sperm (Figure 28C, D) have two axonemes of the 9 + "1" pattern (one complex central rod, nine peripheral microtubule doublets), a nucleus and mitochondrion (although the possibility cannot be excluded that several mitochondria arranged in one row are present), and a single layer of peripheral microtubules interrupted by the axonemes. Two pairs of attachment zones are retained along the axonemes. At the distal end of the spermatozoon, first one and then both of the microtubules in each axonemal doublet, and finally the central rod disappear. At the proximal end, the peripheral microtubules and central cytoplasm (always ?) disappear first and one axoneme terminates before the other.

The type of spermatozoon and spermiogenesis described corresponds to that of most Trematoda Aspidogastrea and Digenea, Monogenea Polyopisthocotylea and many Cestoda.

Sperm ducts of *Austramphilina* are lined by a nucleated epithelium with many surface lamellae and invaginations of the basal lamina and fibrous matrix. Cilia are without a distinct rootlet, and their axonemes lack typical central microtubules (Figure 29) (Rohde and Watson, 1986b).

Xylander (1988a) described vitellogenesis in *Amphilina foliacea*. Yolk follicles are enclosed in a cytoplasmic sheath. Processes of the sheath extend and fill the space between the yolk cells. Immature vitellocytes have few organelles (Figure 30). During maturation, the cells increase in size and an elaborate endoplasmic reticulum and many Golgi bodies develop; shell protein vesicles and lipid droplets are formed. In mature yolk cells, most of the cell body is filled by shell protein vesicles and lipid droplets. The vitelloduct is ciliated and possesses surface lamellae, its nuclei are intraepithelial, but neither lateral cell membranes nor junctions were seen.

According to Coil (1987a), vitelline gland granules compose most of the early egg shell in *Austramphilina elongata*; the granules stain positively with PAS, bromophenol blue and Heidenhain's iron haemotoxylin. The reaction for phenolase is only weak.

3.3.4. *Effects on the Host*

Two papers deal with the effects of *Amphilina foliacea* on the host — Andreev and Markov (1971) and Popova and Davydov (1988). The first authors observed a marked effect on the level of haemoglobin, the number of erythrocytes, Cu, Co, Mn and to some degree Zn contents of *Acipenser gueldenstaedti, A. stellatus* and *A. ruthenus*. The fat contents of the fish decreased, and the index of colloidal stability of serum proteins was affected as well; the Weltman's coagulation band shortened.

In the second paper, the authors report that *Amphilina foliacea* is mostly found in the body cavity of *Acipenser ruthenus* and *A. stellatus*, associated with the liver, although muscles and gonads are also relatively strongly

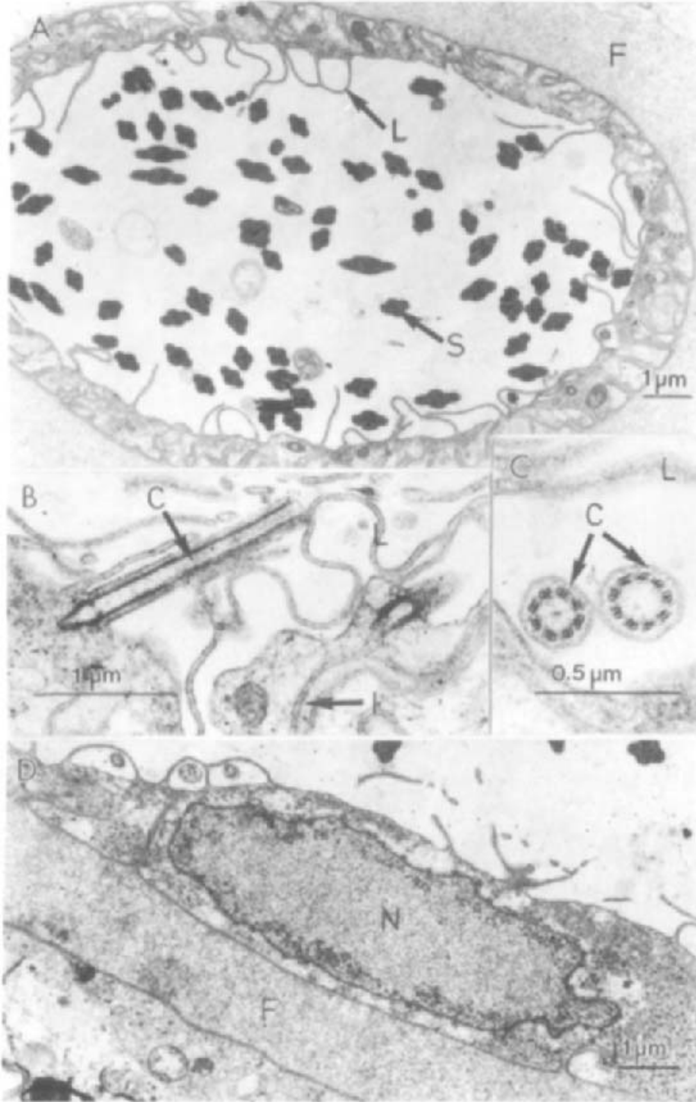


Figure 29 *Austramphilina elongata*, cross-sections through sperm duct. (A) Cross-section through middle-sized sperm duct. Note: invaginations of fibrous matrix into the duct wall, and surface lamellae. (B) Longitudinal section through cilium of sperm duct. Note basal body and lack of distinct root. (C) Cross-section through two cilia of sperm duct. Note: lack of central filaments in one cilium and very faint dense core in the other; eight axonemal doublets in one and nine in the other cilium. (D) Epithelium of sperm duct wall with nucleus. Note: basal lamina and thick underlying fibrous matrix. C, cilium; F, fibrous matrix; I, invaginations by cell membranes; L, lamella; N, nucleus; S, sperm. (From Rohde and Watson (1986b), reproduced with permission of V.E.B. Fischer-Verlag.)

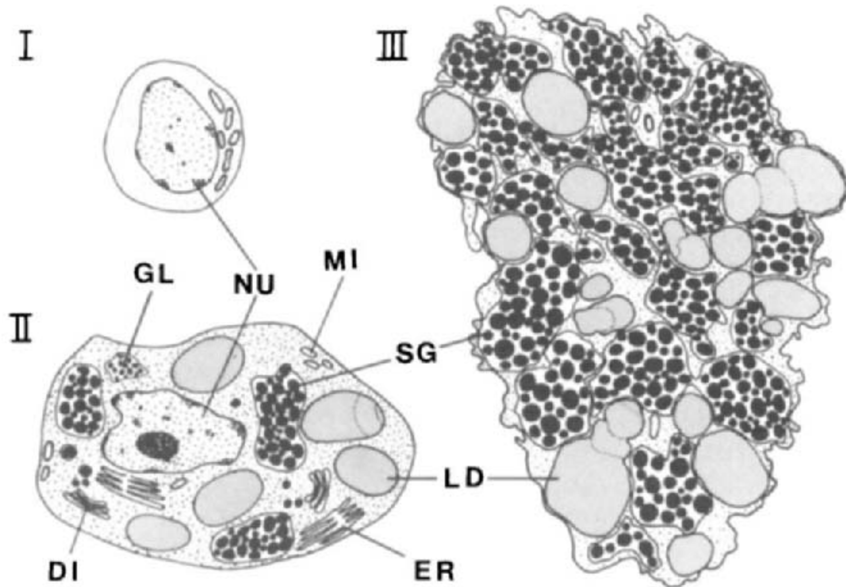


Figure 30 *Amphilina foliacea*, vitellogenesis. (I) Immature vitellocyte. (II) Maturing vitellocyte. (III) Mature vitellocyte. DI, dictyosome (Golgi body); ER, endoplasmic reticulum; GL, glycogen; LD, lipid droplet; MI, mitochondrion; NU, nucleus; SG, shell-protein granule. (From Xylander, (1988a), reproduced with permission of Springer-Verlag.)

infected (Popova and Davydov, 1988). Penetration of tissues by the parasites was accompanied by hyperaemia and haemorrhages followed by extensive inflammation. A leukocyte barrier and granulation tissue were formed around the parasite, followed by a 100–150 µm thick capsule and several layers of fibroblasts containing collagen fibres and capillaries. The outer cell layer contained secretion granules with glycosaminoglycans. Secretion protects the parasite from the immune response of the host. Parasites grow in the capsules and can leave them. The spiny proboscis and frontal penetration glands with two types of secretion help in penetration (Popova and Davydov, 1988).

3.4. Taxonomy and Phylogenetic Relationships

Dubinina (1976, 1978), on the basis of the lack of an intestine, parenchymal concretions, the topography of the reproductive organs, and the structure of the eggs, embryo and the protonephridial system, established the class Amphilinoidea (= Amphilinida) with six genera and eight species. Bandoni and Brooks (1987) revised the Amphilinidea, recognizing eight species and

three genera as valid. The valid genera are *Amphilina* Wagener, 1858, *Schizochoerus* Poche, 1922, and *Gigantolina* Poche, 1922. According to these authors, *Austramphilina elongata*, discussed in detail in this review, becomes *Gigantolina elongata*. I have not used this name for two reasons: (i) more studies using more characteristics including ultrastructural ones, are necessary to support the revised taxonomy; and (ii) it seemed preferable to use names of taxa used in the original, recent papers. Keys to the genera are given by Schmidt (1986), and to the two species found in the Soviet Union by Dubinina (1985).

3.5. Hyperparasitism

Protozoan parasites of uncertain taxonomic status were observed in larvae of *Austramphilina elongata* (Figure 31). Parasites are globular, lined by a cell membrane, with a lobed nucleus, distinct nucleolus, numerous cristate mitochondria, and some electron-dense inclusions (Figure 31A). Figure 31B possibly represents a different developmental stage of the same species.

4. THE GYROCOTYLIDEA

4.1. Development and Life Cycles

It is known that larvae of gyrocotylids hatch from eggs in sea water (in *Gyrocotyle urna* after up to 30 days in sea water) (Malmberg, 1979) and swim

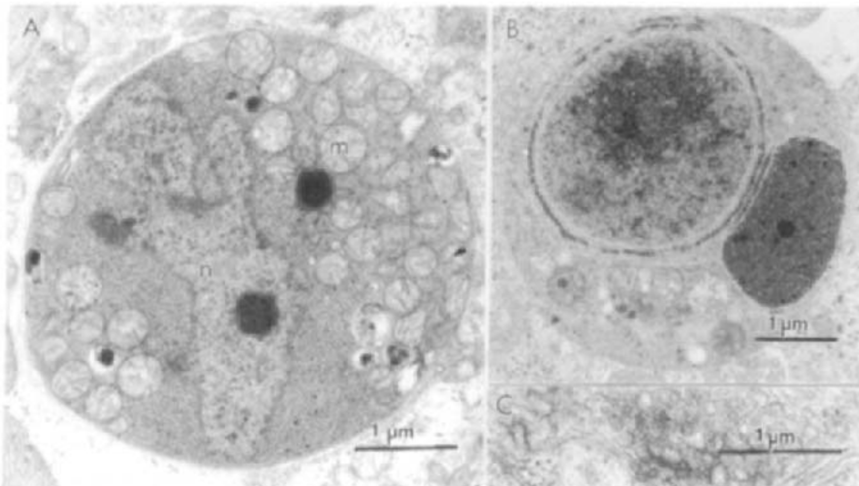


Figure 31 Parasitic protozoans in larva of *Austramphilina elongata*. Note lobed nucleus (n), many mitochondria (m), and Golgi body in C.

around (Manter, 1951, and personal observations), but no one has yet succeeded in infecting intermediate hosts, nor have invertebrate hosts been found to be naturally infected. Thus, Manter (1951) failed in infecting a snail (*Buccinulum multilineatum*), a bivalve (*Aulacomya maoriana*), and a hermit crab with larvae of *Gyrocotyle rugosa*. However, larvae penetrated and remained in large numbers in scrapings of mucus from the spiral valve of the chimaera, *Callorhynchus milii*. Also, larvae penetrated readily into pieces of the spiral valve and usually entered the muscle layers. Within an hour, at least four larvae had entered the blood vessels and travelled within them. Such observations have led some authors to the belief that the life cycle involves only a single, vertebrate host (e.g. Manter, 1951, 1953; Llewellyn, 1970, 1986). Manter (1953) suggested that larvae normally penetrate the gills or skin of chimaeras. Cole (1968), in experiments with *G. fimbriata*, could not demonstrate the behaviour of larvae found by Manter.

Simmons and Laurie (1972) discussed the possibility that chimaeras feed on free gyrocotylid worms (shed by the hosts) containing post-larvae (commonly found in gyrocotylids) and thus become infected. Uniquely, worms of the same species would thus act as "intermediate" hosts. Xylander (1989b) has given several arguments for an indirect life cycle:

1. already the smallest stages from the spiral valve of chimaeras possess a distinct anterior groove (homologous to the groove of Pseudophyllidea, Tetraphyllidea and Amphilinidea which develops in the crustacean intermediate host);
2. young chimaeras feeding on yolk are never infected, indicating that infective stages are ingested with some food items;
3. larvae swim actively and possess a well-developed nervous system, indicative of an active searching for a host; since chimaeras are not actively infected (see 2), an intermediate host must be involved.

Experimental studies to clarify the life cycle are urgently required.

4.2. Structure of the Larva

Xylander (1986a, b, 1987a, b, c, d 1990, 1991) made thorough ultrastructural studies of various organ systems of larval *Gyrocotyle urna*. Larvae have four pairs of different glands (already reported by Simmons, 1974), their perikarya located behind the middle of the body, and they open through discrete ducts at the anterior end (Xylander, 1990). At the posterior end are four ventral and six dorsal hooks, and there are four pairs of flame bulbs (Figure 32).

The epidermis is syncytial and covers the whole body including the caudal cavity into which the hooks protrude (Figure 33) (Xylander, 1987a). Cilia have a single, oblique-horizontal rootlet, there are regularly spaced microvilli (also Simmons, 1974), and various inclusions including mitochondria and

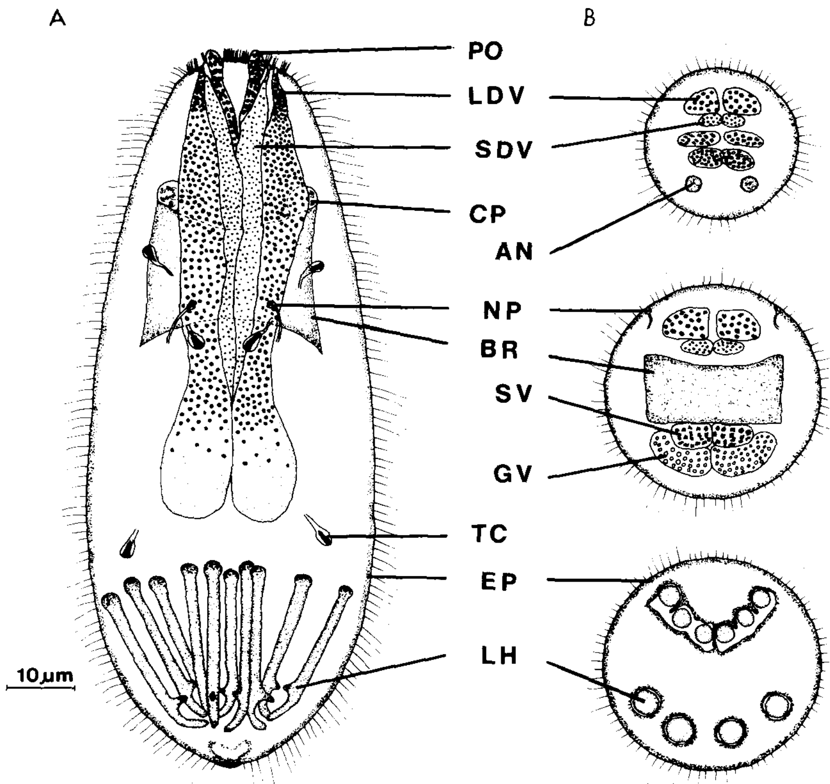


Figure 32 *Gyrocotyle urna*, lycophore larva. (A) Diagram of larva. (B) Cross-sections through the anterior part of the body in front of the brain (upper), the region of the brain (middle) and the posterior end with the larval hooks (lower). AN, anterior nerve chord; BR, brain; CP, ciliary photoreceptor; DI, dictyosome; EP, epidermis; GV, vesicles with amorphous content; LD, lipid droplet; LDV, larger vesicles with electron-dense granules; LH, larval hooks; MT, microtubules; NP, nephroporus; NU, nucleus; NUP, nucleopore; PO, gland pore; PZ, perinuclear cisternae; RER, rough endoplasmic reticulum; SD, septate desmosome; SDV, smaller vesicles with electron-dense granules; SV, vesicles with striped granules; TC, terminal cells. (From Xylander (1990), reproduced with permission of Springer Verlag.)

several kinds of vesicles. The epidermis lacks nuclei (see also Simmons, 1974), Golgi bodies and endoplasmic reticulum. The anlage of the tegument (neodermis) is located below the epidermis (see also Malmberg, 1978), tegumental cell processes are in contact with the basal part of the epidermis by desmosomes. In contrast to the epidermis, the rudimentary tegument is rich in Golgi bodies and cisternae of the endoplasmic reticulum, in addition to coated vesicles and membranous bodies.

Type I gland cells contain electron-dense "striped" granules and open at

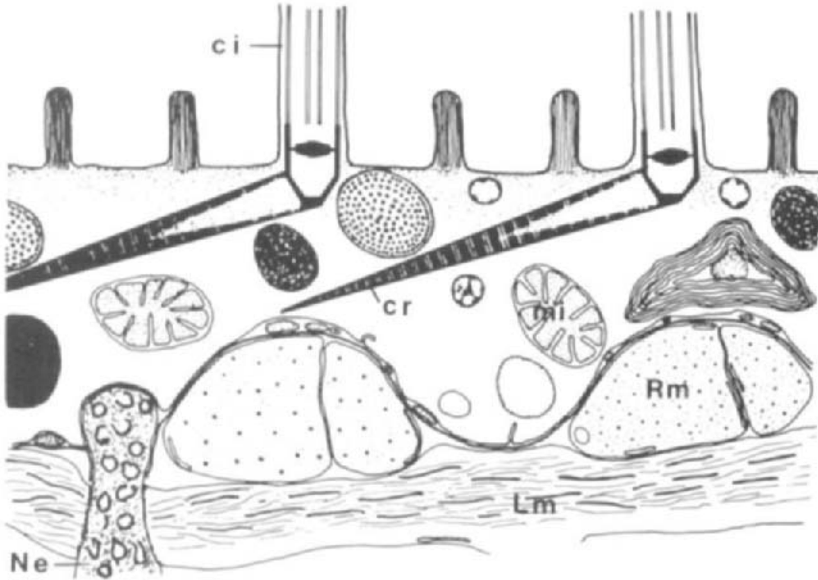


Figure 33 *Gyrocotyle urna*, lycophore larva. Epidermis, tegument and underlying tissue. ci, cilium; cr, ciliary rootlet; Lm, longitudinal muscle fibres; NE, neodermis (tegument); Rm, circular muscle fibre. (From Xylander (1987a), reproduced with permission of Springer-Verlag.)

the very anterior end of the body through two protuberances about 1.8 μm above the adjacent epidermis; the terminal parts of the ducts, as those of the other gland cells, contain peripheral microtubules. Type II gland cells contain vesicles with amorphous content, their perikarya are multinucleate, containing at least three nuclei; the vesicles are interconnected by a fibrous matrix, and their contents become more electron-dense close to the glandular openings, which are more lateral than those of the Type I gland cells and do not rise above the epidermis. Type III gland cells containing vesicles (about 500 nm in diameter) with homogeneous electron-dense granules, are multinucleate and their ducts open dorsally to those of type I and II gland cells. Type IV gland cells contain vesicles similar in structure to the type III vesicles but smaller (about 400 nm in diameter), they are multinucleate and their ducts open laterally to those of type III gland cells (Xylander, 1990).

The brain consists of a general neuropil and perikarya forming various types of vesicles (Xylander, 1987c). The five types of vesicles found differ as follows:

1. 80–115 nm clear vesicles;
2. 50 nm dense vesicles;
3. 20–30 nm vesicles with moderately dense content;

4. 70 nm dense vesicles with a lucent periphery;
5. 65–90 nm vesicles with dark content that is irregularly distributed in the periphery of the vesicles.

Synapses possess a presynaptic membrane covered by a 120 nm broad electron-dense coating, the post-synaptic area has a smaller and less dense covering and fewer vesicles; sometimes there are synaptic contacts to more than one neuron.

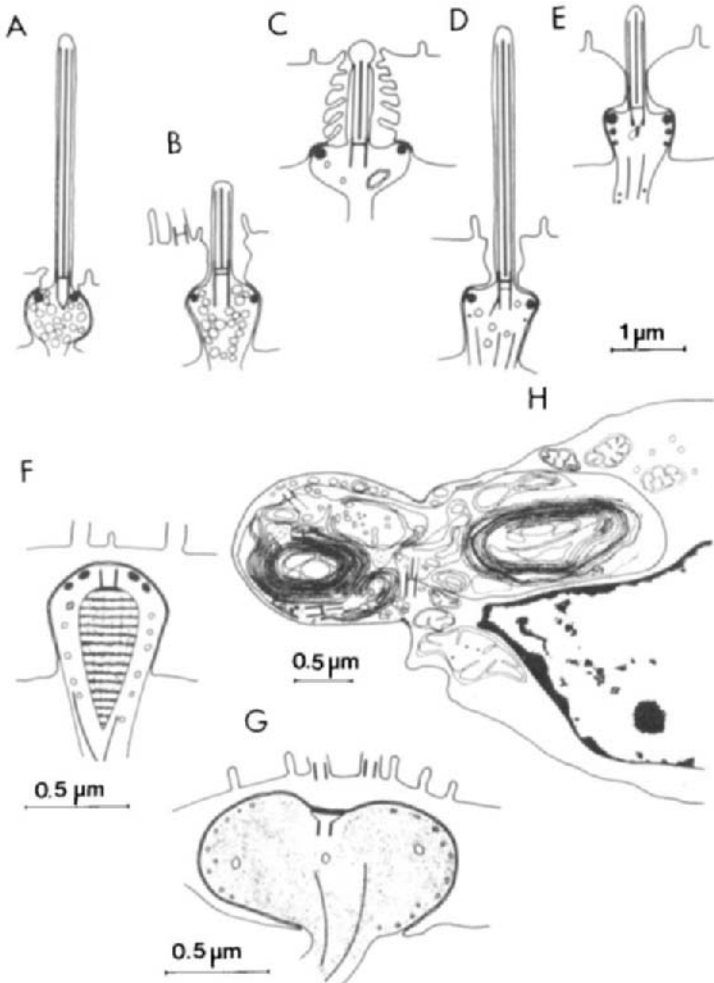


Figure 34 *Gyrocotyle urna*, lycophore larva. Sensory receptors. (A) Type 1 receptor. (B) Type 2 receptor. (C) Type 4 receptor. (D) Type 3 receptor. (E) Type 5 receptor. (F) Type 6 receptor with the enlarged ciliary rootlet. (G) Type 7 receptor. (H) Presumptive ciliary photoreceptor. (From Xylander (1987c), reproduced with permission of VEB Fischer-Verlag, Jena.)

Xylander (1987c) described eight receptor types, five uniciliate and three below the surface. In the ciliate receptors, the diameter of the sensory cilia is larger than that of epidermal and protonephridial cilia, but cilia have the typical 9 × 2 + 2 pattern of microtubules. Unicilate receptors have electron-dense collars as characteristic of sensory receptors of Neodermata.

Uniciliate receptors (Figure 34 A–E) differ in the length and shape of the cilium, presence or absence of a ciliary rootlet, the number of electron-dense collars, and the position on the body. One of the receptors is located in a deep pit of the epidermis which forms many microvilli around the sensory cilium. One non-ciliate receptor (type VI receptor) has an enlarged ciliary cross-striated rootlet and two electron-dense collars (Figure 34F), another type (type VII) possesses a distal basal body (Figure 34G) and a presumptive photoreceptor possesses lamellae derived from membranes of approximately 15 cilia that protrude into an intracellular cavity. The lamellae divide into several flattened branches forming rolled up whorls (Xylander, 1984) (Figure 34H).

The gross morphology of the protonephridial system of larval *Gyrocotyle urna* was described by Malmberg (1974) (Figure 35). Typically, on each side of the body, one pair of anterior and a single posterior flame bulb open into a duct that opens in the anterior third of the body; left and right ducts are connected by a posterior commissure. Rarely there are two anterior and one posterior pair of flame bulbs. The ultrastructure of protonephridia was examined by Xylander (1987d), who found altogether six terminal cells, at least two proximal canal cells, two distal canal cells and two nephridiopore cells. The filtration apparatus (weir) of the flame bulbs consists of internal ribs continuous with the terminal, and of external ribs continuous with the proximal canal cell (Figure 36A, B). The weir lacks longitudinal cords connected by a septate junction, and the proximal canal cell lacks a junction (Figure 36C), but has short microvilli (Figure 36A). The distal canal cell possesses many irregularly shaped microvilli, and the nucleus of the nephridiopore cell is located some distance from the nephridiopore in the interior of the body. Different canal cells are connected by septate desmosomes (Figure 36D).

The hooks of *Gyrocotyle urna* have a blade, guard and handle (Figure 37). Under the electron microscope, an outer homogeneous, moderately electron-dense layer, followed by a more translucent hollow cylinder and a more electron-dense core can be distinguished. Near the area of insertion, which forms a knob-like swelling, the outer layer is subdivided into three sublayers. A comparable but less elaborate swelling is found at the tip of the guard. Hooks are formed in oncoblasts that are degenerate in the free-swimming larva, in which only a thin layer of cytoplasm is left, around the handle and part of the guard, over a length of about 12 μm. It is connected by a cell junction to the "first cavity cell" and this in turn to the epidermis of the caudal cavity (Xylander, 1991).

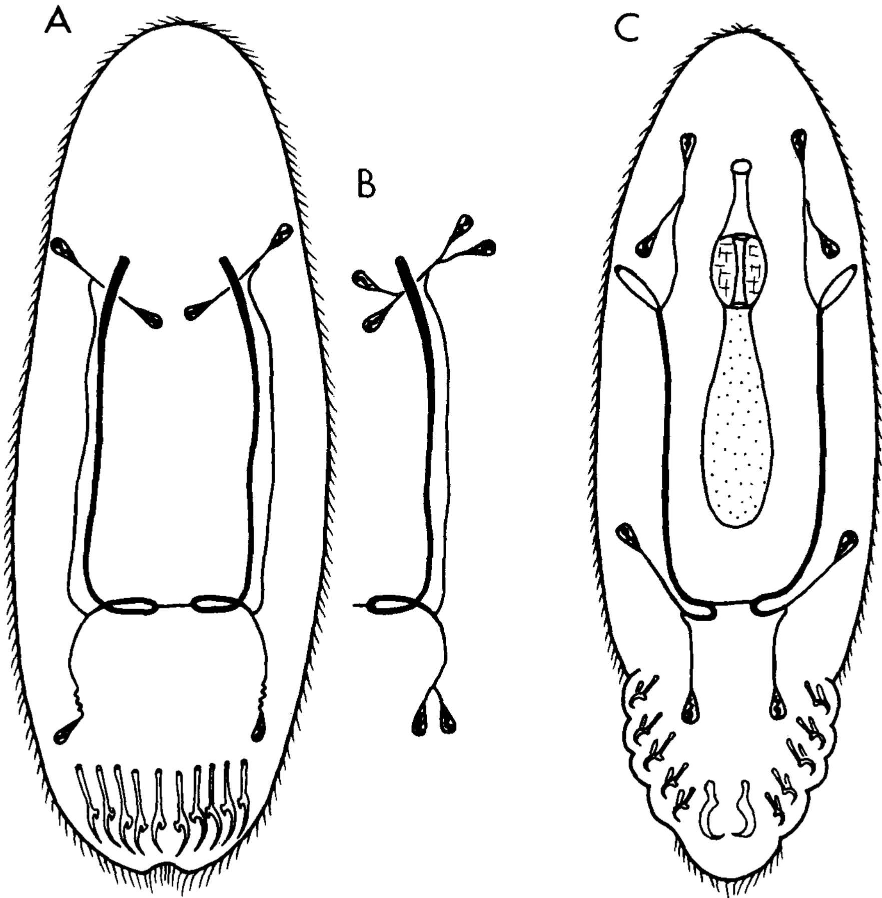


Figure 35 *Gyrocotyle urna*, lycophore larva. (A) Normal protonephridial system. (B) Atypical flame bulb condition, found in one body half in two out of approximately 75 *G. urna* lycophores. (C) The oncomiracidium (monogenoidean larva) protonephridial system, schematized after different authors. Observe the similarities between the posterior system and the *Gyrocotyle urna* protonephridial system and the presence in the oncomiracidium of an anterior system, a mouth, a pharynx and intestine. (From Malmberg (1974), reproduced with permission of the Norwegian Academy of Science and Letters.)

In contrast to larval *Austramphilina*, where the surface layer around the hooks is formed by tegument, the caudal surface layer in *Gyrocotyle* is the epidermis.

The 10 hooks of larval *Gyrocotyle* are simultaneously formed during embryogenesis (Malmberg, 1974; Xylander, 1991). In eggs cultured at 6° C, hooks are not completely formed before the 47th day after dissecting them

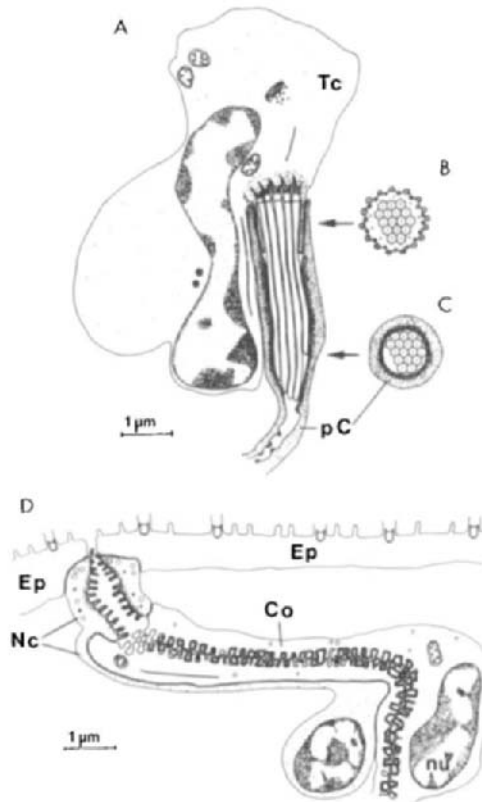


Figure 36 *Gyrocotyle urna*, lycophore larva. Protonephridium. (A) Longitudinal section through the terminal cell. (B) Cross-section through the filter region. (C) Cross-section through the proximal canal. Arrows indicate the level of section. (D) Nephridiopore region and site correlation of the nephridiopore cell and the distal canal. Co, distal canal cell; Ep, epidermis; Nc, nephridiopore cell; nu, nucleus; pC, proximal canal cell; Tc, terminal cell. (From Xylander (1987d), reproduced with permission of Springer-Verlag.)

out of the worm, and development starts at the most distal portions of the hooks.

Interesting and unique is the occurrence of "post-larvae", the smallest of which are hardly larger than free-swimming larvae but have lost their cilia, in the parenchyma of immature and mature worms. Usually only a few are present (e.g. Simmons, 1974; Malmberg, 1979), but in one case up to 101 post-larvae were reported in *Gyrocotyle fimbriata* (Lynch, 1945). Post-larvae undergo development, while in the parenchyma (see review by Williams *et al.*, 1987).

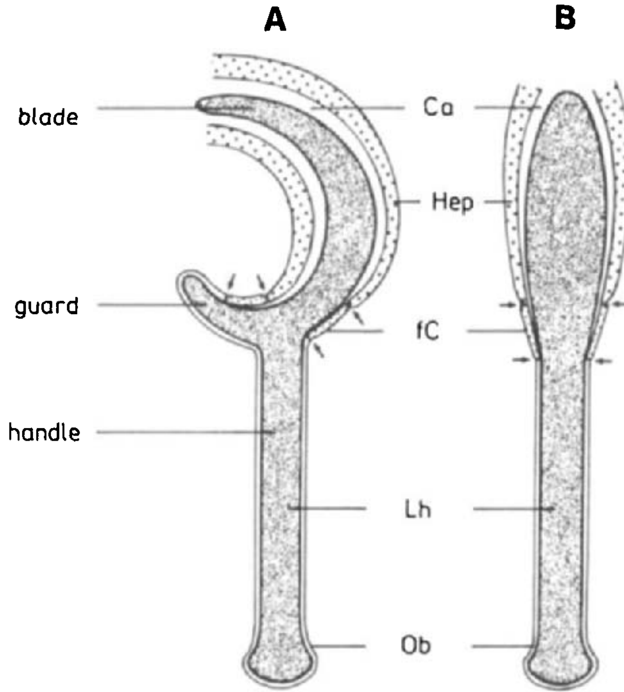


Figure 37 *Gyrocotyle urna*, lycophore larva. Larval hooks and associated tissues. (A) Lateral view. (B) Frontal view. (Arrows indicate the cell junctions between oncoblast, first cavity cell and epidermis of the caudal cavity.) Ca, caudal cavity; fC, first cell of the caudal cavity; Hep, epidermis of the caudal cavity; Lh, larval hook; Ob, oncoblast. (From Xylander (1991), reproduced with permission of Springer-Verlag.)

4.3. Structure of the Adult

Various authors have described the gross-morphology of gyrocotylids (e.g. Haswell, 1902; Lynch, 1945; Manter, 1951; van der Land and Templeman, 1968, Coil, 1991).

The ultrastructure of the tegument of adult *Gyrocotyle urna* was described by Lyons (1969). Subtegumental perikarya are connected by multiple processes containing microtubules to the syncytial surface tegument (neodermis) that has many microvilli. Each microvillus contains a cylinder of electron-dense material just inside the plasma membrane, commencing at the base and terminating just short of a terminal spike. The ventral surface of the rosette is lined by secretory cells secreting PAS positive material. Microvilli on the ventral surface of the rosette are bifurcate. Laurie (1971) showed that *G. fimbriata* and *G. parvispinosa* absorb glucose, galactose and α-methylglucoside

readily, but xylose, mannose, lactose and inulin less readily, apparently through the tegument. An especially effective inhibitor of carbohydrate absorption was phlorhizin but not phloretin. The two species differed in their permeability.

As in *Amphilina* and the eucestode *Diphyllobothrium*, the protonephridial ducts of *Gyrocotyle* form a reticulum (Xylander, 1992a). The flame bulb is similar to that described for eucestodes and *Austramphilina* (see Figure 23; Rohde 1990b, 1991), that is, both a terminal and proximal canal cell contribute to the formation of the weir that lacks longitudinal cytoplasmic cords connected by a septate junction. The proximal canal also lacks a septate junction. The nucleus of the first canal cell is located in the wall of the canal, whereas the perikarya of more distally located canals are subepithelial. Larger capillaries of *Gyrocotyle* have lateral flames (Xylander, 1992a).

Xylander (1992b), in a preliminary notice, reported the occurrence of six types of sensory receptors at the anterior end of *Gyrocotyle urna*, in addition to the types described by Lyons (1969) and Allison (1980). Thus, to date 10 receptor types have been reported. None of the receptors corresponds to the eight receptor types found in larval *Gyrocotyle* (Figure 34).

Light-microscopic studies of spermatogenesis of *Gyrocotyle* were made by Bazitov and Lyapkalo (1982), ultrastructural studies of spermiogenesis in *Gyrocotyle urna* by Xylander (1989a). It is similar to *Austramphilina* and other Neodermata. In early spermatids microtubules appear at the cell periphery, and a median process and two flagella with 9 + "1" axonemes grow out. Flagella are at an angle of about 60° to the axis of the median process and have cross-striated rootlets. The nucleus and mitochondria grow into the median process, and flagella become parallel with the process and are incorporated into it. Mature sperm have neither basal bodies nor rootlets and sperm ducts are lined by a syncytial epidermis and contain cilia (Xylander, 1989a).

The ultrastructure of the vitellaria, vitellogenesis and yolk duct of *Gyrocotyle urna* was described by Xylander (1987b). Yolk follicles are surrounded by an extracellular lamina, whereas vitellocytes and the periphery of the yolk follicles are enclosed by a sheath of cytoplasm. Immature vitellocytes have relatively little cytoplasm. During maturation, the relative volume of cytoplasm increases, and many Golgi bodies, egg-shell granules, lipid droplets as well as endoplasmic reticulum develop. Mature yolk cells are largely filled by lipid droplets and egg-shell granules. Yolk ducts are ciliated, and have intraepithelial nuclei and intraluminal lamellae. Lateral cell membranes were not seen in the wall of the vitelloducts.

4.4. Taxonomy, Ecology and Phylogenetic Relationships

There has been considerable confusion about the taxonomy of gyrocotylids (e.g. Lynch, 1945 and earlier references therein; recent review by Williams

et al., 1987). According to van der Land and Dienske (1968, see also Simmons, 1974), each species is apparently restricted to one chimaerid host species. In each host, a second and much rarer species of *Gyrocotyle* is found. The common species (*urna*-type) of different hosts are related to each other, and so are the rare ones (*confusa*-type). Lynch (1945), on the other hand, states that "although existing data are inadequate for a positive decision, it is very likely that *G. urna* also occurs in *Chimaera ogilbyi*, and the existence of *G. urna* in *Callorhynchus antarcticus* is probable". He reported *G. urna* from its usual host, *Hydrolagus colliei*, which also harbours *G. fimbriata*. Van der Land and Templeman (1968) described two new species, *G. major* and *G. abyssicola*, from *Hydrolagus affinis*, and van der Land and Dienske (1968) described *G. confusa* from *Chimaera monstrosa* and *G. parvispinosa* from *Hydrolagus colliei*, the latter apparently the senior synonym of *G. urna* from this host reported by Lynch (1945). For other records see Johnson and Horton (1972), Khalil (1975), Ichihara (1976) and Hogans and Hurlbut (1984).

Szidat (1968) suggested that *Gyrocotyloides nybelini* may be the male phase during development of *Gyrocotyle urna*. Laurie (1971) found 95 % of approximately 1500 *Hydrolagus colliei* in the San Juan Archipelago infected with gyrocotylids (*G. fimbriata* and *G. parvispinosa*), usually two worms per fish, always of one species only. Williams *et al.* (1987), in their review, stressed that mixed infections occur only very rarely, as reported by a number of authors for several species pairs. They discuss the ecological implications of these findings, suggesting that the most likely explanation is that population structure in *Gyrocotyle* is controlled by homeostatic mechanisms in the parasite infrapopulation (within the host) (see also Halvorsen and Williams, 1967, 1968). Simmons and Laurie (1972) reported *G. parvispinosa* and *G. fimbriata* from *Hydrolagus colliei*, occupying different though overlapping habitats in the intestine (other species pairs infecting one host species do not seem to inhabit different habitats, Williams *et al.*, 1987). They also reported that worms from larger hosts are larger. Allison and Coakley (1973) found two species, *G. rugosa* and *G. maxima*, in *Callorhynchus milii* in New Zealand. Prevalence of infection was 83.8% for the former and 3.1% for the latter species, with some seasonal variation. Sixty-six % of fish had two worms, and infections with three or four mature worms occurred only in fish longer than 50 cm. In heavier infections than four, worms were small and immature. Colin *et al.* (1986) examined 1136 *Chimaera monstrosa* in Norway and recovered altogether 1361 gyrocotylids. They noted a high degree of morphological variation and encountered difficulties in using morphological characters previously considered to be important in segregating the three "forms" (*Gyrocotyloides nybelini, Gyrocotyle urna, G. confusa*). They concluded that *G. nybelini* and *G. confusa* are synonyms of *G. urna*, and suggested further research on the "species pairs" in other chimaerids. Following this suggestion, Bristow and Berland (1988) used electrophoresis

of 15 enzyme systems of these three "forms" that revealed consistently scorable results in five of the systems. According to the authors, "the results clearly show differences at the species level between *Gyrocotyle urna* and *G. confusa* and *Gyrocotyloides nybelini*. The relationship between *G. nybelini* and *Gyrocotyle urna* is less clear but the data support the retention of the species designator while bringing into question the validity of the genus *Gyrocotyloides*".

Berland et al. (1990) studied 16 fatty acids from five gyrocotylids, the three species discussed above from *Chimaera monstrosa*, and two (*Gyrocotyle fimbriata* and *G. parvispinosa*) from *Hydrolagus collei*. They obtained complete separations of the five species, supporting earlier species distinctions based on morphology. Simmons *et al.* (1972) used DNA hybridization to study taxonomic relationships between species of *Gyrocotyle* and concluded that *G. rugosa* and *G. fimbriata* are more closely related to each other than to *G. maxima* and *G. parvispinosa*. Bandoni (1987) revised the Gyrocotylidea and provided a cladistic analysis of the group. She concluded that about half of the observed associations between gyrocotylids and their hosts can be attributed to coevolution, the other half to colonization.

4.5. Physiology

Apparently because of its large size and common occurrence, *Gyrocotyle* has been used for some physiological studies. Koopowitz (1973) described responses to electrical stimulation. Preparations have high thresholds and rather low twitch contractions, and direct stimulation often causes loss of muscular tone. The author attributes this, as well as a reduction in responsiveness when repeatedly stimulated, to the presence of inhibitory neurons. Facilitation of the response is of short duration, and rhythmical spontaneous contractions can be observed. Stimulation of one side of the body leads to the contraction of the other side if the anterior nerve commissure is intact. Keenan and Koopowitz (1982) tested a range of putative neurotransmitter drugs but found responses only to 20 L-glutamate and L-aspartate. Keenan *et al.* (1984) found that conduction velocities in the anterior–posterior direction were greater than in the posterior–anterior direction. They also studied responses to mechanical stimulation. Ammonia formation and amino acid excretion by *G. fimbriata* were studied by Bishop (1975).

5. THE UDONELLIDEA

The morphology of *Udonella* at the light-microscopic level has been described in detail by Ivanov (1952) and van der Land (1967). In particular, Ivanov's

study gives many histological data on the muscles, parenchyma, pharynx, head glands, intestine, protonephridial system including paranephrocytes, nervous system and reproductive organs. Ivanov discussed the relationship of the udonellids with the Monogenea and Temnocephalida and arrived at the conclusion that they represent a class of their own (Udonellidea), ranked at an equal level with the Digenea, Temnocephalida, Monogenea, Gyrocotylidea and Cestoda. Van der Land (1967) did not accept Ivanov's proposal to establish a separate class, but listed the following differences between *Udonella* and the "other" Monogenea: (i) lack of hooks and a ciliated larva; and (ii) a different excretory system. He proposed three subclasses: Udonellida, Monogenea s.str. and Gyrocotylida within the class Monogenea.

Schell (1972) studied the early development of *Udonella caligorum* on *Lepeophtheirus hospitalis* from *Platichthys stellatus* caught near San Juan Islands, Pacific North America. Eggs kept at 8–10°C hatched in 14–16 days. Cleavage began in eggs that had left the uterus.

Kornakova (1983, 1985a, 1987a, 1988) made detailed studies of the tegument, digestive and reproductive systems of *Udonella murmanica*. She considered the "insunk epithelium" of Udonellida and other parasitic platyhelminths as an independently evolved feature and emphasized several peculiarities of Udonellida (Kornakova, 1987b): (i) only one type of anterior gland in *Udonella* (but at least two in Monogenea); (ii) two types of opisthaptor glands represented by modified epithelial cells; (iii) syncytial, insunk gastrodermis; (iv) a combination of a primitive general structural plan with very specialized features in the reproductive system; (v) presence of an ootype (considered to be an adaptation to a parasitic way of life); (vi) presence of paranephrocytes (found in udonellids only, see also Nichols, 1975); (vii) the posterior disc is a terminal (and not ventral) structure; (viii) highly centralized nervous system; and (ix) a peculiar innervation of the posterior disc (different from that of the Monogenea). She concluded that there are no reasons for including the udonellids in the Monogenea, and that udonellids represent an order of Turbellaria, whose ancestors are the Neorhabdocoela, and most probably Typhloplanoida. The parenchyma of the species is composed of three types of cells (Kornakova, 1989).

Xylander (1987e, 1988b), in contrast, on the basis of electron-microscopic studies, pointed out that *Udonella*, like the Neodermata, have a neodermis, sensory receptors with electron-dense collar, spermatozoa with incorporated axonemes but without dense bodies, and a posterior haptor. Hence the Udonellidae must be included in the Neodermata.

Electron-microscopic studies of Rohde *et al.* (1989) support Xylander's view. They verified that sensory receptors, tegument and sperm are indeed of the "neodermatan type". In addition, they showed that the protonephridial system has flame bulbs formed by a terminal and proximal canal cell as in other Neodermata (see also Rohde, 1990b). However, flame bulbs lack longitudinal cytoplasmic cords and proximal canals lack septate junctions

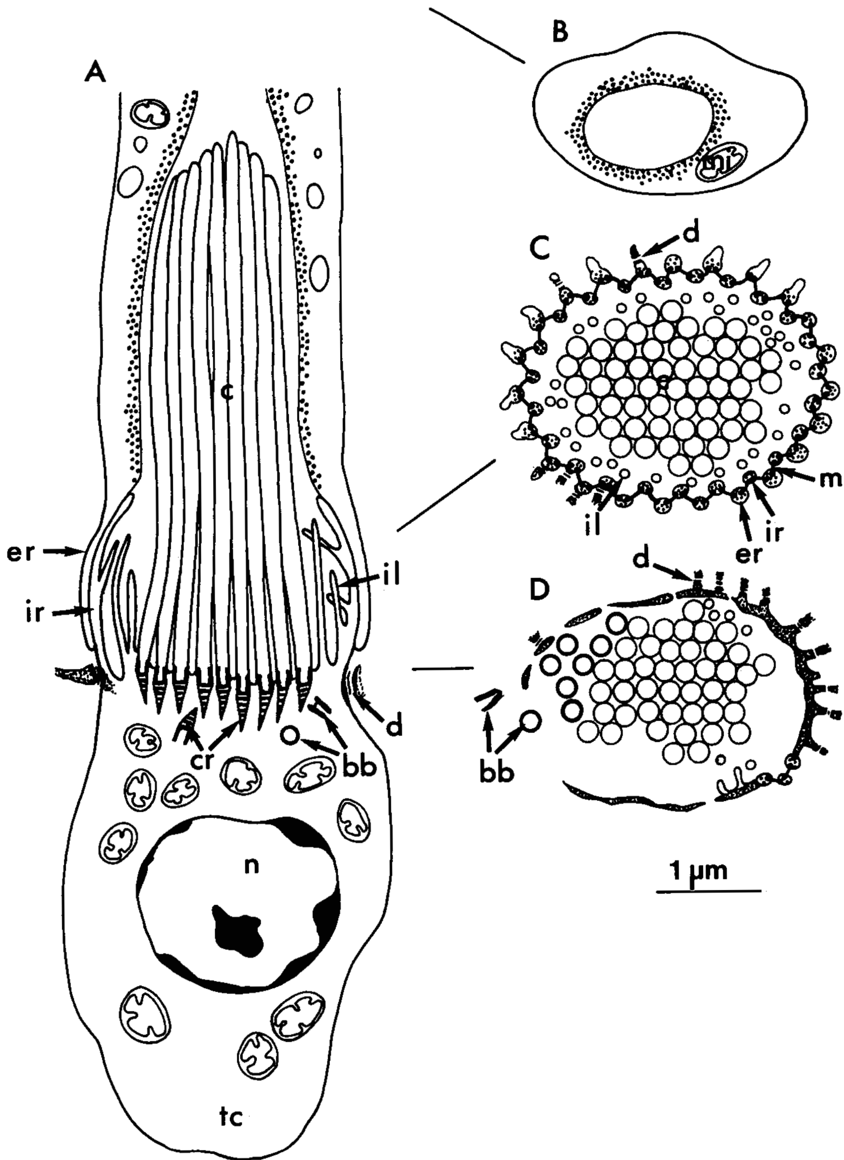


Figure 38 *Udonella*, flame bulb of adult. (A) Longitudinal section. (B) Cross-section at level of proximal capillary. (C) Cross-section at level of weir. (D) Cross-section at level of base of weir. bb, basal body; c, cilium; cr, ciliary rootlet; d, desmosome; er, external rib; il, internal leptotrich; ir, internal rib; m, "membrane". (From Rohde (1990b), reproduced with permission of Pergamon Press.)

(Figure 38). Thus, *Udonella*, in the structure of the protonephridia, resembles the cestodes (Gyrocotylidea, Amphilinidea and Eucestoda), but not the Monogenea and Trematoda (Rohde, 1990b).

Kornakova (1985b) discussed the distribution of udonellids on their hosts, which are Copepoda of the suborder Siphonostomatoida parasitizing fishes of the suborder Percomorpha. She suggested that the original hosts of udonellids were fishes.

Because of the lack of sclerites, species distinctions are difficult and it may well be that the common and widely distributed "species" *Udonella caligorum* is a species complex comprised of several species. In Australia, Byrnes and Rohde (1992) reported *Udonella "caligorum"* from two related copepod genera on two not closely related fish species, *Caligus* on *Acanthopagrus australis* in eastern Australia and *Lepeophtheirus* on *Latris lineatus* in Tasmania. The authors pointed out that there may be more than one Australian species of the genus. Enzyme and DNA studies would be useful to distinguish such "cryptic" species. Specimens thought to be *U. caligorum* were recovered from *Lepeophtheirus hippoglossi* on *Hippoglossus hippoglossus* from northern Norway (Schram and Haug, 1988) and from two caligoids on *Mugil cephalus* from Biscayne Bay, Florida (Skinner, 1975).

Kornakova and Timofeeva (1981) described *U. murmanica* from *Caligus curtus* on *Gadus morhua* on the eastern coast of Sachalin, and van der Land (1967) described *U. papillifera* from *Ceratocolax euthynni* on *Euthynnus aletteratus* from the Gulf of Guinea, western Africa.

Kabata (1973) and Byrnes (1986) examined the distribution of *Udonella* on their copepod hosts (for earlier studies see Sproston, 1946 and Causey, 1961). According to Kabata, who analysed a great number of data, only about 6% of worms on female copepods were carried on the ventral surface and only about 20% were attached to areas from where worms would easily reach tissue debris created by the feeding copepod. Approximately 73% of the worms were attached to the margins of the lateral zones of the dorsal shield, from where they have easy access to the fish. Likewise, Byrnes found that most actively feeding mature worms congregate in areas that do not allow them to take advantage of fish debris produced by the feeding copepod, but most worms have ideal positions to feed directly on the surface tissues of the fish host. According to Timofeeva (1977), mature *U. caligorum* are found only on female copepods (*Caligus curtus* on *Gadus morhua* in the Sea of Murmansk), usually attached to the egg sacs, and eggs are attached to the copepods' egg sacs. Attachment of *Udonella* larvae to nauplius larvae was not seen, and infection of other hosts is thought to be by contact transfer. Minchin (1991) captured three free-swimming *Caligus elongatus* with a plankton sampler, two of which were infected with *U. caligorum*, indicating that infection of other fish hosts may be by infected, free-swimming copepods.

6. PARASITIC TURBELLARIA

Symbiotic (commensal and parasitic) species are found among several groups of Turbellaria. Most attention has been paid to species infecting various echinoderms (e.g. Hickman, 1955; Kozloff, 1965; Cannon, 1982, 1986, 1987, 1990; Shinn, 1983, 1985a, b, 1986; Jondelius, 1986, 1988, 1991, 1992a, b, c; Ubelaker *et al.*, 1986; Jennings and Cannon, 1987; Rohde and Watson, 1988b; Rohde *et al.*, 1988, 1992a; Jennings and Hick, 1990; Li *et al.*, 1992), and to the Fecampiidae (e.g. Christensen and Kanneworff, 1964, 1965; Kanneworff and Christensen, 1966; Bresciani and Køie, 1970; Køie and Bresciani, 1973; Christensen, 1976, 1981a, b, 1988; Shinn and Christensen, 1985; Blair and Williams, 1987; Williams, 1988a, b, 1989, 1990a, b, c, d, 1991; Sluys and van Ginkel, 1989; Watson *et al.*, 1992a, b). Other species and general aspects were discussed by Karling and Nilsson (1974), Newton (1975), Burt and Bance (1981), Jennings (1980, 1981), MacKinnon *et al.* (1981), Pike and Wink (1986), Cifrian *et al.* (1988), Tyler and Burt (1988), Noury-Sraïri *et al.* (1989a, b, c, d, 1990), and Rohde *et al.* (1990). Rieger *et al.* (1991) have given an excellent and comprehensive account of morphological including ultrastructural aspects of Turbellaria. In the present review, I therefore restrict myself to briefly discussing some ultrastructural characteristics that cast light on the phylogenetic relationship of symbiotic turbellarian taxa with the Neodermata (= all non-turbellarian taxa of Platyhelminthes including those discussed in the previous sections).

Most authors (e.g. Ehlers, 1985) assume that the sister group of the Neodermata is to be found among the dalyelliid turbellarians. Brooks *et al.* (1985) Brooks (1989a, b) and Justine (1991) consider the Temnocephalida to be that sister group and Cannon (1986) tentatively suggested that the ancestors of different groups of Neodermata are different symbiotic taxa of Turbellaria. Ehlers (1985) and Rohde (1990b) considered it possible that the Fecampiidae are the sister group of the Neodermata.

Among three species of umagillids (parasitizing echinoderms) examined, one species has vertical and horizontal ciliary rootlets as characteristic of typical Rhabdocoela, in one species the vertical rootlet is only weakly developed, and in a third species only a horizontal rootlet as found in the Neodermata is present (Rohde and Watson, 1988b; Rohde *et al.*, 1988). This might indicate a relationship with the Neodermata. However, ultrastructural studies of the protonephridia of many parasitic and free-living Platyhelminthes have shown that all Neodermata have flame bulbs formed by two cells, a terminal cell and a proximal canal cell. Such flame bulbs, although differing in detail, are also found in most turbellarians as well as in some non-platyhelminth lower invertebrates, and must be considered the plesiomorphic conditions (Figure 39) (Rohde, 1991). All Rhabdocoela including the Dalyelliida and Temnocephalida, in contrast, have flame bulbs formed by a single cell

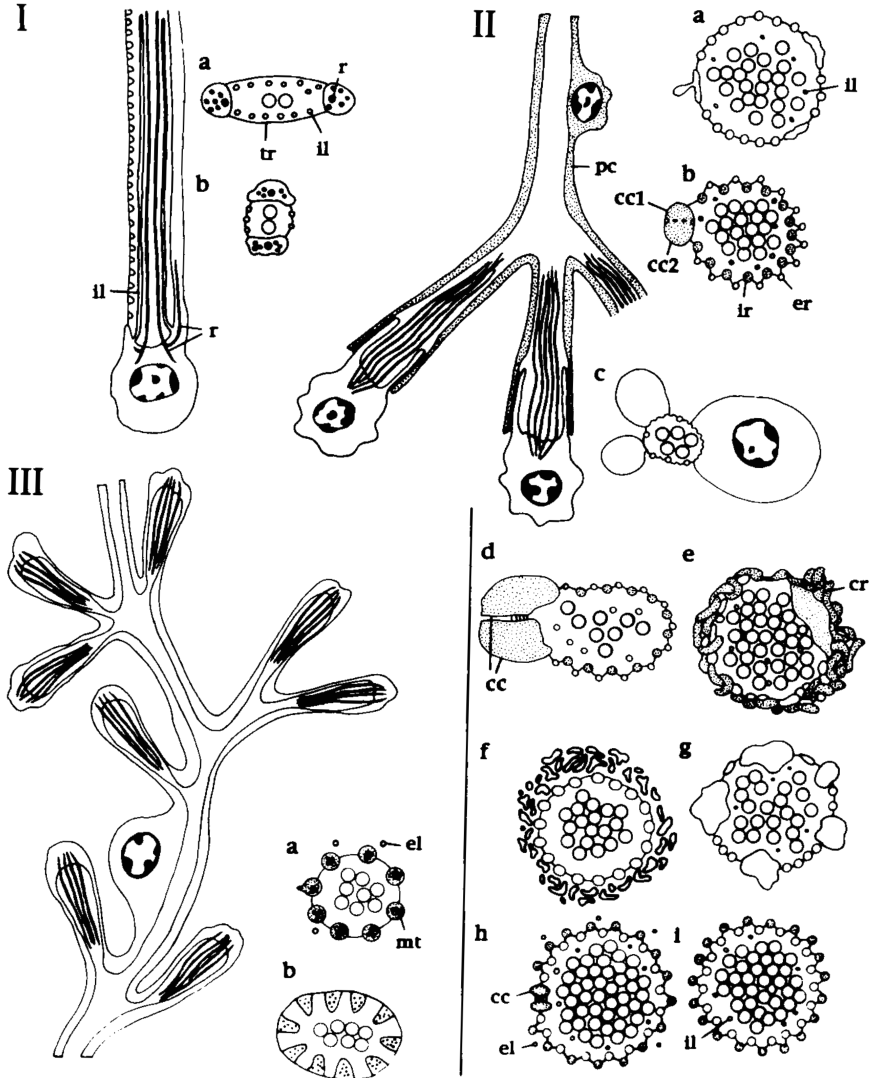


Figure 39 Diagrams of a representative longitudinal section of each of the three main types of flame bulb (terminal part of the protonephridial system), and of cross-sections of various kinds within each main type. I. Flame bulb type I. Catenulida: *Catenula* and *Stenostomum* (Ia), *Retronectes* (Ib). II. Flame bulb type II, Macrostomida: *Microstomum* (IIa), *Macrostomum* (IIb), Polycladida: Götte's larva of *Stylochus* (IIc), Proseriata: *Monocelis* (IId), Prolecithophora: *Urastoma* (IIe), *Archimonotresis* (IIf), Tricladida (IIg), Neodermata: Trematoda/Monogenea (IIh), Gyrocotylidea/ Amphilinidea/Eucestoda (IIi). III. Flame bulb type III. "Turbellarian" Rhabdocoela (IIIa), Lecithoepitheliata (IIIb). cc, cytoplasmic cord; cr, coiled rod; cc_1, first canal cell; cc_2, second canal cell; el, external leptotrich; il, internal leptotrich; mt, microtubule; pc, proximal canal cell; r, rootlet of cilium; tr, transverse rib. (Based on Rohde (1991) with additional data on Catenulida, Polycladida, and Proseriata.)

(and many flame bulbs are formed by one perikaryon, the nucleus located either in the capillary wall or between the flame bulbs (Rohde, 1991; Rohde *et al.* 1992a). Furthermore, the filtration apparatus (weir) of the rhabdocoels consists of a single layer of longitudinal ribs containing bundles of microtubules, whereas the weir of the Neodermata consists of two rows of ribs lacking microtubules. The condition in the Rhabdocoela cannot be plesiomorphic but must be considered synapomorphic for the group (Rohde, 1991). Hence, neither Temnocephalida nor Dalyelliida can be the sister group of the Neodermata, at least not in the sense implied by various authors — that the Neodermata have evolved from a temnocephalid or dalyelliid-like ancestor.

The Fecampiidae are usually included in the Rhabdocoela. However, an electron-microscopic examination of the protonephridia of *Kronborgia* (a genus belonging to the group) has shown that its flame bulbs are not of the rhabdocoel type (Figure 40) (Watson *et al.*, 1992a). Although the weir is not well developed, it resembles that of the Neodermata. Furthermore, Watson *et al.* (1992b) have shown that *Kronborgia* has a photoreceptor known, among the Platyhelminthes, only from polystome Monogenea (Figure 41), and illustrations seem to indicate that larval and female *Kronborgia* of one species may have epidermal cilia with a single, horizontal, rootlet characteristic of the Neodermata (Bresciani and Køie, 1970; Køie and Bresciani, 1973), although larval and male *K. isopodicola* have vertical and horizontal rootlets (Williams, 1990b, 1991; Watson *et al.* 1992a). *Kronborgia*, like the Neodermata, also has sperm with two axonemes incorporated in the sperm body (Williams, 1988b) and fusion of axonemes in spermiogenesis of *K. isopodicola* is proximo-distal as in the Neodermata and not distal-proximal as in the Turbellaria (Watson and Rohde, 1993). Occurrence of several, apparently independent, characteristics may indicate that the Fecampiidae are indeed the sister group of the Neodermata. That they should not be included in this taxon, is indicated by the lack of neodermis and electron-dense collars of their sensory receptors (Køie and Bresciani, 1973).

7. THE PHYLOGENY OF PARASITIC PLATYHELMINTHES

The phylogeny of Platyhelminthes was discussed by Brooks (1982, 1989a,b), Cable (1982), Bazitov (1984), Ehlers (1985, 1986), Joffe (1987), Brooks *et al.* (1985), Rohde (1988, 1990b and further references therein), Ivanov (1991), and Justine (1991), largely based on morphological including ultrastructural data. Recent DNA studies have confirmed that all Neodermata (Trematoda, Digenea and Aspidogastrea, Monogenea, Gyrocotylidea, Amphilinidea, Eucestoda) form one monophylum (Figure 42) (Baverstock *et al.*, 1991; Blair, 1993; Rohde *et al.*, 1993). As suggested by Rohde (1990b), the Dalyellioida (including the Temnocephalida) cannot be considered the sister group of the

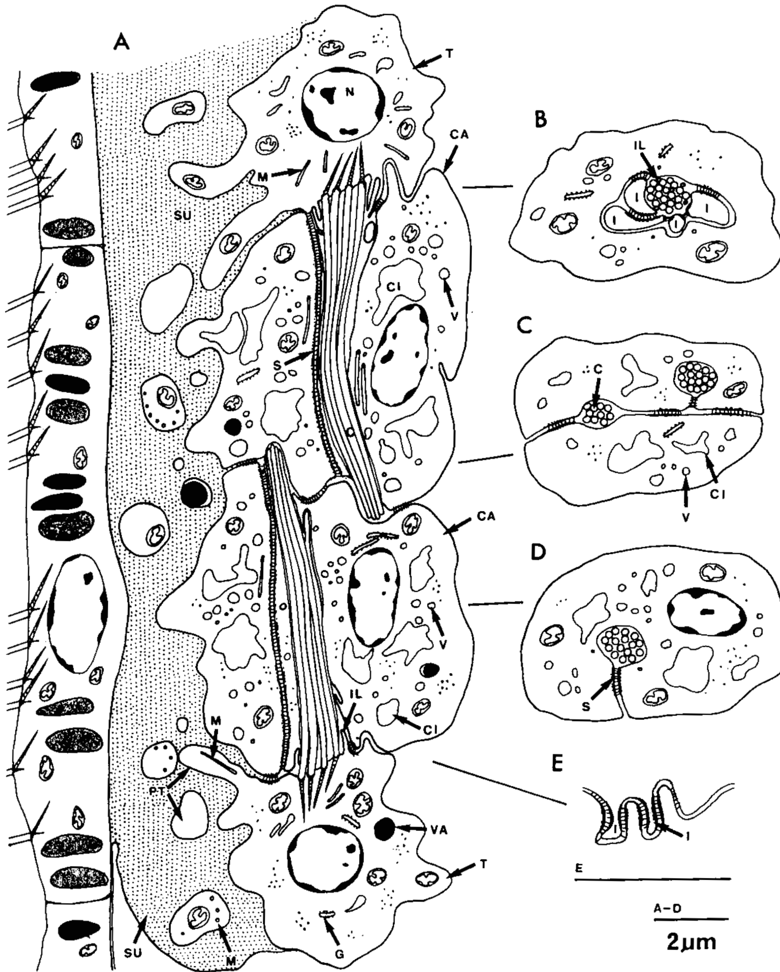


Figure 40 Diagrams of flame bulbs of *Kronborgia isopodicola*. (A) Longitudinal section. (B)–(D) Cross-section at levels indicated. (E) Longitudinal section at level of base of flame. Note epidermal cilia with large horizontal and rudimentary vertical rootlets, large subepidermal space (dotted) separated from lumen of flame bulbs only by septate junctions, and two flames in opposite directions, protruding into intercellular space between two canal cells (A). Each canal cell with longitudinal septate junction, large cisternae, many vesicles, some vacuoles with electron-dense material, mitochondria, granular endoplasmic reticulum, and microtubules. Terminal cells (and probably canal cells) with large cytoplasmic processes protruding into subepidermal space, and large interdigitating processes of terminal and canal cells at base of flame (B, E). (From Watson *et al.* (1992a), reproduced with permission of Editrice Compositori Bologna.)

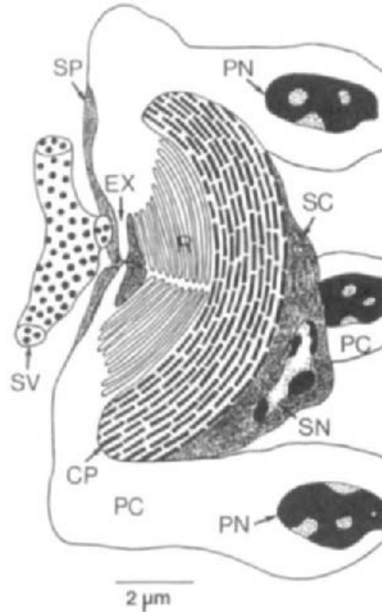


Figure 41 Diagram of photoreceptor of *Kronborgia isopodicola*. Note three receptor cells, each with a nucleus (PN) and rhabdomere (R, only two drawn), one supportive cell (SC) with shrivelled nucleus (SN), several layers of concentrically arranged crystalline platelets (CP), and three processes (SP, two visible) "embracing" the rhabdomeres, their ends connected by a septate junction. Also note branched processes of (neuro-?) secretory cell containing electron-dense secretory vesicles (SV). EX processes of photoreceptor cells (PC). (From Watson *et al.* (1992b), reproduced with permission of Pergamon Press.)

Neodermata, but a larger taxon comprised of the Proseriata, Rhabdocoela and Tricladida may be that sister group. In other words, as suggested by Rohde (1990b), the neodermatan line has split off the turbellarian line much earlier than usually assumed. Blair's (1993) study also has shown that gyrocotylids and amphilinids are closely related to the eucestodes (i.e. that *Gyrocotyle* is *not* a monogenean as claimed by Baverstock *et al.*, 1991), and that the Aspidogastrea are indeed "primitive" neodermatans, representing either the sister group of the other trematodes, or the sister group of all other Neodermata (Figure 43). The latter view is not supported by the findings of Rohde *et al.* (1993).

8. FUTURE RESEARCH

Work on the life cycles of Aspidogastrea other than species of the family Aspidogastridae, and of *Gyrocotyle*, is most urgently required. Ultrastructural

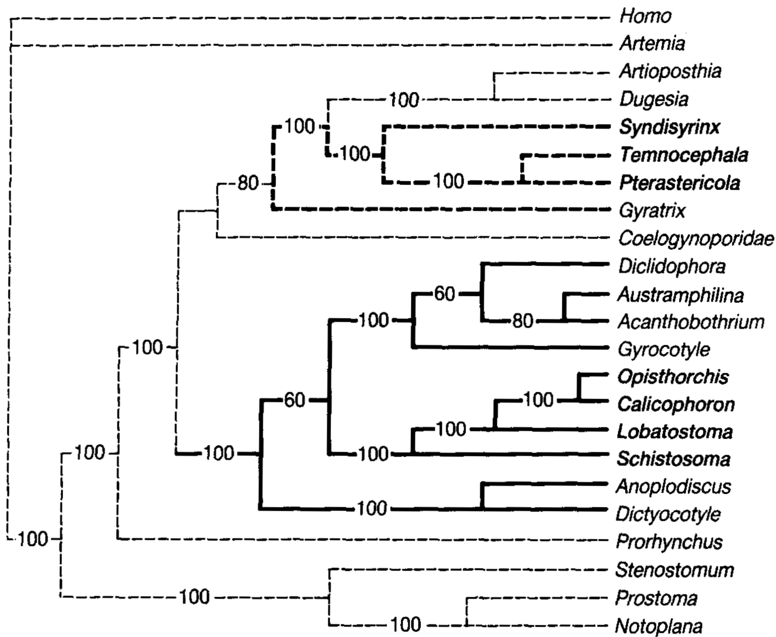


Figure 42 Phylogeny of Platyhelminthes based on partial sequences of 18S ribosomal DNA. Twenty-three taxa, truncated data. Fifty per cent majority-rule consensus of the five equally most parsimonious trees. Numbers indicate proportion (%) of trees supporting a clade. Bold unbroken lines: Neodermata (bold print: Trematoda Aspidogastrea (*Lobatostoma*) plus Digenea); bold broken lines: Rhabdocoela (bold print: Dalyellioida).

studies of various aspects, in particular of spermatogenesis, of the development of the protonephridia and of the pharynges of additional groups of free-living and symbiotic Turbellaria may cast light on the phylogenetic relationships of the various groups, and DNA — studies using longer sequences and additional taxa may lead to a definitive phylogenetic system, if such a system is indeed feasible. Rohde (1990b), among others, has drawn attention to the possibility that, due to horizontal gene transfer, eukaryotic organisms might not evolve as a whole, but that the units of evolution might be character traits, exchangeable between phylogenetic lines and resulting in a process that might be called "mosaic evolution". If such mosaic evolution is a common process, different DNA sequences, as well as different morphological traits of the same species, might yield different phylogenetic trees.

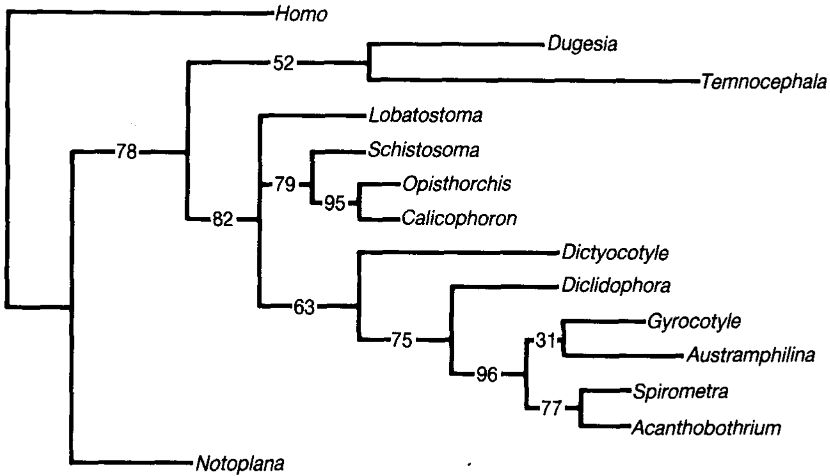


Figure 43 Phylogeny of Platyhelminthes based on partial sequences of 18S ribosomal DNA. Fifty per cent majority rule consensus tree following 100 bootstrap replicates using heuristic search option in PAUP. Tree length 523, CI 0.738 (0.607 if uninformative sites excluded). Numbers on internodes indicate percentage of bootstrap trees which contained the group of taxa to the right of the internode. (From Blair (1993), reproduced with permission of Pergamon Press.)

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Sarcoptes scabiei and Scabies

Ian Burgess

Medical Entomology Centre, University of Cambridge, Cambridge Road, Fulbourn, Cambridge, CB1 5EL, UK

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1. INTRODUCTION

Scabies is an allergic irritant condition resulting from the burrowing of the mite *Sarcoptes scabiei* in the mammalian epidermis. A wide range of species

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is affected and the symptoms associated with the infection vary considerably in intensity, distribution and the resultant morbidity.

The mite itself and the disease of scabies were recognized by a number of scholars of the ancient world including Aristotle and Cicero. However, it was not until 1687 that the true nature of the causal link between the mite and the disease was first recognized by Bonomi and Cestoni in their famous letter to Francisco Redi (Friedman, 1947). Despite this, its translation by Richard Mead and subsequent dissemination after his death, the medical establishment was slow to accept the evidence preferring more esoteric explanations based on Galen's concept of foul "humours" emanating from the body being the cause of symptoms or that the occurrence of mites and symptoms simultaneously was coincidental. Final and universal acceptance was established only in the late 19th century with the publication of the treatise by Hebra (1868).

The history of scabies has been extensively described by Friedman (1947), Heilesen (1946) and Parish (1985). In recent times scabies has been regarded as a disease that follows epidemic cycles ranging from 20 to 30 years between peak levels of infection (Orkin, 1975; Editorial, 1976; Orkin and Maibach, 1978). This concept has arisen from the few records in countries where scabies is a notifiable condition and from the records of military hospitals. Despite suggestions for an international registry of scabies (Andrews, 1981; Alexander, 1984), only Czechoslovakia, Denmark, Norway and Poland currently have an obligatory system for reporting the disease. Of these, only Denmark has records that extend back as far as 1888, some of which are incomplete before 1900 (Christopherson, 1978). Nevertheless, the cyclical nature of the infection is clear in that country and a similar pattern has been observed in Czechoslovakia (Palicka, 1979a), Poland (Zukowski, 1989), Turkey (Tuzun *et al.*, 1980) and the Israeli armed forces (Kimchi *et al.*, 1989).

However, the reasons for such a phenomenon are unclear. It is unfortunate that the principal peaks in Europe and North America this century have coincided with major wars or other significant changes in social behaviour. Friedman (1941, 1947) attempted to attribute all upsurges of scabies to these causes but, as has been observed (Editorial, 1976), in the two World Wars the actual increase began some years before the social upheaval. Alternative suggestions include herd immunity (Shrank and Alexander, 1967; Editorial, 1976) and the inability of physicians to recognize scabies through lack of experience during the periods of low incidence, which results in a subsequent high level of transmission for a few years (Hellier, 1976; Cox and Paterson, 1991).

Such a situation applies strictly only in those countries where diagnosis and treatment are readily available to reduce the prevalence by freeing individuals from the disease. In those countries where there are inadequate medical facilities, a lack of available treatments, or simple ignorance, the underlying prevalence remains high (Christopherson, 1986) and undoubtedly resembles

that found world-wide before the introduction of effective treatments and improved social conditions. Friedman (1947) cites a number of surveys in which more than 30% of a population were scabietic constantly. Such a level would almost certainly mask any epidemic cycles or even prevent them from occurring.

Periodic outbreaks of scabietic mange also occur in a wide variety of mammals other than humans. In some species this may be severely debilitating and reduction of populations of predators such as foxes may result, such that a significant rise occurs in the populations of prey species like hares (Lindstrom and Morner, 1985; Dannell and Hornfeldt, 1987). In other cases the importance is related to morbidity and economic loss in domesticated species including pigs (Soll and Smith, 1987; Wooten-Saadi *et al.*, 1987b; Dalton and Ryan, 1988; Smeets *et al.*, 1989; Hollanders and Vercruysse, 1990; Kambarage *et al.*, 1990), goats (Ibrahim and Abu-Samra, 1985), cattle (Rusbaum *et al.*, 1975; Soll *et al.*, 1987), buffaloes (Tikaram and Ruprah, 1986; Gill *et al.*, 1989) and camels (Nayel and Abu-Samra, 1986a). Wild species affected include bears (Schmitt *et al.*, 1987) and lions (Young *et al.*, 1972; Young, 1975) and a long-term epidemic of scabies has been reported in chamois (Miller, 1986).

2. BIOLOGY

2.1. Anatomy

The scabies mite is a member of the suborder Acaridida (=Astigmata), as it has no detectable spiracles or tracheal system, and of the family Sarcoptidae. The majority of opinion agrees with Fain (1968, 1978) that *Sarcoptes scabiei* is a single species regardless of variations in size, host specificity and anatomical characteristics. However, some workers, notably Kutzer and Onderscheka (1966), Kutzer and Grünberg (1967) and Kutzer (1970), have maintained that mites infecting different hosts are distinct species.

Sarcoptes scabiei shows the typical thin cuticle, without heavily sclerotized shields, of the Acaridida. The coxae are embedded in the ventral surface, and are referred to as epimeres, and the chelicerae have two segments, the smaller hinged in opposition to the larger giving a pincer action, with two segmented palps. The body, or idiosome, is a broad oval shape, subglobose above and flattened ventrally. The idiosome surface is covered with fine striations and dorsally has a number of variable fields of stout setae and spines (Heilesen, 1946; Fain, 1968; Pence *et al.*, 1975). The colour is creamy white with brown sclerotized legs and mouthparts.

The size ranges from 300 to 500 μm long by 230 to 420 μm wide in the female, but the male is only 213–285 μm long by 162–210 μm wide (Fain,

1968). Some of this variation of size appears to be due to differences in preparation and mounting of specimens before measurement (Lunn, 1944).

In both males and females the anterior two pairs of legs (I and II) arise adjacent to the gnathosome and both terminate in a stalked empodium or pulvillus that is often referred to as a sucker (Heilesen, 1946; Mellanby, 1972). The tarsi additionally carry two blade-like claws. In the female both legs III and IV terminate in a long seta but in the male only leg III ends in a seta and leg IV carries a subapical stalked empodium. In males there is only a single blade-like claw on leg IV but two such claws in the female.

The anus is terminal in both sexes. The opening of the copulatory bursa is on the dorsal side just anterior to the anus in the female and stands on a small papilla flanked by two long spines. The genital opening, through which the eggs are laid, is on the ventral surface and consists of a transverse slit mid-way between the anterior and posterior pairs of legs (Pascual *et al.*, 1977). The male genital apparatus is in the ventral mid-line between the anus and the fused epimeres of legs III and IV. Spermatogenesis has been described by Witalinski and Afzelius (1987) and is essentially similar to that in free-living acaridid mites.

2.2. Life Cycle

Early studies on the life cycle of *S. scabiei* were mostly carried out using human cases and volunteers (Heilesen, 1946; Mellanby, 1972). The difficulty with this approach was that relatively small numbers of mites were available and all the procedures were tempered by the conflict between investigation and the ultimate need to treat the patient. Some further information could be gained by examination of mites in epidermal plaques from patients with hyperkeratotic scabies (Van Neste *et al.*, 1981; Van Neste, 1985). However, in the absence of a system of *in vitro* culture, repeatable observations of aspects of the life cycle were not possible. The problem was partially solved by the establishment of a culture colony of *S. scabiei* from dogs on laboratory rabbits (Arlian and Vyszenski-Moher, 1988).

By studying this colony it was possible to establish more accurately the length of each life cycle stage estimated by previous authors. The eggs hatch in 50–53 h followed by a larval stage between 77 and 101 h long. The emerging protonymph duration depended on whether the mite was a male (56–80 h) or a female (approximately 90 min longer). The significant discovery in this study was that not only the females but also the males have a tritonymphal stage which lasts between 53 and 77 h for females and around 5 h longer for males. The failure of previous observers (Munro, 1919; Heilesen, 1946; Gordon and Lavoipierre, 1962; Mellanby, 1972) to observe this stage in males is attributed to its being smaller than the female tritonymph and only slightly larger than the male protonymph.

Several writers have observed various life cycle stages actively walking on the skin surface. Only the ovigerous female does not normally leave its burrow, although Heilesen (1946) observed this occasionally on humans and Arlian and Vyszenski-Moher (1988) on rabbits. The six-legged larvae, after hatching, may make short burrows off that of the parent but frequently tunnel up through the roof of the burrow to the skin (Heilesen, 1946). They then move actively until they find a suitable skin fold or hair follicle and either enter the follicle or else make a short burrow just large enough to conceal the body. This was termed a "moulting pocket" by Gordon and Lavoipierre (1962) and is produced at each developmental moulting stage (Heilesen, 1946).

All stages of the mite normally choose a skin crease to start their burrow (Heilesen, 1946; Mellanby, 1972). Entry to the skin is achieved by a combination of chewing with the chelicerae and a side to side motion of the gnathosome. As fragments of skin are removed, and a flap of skin is formed, it is pushed upwards by the mite forcing the gnathosome underneath and levering it upward. At the same time the hole is enlarged by the action of legs I and II using the blade-like claws. The mite scrapes at the skin with these, first on one side and then the other. Some skin is eaten but the rest of the debris is merely pushed aside. The forward movement is partly side to side as the mite turns slightly to bring the claws on the front limbs to bear on the end of the burrow. Once it is able to insert the whole of the body into the hole it pushes forward and upward with the hind limbs, which allows greater purchase by the digging appendages, and forces the stout spines on the dorsal side against the roof of the burrow to prevent backward movement.

The adult male is more active than the female (Heilesen, 1946) and runs around on the skin surface seeking a mate. Heilesen observed males entering female burrows and pushing their anterior ends between the females and the dorsal side of the burrows. After a short time the male mite rotated through 180° so that it was facing the opening of the burrow but with its posterior ventral surface, and the genital apparatus, in apposition to the posterior dorsal surface of the female where the copulatory bursa is found. He was never able to observe copulation directly but concluded it had occurred because the females began to lay eggs.

The adult female burrows in a downward direction and appears to penetrate the spinous cell layer, at least with her mouthparts (Ackerman, 1985; Van Neste, 1986). However, due to the continual outgrowth of the epidermis, the rest of the burrow together with the mite faeces, eggs, hatched shells and any newly hatched larvae are found in the cornified layers.

The female mite lives from 4 to 6 weeks during which time the burrow advances between 0.5 and 5 mm each day (Mellanby, 1985). The older parts of the burrow are lost as the keratinized epidermis is rubbed off, so the full length cannot be determined. However, some measure of the age of the burrow can be made by the number of eggs present since the mite lays two

to four each day, often in groups. These appear to be stuck to the floor of the burrow with amorphous material (Van Neste and Lachapelle, 1981). The longest burrow recorded in biopsy specimens by Friedman (1947) contained 38 ova of which seven still contained embryos. Once development is completed the larvae cut their way out of the egg shells using chelicerae and forelimbs (Van Neste, 1981).

3. PARASITOLOGY

Few studies of the parasitology of *S. scabiei* have been made in humans. The most thorough work was performed by Mellanby and his co-workers during the early 1940s. In these experiments more than 50 previously uninfected volunteers were deliberately infected with fecund female mites and the course of the infections followed over periods of 200 days or more. In all cases there was no itching during the first month of the infection. Irritation set in between 6 and 10 weeks after infection at which time the subjects all showed normal signs of clinical scabies (Mellanby, 1944a). Although they expected to find new adult female burrows by about 10–14 days after the initial infection, no new female was identified until the 21st day, from which Mellanby concluded that wastage of developmental stages was very high even in non-immune subjects. The subsequent parasite burdens increased rapidly and generally peaked at around 3–4 months, with over 380 adult female mites on some subjects, followed by a rapid reduction in numbers of mites. Such a pattern is probably determined by a variety of factors including the development of an immune response, which in turn influences the degree of excoriation of the burrows, and removal of the mites by scratching and the development of secondary infections (Johnson and Mellanby, 1942). When the same volunteers were cured and reinfected at a later date not only did the itch develop almost immediately but many of the mites failed to establish. None of the reinfected subjects harboured more than 40 mites even after several months (Mellanby, 1944a). These findings help to explain the earlier work of Johnson and Mellanby (1942), in which they endeavoured to extract every adult female mite from the bodies of 886 men during several examinations. They actually obtained 9978 mites giving an average parasite burden of 11.3. Clearly, since only six of their subjects had over 100 mites and only 50 had more than 40 mites, the majority of those studied were either recently infected or had suffered the disease for some considerable time. Over half the group had fewer than five mites present. A similar picture was obtained in an assessment of 119 women patients, who had an average parasite burden of 12.5 (Bartley and Mellanby, 1944). Most significant in both studies was that over 80% of individuals were found to have mites on their hands, with 63.1% of mites occurring there on men and 74.3% on women. In the case of children every

one of 18 cases was infected on the hands, with this site bearing 62.3% of the mites. Comparable findings were made by Heilesen (1946) on 172 first-time and 62 reinfected patients, although he found a greater percentage of women infected on their hands and some variation of percentage distribution on the less frequently infected parts of the body.

A curious characteristic of Heilesen's (1946) findings was that he found no clear inverse correlation between the numbers of mites and the intensity and number of lesions. This led him to conclude that the mite burden and the reaction to them were actually linked. The more recent work of Baweja *et al.* (1986) correlates more closely with that of Mellanby (1944a), as they found a similar long delay before the development of a skin test reaction. Interestingly, Baweja and colleagues (1986) found their highest mite counts in asymptomatic contacts which contrasts to Mellanby's group, all of whom had started to show symptoms before reaching their highest parasite burdens. If Baweja *et al.* (1986) are correct in this finding it may mean that some people remain asymptomatic carriers for a longer time, which has serious implications for contact tracing procedures in control of outbreaks of the disease and emphasizes the importance of treating all contacts, whether symptomatic or not. Such a situation would offer a more satisfactory explanation for the cases described by Palicka (1982), for whom no contacts could be traced, rather than the explanation given at the time that fomites were the source of infection.

4. PATHOLOGY

4.1. Histopathology

A number of cytological changes occurs as the mites burrow through the epidermis. The early part of the burrow, where the mite passes through dead keratinized cells, shows no change in the structure other than that caused by the physical activities of the mite and the cells remain orthokeratotic (Van Neste and Lachapelle, 1981). When the mites approach the living parts of the epidermis cellular changes occur which may be due to cytolytic secretions in saliva, although no substance of this type has yet been identified (Van Neste, 1981). This cytolysis can continue across the dermo-epidermal junction but does not affect the collagen fibres of the dermis. Cells adjacent to the necrotic area appear to be disturbed in their keratinization process and become parakeratotic. In the upper levels of the epidermis this is found in the floor of the burrow only, but as the mite progresses deeper the zone of parakeratosis advances up the walls of the burrow until the whole burrow is surrounded by parakeratotic cells as it passes through the Malpighian layer (Van Neste and Lachapelle, 1981; Van Neste, 1982, 1985). These events are most easily seen

in the hyperkeratotic form of the disease but similar reactions occur in the classical papular form (Falk and Eide, 1981; Ackerman, 1985; Hayashi, 1986b; Head *et al.*, 1990). In the papular form a mixed perivascular infiltration of the dermis occurs, consisting of lymphocytes, histiocytes and eosinophils. This sometimes extends as far as the subcutaneous fat layer (Ackerman, 1985) and in some cases causes vasculitis, especially of the deep plexus, in which vessel walls are found to contain inflammatory cells and fibrin. Occasionally thrombi are found in vessels of the superficial plexus.

Spongiotic lesions in the epidermis and dermis occur beneath the burrows and often in the vicinity of mite faeces or eggs (Ackerman, 1985; Van Neste and Lachapelle, 1981). This is particularly so in the hyperkeratotic form of the disease where the epidermis becomes markedly psoriasiform, in which case the spongiosis is more extensive with increased pustule formation and development of neutrophilic abscesses within the spinous zone (Ackerman, 1985). Such abscesses are more common in pigs than humans (Van Neste and Staquet, 1986).

4.2. Immunopathology and Immunology

4.2.1. *Immunology of the Dermis and Epidermis*

Infiltrating mononuclear cells within the dermis are principally T lymphocytes, followed by macrophages and B lymphocytes (Falk and Matre, 1982; Reunala *et al.*, 1984). In scabies these T lymphocytes produce only the lymphokine leucocyte migration inhibition factor when stimulated with phytohaemagglutinin (Kozin *et al.*, 1990), which would explain the apparently selective infiltration of cells into the dermis.

Several studies have investigated the immunological aspects of scabies lesions by biopsy and incubation with specific antisera. However, the results were somewhat equivocal since only a small proportion of specimens showed positive immunofluorescence results. Frentz *et al.* (1977) found the third component of complement (C3) and immunoglobulin (Ig) M at the dermo-epidermal junction in one patient and C3 only in another. They also found C3 on the body of a mite. Similar deposits of C3 and IgM were found by Hoefling and Schroeter (1980) at the dermo-epidermal junction and C3 alone has been identified by Salo *et al.* (1982) and Van Neste and Lachapelle (1981). Salo *et al.* (1982) also reported C_3, IgA and IgM in dermal vessel walls. Hayashi (1986b) found IgA-, IgG- and IgM-positive plasma cells but no C3 in humans. Morsy and Gaafar (1989) found a predominance of IgG cells in young pigs but mostly IgM plasma cells in older animals. A problem arises in interpretation of any of these results, partly because relatively few subjects showed the same reactions and partly because some deposition was associated with other causes such as IgM of rheumatoid factor, as found in five of 15

patients by Salo *et al.* (1982). Frentz *et al.* (1977) found deposits of IgE in perivascular areas and also in the vessel walls of four of 11 patients both in the vicinity of burrows and in papules. IgE-positive mast cells were identified in 16 of 22 cases (Hayashi, 1986b) and 12 of 18 cases (Hayashi *et al.*, 1986). In both studies IgE was also found on the surface of mites and clusters of granules resembling mite faeces.

4.2.2. *Humoral Response*

Evaluation of the humoral response to scabies is less difficult, and greater numbers of subjects can be enrolled in each study. Reduced levels of IgA have been found during active scabies infection (Hancock and Milford-Ward, 1974; Falk, 1980; Allevato *et al.*, 1987b), which subsequently returned to normal following treatment. In two of the studies levels of IgG and IgM were found to be higher during the infection and decreased after cure (Hancock and Milford-Ward, 1974; Falk, 1980), whereas Allevato *et al.* (1987b) found no difference in the levels of these globulin classes; they attributed this to their playing no part in the immunological control of the infection in humans.

Levels of specific IgG may fall even during the infection. In one study three patients who had shown symptoms for less than 1 month had higher levels than 11 whose symptoms were evident for longer (Hayashi *et al.*, 1985). In contrast, Ezeoke (1985) found raised levels of all classes of immunoglobulins in 57 Nigerian pre-school children with scabies, compared with matched controls. The presence of specific and raised levels of serum IgE in scabies has been found in a number of investigations. The early work of Hancock and Milford-Ward (1974) failed to show any elevation in 99% of patients; however, it is likely that test methods at that time were insufficiently sensitive. Later work demonstrated raised IgE in 15% (Araujo-Fontaine *et al.*, 1977), 35% (Christensen *et al.*, 1985), 41% (Falk, 1980), 45% (Falk, 1981), and 93% of patients (Galosi *et al.*, 1982). The onset of increase in IgE occurs within the first few weeks of infection (Galosi *et al.*, 1982) and it has been suggested to have a protective role in the early months of the infection (Lo-Socco *et al.*, 1988). After treatment the levels of IgE fall rapidly within 1 month (Allevato *et al.*, 1987a) and are significantly lower after 1 year (Falk, 1981; Lo-Socco *et al.*, 1988). No correlation was found between the IgE levels, the duration of pruritus, and the values obtained by radioallergosorbent assay (RAST) (Christensen *et al.*, 1985).

4.2.3. *Specificity and Cross Immunity*

The specificity of the IgE can be shown by passive transfer in some patients up to 1 year after treatment but beyond that time the reaction was lost (Falk and Bolle, 1980b), and specific circulating IgE antibodies were also demonstrated in 30% of subjects by Christensen *et al.* (1985) using extracts of

porcine scabies mites. However, cross-reactions with other mite species, particularly the house dust mites *Dermatophagoides pteronyssinus* and *D. farinae*, have been found, which may render the specificity in some cases less certain. Falk (1981) showed a cross-reaction with *D. pteronyssinus* antigen up to 1 year after a scabies infection, which diminished more rapidly in non-atopic patients. In a further series of 135 scabies patients, Falk and Bolle (1980a) found elevated IgE in 45% of whom 33% also gave a positive RAST reaction to *D. pteronyssinus*, contrasted with a control group in which only 4% had elevated IgE and 2% gave positive RAST reactions. Significantly, 23% of the scabies patients were atopic, compared with only 9% of controls, suggesting a correlation between scabies and atopic disease.

Further investigation of the antigens of dust and scabies mites by crossed immunoelectrophoresis (CIE) by Falk *et al.* (1981) showed 25 antigens in *D. farinae*, of which four were common with *S. scabiei*. By tandem CIE they found three common antigens. However, when examining pooled sera from dust mite allergic patients, they found no IgE active against scabies mites. This contrasts with the finding of Arlian *et al.* (1990a) that a definite IgE cross-reaction was found in such patients.

A major problem with much immunological work on scabies has been the difficulty of obtaining scabies mites in sufficient quantities to provide a regular and more or less standard supply of antigen. This problem was partly solved by Christensen *et al.* (1985), who used mites from pigs that had developed a hyperkeratotic type reaction. The most successful procedure hitherto has been the establishment of a colony of canine scabies mites on rabbits (Arlian *et al.*, 1984a). Using this source, extracts of mites were found by crossed immunoelectrophoresis to contain nine antigens, of which seven moved anodically and two cathodically (Arlian *et al.*, 1985). Sera from rabbits heavily parasitized with canine scabies mites were found to contain antibodies that cross-reacted with six somatic antigens of *Dermatophagoides farinae* and three from *D. farinae* faeces. Conversely six of the nine antigens from *S. scabiei* were cross-reactive with *D. farinae* antisera and using sodium dodecyl sulphate-polyacrylamide gel electrophoresis 30 protein and peptide bands were found in *S. scabiei*, of which 15 bound *D. farinae* specific IgE from patients with positive skin tests. Control subjects showed no IgE activity against either mite (Arlian *et al.*, 1988d). A similar cross-reactivity was shown to *D. pteronyssinus*, using immune rabbit serum (Arlian *et al.*, 1991).

It has long been suspected that antigens from mite bodies, faeces or hatching and moulting fluids are responsible for the allergic reaction to scabies (Mellanby, 1972). The oedematous infiltration into the dermis and epidermis, demonstrated by histology, would bring serous fluids into contact with the mites and their products in the burrows. Any water-soluble components of faeces, mite bodies and mite saliva could, therefore, diffuse out into the tissues from the burrows and this could explain why papulovesicles and spongiotic and bullous reactions develop usually beneath burrows behind the mites.

4.3. Pathophysiology

Apart from the immunological effects, other physiological changes occur in scabies infections, the severity of which is often associated with the extent of chronic infection. The morbidity that results is more easily measured in mammal species other than humans and is usually due to severe dermatitis, alopecia and dermal pathology leading to loss of condition or even death (Dannell and Hornfeldt, 1987; Gill *et al.*, 1989; Ahmad *et al.*, 1990; Hollanders and Vercruysse, 1990). Experiments using infected laboratory rabbits have shown that the loss of condition and failure to gain weight in heavily parasitized animals were not specifically due to energy loss resulting from the ingestion of host skin and serous fluids (Arlian *et al.*, 1988b). Each female mite requires only 2 nl, and each male 0.76 nl, of oxygen per hour. Such an oxygen consumption related to the number of mites present was found to represent only 1.9% of the weekly weight gain of uninfected growing rabbits. The loss of weight was, therefore, attributed to indirect energy loss by processes such as hyperplasia and sloughing of stratum corneum. Some loss may result from increased scratching, as observed in pigs by Davis and Moon (1990), although the growth performance of pigs was found to vary in different studies with one group of more heavily infected animals showing increased food intake and growth during the first few weeks followed by a levelling off to match controls and a second group performing similarly to their control groups (Wooten-Saadi *et al.*, 1987a). Comparison of infected and treated farrowing sows with an untreated group showed that the untreated animals ate more but produced smaller litters and the piglets themselves were less efficient at gaining weight (Arends *et al.*, 1990). Nevertheless, a general improvement in weight gain is shown following treatment (Sheahan *et al.*, 1974).

Prolonged and heavy infections with scabies induce changes in blood chemistry and cytology and have effects on organs other than the skin. A severe anaemia develops in rabbits, dogs and pigs showing reduced total haemoglobin, haematocrit and cellular haemoglobin (Sheahan, 1974; Ferreira *et al.*, 1987; Arlian *et al.*, 1988e). Similar significant reductions were found in Nigerian pre-school children (Ezeoke, 1985). Sheahan (1974) explained the anaemia by the reduced ability of the animals to absorb iron from food and increased loss of iron in desquamated skin. However, Arlian *et al.* (1988e) found only insignificant reductions in iron levels compared with controls. Using an alternative approach Sheahan (1974) found that animals fed a complete diet developed equally severe scabietic lesions but that iron deprivation contributed considerably to loss of weight gain and anaemia.

The levels of circulating leucocytes in different species vary following infection. Arlian *et al.* (1988e) found an overall reduced white cell count with low levels of lymphocytes, eosinophils and basophils but elevated neutrophil numbers. Ferreira *et al.* (1987) found no difference in lymphocyte counts in

infected dogs which conforms with the findings in pigs by Wooten *et al.* (1986), in which total and differential counts were unaltered following a single artificial infection, and in wild coyotes with natural infections (Pence *et al.*, 1983). In contrast, Falk and Eide (1981), Hayashi (1986a) and Deshpande (1987) found eosinophilia in some human patients. Two separate studies by Wooten *et al.* (1986) and Wooten-Saadi *et al.* (1988) examined the blastogenic response of T and B lymphocytes in infected and treated pigs and found that their response to mitogens was unaltered under cold or ambient conditions.

Extensive comparison of blood biochemical concentrations has been performed in only two studies (Pence *et al.*, 1983; Arlian *et al.*, 1988e). Of the quantities measured only albumin concentrations behaved similarly in both studies and were reduced, whereas in humans no reduction of serum albumin was found by Ezeoke (1985). Levels of mineral ions in serum were mostly normal although calcium was reduced in both rabbits and coyotes. Levels of vitamin A, in the form of retinol, and vitamin E are both reduced in dogs (Ferreira *et al.*, 1985, 1987).

Apart from dermatological changes, other organs are affected in heavily infected rabbits (Arlian *et al.*, 1990b). The principal histopathological findings are amyloid deposits in kidney glomeruli, liver, red pulp of the spleen, intestine and tongue. Such chronic changes to glomerular structure often result in loss of serum proteins, particularly albumin, in the urine. Albuminuria has been reported in humans with scabies (Nicholas and Jambon, 1908; Hayman and Fay, 1921) which in some cases developed to a clinical nephritis but which appears to be distinct from bacterially induced acute glomerular nephritis (see Section 4.4.2). Similar changes have been observed in other severe chronic parasitic infections caused by immune complex deposition. Since immune complexes have been identified in scabies (Van Neste and Salmon, 1980) and have been postulated as being the aggravatory trigger of other inflammatory lesions including dermal nodular reactions (Van Neste, 1986), they are one likely cause of deep tissue changes. That circulating antigens, and presumably complexes, occur at high levels in human scabies was indicated by Kleiner-Baumgarten *et al.* (1986) in their observations of a patient with angioimmunoblastic lymphadenopathy concurrent with scabies which regressed on treatment of the mite infection and reappeared on reinfection.

4.4. Secondary Infections

4.4.1. *Pyoderma*

Impetigo is a common sequela of scabies if the mite infection is left untreated for any length of time (Mellanby, 1972; Alexander, 1984). The extent and range of sites showing bacterial invasion is highly varied (Borda, 1954) and

may result in cellulitis, lymphangitis or acute glomerulonephritis according to the causative bacterial organisms. In warmer countries pyoderma is common and may follow any minor skin trauma such as an insect bite (Poon-King *et al.*, 1967; Taplin, 1981; Ide, 1989). The majority of these lesions are primarily caused by *Streptococcus pyogenes* with secondary invasion by *Staphylococcus aureus* (Poon-King *et al.*, 1967; Taplin *et al.*, 1973; Allen and Taplin, 1974; Taplin and Allen, 1974) and may achieve prevalences of between 10 and 30%. However, if scabies is present bacterial infection levels may increase dramatically (Montgomery, 1985; Taplin *et al.*, 1991).

4.4.2. *Acute Glomerulonephritis*

The first recorded association of infected scabies with acute glomerulonephritis (AGN) was made by Borda (1954), although it is possible that the earlier reports of scabies patients with albuminuria (Nicholas and Jambon, 1908; Hayman and Fay, 1921) were actually cases of AGN rather than being due to direct effects of scabies or the toxic treatments applied to some patients. However, although pyoderma had become associated with the occurrence of epidemic AGN by the early 1960s (Symonds, 1960; Blumberg and Feldman, 1962; Poon-King *et al.*, 1967), it was not until the now classical study of the situation in Trinidad was well under way that the link between scabies and epidemic AGN was established.

Even following the first epidemic of AGN in Trinidad very few patients were identified with scabies (Ortiz *et al.*, 1970) but, during 1970, as the incidence of scabies increased so did the incidence of AGN (Svartman *et al.*, 1972, 1973) such that by mid-1971 64% of AGN patients had scabies. There was some suspicion that the scabies and the nephritogenic strains of streptococci were linked with the large numbers of mangy dogs on the island and that either mites or bacteria or both were passed back and forth between humans and animals (Potter *et al.*, 1985). Although a control programme for dogs was instituted and control of the epidemic coincided with it, there was no causal link established. During the second half of 1971 very high incidences of both scabies and AGN occurred in which the two conditions almost exactly paralleled each other in frequency. What was most important was the reappearance of M-type streptococcal strains and particularly M-55 (Poon-King *et al.*, 1973; Potter *et al.*, 1974).

Interestingly, this epidemic conformed with the seasonal increase of activity of the scabies mite, since peak incidence was from June to December (Section 6.2.4). From the records of Svartman *et al.* (1973), the spread of M-55 streptococci appeared to increase dramatically about every 2 months which in turn coincided with increases in the number of cases of scabies identified. Such an irregularity in scabies incidence has been described by Hernandez-Perez (1971) and is due to the asymptomatic period that follows infection with the mites.

Shelley *et al.* (1988) have isolated staphylococci not only from burrows but also from mite faecal pellets; consequently it may be possible that the spread of M-55 streptococci, and subsequently AGN, during the Trinidad outbreak could have been brought about by the mites themselves. Should such a similar epidemic recur, culture of scybala for bacterial serotypes and contact tracing may help to clarify the links between scabies and AGN.

After December 1971, the occurrence of both scabies and AGN diminished rapidly in Trinidad and remained low for a number of years (Potter *et al.*, 1985). A similar outbreak occurred between 1986 and 1988 as part of a periodic upsurge throughout that part of the Caribbean (Reid *et al.*, 1990a), during which it was found that, in the first few months, AGN was due to new M-types of streptococci for the area: M-73, M-48 and a provisional type PT-5757. However, after about 5 months, when the epidemic was more intense, M-55 reappeared as the predominant type (Reid *et al.*, 1990b).

At other times pyoderma does occur in scabies patients on the island but it is principally related to *Staphylococcus aureus* with only around 30% of cultures showing type A and type B haemolytic streptococci (Suite, 1990). A similar pattern was found in Papua New Guinea by Montgomery (1985), although there type A streptococci predominated in scabies pyoderma and exceeded the frequency of staphylococci.

Association of AGN with pyoderma in scabies has also been reported from Ghana (Acheampong *et al.*, 1988); USA (Anthony *et al.*, 1967); Taiwan (Ferng *et al.*, 1982); South Africa (Hersch, 1967); India (Jerath *et al.*, 1979; Verma *et al.*, 1983); Israel (Lasch *et al.*, 1971); Canada (Robinson, 1977); Zimbabwe (Seggie, 1981); New Caledonia (Thevenieau, 1981), and Nigeria (Whittle *et al.*, 1973). Although the majority of these outbreaks have been in communities a few individual cases have also been reported, mostly in temperate countries including France (Moulin *et al.*, 1977; Charmes *et al.*, 1978) and Japan (Takiguchi *et al.*, 1987), and also from Algeria (Barabe *et al.*, 1978).

Infected scabies has also been identified as the source of an attack of acute haematogenic osteomyelitis in one case (Gaida *et al.*, 1985). No doubt future studies will implicate this pyoderma as the cause of other bacterial pathology.

5. CLINICAL ASPECTS

5.1. Clinical Presentation

The diagnosis of scabies in humans is theoretically straightforward (Mellanby, 1972; Epstein and Orkin, 1985) yet so many other factors influence its clinical manifestations that, like syphilis, scabies has come to be known as the "great imitator".

5.1.1. *Classical Scabies in Adults*

The typical presentation of the disease is a pruritus that may affect all parts of the body except the head and the middle of the back. This frequently is more intense at night, particularly soon after retiring to bed. Two forms of skin eruption occur concurrently. The first is associated with the sites, or the vicinity, of burrows and consists of papular and vesicular lesions which in some examples become pustular or bullous (see Section 4.1). A more generalized itchy papular eruption, not associated with the sites of election of the mites, also occurs which is bilaterally symmetrical in distribution. It affects the midriff, the insides of the thighs and lower buttocks, the axillae, the lower legs and ankles and the wrists extending along the forearms to the elbow regardless of the number or distribution of the mites (Mellanby, 1972). In many cases the rash is sparse, pale and diffuse and it is often overlooked.

In some cases, particularly males and children, a nodular reaction occurs which may persist for months after successful treatment of the disease (Hurwitz, 1985). The nodules are typically dark pink to brown, firm and extremely pruritic. They develop most frequently on the anterior fold of the axillae, around the groins, the penis or scrotum, on the buttocks, and around the navel (Hayashi, 1986b).

5.1.2. *Classical Scabies in Young Children*

The development of scabies in infants and young children, although following essentially the same course as in older children and adults, often presents with a different distribution of lesions and symptomatic variations that may result in failure to diagnose. It has been suggested by Hurwitz (1985) and Madsen (1965) that the variations observed may be due to differences of distribution of pilosebaceous units in the skin and thickness of the stratum corneum, and the discovery that reduction of sebum production exacerbates scabies appears to confirm this (Zlotogorski and Leibovici, 1987). Involvement of the face, neck, scalp and, particularly, the post-auricular fold is common (Hurwitz, 1982, 1985; Cambazard, 1987; Niguez-Carbonell *et al.*, 1988; Wakhlu *et al.*, 1988; Sterling *et al.*, 1990) and the overall clinical presentation is often obscured by the differences of bathing and hygiene practices performed on young children (Hurwitz, 1985).

As with adults, the nail plates of infants may be affected and harbour mites which are overlooked during treatment (Sokolova and Sizov, 1989). Mites are certainly found on the scalp (Elmros and Hornqvist, 1981; Gogna *et al.*, 1985) but there is some doubt whether they occur on the face itself (Burns *et al.*, 1979; Dolezai and Plamondon, 1980; Haas and Stuttgen, 1987b). Since it is believed that the papular reaction is a result of the temporary burrows and pockets made by larvae and nymphs (Hurwitz, 1985), it is likely to be necessary to treat those areas of the face so affected despite the assertion by

Haas and Stuttgen (1987b) to the contrary and this is especially important in humid tropical zones where facial involvement is more widespread. The effective treatment achieved by Taplin *et al.* (1991) can be attributed in part to their routine treatment of the faces of children.

5.1.3. *Infantile Acropustulosis*

In recent years a further complicating factor in the diagnosis of scabies in infants has emerged in the form of acropustulosis of infancy (AI). This condition presents as pruritic acral papules and vesicopustules. Early cases were all described in black male infants (Jarratt and Ramsdell, 1979; Kahn and Rywlin, 1979), who still constitute the majority. However, cases have also been described from Britain (Newton *et al.*, 1986), France (Prigent, 1987), Argentina (Pizzi-de-Parra *et al.*, 1987) Israel (Ingber *et al.*, 1988), Senegal (Ball and Arborio, 1988) and south-east Asia (Bjornberg and Friis, 1978). The condition may arise between birth and 10 months of age (Newton *et al.*, 1986) and in most cases resolves spontaneously before the fourth year (Jarratt and Ramsdell, 1979; Kahn and Rywlin, 1979; Lucky and McGuire, 1982; Jennings and Burrows, 1983; Newton *et al.*, 1986). The lesions are sterile and neutrophilic, although Lucky and McGuire (1982) found eosinophils in the pustules of their case.

The condition often does not respond to antihistamines or corticosteroids but some cases have been successfully treated with sulphones or sulphonamides (Kahn and Rywlin, 1979, Newton *et al.*, 1986, Pizzi-de-Parra *et al.*, 1987). Some cases clearly develop as a sequela of scabies and constituted 13 of the 32 patients seen by Bjornberg and Friis (1978) and four of eight described by Elpern (1984). Additionally, the single case described by Lucky and McGuire (1982), the two by McFadden and Falk (1985) and two of the four described by Newton *et al.* (1986) had eczema, atopic symptoms, or high levels of IgE. Consequently AI may be an abnormal reaction to scabies that persists after the infection has passed, as postulated by Newton and colleagues (1986). Alternatively, it may be related to an atopic reaction. Since cross-antigenicity has been shown between scabies mites and house dust mites (Section 4.2), it is possible that AI patients may be reacting to dust mite allergen and in some cases sarcoptid mites act as an alternative or additional trigger.

5.2. Crusted Scabies

5.2.1. *Presentation*

Hyperkeratotic or crusted scabies is also commonly known as Norwegian scabies following the description of the condition by Boeck and Danielssen in leprosy patients in Norway (Hebra, 1852). The condition is characterized

by the development of hyper- and parakerototic crusts which may be loose, and either scaly or flaky, or dense, thickened and adherent. This crusting may also be accompanied by an irregular and diffuse erythroderma (Frost and Parker, 1988). Plaques that are loose are more commonly found on non-flexor surfaces such as the chest, back and head, particularly behind the ears. Plaques found on flexor surfaces are generally more dense and may acquire considerable thickness (Alexander, 1984).

There is frequently involvement of the nail beds or nail plates on either the hands or feet (Hebra, 1852; Koscard, 1984; De Paoli and Marks, 1987) and such sites may be the only abnormal manifestations of the disease on patients presenting with otherwise normal scabies. Such involvement will often result in treatment failures (Scher, 1983, 1985; Witkowski and Parish, 1984).

Crusted scabies almost certainly begins as classical scabies in the majority of cases but because of some predisposing factor it is transformed at a later stage. In most cases the condition has been present some time before specialist help is sought, so the early stages in such patients are rarely, if ever, observed. However, unless similar predisposing conditions prevail in contacts of the patient with crusted scabies, all other cases contracted as a result of contact with a crusted scabies patient will be of the classical form with normal vesiculo-papular eruptions (Carslaw *et al.*, 1975; Burns, 1987b). Once crusted scabies is suspected, examination of the crusts microscopically allows a simple diagnosis. The crusts are relatively soft and spongy, being honeycombed with burrows. Preparation in potassium hydroxide of even a small fragment of scale normally reveals large numbers of eggs, developing larvae and nymphs and adult female mites (Van Neste and Lachapelle, 1981; Van Neste *et al.*, 1981; Van Neste, 1985; Van Neste and Staquet, 1986).

There has been some debate about whether the mites that cause crusted scabies may be different in some way. Although Fain (1978) found that the bare patch in the field of scales on the back of the female was larger, he was unable to detect any other difference. Furthermore, since the majority of contacts of crusted scabies patients develop normal scabies (Carslaw *et al.*, 1975; Burns, 1987b) any suggestion that the mites may be different is difficult to justify. The answer is rather in the difference between patients, as originally suggested by Hebra (1852).

5.2.2. *Predisposing Factors*

Alexander (1984) has roughly analysed a series of reports and found that, of the predisposing conditions reported to that time, Down's syndrome had been recorded approximately five times more frequently than any other causal link. This is indicated clearly in the reports by Calnan (1950) and Hubler and Claybaugh (1976), in which patients with Down's syndrome constituted almost the totality of those with crusted scabies. Amongst other

predisposing conditions neurological and immunological disorders made up the majority of the remainder. At the time Alexander was writing the total number of cases of crusted scabies reported was a little over 300. Since then numbers have increased considerably. Patients continue to be recognized suffering from crusted scabies and Down's syndrome (Ramsay and Powell, 1987; O'Donnell *et al.*, 1990; Scherbenske *et al.*, 1990) and Bloom's syndrome (Dick *et al.*, 1979). There is an increased association with the elderly and handicapped (Carslaw *et al.*, 1975; Juranek *et al.*, 1985; Burns, 1987b; Gallina *et al.*, 1987; Wolf and Krakowski, 1987b; Schewach-Millet *et al.*, 1990); there are also links with metabolic disorders including diabetes (Klein and Cole, 1987; Yarbrough and Iriondo, 1987; Lodi *et al.*, 1990), hypoparathyrosis (Sokolova *et al.*, 1990) and kwashiorkor (Strobel *et al.*, 1982), psoriasis (Keipert, 1986; Verner *et al.*, 1987), atopic eczema together with IgA linear bullous dermatosis (Leroy *et al.*, 1980), and various forms of malignant disease (Monti *et al.*, 1983; Savall *et al.*, 1985; Suzumiya *et al.*, 1985; Wolf and Krakowski, 1987b; Lang *et al.*, 1989; Wada *et al.*, 1989).

Immunosuppression, systemically for transplant surgery or locally using radiotherapy or topical steroids, plays an increasing role in development of crusted scabies (Lipitz *et al.*, 1981; Youshock and Glazer, 1981; Mikheev, 1984; Tomono *et al.*, 1985; Van Neste *et al.*, 1985; Wolf *et al.*, 1985; De Heredia *et al.*, 1986; Barnes *et al.*, 1987; Wolf and Krakowski, 1987a; O'Donnell *et al.*, 1990). Concurrent development of crusted scabies with *Pneumocystis carinii* pneumonia has been described twice but with no apparent underlying factor that would have immunosuppressed the patients sufficiently to allow pneumocystosis to develop (Kobayashi *et al.*, 1982; Arata *et al.*, 1984). It is possible that the scabies may have had a sufficiently immunodepressive effect on the patient to permit *P. carinii* to gain a foothold but if this were the case such concurrent infections would be expected more often. Since those reports, the widespread dissemination of human immunodeficiency virus (HIV) and its general immunodepressive effects have resulted in frequent infections with *P. carinii* and HIV is now also often associated with crusted scabies (Rau and Baird, 1986; Sadick *et al.*, 1986; Drabick *et al.*, 1987; Glover *et al.*, 1987; Dillon, 1989; Hall *et al.*, 1989; Jucowics *et al.*, 1989; Inserra and Bickley, 1990; Jessurun *et al.*, 1990; Rostami and Sorg, 1990; Sirera *et al.*, 1990). The association of crusted scabies with HIV and acquired immune deficiency syndrome has now become so common that the majority of cases go unreported, although various personal communications suggest that the index of suspicion of the risk of scabies in these patients is still relatively low in some places and some cases remain undiagnosed for months.

There have been some cases of crusted scabies in Australian Aboriginals in whom no predisposing condition was recognizable (Gogna *et al.*, 1985). Two of the three cases suffered multiple relapse of the condition following their return to endemically infected villages. In two cases, a mother and infant daughter, there appeared to be a hereditary link since there was strong

suspicion that the infant's grandmother was similarly affected. However, in none of the cases was a metabolic, neurological or immunological defect found.

5.3. Diagnosis

5.3.1. *Differential Diagnosis*

The practical diagnosis of scabies is fraught with difficulties and, apart from other dermatological conditions, alternative possible arthropods need first to be eliminated as causes. This is especially so in the family or in institutional settings when an apparently contagious outbreak occurs. Common causes are mites of the genera *Dermanyssus*, which parasitize pigeons or other roosting birds and enter buildings through the cracks around window openings (Bardach, 1981; Dietrich and Horstmann, 1983; Regan *et al.*, 1987), and *Cheyletiella*, which are found on dogs, cats and rabbits (Shelley *et al.*, 1984; Maunder, 1989). A variety of other parasitic, predatory and free-living mites may also result in misdiagnosis (Blankenship, 1990) as can some insect bites (Burns, 1987a) and allergic reactions to faeces of house dust mites and psocids and friable hairs from beetle larvae (unpublished personal observations). Discovery of a mite burrow is the clear aim in diagnosis of scabies. In clinics in temperate climates the success rate may be high, ranging from 60% of cases (Orkin, 1985b) up to nearly 90% (Alexander, 1984). However, under tropical conditions the development of clearly defined burrows is less common and they may be detectable in possibly as few as 3% of patients, as in Panama (Taplin *et al.*, 1983a, 1986). A similar low incidence, around 7%, was found by Sehgal *et al.* (1972) and Wakhlu *et al.* (1988) in India, and personal experience in Bangladesh suggested that burrows were very rare under conditions of high humidity (Burgess *et al.*, 1986 and unpublished observations). Finding burrows in young children is particularly difficult (Hurwitz, 1985). Even on adults there are generally few burrows since the number of adult female mites averages only about 11 per patient (Mellanby, 1972). Therefore, it is not surprising that burrows are difficult to find especially if excoriation, eczematization or pyoderma have obscured the presentation.

5.3.2. *Identification of Mites and their Products*

The burrow is described as "a greyish line resembling a pencil mark" by Alexander (1984). In general it shows as a discoloured irregular track that may be paler than the surrounding skin especially near the entrance to the burrow. On pale skin the faecal pellets maybe seen as dark specks and the mite itself may be seen as a darker spot at the blind end of the tunnel. On black skin the burrow may be paler and the mite appears as a white spot

(Alexander, 1984; Orkin, 1985b). Scabies is often overdiagnosed in the United States (Pariser and Pariser, 1987), whereas in Britain the tendency to underdiagnose seems more widespread (various personal communications). As a result several techniques have been developed to aid the primary diagnosis of scabies by general medical practitioners. In each case the purpose is to find mites, eggs or faecal pellets.

5.3.2.1. *Mites.* A traditional method that has been used for centuries as a control method by peasants, and which was described by Bonomo and Cestoni (Parish, 1985), is to remove mites from the head of the burrow using a needle. This method was used extensively by Mellanby (1972) who found that both dead and live mites stuck to the needle point when they came into contact with it, thereby refuting the suggestion that mites grasp the needle. This method was used by assistants to Taplin *et al.* (1986, 1990, 1991). However, in the absence of obvious burrows other methods of detection are necessary.

5.3.2.2. *Burrows.* Suspected burrows may be tested for penetration of contrasting media. In the burrow ink test (BIT), described by Woodley and Saurat (1981), ink is rubbed over the area under examination directly from a pen. The ink is immediately removed with an alcohol swab but any that has spread into the burrow is left behind and shows up as a dark irregular track. Although possibly useful with nervous or uncooperative patients, the BIT gives in excess of 30% false negative results and is very time consuming. An alternative test, using the same principle, employs a liquid tetracycline preparation which is then allowed to dry. The superficial material is removed using an alcohol swab and any that has penetrated burrows can be detected by its fluorescence under Wood's light (Estes, 1982).

5.3.2.3. *Biopsy techniques.* Investigation of other lesions such as papules and vesicles may also reveal mites and their products. Friedman (1947) obtained samples by a variety of biopsy methods including snipping the epidermal roof from large vesicles with scissors. Smaller papules may be sampled by epidermal shave biopsy in which the papule is raised by pinching and the top removed using a scalpel blade held parallel to the skin surface (Itani, 1970; Martin and Wheeler, 1979). A similar result can be achieved using a cuvette (Orkin, 1985b) to remove just the cornified layer. In some cases punch biopsy followed by serial sectioning has been used successfully (Ackerman, 1985). Two alternative techniques have been tried in which superficial epidermal layers are successively stripped away using adhesives. Glue stripping employs methacrylate or cyanoacrylate glue spread on a glass microscope slide which is then pressed against the lesion to be investigated. When the glue has set the slide is quickly lifted to strip off the epidermal surface (Estes, 1982). A similar approach is to use clear cellulose adhesive tape, which is applied to the skin after swabbing with ether or acetone. The strip of tape is transferred to a microscope slide for examination (Orkin, 1985b). The principal disadvantage is that both techniques require three to six strippings of epidermis before the burrow is adequately exposed.

5.3.2.4. *Contact microscopy.* Recent suggestions for non-invasive examination of lesions have been to use direct or contact microscopy which allows the examination of a large number of lesions rapidly but without discomfort to the patient. This approach was thought to be particularly suitable for use on children (Kolpakov and Prokhorenkov, 1985; Haas, 1987).

5.3.2.5. *Skin scraping.* The most widely used technique for investigation of scabies lesions is skin scraping using a round-bladed scalpel (Palicka *et al.*, 1979; Alexander, 1984; Orkin, 1985b). The blade is held at right angles to the skin and scraped back and forth along the suspect burrow or used to remove the surface of a papule. Some controversy exists over whether oil should be applied to the blade, so that the resulting slurry of skin cells and mite products can be picked up and mounted directly in the oil on a microscope slide for observation, or whether saline should be used for scraping and the product mounted in potassium hydroxide solution or lactic acid, which both clear the specimen and soften keratin (Alexander, 1984). Very largely, the medium used is a matter of personal choice although the faecal pellets, which are much more numerous than mites or eggs and are often the only detectable sign of infection, are dissolved by potassium hydroxide. Consequently a thorough examination would wisely utilize both techniques.

Some practitioners have expressed concern, and some discussion has ensued, about the use of mineral oil since it permits the survival of any mites removed from lesions for some time by protecting them from dehydration (Stone *et al.*, 1980; Estes and Arlian, 1981; Ong and Bhatia, 1981, 1982). This was confirmed by Davis and Moon (1987), who showed that mineral oil was the best of three media for survival of mites. However, such fears as were expressed, over the health and safety risks to laboratory personnel, are irrelevant if good practice is followed and specimens are properly and safely discarded.

6. TRANSMISSION AND EPIDEMIOLOGY

6.1. Transmission

Some early writers on scabies suggested that fomites played a significant role in transmission of the mites. However, a number of studies, including those by Hebra (1868), indicated that inanimate objects were of no consequence and that only physical contact with an infected person permitted transmission. Nevertheless the controversy about the role of fomites continues to this day.

During the two World Wars enough soldiers were rendered unfit for duty by scabies for a special study to be set up by the British Government. Using volunteers, Kenneth Mellanby investigated transmission by a variety of

means (Mellanby, 1941b, 1942, 1973). Although occasional infections might be acquired from shared clothing or bedding, he found that the only likely mode of transmission under normal circumstances was by direct physical contact. Similar results were obtained independently by Heilesen (1946).

6.1.1. *Survival of Mites*

That mites are capable of survival for short periods off the host has been shown for *S. scabiei* of human origin by Mellanby *et al.* (1942b), Heilesen (1946) and Arlian *et al.* (1984b, 1988f). However, the temperatures required were generally low and relative humidity needed to be close to saturation. At the best temperatures for survival, mites were often incapable of movement. Even under optimum conditions morbidity and mortality of the mites were high and survival beyond 48 h at normal ambient temperatures was rare. Using mites derived from rabbits infected with canine *S. scabiei*, longer survival periods up to 3 weeks were observed in females and nymphs at temperatures between 10 and 15°C, provided humidity remained near saturation. Survival diminished with reduced humidity and/or increased temperature to around 2 days at 20°C or more and 25% relative humidity (Arlian *et al.*, 1989). Such a variation of survival capacity may be related to strain differences and the physiology and pathology of the particular host–parasite systems. In all cases survival limitation away from the host is related to the rate of dehydration and with the dog strain it was found that the mites were incapable of absorbing sufficient water from unsaturated air despite possessing a mechanism for active water uptake (Arlian *et al.*, 1988c). Under normal conditions in the burrow it is likely that the mites not only live in nearly saturated atmospheric conditions but actively feed on tissue fluids oozing into the burrows (Van Neste, 1986; Arlian *et al.*, 1988c).

Any mites that survive on fomites may be capable of infecting a new host provided they can gain access to the host's skin and burrow into it. A form of host-seeking behaviour has been observed in which mites move toward warmer conditions along a temperature gradient (Mellanby *et al.*, 1942b; Arlian *et al.*, 1984c) and also towards host odour, which is non-specific with respect to the host origin of the mites (Arlian *et al.*, 1988a). Live mites recovered from homes of scabietic patients were in some cases capable of penetrating the skin of a new host. However, because these mites were partially dehydrated and starved, they took longer than normal to penetrate the skin as a result of their weakened state, despite starting their burrow sooner than normal after being placed on the skin. In reality the risk of infection is actually quite low from mites that do survive. In Mellanby's (1942) studies only four of 272 attempts to infect volunteers who climbed into warm beds just vacated by heavily infected patients resulted in new cases, with only two further transmissions during 38 changes of underclothes. In contrast,

there is considerable evidence to support physical contact as the only epidemiologically significant mode of transmission.

6.1.2. *Transfer of Mites*

Johnson (1943) and Mellanby (1944a) stated that infections are contracted by the transfer of newly mated female mites which usually wander around on the skin surface before making a permanent burrow. Mellanby (1944a) also suggested that older females may occasionally be transferred if their burrows are scratched open but the probable coincidence of such an event with exposure to a new host is low. He was unable to obtain infections by transferring immature stages to a new host, but this was achieved by Munro (1919). However, Mellanby (1944a) was able to demonstrate that, if sufficient egg-laying females were present on a host but were subsequently removed by physical extraction, the infection would continue. In some cases, with low parasite burdens, the infection died out after removal of the females, suggesting that mortality amongst larvae and nymphs is normally very high (Mellanby, 1944a). Nevertheless, during the peak period of infection the immature mites are normally very numerous and spend a high proportion of their time on the skin surface, so they have a much greater chance of transferring to a new host (Alexander, 1984). Some inevitably survive to start new infections.

6.2. Epidemiology of Human Scabies

6.2.1. *Cycles of Infection*

Scabies is found in almost all parts of the world, with the exception of a few isolated communites. Friedman (1947) cited numerous reports of the levels and extent of infections from around the globe indicating that high prevalence existed in almost all populations in the period before the development of effective treatments. Nevertheless, even where prevalence is high, and in the absence of adequate treatments, some people have always failed to contract the disease

The apparent epidemic cycles of scabies have been attributed to the development of "herd immunity" (Mellanby, 1944a; Shrank and Alexander, 1967) in which the proportion of the population already having been infected slowly increases through the peak period of incidence and eventually the "immune" population becomes so large that it induces a reduction of transmission, with a subsequent significant decrease in prevalence, so that the disease may for a time disappear. Once a new susceptible population has grown up, a new wave of infection can begin. Shrank and Alexander (1967) estimated this period to be around 15 years for both the peak and trough of the cycle.

That such a variation occurs was demonstrated by the number of new scabies cases attending a private clinic (Epstein, 1955; Epstein and Orkin, 1985). During the period from 1937 to 1983 incidence increased during the first few years to a peak of 5.4% of all dermatology patients in 1945, followed by a reduction to zero in 1955 and 1959. Throughout the 1950s and early 1960s incidence remained below 1% but steadily increased from the middle of the decade to a new peak of 3.6% in 1976 with another subsequent decline in numbers.

How the infection may be limited by an immune population is unclear. Mellanby (1944a) showed that some previously infected patients did eliminate a second infection, although there was no evidence that mites were actually killed by the host's immune reaction. However, second or subsequent infections failed to produce parasite burdens as high as those found in primary infections. In certain cases he found that, because of the almost immediate itch reaction to a second infection, many of the mites were eliminated by scratching before they were able to establish themselves. Since a low number of adult female mites will produce relatively small numbers of offspring, it may be that transmission ultimately fails because insufficient mites are passed on to new hosts.

6.2.2. *The Role of Race*

In countries where the disease is endemic the epidemic cycle has not been reported in the same way but this may be due to lack of surveillance rather than the non-occurrence of the phenomenon. There have also been suggestions that some racial groups may be more susceptible to infection. Friedman (1947) suggested that Whites may be less susceptible than others but contradicted himself with his own evidence that amongst American soldiers during two World Wars the percentage of Blacks contracting scabies was less than half that of Whites. An even more pronounced difference was noted by Alexander (1978) in his clinic in Maryland, USA. In a population consisting of 48% Blacks, 36 of 37 patients with scabies were White. Similar observations of an overall skewed ethnic distribution of the infection were made in subsequent correspondence from other parts of the USA (Knox *et al.*, 1979; Lomholt, 1979; Rietschel *et al.*, 1979). In contrast to this, Hersch (1967) found a higher incidence in South African Blacks than in other races. Similar variations of distribution have been found in other multi-ethnic communities. Lines (1977) and Andrews (1979) found the highest prevalence in Polynesian immigrants to New Zealand, followed by Maoris, with the lowest levels in those of European origin. Funaki and Elpern (1987) found a higher prevalence amongst Whites and Hawaiians on the island of Kauai, Hawaii, than in Japanese or Philippinos. Such differences may reflect levels of affluence or family and social structure, as suggested by Lines (1977). Alternatively, there may be some characteristics of mite behaviour or physiology that reduce their

ability to transfer between races which have not yet been investigated. None of the racial groups mentioned appears to have any special protective characteristic, and they may be protected in some of the examples only by a low level of social contact with people of different ethnic origins. However, there is evidence that some individuals may be predisposed to infection with *S. scabiei*. Falk and Thorsby (1981) investigated the distribution of 33 histocompatibility antigens of the HLA-A, B and C series in 66 scabies patients. Of these antigens, only HLA-A_{11} was present in significantly more patients than healthy controls, with distributions of 28.3% and 10.4% respectively. A similar correlation was found with the same antigen in a study of 62 patients by Morsy *et al.* (1990) in Egypt. It has also been suggested that atopic patients sensitive to house dust mites may be more susceptible to scabies infection (Falk and Bolle, 1980a; Arlian *et al.*, 1991). Both of these features would conform with the suggestion of a genetic predisposition to scabies made by Christopherson (1986). Such aspects of the disease require extensive investigation and may provide an insight into some of the variations of infection within multi-ethnic communities mentioned previously.

6.2.3. *Community Risk Factors*

A variety of other factors influences the incidence of scabies within communities but the assessments are not always clear. Surveys are often not comparable, being based on different criteria such as national or local geographical zones, socio-economic groups, or attendance at clinics either specifically for scabies or as part of some other clinical programme. Particularly in the latter group the data may have no validity at all since often the personnel involved are not specialists in dermatology or skin parasitology and in many parts of the world the term "scabies" has become a generic term for any skin lesion of a persistent nature, with or without pruritus, and is often associated with pyoderma. The assessment of dermatological diagnosis in Jamaica by Badame (1988) concluded that scabies was the first thought in diagnosis by non-specialists and other dermatological diagnoses were made only after the exclusion of scabies, often with no alternative offered.

6.2.3.1. *Hygiene.* In several published surveys the data acquired were provided either by untrained observers, often responding to questionnaires with little trained support, or else prospectively. In many cases episodes of infection were too brief to be real scabies, as in surveys of pre-school children in urban Nigeria (Goyea, 1988) and rural India (Damodoran *et al.*, 1979). In the Nigerian study it was clear from the text that scabies was based on any case of "bad skin". Similarly in the studies of Stanton *et al.* (1987a, b) in Bangladesh, the attacks were often too brief and, although undoubtedly many real cases existed within the study community, because the observations were obtained mostly from subjects themselves, numerous other skin problems must have been included. Having worked in the same community

I have personal experience of how misleading such information supplied by patients can be.

Hygiene practices as controlling factors for infection were prominent in the work of Stanton and co-workers (1987a, b) whose studies were primarily concerned with diarrhoeal disease. An extension of the same hygiene concept was applied in assessment of the effects of availability of fresh water on the control of scabies in communities in Panama (Ryder *et al.*, 1985) and India (Verma and Srivastava, 1990). None of the parasitological evidence suggests that scabies is in any way influenced by hygiene or the availability of water *per se*, although continual or excessive washing may alter the symptomatic presentation (Orkin, 1985a) and my own observations of children in Bangladesh showed that children who were playing in and out of water throughout the day were as likely to be infected as were those for whom water was scarce. In clinical trials on these same subjects we were more concerned that swimming may have adversely affected the efficacy of treatments, because they were washed off, rather than having any effect on the scabies itself (Burgess *et al.*, 1986). The extensive survey by Palicka (1982) in Czechoslovakia suggested that increased personal hygiene merely served to delay the onset and reduce intensity of symptoms, and Bartley *et al.* (1945) found that scabies continued to increase in a community washing regularly, unless other control measures were effected.

6.2.3.2. *Age.* Without the influence of other predisposing factors the greatest incidence of scabies would be expected in the youngest sector of the population and such is the case, although the highest levels vary with the survey. Montgomery (1985) found the highest prevalence in children up to 2 years old and Wakhlu *et al.* (1988) reported over 81% of cases below 6 years. However, most workers have not found such a specific distribution and high incidences in children and young adults up to 20 years of age are usual (Sehgal *et al.*, 1972; Nair *et al.*, 1973; Church and Knowlesden, 1974; Gulati *et al.*, 1977; Desai and Nair, 1978; Palicka, 1979a, b, 1982; Tonkin and Wynne-Jones, 1979; Desai *et al.*, 1985; Baweja *et al.*, 1986; Andrews and Tonkin, 1989). Such an incidence would be expected if the herd immunity theory of Shrank and Alexander (1967) were true, since those under 20 years old would have been unaffected by previous epidemic peaks. However, in some studies the incidence appears unaffected by age up to around 45 years (Nathan, 1945; Christophersen, 1978). Some studies have shown a decline in prevalence with age, although the rate is variable and may simply reflect cultural differences (Nair *et al.*, 1973; Masawe and Nsanzumuhire, 1975; Andrews and Tonkin, 1989). Few studies have examined people over 45 years old as part of a whole population and the indications are that they are relatively free of the infection (Christophersen, 1978). However, there has been a recent upsurge of scabies in the elderly in nursing homes and other long stay care facilities (Parish *et al.*, 1983; Juranek *et al.*, 1985; O'Keefe *et al.*, 1985; Burns, 1987b; Coenraads, 1987; Hitzig *et al.*, 1987; de la Rue Browne, 1988; Lomholt, 1988; Meyers,

1988; Taplin and Meinking, 1989; Meinking and Taplin, 1990; Mocsny, 1990; Myint, 1990). These outbreaks are normally associated with one or more cases of hyperkeratotic scabies that have not been recognized and therefore continue to reinfect patients and staff despite treatment measures (Burns, 1987b; Hopper *et al.*, 1990). Often the problem is exacerbated by the transfer of patients between institutions (Editorial, 1988) and treatment regimes that are ineffective (Reilly *et al.*, 1985). In some cases the presentation may be obscured by other medication (Parish *et al.*, 1983; Coenraads, 1987), leading Parish and colleagues (1983) and Cox and Patterson (1991) to recommend the use of topical steroids when presented with obscure symptoms, or after treatment, either to eliminate the prolonged itch or else to make detection of any surviving mites easier by exacerbating the condition. Such an approach should always be taken with caution and only on patients under constant supervision, otherwise new cases of crusted scabies could be induced.

6.2.3.3. *Contact tracing.* Tracing the source of an infection with scabies is as important epidemiologically as with other infectious diseases. However, because of the time lapse between infection and the development of symptoms the actual event or circumstance is often not clear. Thus the prevalence amongst combatant soldiers in World War II was initially blamed on transmission in barracks rather than on contagion of soldiers by their families during leave of absence, as was shown by Mellanby (1942, 1972). Since then several studies have emphasized the role of the family in the spread of contagion but have equally shown that once scabies is introduced not all family members contract the disease, a phenomenon for which no satisfactory explanation has yet been supplied. Hyde (1905) was the first to propose the family as the primary epidemiological unit, yet this aspect of the disease was largely ignored for over six decades. Progress of the infection through families was observed by Hernandez-Perez (1971), showing that dealing with individual symptomatic patients is ineffectual. Extensive studies in Czechoslovakia by Palicka and Merka (1971), and Palicka (1982) showed that the incidence in families could be as high as 22%, when that in the general population was only 1.3%. Examination of 6000 families with a history of scabies showed only one person becoming infected in 63% of households, without further transmission to family members. In the remainder at least one other member of the household became infected but the infection appeared to be restricted to the family in almost all cases. These results are comparable with those of Church and Knowlesden (1978), in England, in which 38% of households had more than one case. Introduction of the disease to the family was by young people between 15 and 20 years old in approximately 60% of cases, children at school in around 30% of cases, and in only 10% was a pre-school child the index case. The late teenage group were those most likely to bring the infection to the household and did so eight times more frequently than their representation in the population would suggest. Schoolchildren were the index case twice as often as expected. The conclusion was that young children

were more likely to contract the disease from older family members, a finding also made by Sheila *et al.* (1982) working in Madras in southern India. High levels of intrafamilial transmission have also been indicated by Wakhlu *et al.* (1988), who found that 73% of 126 infected children came from a family where other members had the disease, and Sharma *et al.* (1984), who examined a whole rural community in which prevalence was 13% of individuals but 30.9% of households. In this study each index case was judged to have infected an average of 1.9 other people and the proportion of infected households increased linearly with household size. Similar findings regarding scabies and family size were reported by Gulati *et al.* (1977) and Nair *et al.* (1973), but not by Andrews and Tonkin (1989) or Sachder *et al.* (1982).

A concept of disease foci has been suggested by Sokolova *et al.* (1989a) based on the "potential focus" with only one patient, and the "radiating focus" with two or more cases. In practice over 80% of radiating foci are family groups, whereas 85% of potential foci are either organized or collective bodies such as schools and work-places. Incidence within the groups depends on time and intensity of contact rather than the size of the focus and conforms with other analyses of transmission in the community.

6.2.3.4. *Crowding.* The problem of overcrowding has often been linked with high prevalence of scabies. Nair *et al.* (1973) and Sachder *et al.* (1982) indicated that a relative increase of affluence resulted in less crowding of sleeping quarters and subsequent reduced transmission. Overcrowding certainly accounts for increased incidence in some hostels (Bhavsar and Mehta, 1985) and Alexander (1984) cited a number of examples of temporary overcrowding and population migrations that appeared to have increased the incidence of scabies. Friedman (1947) went to great lengths to attribute any increase in the disease to some population movement and no doubt such circumstances have played a part, especially when individuals from an endemically infected population have been moved into a relatively scabies-free, and thus presumably susceptible, one (Bjornberg and Friis, 1978; Fain, 1978; Orkin, 1985a; Haas and Stuttgen, 1987b). However, the relative effects of crowding on incidence are not clear. Mellanby (1941a) showed that the increase of scabies in British cities, where people were often crowded together for long periods in air raid shelters, was closely matched by the increase in other towns, where bombing was not a problem and the inhabitants remained in their homes. Consequently the relationship between scabies and relative levels of crowding in the community and the household is likely to be complex and requires more investigation of the susceptibility of individuals exposed as well as assessment of the importance of other exposure risks.

6.2.4. *Seasonality*

It is generally accepted that the incidence of scabies increases through the autumn into the winter, with a subsequent decline in spring. This has been a consistent feature in the records maintained in Denmark and a month by

month comparison of the years 1899–1908 with 1960–1969 shows a close correlation in incidence of the disease for the two periods (Christopherson, 1978). Such a seasonal rise has been attributed to the tendency of people to huddle together for warmth in winter leading to greater transmission (Friedman, 1947; Mellanby, 1972), and Alexander (1984) suggested that this would explain the increase during the South Asian monsoon period (Sehgal *et al.*, 1972). In my experience in Bangladesh there was certainly a rise at that time but not because people huddled together for warmth. On the contrary most people have problems keeping cool then, whereas they do come into closer contact in January and February when temperatures fall at night but when scabies is beginning to decline. Recent studies show a general trend for an increased incidence of scabies in the winter (Hayashi, 1986a; Kimchi *et al.*, 1989). Some authors are more specific. Sato *et al.* (1989) found the highest number of new patients from October to March with a peak in November, and Sheila *et al.* (1982) found that two peaks occurred: the highest incidence in adults was in January but in children the peak appeared in March. Whether such regional variations are due to differences in transmission or are artefacts of reporting is hard to determine; however, a new approach by Sokolova *et al.* (1989b) may help to clarify the situation. These authors counted the number of eggs in burrows over the seasons and showed that from September to December the number of ova was 11.5 ± 1.0 up to 12.6 ± 1.3, whereas during the period January to July only 6.0 ± 0.6 to 8.9 ± 1.1 eggs were found. This they termed the fertility index. They also found that some female mites entered a latent phase between December and July when oogenesis was suspended.

If mite egg production was low during the January to July period the numbers of mites developing, and the subsequent transmission rates, would be correspondingly lowered. However, once the females increased their egg production there would be a lag phase during which mite numbers increased and successful transmission to new hosts also increased, but any new hosts would not develop a symptomatic response for up to 2 months. Consequently a rise in the number of diagnoses would start in September. Similarly, after the mites have entered the more latent phase in December there would be a corresponding delay in decline of incidence until all new cases had developed symptoms. This delay could explain the late peak of incidence in adults observed by Sheila *et al.* (1982) but not that in children. It is possible that the mites in newly infected hosts in this study retained a higher rate of fertility for longer, thus allowing a further wave of transmission.

6.3. Animal Scabies

6.3.1. *Cross-infectivity*

Mites from one species of mammal may transfer occasionally to other species. Experimental work by Arlian *et al.* (1984a, 1988a) shows that mites from dogs

or pigs are able to infect a variety of alternative host species with varying success, ranging from survival for as little as 1 or 2 days for dog-derived mites placed on mice, up to 13 weeks of infection with the same mites on goats. The most successful transfer was of mites from dogs placed on rabbits, from which the authors were able to establish a long-term laboratory colony. Similar results have been obtained by others, with successful transfer of goat mites to donkeys (Abu-Samra *et al.*, 1985), sheep mites to dromedary camels (Nayel and Abu-Samra, 1986b), and goat-derived mites to desert sheep (Ibrahim and Abu-Samra, 1987).

6.3.2. *Transfer to Humans*

It is not uncommon for humans working with domesticated animals to become infected with their mites. This has given rise to terms like "cavalryman's itch" and "pig-handler's itch" to describe the subsequent irritation. Experimental transfer of canine mites to humans induced severe itch within 24 h (Estes *et al.*, 1983). The mites were able to feed and lay eggs but did not survive for more than a few days. The distribution of lesions on humans infected with dog scabies is generally distinctively different from that of human scabies, with most lesions appearing on the trunk and arms. Characteristically, few lesions occur on the hands (Smith and Claypole, 1967). The infection is commonly acquired when a family obtains a new dog (Orkin, 1985a; Meijer and Van Voorst-Vader, 1990). Since dogs tend to develop a crusted form of the disease it is easy for crusts and scales bearing mites to become detached in the house as a potential source of infection to the occupants (Chang and Cho, 1990) in a similar way to the crusts derived from human hyperkeratotic scabies patients (Carslaw *et al.*, 1975), or even free in the environment of patients, as discovered by Arlian *et al.* (1988f).

Since the infection is self limiting, canine scabies in humans generally requires no treatment provided the causative animals are cured. However, in some cases Smith and Claypole (1967) found that new lesions continued to appear for up to 5 weeks and would benefit from specific therapy.

Transmission of scabies from pigs to humans occurs readily in those handling the animals and infections have been found in pig herders (Chakrabarti, 1990) and slaughterers who were skinning the animals (Shoji *et al.*, 1985). In both cases the infections were self limiting and occurred mainly on hands, legs and, in several cases, on the trunks of the slaughterers. Mites were not detected in the lesions examined by Shoji and colleagues (1985) but Chakrabarti (1990) found that the reaction could last up to 3 weeks after exposure ceased. A similar reaction was found in farmers handling infected water buffaloes (Chakrabarti *et al.*, 1981).

7. TREATMENT AND CONTROL

7.1. Scabicides

Numerous materials have been used for the treatment of scabies over the centuries and it has long been recognized that, for the majority of active substances, topical application is the only effective means of delivery. The choice of material is largely based on the personal preference of the prescriber but considerations of age of the patients, their state of health, degree of excoriation or eczematization, and availability and cost of the product will also influence the choice (Burns, 1991), apart from efficacy or potential toxicity.

7.1.1. *Sulphur*

For many years sulphur in some form was the mainstay of treatment. In practice the only useful form of this material is as an ointment of precipitated sulphur in petroleum jelly or some similar base (Rees, 1985) and it is still the treatment of choice for more conservative dermatologists or in places where cost or lack of availability of more sophisticated forms of treatment make it the only feasible scabicide. A concentration of 6–10% is normally used for three consecutive days (Mellanby *et al.*, 1942a; Borda, 1954; Alexander, 1984), but excessive use may cause an irritant dermatitis (Mellanby *et al.*, 1942a; Orkin and Maibach, 1985; Burns, 1991), especially in those with a tendency for xerosis or atopic dermatitis (Rees, 1985). Lower concentrations are used in young children. Sulphur itself is not toxic to mites, since Mellanby *et al.* (1942a) were able to keep mites alive for several days embedded in sulphur ointment on a microscope slide. It appears that organic or metabolized products of sulphur, possibly pentathionic acid, constitute the active form produced either by skin microorganisms or epidermal cells (Rees, 1985).

7.1.2. *Benzyl Benzoate*

Benzyl benzoate was first used in the form of "balsam of Peru", of which it forms the active constituent and which is still used in some countries with the attendant risk of contact irritancy (Temesvart *et al.*, 1978). The first real trials of benzyl benzoate itself were performed by Kissmeyer (1937), but its use was largely established by the trials conducted by Mellanby and co-workers (Mellanby *et al.*, 1942a; Mellanby, 1972, 1973). At therapeutic concentrations it is a skin irritant and may cause severe stinging, especially of excoriated skin, and consequently was soon not recommended for use on children (Bradshaw, 1944). Dilution to make it more acceptable seriously reduces its

efficacy, as does incomplete application or not making the generally accepted two or three applications (Alexander, 1984; Burns, 1991). In contrast, Mellanby *et al.* (1942a) obtained nearly 100% effectiveness with a single application. This treatment has made a brief come-back in some parts of eastern Europe and countries of the former Soviet Union in recent years (Levkov, 1980; Fedorovskaia *et al.*, 1986) as part of more concentrated efforts to control the disease.

7.1.3. *Monosulfiram and Related Compounds*

At around the same time that benzyl benzoate was undergoing trials, monosulfiram was also being tested (Percival, 1942; Clayton, 1943; Bradshaw, 1944). All three authors claimed it was 100% successful, although a reappraisal of their data indicates rather less and the cases which they deemed to have been reinfected were more probably relapses. After these trials it was suggested that the emulsion form was particularly suitable for the treatment of children, but most of those involved were too young to complain so it is probably hard to justify the claim of suitability. Since the material is dissolved in industrial methylated spirit, which is then diluted to about three times its volume, it is certain to cause discomfort to excoriations. Monosulfiram is used in conjunction with benzyl benzoate in some formulations (Landegren *et al.*, 1979; Blanc and Deprez, 1990) and a similar molecule, dixanthogen, is now no longer available (Brandrup and Weismann, 1990).

A further complication with this material is its chemical similarity to disulfiram (Antabuse®), which is used in the treatment of alcoholics. Both monosulfiram and alcohol readily cross the dermis to enter the bloodstream which, after two or three applications on successive days, may induce an "Antabuse® reaction" with dermal oedema, flushing, sweating and tachycardia (Alexander, 1984). This is more likely if the patient imbibes alcohol (Gold, 1966; Morley, 1967; Plouvier *et al.*, 1982; Blanc and Deprez, 1990), but has been observed in a normal subject following a single application of monosulfiram emulsion (Burgess, 1990). This person was unable to metabolize effectively the alcohol that crossed the dermis after treatment.

7.1.4. *Crotamiton*

Crotamiton has been widely used for scabies, particularly on children because it also has antipruritic properties. However, some doubt about both its scabicidal and antipruritic attributes exists and results are extremely variable and, in the main, unsatisfactory (Borda, 1954; Cubela and Yawalkar, 1978; Coskey, 1979; Konstantinov *et al.*, 1979; Wolf and Wolf, 1988; Taplin *et al.*, 1990; Roth, 1991). It has been recommended for children (Hurwitz, 1985) but, since it may require several applications (Cubela and Yawalkar, 1978; Konstantinov *et al.*, 1979), there are inherent drawbacks, especially since

multiple applications were suspected to have induced methaemoglobinaemia in one child (Arditti and Jouglard, 1978).

7.1.5. *Lindane*

Although some quite remarkable success was achieved with early scabicides, the best prospects for control and eradication arrived with the advent of synthetic insecticides and the prospect of one-application treatment cures became a real possibility. The first of these, lindane (γ-hexachlorocyclohexane), was used successfully by Cannon and McRae (1948) and Wooldridge (1948). The success was dependent on concentration and a reduction from 1% to 0.5% active ingredient reduced success equivalently (Kornblee and Combes, 1950); however, efficacy is apparently not particularly dependent on time, provided enough time is allowed for the material to pass into the skin (Hayden and Caplan, 1971; Taplin *et al.*, 1983b).

Lindane is detected in the blood stream soon after application, from whence it is excreted in the urine (Lange *et al.*, 1981). Some of the material is stored dissolved in body fat (Zesch *et al.*, 1982), and may be subsequently excreted in breast milk (Senger *et al.*, 1989). Considerable controversy has surrounded the use of lindane in recent years, with reports of neurotoxic and other side effects following therapy, especially in children. A number of children, and a few adults, have suffered a wide range of symptoms from irritability through to convulsions and death, in relatively acute exposures, and aplastic anaemia and other dyscrasias chronically, but in most cases these followed over-use of the product and ingestion or other inappropriate application, especially if the epidermal barrier was already compromised (Ginsberg *et al.*, 1977; Lee and Groth, 1977; Pramanik and Hansen, 1979; Mead, 1982; Saurat, 1982; Telch and Jarvis, 1982; Davies *et al.*, 1983; Berry *et al.*, 1987; Friedman, 1987; Rauch *et al.*, 1990; Tenenbein, 1991). Analysis of some 26 cases suggested that the evidence for toxicity in some of the cases was insufficient and that others were due to inappropriate application (Kramer *et al.*, 1980). Considerable efforts have been made to preserve the use of what is in other respects a useful, reliable and commercially successful scabicide by the formulation of guide-lines for its use (Rasmussen, 1981, 1987; Shacter, 1981). Whatever the outcome, it is probably wise to avoid its use in children under 10 years old (Orkin and Maibach, 1985), those with reduced body fat (including anorexics, geriatrics and some athletes), and anyone with a history of fits or other siezures.

7.1.6. *Malathion*

Malathion (0.5% in an aqueous base) is in use in Britain and cure rates in trials were comparable with other effective treatments such as lindane (Hanna *et al.*, 1978; Burgess *et al.*, 1986; Myint, 1990). A single trial of

malathion in an alcoholic base has been reported in which it proved effective in 20 of 24 cases (Thianprasit and Schuetzenberger, 1984). However, the view of the authors that the formulation was "well tolerated", despite the burning sensation experienced by all but one subject, should be considered somewhat optimistic in view of the irritant characteristics of other scabicides containing less alcohol. Malathion used to treat scabies must be pure, since toxic reactions may occur if low-grade agricultural material is used, especially with children (Petros, 1990).

7.1.7. *Pyrethroids*

Pyrethroid insecticides have only recently begun to be used as antiscabies agents. One problem with this group of compounds is that they can be rapidly metabolized by mammals and, although this is favourable in terms of low mammalian toxicity, it is unfavourable as the materials may be degraded before they have time to act. These problems may be overcome either by effective formulation, which enhances the rapidity of effectiveness, or by using more stable forms of the molecules incorporating chlorine or other substitutions that reduce the rapidity of cleavage. The first trials involved the use of S-bioallethrin (0.3%), an unsubstituted molecule, in an aerosol form (Belaube *et al.*, 1984; Jean-Pastor and Belaube, 1984). A single trial has also been reported using decamethrin (0.02%) in a lotion formulation (Schenone *et al.*, 1986). Both formulations achieved over 90% cures with more than one application, and S-bioallethrin was successful in 81% of cases when used only once (Jean-Pastor and Belaube, 1984).

More recently permethrin, a chlorinated molecule, has been widely tested in comparison with other scabicides and proved acceptable and effective with rates of cure around 90% or more when assessed 3–4 weeks after treatment (Hansen *et al.*, 1986; Taplin *et al.*, 1986, 1990, 1991; Haustein and Hlawa, 1989; Van der Rhee *et al.*, 1989; Yonkosky *et al.*, 1990; Schultz *et al.*, 1990).

7.1.8. *Oral Treatments*

Some interest has been shown in the possibility of oral treatment of scabies. Hebra (1868) showed that sulphur was ineffective taken in this way, but Hernandez-Perez and Bechelli (1969) demonstrated that the anthelmintic thiabendazole was effective for some patients at a dose of 50 mg kg^{-1} daily for 5 days. Better results were obtained with 25 mg kg^{-1} over 10 days, when six of seven patients were cured and the other required a further 10 days' treatment (Hernandez-Perez *et al.*, 1969). De Mello and Uthida-Tanaka (1970) were able to obtain only about 50% success with thiabendazole; however, they detected fewer side effects using a syrup formulation than with tablets. Only partial success was achieved by Raoult *et al.* (1982) using the similar molecule, flubendazole. Thiabendazole is also available as a cream and has been tried by Biagi *et al.* (1974) and Hernandez-Perez (1976). The

former used a 5% formulation applied twice daily for 5 days and cured 14 of 19 patients, with success in the remainder after a further 5 days' application. Hernandez-Perez (1976) used 10% and obtained 80% cure after 5 days.

Some success has been achieved using metriphonate, an organophosphorus compound, by Barranco *et al.* (1976) at a dose of 10 mg kg^{-1} on each of 2 days each week for 4 weeks, but care must be taken in monitoring patients for choline esterase inhibition. Cotrimoxazole also has some effect (Shashindran *et al.*, 1979).

7.1.9. *Treatments for Animals*

Treatment of scabies in domestic animal species has employed similar materials to those used on humans and many of those used clinically were first employed on other mammalian species. Current treatments may employ acaricides such as amitraz, which is used on pigs (Gaafar *et al.*, 1986) and dogs (Yathiraj *et al.*, 1990) or organophosphorothioates such as phoxim on pigs (Oksanen and Tuovinen, 1990) or phosmet on pigs (Hewett, 1985; Kambarage, 1991) or cattle (Henderson *et al.*, 1987). However, the treatment that has attracted most interest recently is ivermectin. It can be administered topically at a rate of 500 mg kg^{-1}, which is wholly effective (Barth and Preston, 1988); orally, which is only partially effective (Schroder *et al.*, 1985); or by subcutaneous injection at a rate of 200 mg kg^{-1}, which is often wholly effective with a single injection, for camels (Chellappa *et al.*, 1989), goats (Manurung *et al.*, 1990), pigs (Ohba *et al.*, 1989), sheep (Pangui, *et al.*, 1991), dogs (Rai and Yathiraj, 1988) and cattle (Schroder *et al.*, 1985). This material circulates in the blood and becomes incorporated into the epidermal cells, rendering them toxic to the mites that eat the cells.

7.2. Method of Treatment

Most problems with scabies treatment arise because it is applied incorrectly in some way. For many years a pre-treatment bath was recommended (Borda, 1954; Mellanby, 1972), often associated in practice with scrubbing to "open the burrows", a practice condemned by Borda (1954). Generally this had a counterproductive effect because the induced vasodilation in the skin increased the uptake of the scabicide by the blood (Solomon *et al.*, 1977; Lange *et al.*, 1981; Zesch *et al.*, 1982). Such an effect is enhanced in those areas of skin that are already more susceptible to penetration by pesticide due to their relatively good blood supply or thinness of epidermis (Maibach *et al.*, 1971; Feldman and Maibach, 1974). In all cases a patient will require the assistance of somebody else to apply the treatment, especially to the back (Burns, 1991), unless dipped in the manner used by Fernandez *et al.* (1984). Many formulations when introduced were intended for use in clinics and consequently the attendant instructions are scant and sometimes misleading

for domiciliary use by persons unfamiliar with the subject. Alexander (1984) and Orkin and Maibach (1985) gave examples of the precise instructions that should be provided by the prescriber. The most important features are that the patient should treat the whole body surface from the neck down in classical scabies, including the soles of the feet, and pay particular attention to the hands. In the light of the study by Scher (1983), this should include brushing formulation under the ends of the nails. With children and elderly patients the face, head and, especially, the post-auricular fold should be included (Hurwitz, 1985; Taplin and Meinking, 1989; Maunder, 1992). However, with patients living in more tropical conditions, which could include the microclimate of some long-stay care facilities, mites can be found particularly in the post-auricular folds of normal adults (Taplin, 1983; Taplin *et al.*, 1991; Burgess, 1992), and this site should be suspected in any case of treatment failure.

The persistent itch often misleads patients into believing the treatment has failed. They may subsequently treat themselves repeatedly, resulting in a contact dermatitis reaction to the scabicide that confuses subsequent management of the case (Meneghini *et al.*, 1982; Farkas, 1983; Fiumara and Kahn, 1983). In order to avoid such problems, Orkin and Maibach (1985) recommended limiting the availability of scabicide by supplying a non-refillable prescription for each patient treated.

Even after successful treatment, symptoms will persist for some days or weeks. Itching may even intensify for a short time, and can be relieved by the use of oral antihistamines for a few days (Orkin and Maibach, 1985) and the application of non-steroid antipruritic agents such as calamine or crotamiton (Burns, 1991; BMA and RPS, 1992). Since lesions may persist for more than 2 weeks, especially if secondary infection is present, the practice of recording their sites at initial diagnosis and at follow up examinations is recommended. The use of Polaroid® photographs can prove invaluable as a record of the position of original lesions and for identification of new ones up to a month after the initial treatment (Taplin *et al.*, 1986, 1990, 1991).

Crusted scabies is treated using the same materials as classical scabies. However, because of the massive thickening of the hyperkeratotic plaques in some cases there is a problem of penetration of the active ingredient in sufficient quantity. In such cases several applications of scabicide may be required (Burns, 1991; Burgess, 1992; Maunder, 1992). In treating a particularly difficult case, Ward (1971) found that methotrexate assisted in eradication of the mites.

7.3. Control, Eradication and Future Prospects

Alexander (1984) stated: "Ideally a worldwide mass treatment campaign should eradicate scabies". Such should be our aim, but a number of initial

hurdles needs to be crossed including adequate diagnosis, selection of treatment and treatment regimes, and poverty. Many countries still have little treatment available at an affordable price for those most in need. Even in developed countries scabies still manages to throw the health authorities into confusion and the frequent outbreaks reported to the Medical Entomology Centre at Cambridge (UK), and referred to by Juranek *et al.* (1985), Taplin and Meinking (1989) and Burns (1991), testify to this.

The obvious first step, which many health authorities seem to avoid for reasons of public sensitivity, is to set up national and international reporting schemes on the lines of those in Denmark. Once the epidemiological basis was in place, a process of eradication could begin.

Alexander (1984) also suggested that mass trials of preventive measures could be instituted in populations in endemic areas as part of an internationally sponsored programme. Such attempts were made, on a limited scale, with some success by Gordon *et al.* (1944) and Bartley *et al.* (1945), using monosulfiram soap, and on a large scale by Mellanby (1944b) with benzyl benzoate used prophylactically on the hands and wrists of schoolchildren at intervals of 2 weeks. When the 13 000 test subjects and nearly 6000 controls were assessed after 12 weeks, both groups showed a similar degree of reduction of incidence of the disease, primarily because all who were found to have scabies were treated. Consequently it was concluded that inspections and vigilance play a greater potential role in scabies control and eradication than any prophylactic measures. The role of health education of public and professionals was stressed by Percival (1941), Mellanby (1942) and Borda (1954), and there is recent evidence that health education combined with treatment results in a greater reduction in scabies than is achieved by treatment alone (Antony, 1982). This is to be expected since knowledge of the disease, even in developed countries, amongst the general population is scant and that amongst most health care professionals could be dramatically improved. A similar study by Reid and Thorne (1990) promised a similar improvement, but their data cover too short a period of intervention for any real conclusions to be drawn.

Until scabies can be controlled or eradicated in developed countries there is little hope of success in developing ones. Simply moving in with a team and applying scabicide produces little long-term effect (Taplin *et al.*, 1983a, b), and even after a long-term surveillance and treatment programme any interruption of vigilance or the supply of materials for treatment results in a rapid and significant increase in incidence (Taplin *et al.*, 1991).

The problem in developed countries is exacerbated because the presence of scabies is often not suspected and eradication measures, once instituted, are often half-hearted, inadequate and inconsistent. Cases of crusted scabies, often the primary cause of outbreaks in institutions, are frequently overlooked and, unless treated, will remain as reservoirs of infection and reinfection of patients, staff and visitors over a long period (Carslaw *et al.*,

1975; Juranek *et al.*, 1985; Burns, 1987b, 1991). Institutional scabies often provokes a flurry of unnecessary peripheral activities that fail to reach the cause of the problem. Although cases of crusted scabies may shed infective keratotic plaques into the environment (Carslaw *et al.*, 1975), the distribution of these within the institution is normally quite restricted (Burns, 1987b). Nevertheless, care should be taken with bed sheets and personal clothing of these patients since they may inadvertently transport infected mites outside the institution in exceptional cases (Thomas *et al.*, 1987) and in one instance two patients were infected from contaminated medical instruments (Kim *et al.*, 1990). However, studies in hospitals and communal homes where there had been scabies outbreaks found considerably fewer viable mites in the environment than in domestic premises of scabies patients, due to the higher temperature and lower humidity in the hospitals and homes (Arlian *et al.*, 1988f). Nevertheless, in some circumstances it has been thought necessary to perform specific acaricidal treatments of beds and other furnishings (Burns, 1991).

A further complication of scabies control appears to be that not all cases respond to treatment. This may be due to incomplete treatment, as proposed by Taplin (1983) in reply to the suggestion by Hernandez-Perez (1983) that he had detected cases of lindane resistance in El Salvador. Only if the investigator had personally applied the treatment with head to toe applications and if clustering of non-responsive cases occurred could resistance be confirmed, according to Taplin (1983). Such criteria are lacking in many reports of suspected resistance and some difficulty arises from the use of unpredictable treatments such as crotamiton (Coskey, 1979; Roth, 1991). However, Roth (1991) also found a lack of response to lindane when applied under supervision, as occurred with the cluster of cases described by Reilly *et al.* (1985). Such clusters have now been identified by Taplin *et al.* (1986) in Panama and other clusters have been reported from across North America (Meinking and Taplin, 1990). In Britain there have been a few reports of well supervised and, in some cases, clustered instances of lindane failure (various personal communications) but, as with any parasitic arthropod infection, definitive determination of resistance status is difficult. The situation is such now that any further achievement in the eradication of scabies will require a political input in addition to clinical effort in order to bring about effective preventive and therapeutic measures for both the developed and developing worlds.

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